Background: Cholesterol transporter NPC1L1 is expressed in small intestine but not in colon.
Results: DNA in the mouse NPC1L1 gene is hypermethylated in colon as compared with small intestine. DNA methylation decreases the promoter activity of NPC1L1.
Conclusion: DNA hypermethylation may be responsible for silencing NPC1L1 expression in the colon.
Significance: Altering DNA methylation may represent a novel mechanism to modulate NPC1L1 expression and cholesterol absorption.

Intestinal NPC1L1 transporter is essential for cholesterol absorption and the maintenance of cholesterol homeostasis in the body. NPC1L1 is differentially expressed along the gastrointestinal tract with very low levels in the colon as compared with the small intestine. This study was undertaken to examine whether DNA methylation was responsible for segment-specific expression of NPC1L1. Treatment of mice with 5-azacytidine (i.p.) resulted in a significant dose-dependent increase in NPC1L1 mRNA expression in the colon. The lack of expression of NPC1L1 in the normal colon was associated with high levels of methylation in the area flanking the 3-kb fragment upstream of the initiation site of the mouse NPC1L1 gene in mouse colon as analyzed by EpiTYPER® MassARRAY®. The high level of methylation in the colon was observed in specific CpG dinucleotides and was significantly decreased in response to 5-azacytidine. Similar to mouse NPC1L1, 5-azacytidine treatment also increased the level of human NPC1L1 mRNA expression in the intestinal HuTu-80 cell line in a dose- and time-dependent manner. Silencing the expression of DNA methyltransferase DNMT1, -2, -3A, and -3B alone by siRNA did not affect NPC1L1 expression in HuTu-80 cells. However, the simultaneous attenuation of DNMT1 and -3B expression caused a significant increase in NPC1L1 mRNA expression as compared with control. Also, in vitro methylation of the human NPC1L1 promoter significantly decreased NPC1L1 promoter activity in human intestinal Caco2 cells. In conclusion, our data demonstrated for the first time that DNA methylation in the promoter region of the NPC1L1 gene appears to be a major mechanism underlying differential expression of NPC1L1 along the length of the gastrointestinal tract.

Intestinal cholesterol absorption occurs mainly in the proximal small intestine and positively correlates with levels of plasma cholesterol (1, 2). The Niemann-Pick C1-like 1 (NPC1L1) protein, localized to the brush border membrane of intestinal epithelial cells, is shown to be essential for cholesterol absorption (3). The loss of NPC1L1 in mice causes a remarkable decrease in cholesterol absorption, and NPC1L1 protein has been shown to be the molecular target for ezetimibe (3). Also, studies have shown that the knock-out of NPC1L1 prevented atherosclerosis in apoE knock-out mice (4). These observations suggest that NPC1L1 is an attractive target for the treatment of hypercholesterolemia and the prevention of atherosclerosis (3).

The expression of NPC1L1 exhibits species- and tissue-specific patterns. For example, NPC1L1 has been shown to be expressed in the liver of humans but not in mice and rats (5). Also, the expression of NPC1L1 mRNA in humans, mice, and rats is significantly higher in the small intestine as compared with the colon demonstrating differential expression along the length of the gastrointestinal tract (5). Nevertheless, the mechanisms underlying the segment-specific expression of NPC1L1 have not yet been investigated.

Tissue-specific expression of genes is determined by different mechanisms, including differential expression of certain transcription factors or by involvement of epigenetic mechanisms (6). In this regard, NPC1L1 expression has been shown to be modulated at the transcriptional level. For example, SREBP2 and HNF4α transcription factors have been shown to stimulate NPC1L1 promoter activity and increase its mRNA expression (7, 8). Because the expression of these transcription factors is not intestinal segment-specific (9, 10), their effects on gene transcription may not explain the region-specific expression of intestinal NPC1L1 mRNA. This study investigated the role of epigenetic mechanisms such as DNA methylation in dictating region-specific expression of NPC1L1. DNA methylation is a process mediated by DNA methyltransferases by which methyl groups are covalently added to the 5’-position of cytosine in the CpG dinucleotide (11). DNA methylation is often associated
with suppression of gene expression (12). The modification of DNA by methylation alters gene transcription by either blocking the access of certain transcription factors to their consensus sequences on the promoter region (13) or by allowing the binding of methyl-CpG-binding proteins that recognize methylated DNA and recruit protein partners to suppress gene expression (14). In this regard, previous studies have shown that the knock-out of the methyl DNA-binding protein MBD2 induced the expression of certain genes in the colon that are specifically expressed in the duodenum and pancreas (14). These observations indicated that DNA methylation might play a role in suppressing the colonic expression of genes that are usually expressed in the small intestine.

Our data show for the first time that the 5’ upstream regulatory region of mouse NPC1L1 was hypermethylated in the colon as compared with the small intestine. Treatment with the hypomethylating agent 5-azacytidine significantly increased NPC1L1 expression in the colon concomitant with a decrease in DNA methylation at specific CpG dinucleotides in the NPC1L1 gene. Furthermore, in vitro methylation of the human NPC1L1 promoter caused a decrease in the promoter activity in the human intestinal Caco2 cells. These findings clearly indicate the essential role of DNA methylation in controlling the expression of NPC1L1 along the length of the gastrointestinal tract. Harnessing alterations in DNA methylation by dietary manipulations or environmental stimuli may prove to be of tract. Harnessing alterations in DNA methylation by dietary manipulations or environmental stimuli may prove to be of tract. Harnessing alterations in DNA methylation by dietary manipulations or environmental stimuli may prove to be of tract. Harnessing alterations in DNA methylation by dietary manipulations or environmental stimuli may prove to be of tract. Harnessing alterations in DNA methylation by dietary manipulations or environmental stimuli may prove to be of tract. Harnessing alterations in DNA methylation by dietary manipulations or environmental stimuli may prove to be of tract. Harnessing alterations in DNA methylation by dietary manipulations or environmental stimuli may prove to be of tract. Harnessing alterations in DNA methylation by dietary manipulations or environmental stimuli may prove to be of tract. Harnessing alterations in DNA methylation by dietary manipulations or environmental stimuli may prove to be of tract. Harnessing alterations in DNA methylation by dietary manipulations or environmental stimuli may prove to be of tract. Harnessing alterations in DNA methylation by dietary manipulations or environmental stimuli may prove to be of tract. Harnessing alterations in DNA methylation by dietary manipulations or environmental stimuli may prove to be of tract. Harnessing alterations in DNA methylation by dietary manipulations or environmental stimuli may prove to be of tract.Harnessing alterations in DNA methylation by dietary manipulations or environmental stimuli may prove to be of tract. Harnessing alterations in DNA methylation by dietary manipulations or environmental stimuli may prove to be of tract. Harnessing alterations in DNA methylation by dietary manipulations or environmental stimuli may prove to be of tract. Harnessing alterations in DNA methylation by dietary manipulations or environmental stimuli may prove to be of tract. Harnessing alterations in DNA methylation by dietary manipulations or environmental stimuli may prove to be of tract. Harnessing alterations in DNA methylation by dietary manipulations or environmental stimuli may prove to be of tract. Harnessing alterations in DNA methylation by dietary manipulations or environmental stimuli may prove to be of tract. Harnessing alterations in DNA methylation by dietary manipulations or environmental stimuli may prove to be of tract. Harnessing alterations in DNA methylation by dietary manipulations or environmental stimuli may prove to be of tract. Harnessing alterations in DNA methylation by dietary manipulations or environmental stimuli may prove to be of tract. Harnessing alterations in DNA methylation by dietary manipulations or environmental stimuli may prove to be of tract. Harnessing alterations in DNA methylation by dietary manipulations or environmental stimuli may prove to be of tract. Harnessing alterations in DNA methylation by dietary manipulations or environmental stimuli may prove to be of tract. Harnessing alterations in DNA methylation by dietary manipulations or environmental stimuli may prove to be of tract. Harnessing alterations in DNA methylation by dietary manipulations or environmental stimuli may prove to be of tract. Harnessing alterations in DNA methylation by dietary manipulations or environmental stimuli may prove to be of tract. Harnessing alterations in DNA methylation by dietary manipulations or environmental stimuli may prove to be of tract.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Materials**—Human duodenal HuTu-80 and human intestinal Caco-2 cell lines were obtained from the ATCC and grown routinely in T-150-cm² plastic flasks at 37 °C in a 5% CO₂, 95% air environment. The HuTu-80 cells were cultured in minimum essential medium (Eagle’s) containing 2 mM L-glutamine, Earle’s balanced salt solution adjusted to contain 1.5 g/liter sodium bicarbonate, 0.1 mM nonessential amino acids, and 1.0 mM sodium pyruvate and supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml gentamicin (Invitrogen). Caco-2 cells were grown in the same minimum essential medium supplemented with 20% FBS. 5-Azacytidine, inhibitor of DNA methylation, was purchased from Sigma. All the siRNAs were obtained from Qiagen (Valencia, CA). DNMT1 antibodies were obtained from cell signaling; antibodies for DNMT3B were obtained from Abcam, and antibodies for DNMT3A were from Santa Cruz Biotechnology. All chemicals were of reagent grade and obtained from commercial sources.

**Inhibition of DNA Methylation and RNA Extraction**—HuTu-80 cells and Caco2 cells were seeded 1 × 10⁶ cells/well in 6-well plates (Transwell inserts for Caco2) and, cells were treated with different concentrations (1, 5, and 10 μM) of the DNA methylation inhibitor (5-azacytidine) for 48 and 72 h. RNA was isolated by using Qiagen RNeasy mini kit (Qiagen) according to the manufacturer’s instructions.

**Real Time PCR Analysis**—Equal amounts of RNA from control and treated samples were reverse-transcribed and amplified in a one-step reaction utilizing Brilliant SYBR Green QRT-PCR master mix kit (Stratagene, Clara, CA). Human NPC1L1 was amplified with gene-specific primers as follows: sense primer, 5’-TATTTCCCTTGTTCTCAGACG-3’, and antisense primer: 5’-CCCGAGACTTCTGGTATACTC-3’. GAPDH was amplified as an internal control utilizing gene-specific primers as follows: sense primer, 5’-GAAATCCCATCACCATTCT-3’, and antisense primer, 5’-AAATGAGCC- CAGCTTCTT-3’. The quantification was expressed as a ratio of ΔCt-NPC1L1/ΔCt-GAPDH, where ΔCt-NPC1L1 and ΔCt-GAPDH represent the difference between the threshold cycle of amplification of treated and control RNA for NPC1L1 and GAPDH, respectively. All real time qPCRs were performed in triplicate.

**Cloning and in Vitro Methylation of pCpG Free-L1 Vector**—For the construction of pCpG free L1 vector, the DNA fragment containing −1741/+56 region of the human NPC1L1 promoter was amplified using forward 5-ATCGATGCAGTAC- TTGGACTCTATCTCTCTGTGG-3’ and reverse 5-ATCGATGCGAAGCTTCCCAGGTCTGGGAAGGGGTCA-3’ primers, and the amplified promoter fragments were then inserted into a promoterless CpG free vector (pCpG free basic lucia, Invivo-Gen, San Diego) upstream of the lucia reporter gene. Cloned vector was then methylated in vitro utilizing SsI methylases (New England Biolabs, Frankfurt, Germany) according to the manufacturer’s instruction. Briefly, 1 μg of plasmid DNA was added to a reaction containing CpG methyltransferase (SssI, 4 units/μl) in the presence of 160 μM S-adenosylmethionine (New England Biolabs) and incubated for 4 h at 37 °C, and S-adenosylmethionine was replenished after every 2 h. Unmethylated control reaction contained the NPC1L1 promoter construct and methylases but not S-adenosylmethionine. Plasmid DNA was then purified by using QiaGen miniprep kit and quantified using a spectrophotometer.

**Transient Transfections and Luciferase Assay**—Caco2 cells were seeded at a density of 1.5 × 10⁶ cells/well on 24-well plates and cotransfected while still in suspension, with the NPC1L1 promoter in the pCpG free vector along with β-galactosidase mammalian expression vector (to control for transfection efficiency) utilizing Lipofectamine 2000 reagent (Invitrogen). A pCpG free lucia vector with EF1 promoter (pCpG free basic lucia, Invivo-Gen, San Diego) was used as a control for the methylation experiment. After 48 h of transfection, cells were washed with phosphate-buffered saline and lysed using a passive lysis buffer from Promega (Madison, WI). The activities of both lucia and β-galactosidase were measured using QUANTI-Luc (lucia assay reagent) from InvivoGen and β-galactosidase luminescent detection system from Clontech, respectively, according to the manufacturer’s instructions in a luminometer (Promega). The promoter activity was expressed as a ratio of lucia to β-galactosidase activity in each sample.

**siRNA Transfection**—Expression of DNMTs² (DNMT1, -2, -3A, and -3B) in HuTu-80 cells was selectively silenced utilizing specific siRNAs (Qiagen). Scrambled siRNA was used as a negative control. HuTu-80 cells were plated in 6-well culture plates, and the next day cells were transiently transfected with

² The abbreviations used are: DNMT, DNA methyltransferase; q-RT, quantitative RT.
100 pmol of siRNA duplexes using Lipofectamine (Invitrogen). Cells were harvested 72–96 h after the transfection. Silencing was validated by real time PCR utilizing DNMT-specific primers as well as by Western blotting using DNMT-specific antibodies.

Western Blotting—Control or siRNA-transfected HUTU-80 cells were first rinsed with ice-cold 1× PBS and then lysed with a cell lysis buffer containing 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1× protease inhibitor mixture, and 0.1% SDS. The cells were incubated with this mixture for 15–20 min on ice and further lysed by sonication in inhibitor mixture, and 0.1% SDS. The cells were incubated with this mixture for 15–20 min on ice and further lysed by sonication.

mFab blocking buffer in 1× PBS and 0.1% Tween 20. Furthermore, membranes were probed with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody for 1 h, and the bands were visualized with enhanced chemiluminescence detection reagents.

Animal Studies—Animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at the Jesse Brown Veterans Affairs Medical Center. 8-week-old C57BL6/J mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and were acclimatized for 5 days with free access to food and water and 12:12 h light/dark cycles. Mice were then divided into four treatment groups with controls were analyzed by one-way analysis of variance. A p value of 0.05 or less was considered statistically significant.

RESULTS

DNA Methylation Analysis by EpiTYPER® MassARRAY®—The EpiTYPER® MassARRAY® from Sequenom was utilized to measure the levels of the DNA methylation in mouse NPC1L1 gene in jejunum, ileum, and colon. Genomic DNA was isolated from different regions of the gastrointestinal tract utilizing the DNeasy blood and tissue kit (Qiagen). The EpiTYPER® MASS-ARRAY® assay was performed by Sequenom Inc. (San Diego). We have focused on a 3-kb fragment of mouse NPC1L1 gene upstream of the translation initiation site (that was considered as +1). Briefly, the DNA was treated with bisulfite, and five fragments were then amplified from the 3-kb NPC1L1 fragment using bisulfite-specific PCR primers (Table 1). The reverse PCR primers contained a 10-mer tag, and the forward primers contained a T7 promoter tag for the subsequent in vitro transcription. The products of the in vitro transcription were then cleaved by ribonuclease, and mass spectra were then acquired to measure the different levels of guanine and adenine in the cleavage products reflecting the level of DNA methylation. The investigated 3-kb region of the NPC1L1 gene contains 32 CpG dinucleotides, and the assay generated successful data for 25 CpG sites. The assay, however, failed to generate successful data regarding the methylation of seven CpG sites due to either low mass or high mass of the cleavage products.

Statistical Analysis—Results are expressed as mean ± S.E. Student’s t test was utilized for statistical analysis. Comparisons of multiple treatment conditions with controls were analyzed by one-way analysis of variance. A p value of 0.05 or less was considered statistically significant.

TABLE 1

| Primer      | Sense primer                        | Antisense primer                        |
|-------------|-------------------------------------|----------------------------------------|
| Primer 1    | GTTGTGTTCTGTTGTTATATTTTT            | CTCCTATTCTCCCAACACATTCCA               |
| Primer 2    | TTTGACTTCTGTGGACAGTAAGA             | CAGAACCCAAACATCATATACCCA               |
| Primer 3    | GGATTTAAGGGATTTTGCTTGTG            | AAAGCTTAAAGCTTACCTTCCACC               |
| Primer 4    | GATTTGATAAGTGTGGATGGGTATTAT         | TACATATTCACGCCAAATCTCCCC               |
| Primer 5    | GGATTTAAGGGATTTTGCTTGTG            | CATCAATATATACCACCTTCCATCTC            |
Data presented in Fig. 1, D–F, show that these enzymes responsible for DNA methylation are differentially expressed in jejunum, ileum, and colon. Assessment of DNA Methylation of Mouse NPC1L1 Promoter in Colon—DNA methylation in the promoter region of genes usually suppresses their expression (12). We sought to investigate the relative methylation of 5′ upstream regulatory region of the mouse NPC1L1 gene in colon as compared with small intestine. In silico analysis of the 3000-bp fragment upstream of the translation initiation site of the mouse NPC1L1 promoter revealed the presence of 32 CpG dinucleotides that are potential targets for methylation (Fig. 2A). The methylation of these CpG sites in the mouse NPC1L1 promoter was analyzed by the EpiTYPER® MASSArray® (Sequenom). Because of inherent limitations of the assay (16), 7 of the 32 CpG dinucleotides were not analyzed by this approach, including −54, −122, −151,
The percent of methylation was examined in the jejunum, ileum, and colon, both in control and 5-azacytidine-treated mice (10 mg/kg body weight). The results showed that the overall DNA methylation level is higher in colon as compared with jejunum and ileum. The difference in DNA methylation from the available data reached statistical significance with higher levels in the colon as compared with jejunum alone or jejunum and ileum at CpG sites as follows: −253, −428, −607, and −2529 (the translation initiation site is designated as +1). The percent of methylation was examined in the jejunum, ileum, and colon, both in control and 5-azacytidine-treated mice (10 mg/kg body weight). The results showed that the overall DNA methylation level is higher in colon as compared with jejunum and ileum. The difference in DNA methylation from the available data reached statistical significance with higher levels in the colon as compared with jejunum alone or jejunum and ileum at CpG sites as follows: −239, −263, and −299 (Fig. 2B); −327, −423, and −525 (Fig. 2C); −909 (Fig. 2D); −2271 and −2301 (Fig. 2F); −2655, −2673, and −2714 (Fig. 2G); and −2724 and −2808 (Fig. 2H). The methylation of CpG sites −2840 and −2887 (Fig. 2H) was more in the colon as compared with ileum and jejunum, although the difference did not reach statistical significance. Also, there were CpG sites that exhibited equal levels of methylation in the colon, ileum, and jejunum, including the following: −1242, −1143, and −930 (Fig. 2D); −1808, −1689, −1666, and −1479.

**FIGURE 2.** DNA methylation of mouse NPC1L1 promoter in the small intestine and colon. A, schematic representation of the 3000-bp fragment of mouse NPC1L1 promoter region (+1 represents the translation initiation site). The CpG dinucleotides are indicated as dark circles. B–H, genomic DNA was isolated from jejunum (Jej), ileum (Ile), and colon (Col) of control and 5-azacytidine-treated mice. DNA methylation was investigated by EpiTYPER® MASSArray® assay in a 3000-bp fragment of mouse NPC1L1 gene. The % of methylation of each of the 25 CpG sites that were covered by the assay is shown in the figures. The 1st bar for each intestinal segment represents control mice, and the 2nd bar represents mice treated with 5-azacytidine. Data are presented as mean ± S.E. from three samples of each group. *, p < 0.05 as compared with jejunum; #, p < 0.05 as compared with ileum; $, p < 0.05 as compared with colon from untreated mice; ##, p < 0.05 as compared with jejunum of untreated mice; $$, p < 0.05 as compared with ileum of untreated mice.
incubation with 10

NPC1L1 mRNA expression in HuTu-80 cells was time-depen-

dient with almost 25-fold stimulation occurring after 72 h of

Moreover, the increase in mRNA in a dose-dependent manner. Moreover, the increase in

A

5-azacytidine (1, 5, and 10 µM) for 48 h (white bars) and 72 h (dark bars). B, Caco2 cells grown on filter support were treated with 5-azacytidine for 48 h. Cells were then harvested, and RNA was isolated. NPC1L1 mRNA expression relative to GAPDH mRNA expression was assessed by q-RT-PCR. Results are presented as fold increase as compared with untreated control that is set as 1. Data represent mean ± S.E. of three different experiments. *, p < 0.05 treated versus control untreated cells.

We next examined the effect of DNA demethylation on the

DNA Methylation Decreases NPC1L1 Promoter Activity—

We next investigated the direct effects of DNA methylation on the promoter activity of human NPC1L1. We have previously cloned and characterized a promoter fragment flanking the region between −1741 and +56 (+1 is the transcription initiation site) of the human NPC1L1 gene (7). To examine the effects of DNA methylation, the NPC1L1 promoter fragment was subcloned into the CpG-free-basic lucia vector and then subjected to in vitro DNA methylation as described previously (18). The methylated construct of NPC1L1 promoter along with the expression vector for β-galactosidase (control for transfection efficiency) were transfected into human Caco2 cells, and the promoter activity was evaluated by assessing the ratio of lucia to β-galactosidase activities. As shown in Fig. 5A, NPC1L1 promoter activity was significantly higher (~7-fold) than the activity of empty vector alone. Fig. 5A also shows that in vitro DNA methylation before transfection significantly reduced NPC1L1 promoter activity to the levels of empty vec-

tor. The activity of the control promoter (EF1) in the same CpG-free vector was not affected by in vitro DNA methylation indicating that the decrease observed in NPC1L1 promoter activity by in vitro DNA methylation was specific (Fig. 5B).

These findings clearly show that the promoter activity of human NPC1L1 gene is suppressed by DNA methylation.

**DISCUSSION**

NPC1L1 mRNA and protein are differentially expressed along the length of the gastrointestinal tract. Indeed, the NPC1L1 protein is absent in the colon, and the mRNA is minimally expressed. Our data showing that the level of NPC1L1 mRNA is remarkably lower in the mouse colon by ~50-fold as compared with ileum and jejunum are in agreement with the recent findings reported by Xie et al. (15). This segment-specific expression raises the following important physiological question. What is the mechanism responsible for the lack of NPC1L1 expression in colon?
Intestinal segment-specific patterns of gene expression have long been investigated, and a number of mechanisms have been suggested. Different levels of expression of transcription factors (stimulator or suppressor) may be involved, as is the case for GATA-4, which recently has been shown to be essential for defining the jejunal phenotype in the small intestine (19, 20). Epigenetic mechanisms, including histone modifications and/or DNA methylation or regulation by miRNA, may also be involved (21–23). Indeed, in earlier studies a possible role of DNA methylation was suggested based upon the fact that the lack of the methyl DNA-binding protein MBD2 in knock-out mice resulted in stimulation of expression of a set of pancreatic and duodenal genes in the colon (14). It is also interesting to note that the genes induced in the colon of these mice were generally involved in nutrient digestion and absorption in the small intestine (14). This report suggests that DNA methylation represents one of the major mechanisms responsible for suppressing nutrient digestion and absorption processes in the colon. Because NPC1L1 mediates cholesterol absorption, it is, therefore, logical to consider DNA methylation as one of the potential mechanisms responsible for the suppression of its expression in the colon. Indeed, our data showed that NPC1L1
mRNA expression in mouse colon is remarkably induced by the DNA methyltransferase inhibitor 5-azacytidine. The 5-azacytidine is an aza-nucleoside that enters the cells and is subsequently converted to 5-aza-dCTP and incorporated into the DNA (24). The incorporated aza-cytosines in the CpG dinucleotides are recognized as natural substrates for the DNA methyltransferases. The binding of the methyltransferases to the aza-cytosine is covalent resulting in degradation of the enzyme and loss of the DNA methylation mark (24). The dose and duration of 5-azacytidine treatment utilized in current studies in mice have been previously shown to be effective in decreasing DNA methylation and stimulating gene expression (25).

The fact that 5-azacytidine significantly increased colonic expression of NPC1L1 mRNA in mice strongly suggests that Npc1l1 gene expression is silenced in the colon by DNA hypermethylation.

The mapping of methylation sites in the NPC1L1 gene in mouse intestine revealed valuable information supporting the role of DNA methylation in controlling segment-specific expression of mouse NPC1L1. Our data showed that the overall level of DNA methylation of CpG dinucleotides present in the 3-kb region of the Npc1l1 gene upstream of the translation initiation site was similar between ileum and jejunum and significantly higher in the colon as compared with both ileum and jejunum. It is important to note that methylation of some of the CpG sites analyzed were similar between the colon and the small intestine suggesting that the methylation of specific CpG dinucleotides in the NPC1L1 promoter appears to be crucial for suppressing the expression of NPC1L1. The methylation of these sites with higher levels in the colon was significantly decreased by treatment with 5-azacytidine. The decrease in DNA methylation in a number of CpG sites by 5-azacytidine also occurred in a number of CpG dinucleotides in ileum and jejunum as well, but it was not associated with any increase in NPC1L1 expression in these regions. Only three CpG dinucleotides, i.e. −263, −909, and −2724 (relative to the translation initiation site), in the NPC1L1 gene showed a statistically significant decrease in DNA methylation by 5-azacytidine that was concurrent with the increase in NPC1L1 mRNA expression in the colon. Altogether, our initial mapping of DNA methylation sites on the mouse NPC1L1 promoter demonstrated for the first time a strong correlation between the methylation of specific CpG sites and NPC1L1 mRNA expression. Furthermore, it appears that the high levels of DNA methylation of NPC1L1 promoter are strongly associated with the suppression of mRNA expression in the colon. The level of DNA methyltransferase DNMT1 is significantly less in the colon as compared with the small intestine. However, the mRNA expression of DNMT3A and DNMT3B is significantly higher in the colon as compared with the jejunum. It should be noted, however, that the levels of DNMT3A and -3B are similar in both the ileum and colon. These data suggest that DNA methylation of the NPC1L1 promoter does not correlate with the expression of DNMTs. The findings suggest that NPC1L1 gene methylation may be dependent on other factors beside DNMTs such as DNA demethylases.

Similar to mouse NPC1L1, DNA methylation appears to play a critical role in the expression of human NPC1L1 mRNA according to our findings in human intestinal HuTu-80 cells. DNA methylation is mediated by DNMTs that catalyze the transfer of methyl groups from S-adenosylmethionine to the cytosine residue in the CpG dinucleotides (17). Three distinct families of DNMTs, DNMT1, DNMT2, and DNMT3, have been identified in mammals. DNMT1, known as the maintenance DNMT, mediates the methylation of the hemimethylated double-stranded DNA. DNMT3 is responsible for the de novo methylation, as it methylates the unmethylated and hemimethylated double-stranded DNA. DNMT3 family consists of three members as follows: DNMT3A, DNMT3B, and DNMT3L. DNMT3L lacks the cytosine methylation activity and functions as a regulatory factor in germ cells (17). The functional roles of DNMT2 are slowly emerging, and recent studies suggested its involvement in tRNA methylation (17). Our findings show that the attenuation of either DNMT1 or DNMT3B alone did not affect NPC1L1 mRNA expression. This is not surprising as previous studies showed functional redundancy between these different isoforms of DNMTs and that DNMT1 and DNMT3B cooperate to maintain DNA methylation in human cancer cell lines (26–28).

The dose and duration of 5-azacytidine treatment utilized in current studies in mice have been previously shown to be effective in decreasing DNA methylation and stimulating gene expression (25). The fact that 5-azacytidine significantly increased colonic expression of NPC1L1 mRNA in mice strongly suggests that Npc1l1 gene expression is silenced in the colon by DNA hypermethylation.

The data from this study not only unravel a mechanism involved in the segment-specific expression of intestinal NPC1L1, but also demonstrate a novel means to modulate its expression. In this regard, the increase in NPC1L1 expression has been shown in diseases associated with hypercholesterolemia such as diabetes mellitus (29). Also, the inhibition of NPC1L1 expression was shown to be beneficial in decreasing the levels of blood cholesterol (3). Moreover, hepatic NPC1L1 was recently shown to be involved in infection with hepatitis C virus and other hepatic disorders such as steatosis (30). Therefore, NPC1L1 appears to be an attractive target for inhibition in the treatment of a number of disorders, including infection with hepatitis C virus and hypercholesterolemia. DNA methylation was recently shown to be a dynamic process that is altered in somatic adult cells in response to environmental factors such as exercise (31). Also, recent investigations have shown that dietary supplements containing betaine, metafoline, creatine, and vitamin B12 could alter DNA methylation (32). Future studies will aim at examining the effects of these types of dietary supplements on DNA methylation of the NPC1L1 gene and its expression in the intestine and liver. In conclusion, our current findings suggest that increasing DNA methylation of the NPC1L1 gene may suppress NPC1L1 expression.
expression in the small intestine and liver. The increase in DNA methylation of NPC1L1 by dietary manipulation may therefore provide a novel therapeutic means for the treatment of a number of disorders such as hypercholesterolemia, hepatic hepatitis C virus infection, and hepatic steatosis.

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