DNA methylation contributes to expression of the human neurotensin/neuromedin N gene

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Dong, Zizheng, Xiaofu Wang, Qingzheng Zhao, Courtney M. Townsend, J.R., and B. Mark Evers. DNA methylation contributes to expression of the human neurotensin/neuromedin N gene. Am. J. Physiol. 274 (Gastrointest. Liver Physiol. 37): G535–G543, 1998.—The gut and liver share a common embryological origin. The gene encoding the gut hormone neurotensin/neuromedin N (NT/N) is expressed in the adult small bowel, and NT/N is transiently expressed in the fetal liver, suppressed in the adult liver, and reexpressed in certain liver cancers. In our present study, we found that the NT/N gene was expressed at high levels in the human hepatoma cell line Hep 3B but was not expressed in Hep G2 cells. To further determine the mechanisms regulating NT/N expression, we performed Southern blotting and gene cloning techniques. Neither alteration nor mutation of the NT/N gene was responsible for this differential NT/N expression pattern. Human NT/N promoter constructs were transfected into either Hep 3B or Hep G2. Both cell lines supported NT/N transcription, indicating that the absence of NT/N expression in Hep G2 cells was due to mechanisms other than the absence of positive transcription factors. The role of DNA methylation was next assessed. Methylation of NT/N promoter constructs in vitro resulted in a 67-fold reduction in promoter activity, whereas treatment with the demethylating agent 5-azacytidine induced NT/N expression in Hep G2 cells, thus suggesting that DNA methylation plays a role in the expression of the gut endocrine gene NT/N. Defining the mechanisms regulating NT/N expression in these hepatic-derived cell lines will provide not only a better understanding of cell-specific and developmental regulation of a gut endocrine gene but also possible insight into liver cell lineage patterns and the derivation of certain hepatocellular cancers.

endocrine gene expression; differentiation; hepatocellular cancer

THE HUMAN GASTROINTESTINAL (GI) tract forms during the fourth week of fetal development (44). The endoderm of the primitive gut gives rise to the epithelial lining of the small intestine and colon and the parenchyma of the solid organs (e.g., the liver). This common embryological origin of the liver and gut suggests the presence of shared ancestral stem cells that are capable of multidirectional differentiation. Findings that support this hypothesis include the identification of intestinal markers in hepatoma cell lines (63), the ability of “oval” cells, induced in the rat liver after various chemical and surgical manipulations, to undergo intestinal metaplasia (24, 51, 57, 60), the association of hepatoblastoma with the polyposis coli syndromes (32, 36), and a “hepatoid” differentiation pattern noted in certain colon cancers (28). As fetal development proceeds, however, these stem cells become committed to differentiate into a given cell type with a specialized function. For example, a fixed stem cell population in the proliferating crypts of the small intestine gives rise to four primary cell types (absorptive enterocytes, goblet cells, Paneth cells, and enteroendocrine cells) (11), which express specific genes in a defined pattern along both the vertical and longitudinal gut axes. The molecular basis for this strict tissue-specific pattern of gene expression in the GI tract remains unclear.

Neurotensin (NT), a tridecapeptide originally isolated from bovine hypothalami by Carraway and Lee (7) and subsequently localized to specialized endocrine cells (N cells) of the adult small bowel (reviewed in Ref. 53), facilitates fatty acid translocation (2), affects gut motility and secretion (53), and stimulates the growth of normal gut mucosa (13, 62) and certain colon and pancreatic cancers (29, 64). In addition, NT enhances hepatocarcinogenesis in rats given N-nitrosomorpholine and augments epidermal growth factor- and transforming growth factor-α-mediated DNA synthesis in normal rat hepatocytes (27, 45). We have shown that the gene encoding NT and the structurally related hexapeptide neuromedin N (designated NT/N) are developmentally regulated in the gut of both rats and humans in a distinctive temporal- and spatial-specific pattern (19, 20). NT/N expression is initially low in the fetus but rapidly increases after birth to assume the distinctive adult topographical distribution of increasing NT/N expression along the longitudinal axis of the small bowel. In addition to NT/N expression in the small bowel, we have demonstrated NT/N expression in the fetal human liver; expression was not apparent in the normal adult liver (18). The expression of the gut endocrine gene NT/N in the fetal liver provides yet another example of the close developmental relationship between the liver and the gut.

In our present study, we have cloned the full-length human NT/N cDNA and analyzed the human hepatoma cell lines Hep 3B and Hep G2 to further define mechanisms that contribute to NT/N gene expression. We found that Hep 3B, but not Hep G2, cells express high levels of the NT/N gene; these differences in NT/N expression were not secondary to deletion, mutation, or changes in gene structure. However, Hep G2 cells support NT/N transcription from reporter gene constructs, suggesting that the absence of NT/N expression in this cell line is due to mechanisms other than the absence of positive transcription factors. We next assessed the role of DNA methylation in the control of NT/N expression and found that the activity of enzymatically methylated NT/N reporter constructs was dramatically reduced in Hep G2 cells. Furthermore, treatment of Hep G2 with the demethylating agent 5-azacytidine (5-azaC) resulted in NT/N activation, demonstrating that the suppression of NT/N expres-
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Detection in Hep G2 cells is mediated, at least in part, by DNA methylation. The high-level expression of NT/N in the Hep 3B cell line and the ability of Hep G2 cells to support NT/N transcription provide additional evidence that the liver, at some point during development, expresses what traditionally have been considered “gut-specific” genes. The NT/N gene will provide an important molecular model to further delineate the cellular mechanisms leading to the early differentiation and subsequent maturation of the liver and possible derivation of certain hepatocellular cancers.

MATERIALS AND METHODS

Cell culture and tissue procurement. Hep G2 and Hep 3B, human liver cancer cell lines obtained from American Type Culture Collection (ATCC; Rockville, MD) (1, 35), were cultured in Dulbecco’s modified Eagle’s medium (DMEM) and F12K at 1:1 supplemented with 5% FBS. All cells were maintained in a humidified atmosphere of 95% air and 5% CO₂ at 37°C.

For Northern blot analysis, poly(A)⁺ RNA was electrophoresed in 1.2% agarose-formaldehyde gels, transferred to nitrocellulose, and hybridized with a cRNA probe (pHNT-G536 DNA METHYLATION AND NT/N GENE EXPRESSION) then hybridized with the full-length NT/N cDNA probe labeled by a random primer procedure. After the first round amplification using the dT primer and primer I, the PCR reaction mixture was diluted to 1 ml and the cDNA fragments were amplified using primer II, the PCR product was purified by agarose gel electrophoresis and cloned into the pGEM4 vector (3). Hybridization and washing conditions were as described previously (23). Blots were stripped and reprobed with the human albumin gene (43) and from cell lines as described previously (23) except UTMB. Ribonuclease (RNase) protection analyses were performed as described previously (23) using the RPA-II kit (Ambion, Austin, TX). Ribonuclease (RNase) protection analyses were performed as described previously (23) using the RPA-II kit (Ambion, Austin, TX).

For reverse transcription-polymerase chain reaction (RT-PCR), the relative location of the primers used for the RT reaction are shown in Fig. 1. The sequences of the primers used for cloning the full-length human NT/N cDNA were as follows: 5'-ATTG CGGCCCCCA(C)₃; 5’ primer I, 5’-TCCCGCGGACTTGCTTGTGTAACGGCT-3’; and P₈, 5’-GCCAATTCGAAA GGAGGTCG TGCA-3’. We used 1 µl of BON cell poly(A)⁺ RNA for the RT reaction and PCR as described above. After the first round amplification using the dT primer and primer I, the PCR reaction mixture was diluted to 1 ml and the cDNA fragments were amplified using primer II, the PCR product was purified by agarose gel electrophoresis and cloned into the pGEM4 vector (3). For Northern blot analysis, poly(A)⁺ RNA was electrophoresed in 1.2% agarose-formaldehyde gels, transferred to nitrocellulose, and hybridized with a cRNA probe (pHNT-G536 DNA METHYLATION AND NT/N GENE EXPRESSION) then hybridized with the full-length NT/N cDNA probe labeled by a random primer procedure. After the first round amplification using the dT primer and primer I, the PCR reaction mixture was diluted to 1 ml and the cDNA fragments were amplified using primer II, the PCR product was purified by agarose gel electrophoresis and cloned into the pGEM4 vector (3). Hybridization and washing conditions were as described previously (23). Blots were stripped and reprobed with the human albumin gene (43) and from cell lines as described previously (23) except UTMB. Ribonuclease (RNase) protection analyses were performed as described previously (23) using the RPA-II kit (Ambion, Austin, TX). Ribonuclease (RNase) protection analyses were performed as described previously (23) using the RPA-II kit (Ambion, Austin, TX).

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RESULTS

NT/N gene is constitutively expressed in human hepatoma cell line Hep 3B; the lack of NT/N expression in Hep G2 cells is due most likely to regulation at the level of gene transcription. We have previously shown that NT/N is transiently expressed in the fetal human liver (18). In the present study, we determined whether NT/N is expressed in the hepatic-derived human cell lines Hep 3B and Hep G2 and found, surprisingly, that Hep 3B cells abundantly express the NT/N gene (Fig. 2A). In fact, NT/N expression was as high or higher in Hep 3B cells compared with BON, a cell line that we have previously shown expresses high levels of NT/N mRNA and synthesizes and secretes NT peptide in a fashion identical to the normal intestine (9, 22). In contrast, expression of NT/N was not detected in Hep G2 cells. As controls, the blot was reprobed with human albumin gene with expression noted in both Hep 3B and BON cells. As confirmation of NT/N expression in Hep 3B cells, RNA was analyzed by RNase protection (Fig. 2B). The expected 180-bp NT/N protection product corresponding to exon 1 was noted in both Hep 3B and BON cells but not in Hep G2 cells. This result identifies high-level expression of the gut endocrine gene NT/N in the human hepatocellular cancer cell line Hep 3B. In addition, we have recently analyzed a hepatocellular cancer (nonfibrolamellar) resected from a 59-yr-old Asian male. NT/N was expressed in this hepatocellular cancer as noted by RNase protection (data not shown), thus establishing that NT/N is not only expressed in the Hep 3B cell line but also in a hepatocellular cancer in vivo.
In contrast to Hep 3B cells, NT/N was apparently not expressed in Hep G2 cells. We next utilized the more sensitive RT-PCR assay to determine whether Hep G2 cells express NT/N at levels that are not detectable by either Northern blot or RNase protection. In the first analysis, primers P1 and P2, located in the first and third exons respectively, were used to amplify a cDNA fragment of \(-380\) bp. Using this procedure, we identified the predicted NT/N fragment in both Hep 3B and BON cells; no apparent amplification product was detected in this reaction using RNA from Hep G2 cells (Fig. 2C, left).

In a second assay, primers P3 and P4, located in the 5'-untranslated region of exon 1 and intron 1, respectively, were used to assess whether this absence of NT/N expression occurs at the level of gene transcription. Using this strategy, which was previously described for the thymidine kinase (40) and sucrase isomaltase (42) genes, we identified pre-mRNA transcripts. Although this represents an indirect method of assessing transcription, the absence of unspliced NT/N pre-mRNA in Hep G2 cells would suggest that the NT/N gene is not transcribed. Performance of this RT-PCR reaction yielded a predicted DNA fragment of \(-370\) bp in both Hep 3B and BON cells but not in Hep G2 cells (Fig. 2C, right). As a control, all reactions contained primers for GAPDH; amplified fragments of \(-1.0\) kb were detected in all samples (including Hep G2), thus confirming that the RT reaction worked. Because of the increased sensitivity of the RT-PCR, these data provide strong evidence that NT/N is not expressed in Hep G2 cells, and, furthermore, the findings are suggestive that this lack of expression is a result of transcriptional regulation.

The NT/N gene is intact in both Hep 3B and Hep G2 cells. Possible explanations for the absence of NT/N expression in the Hep G2 cell line include chromosomal deletions or mutations resulting in a defect of the NT/N gene itself. Bean et al. (3) previously described a human NT/N genomic clone encompassing exons 1 through 3; however, exon 4, which encodes the NT and neuramidin-D N peptides, was not included in this partial NT/N clone. To accurately assess the entire coding region of the human NT/N gene, we cloned the full-length human NT/N cDNA to utilize as a probe for Southern blots.

Cloning the full-length cDNA was accomplished using BON cell RNA as a template, oligo(dT)\(_{17}\) as a 3'-end primer, and a 5'-end gene-specific primer in a two-round amplification similar to a nested PCR reaction (25). Nucleotide sequence analysis of the positive clones demonstrated a cDNA fragment of 756 bp that, similar to the canine NT/N prepropeptide (16), contains an open reading frame encoding a predicted precursor protein of 170 amino acid residues (Fig. 3A). In contrast, the precursor NT/N peptide in cows (8) and rats (33) is composed of 169 amino acids. Of the 169 comparable positions, the human NT/N protein is 90% identical to that of both dogs and cows and 78% identical to rat NT/N sequences (Fig. 3B). The nucleotide level, the human NT/N precursor is 92%, 91%, and 81% identical to the dog, cow, and rat nucleotide sequences, respectively. Taken together, these results indicate a high degree of conservation of the NT/N coding region among the four species.

This full-length clone was then labeled and used to probe genomic DNA extracted from Hep G2, Hep 3B, and BON cells. The pattern of hybridization was the same in all three cell lines (Fig. 3C), suggesting that the NT/N gene was present and that there was no gross rearrangement in the coding region. Next, PCR was used to analyze the proximal NT/N promoter region. DNA extracted from the cell lines was amplified, and the resulting fragment was subcloned and sequenced. The sequence of the proximal NT/N promoter was identical in all three cell lines and the same as previously published for the human NT/N gene (3) (data not shown).

Both Hep 3B and Hep G2 cells possess the requisite cellular factors to transcribe the NT/N gene. Previously, we have shown that the proximal promoter region (216 bp upstream from the transcriptional start site) of the rat NT/N gene is sufficient to direct high-level expression in the BON endocrine cell line (22). This proximal promoter region, which is highly conserved in the human NT/N gene (3), contains a crucial AP-1/CRE site located \(-40\) bp from the transcriptional start site. Other more distal regions include near-consensus CRE and AP-1 sites that are important for NT/N induction in PC12 cells (34) but are not as important for constitutive NT/N expression in BON.

We next determined whether Hep 3B or Hep G2 cells could support transcription of human NT/N promoter constructs. A \(-373\) promoter construct contains the proximal AP-1/CRE site and more distal CRE and AP-1 sites (3); the \(-122\) deletion construct contains the proximal AP-1/CRE site, and the \(-42\) construct contains the NT/N TATA box. Transient transfections into Hep 3B cells resulted in high-level NT/N activity for both the \(-373\) and \(-122\) promoter deletions compared with the promoterless control vector pXP1; the NT/N promoter was silenced with deletion to \(-42\) (Fig. 4A). Surprisingly, transfection of the \(-373\) NT/N construct into Hep G2 cells resulted in NT/N promoter activity similar to that noted in the NT/N-expressing Hep 3B cell line (Fig. 4B). Also similar to Hep 3B, deletion to \(-42\) resulted in near silencing of the NT/N promoter in Hep G2 cells. Deletion to \(-122\) resulted in a greater decrease in NT/N promoter activity in Hep G2 compared with Hep 3B cells. The significance of this finding is not known but may indicate the importance of the near consensus CRE and AP-1 sites for NT/N promoter activity in Hep G2 cells. Taken together, our results demonstrate a disparity between expression of NT/N mRNA in the two liver-derived cell lines and the ability of the cells to transcribe from the NT/N promoter. These findings suggest that the requisite cellular machinery to transcribe the NT/N gene is present in both cell lines; however, other mechanisms are responsible for NT/N gene suppression in Hep G2 cells.
NT/N gene suppression in Hep G2 cells is mediated, in part, by gene methylation. The possible role that gene methylation plays in the expression of NT/N in these two cell lines was next assessed. As a first step, methylation of the NT/N promoter (−373/+26) construct in vitro with the CpG methylase SssI was performed, and the methylated constructs were transfected into Hep G2 cells. Results were compared with methylation of a pSV2Luc control vector. The completeness of CpG methylation was verified by digesting with HpaII with fully methylated plasmids resistant to HpaII digestion (Fig. 5A). CpG methylation of the human NT/N promoter produced a 67-fold reduction of transcriptional activity (Fig. 5B). In contrast, similar to the findings of others (4, 26), the simian virus 40 enhancer is only weakly sensitive to methylation as noted by only a threefold reduction in transcriptional activity of the pSV2Luc control vector (Fig. 5B). Therefore, these results demonstrate that transcriptional activity of the human NT/N gene is downregulated by DNA methylation in a preferential and dramatic fashion. This repression was not simply due to methylation of the luciferase gene or of plasmid DNA, since the relatively methylation-insensitive SV40 enhancer and promoter was much less affected by the same treatment.

The role of methylation in regulating NT/N gene expression was further assessed by treating Hep G2 cells with the demethylating agent 5-azaC (Fig. 6A). Cells treated with 3 µM 5-azaC for 4 days demonstrated a low level of NT/N expression (Fig. 6A, lanes 3 and 4) compared with untreated Hep G2 cells (Fig. 6A, lane 2). Addition of 8 µM 5-azaC for 4 days (Fig. 6A, lanes 5 and 6) resulted in higher NT/N expression.

Fig. 3. Cloning full-length human NT/N cDNA and analysis of genomic DNA. A: composite sequence determined from human full-length cDNA clone and predicted amino acid sequence of human preproneurotensin/neuromedin N. The neuromedin N and NT coding regions, located in tandem on exon 4, are boxed by solid lines. B: comparison of the putative amino acid sequence of human NT/N to known sequences of cow, dog, and rat; —, identical sequences; +, similar sequences. C: genomic DNA (5 µg) from Hep 3B, BON, and Hep G2 cells was digested with XbaI (X), HindIII (H), or BglII (B) and transferred to nitrocellulose and hybridized with full-length NT/N cDNA probe.
compared with 3 µM 5-azaC (Fig. 6A, lanes 3 and 4). Finally, cells were treated with 8 µM 5-azaC for 4 days, at which time the medium was changed to fresh medium with 3 µM 5-azaC for an additional 4 days (Fig. 6A, lane 7). NT/N was expressed, but at lower levels than with treatment with 8 µM alone for 4 days. BON cell RNA (Fig. 6A, lane 8) was added as a positive control and demonstrated high-level NT/N expression. A separate RNase protection gel was performed, using the human GAPDH probe to confirm that RNA samples were intact (Fig. 6B). Treatment of Hep 3B cells with 5-azaC produced no apparent changes in NT/N expression levels (data not shown). Taken together, the reduction of NT/N promoter activity by methylation and the induction of NT/N expression by 5-azaC demonstrate that DNA methylation plays a role in NT/N gene suppression in Hep G2 cells.

**DISCUSSION**

In the present study, we demonstrate high-level expression of the terminally differentiated endocrine gene NT/N in the human hepatoma cell line Hep 3B. In fact, expression levels of NT/N by Northern blot were as high in Hep 3B cells as in the BON endocrine cell line. Earlier reports have described NT/N gene expression in the rare fibrolamellar hepatic tumor (14, 18, 52), which is characterized by a different morphology, no causal association with the hepatitis virus, and an overall better prognosis than the more common hepatocellular cancer (reviewed in Ref. 15). Our results, however, suggest that NT/N gene expression is not unique to the fibrolamellar variant and can also be associated with other hepatocellular cancers.

Expression of the NT/N gene may provide insight into the particular cell lineage that gives rise to the hepatocellular cancer. A prevailing hypothesis is that tumor cells are transformed stem cells or early precursor cells (50, 51, 56, 57). Therefore, the expression of NT/N may not simply reflect activation or induction by a transformation event but rather a normal gene that is representative of an immature cell population. Consistent with this notion is the finding of NT/N gene expression in the human liver during early fetal development (18). Although Wang et al. (61) have shown that gastrin, another gut hormone, is expressed in the liver of transgenic mice containing the human gastrin minigene, our results are, to our knowledge, the only demonstration of expression of a gut endocrine gene in not only the normal fetal liver but also hepatocellular cancers. Thus it is clear that the liver can support transcription of gut genes traditionally regarded as localized only to enterodocrine cells.

In marked contrast to in Hep 3B cells, increasingly sensitive techniques failed to detect NT/N expression in untreated Hep G2 cells. Both Hep 3B and Hep G2 hepatoma cells are epithelial in morphology and retain the capacity to synthesize plasma proteins including albumin, but differ in that Hep 3B cells synthesize and secrete the hepatitis B surface antigen and produce tumors when injected into athymic nude mice (1, 35). At least three general possibilities exist to explain silencing of gene expression: 1) gene mutation or deletion, 2) lack of necessary cellular factors to activate transcription.
tion (i.e., positive-acting transcription proteins), and 3) mechanisms that block transcription (e.g., gene methylating or repressor proteins that bind the promoter) (17, 47, 59).

The absence of NT/N gene expression in Hep G2 cells was not due to an apparent deletion of the gene, alteration in the gene structure, or mutation of the promoter region as demonstrated by Southern blotting and gene cloning techniques. Moreover, Hep G2 cells support NT/N transcription from reporter gene constructs, suggesting that these cells possess the necessary factors to activate the NT/N promoter. Therefore, our results suggest that the absence of NT/N expression in Hep G2 cells may be due to mechanisms that can block gene transcription.

In the present study, we demonstrate by independent and complementary approaches that DNA methylation plays a role in NT/N gene suppression in Hep G2 cells. Partial activation of the suppressed NT/N gene was induced by short-term treatment of Hep G2 cells with the demethylating agent 5-azaC. Moreover, in vitro methylation of the NT/N promoter sequences markedly inhibited NT/N activity in Hep G2 cells. Although it is becoming increasingly evident that DNA methylation ensures the silencing of certain tissue-specific genes in nonexpressing cells (4, 30, 37, 41, 47, 58, 59), this is the first demonstration that methylation may play a role in the expression of a gut endocrine gene. Previously, we have shown that the high-level NT/N expression noted in BON cells is dependent on a crucial AP-1/CRE proximal promoter element that binds both AP-1 and CREB/ATF proteins (22). Therefore, one possibility is that DNA methylation interferes with NT/N expression by affecting the binding of these transcription factors to the NT/N proximal promoter. Another possibility is that DNA methylation can inhibit transcription through methyl-C-binding proteins that bind specifically to methylated, but not unmethylated, DNA (46, 59). Finally, DNA methylation could alter the chromatin structure, thus influencing gene accessibility (31, 59).

Although we did not specifically address the question of which of these mechanisms is involved in NT/N gene regulation, the results of our present study using the transiently transfected methylated plasmids would argue in favor of one of the first two mechanisms.

Gene methylation appears to be involved in several crucial cellular processes, including differentiation, development, and carcinogenesis (10, 30, 38, 39, 59). The differences in NT/N gene expression in Hep 3B and Hep G2 cells suggest derivation of these lines from different cell lineages in the liver or, alternatively, may represent different levels of cellular maturation or differentiation.

The results from our present study suggest that DNA methylation plays a role in NT/N gene regulation; however, gene methylation alone does not fully account for the marked suppression of NT/N in Hep G2 cells. We suspect that the strict tissue-specific regulation of NT/N gene expression is dependent on the combinatorial effects of not only gene methylation but also enhancer and/or repressor proteins that bind to the proximal NT/N promoter (22) and possible additional sites outside of the immediate 5'-flanking region (e.g., the first intron). Further studies should provide important insight regarding the interplay between DNA methylation and the complex network of ubiquitous and tissue-limited transcription factors that govern differentiation and the specific developmental expression pattern of NT/N in the gut and liver. In addition, these hepatic-derived cell lines, which differ greatly in their ability to express NT/N, should provide useful models to better characterize the mechanisms regulating cell-specific expression of the NT/N gene.

In conclusion, the important points from our present study include 1) the identification of NT/N gene expression in hepatocellular cancers, which, in combination with our previous findings of expression in the fetal liver, further emphasizes that the liver can support transcription of genes traditionally regarded as only localized to certain cells of the gut; 2) the cloning of the entire human NT/N cDNA, including exon 4, which...
encodes both the NT and neuromedin N peptides; and 3) the demonstration that DNA methylation contributes to the regulation of expression of a gut endocrine gene. Finally, the NT/N gene will not only provide a useful model to delineate mechanisms contributing to gut development but may also be useful in determining liver cell lineage patterns and derivation of certain hepatocellular cancers.

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