NF1 transcriptional factor(s) is required for basal promoter activation of the human intestinal NaPi-IIb cotransporter gene

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Xu, Hua, Jennifer K. Uno, Michael Inouye, James F. Collins, and Fayez K. Ghishan. NF1 transcriptional factor(s) is required for basal promoter activation of the human intestinal NaPi-IIb cotransporter gene. Am J Physiol Gastrointest Liver Physiol 288: G175–G181, 2005; First published September 30, 2004; doi:10.1152/ajpgi.00396.2004.—The human intestinal type IIb Na\(^+\)-P\(^-\) cotransporter (hNaPi-IIb) gene promoter lacks a TATA box and has a high GC content in the 5’-flanking region. To understand the mechanism of hNaPi-IIb gene transcription, the current study was performed to characterize the minimal promoter region and transcriptional factor(s) necessary to activate gene expression in human intestinal cells (Caco-2). With the use of progressively shorter promoter constructs, a minimal promoter extending from bp \(-58 \to +15\) was identified and shown to direct high levels of hNaPi-IIb cotransporter expression in Caco-2 cells. Gel mobility shift assays (GMSAs) indicated that two regions could be bound by nuclear proteins from Caco-2 cells: region A at bp \(-26 \to -23\) and region B at bp \(-44 \to -35\). The introduction of mutations in region A abolished promoter activity, whereas mutations in region B had no effect. Deletion mutants of the same regions showed identical results. Furthermore, DNase I footprinting experiments confirmed the observation made by GMSAs. Additional studies, which used a specific nuclear factor 1 (NF1) antiserum, demonstrated that NF1 protein(s) binds to the minimal promoter at region A. These results indicated that the NF1 protein(s) is required to activate the basal transcription of hNaPi-IIb gene under normal growth conditions. This study has thus identified a new target gene in the small intestinal epithelium that is directly regulated by NF1 transcriptional factor(s).

NF1 transcription factors are ubiquitously expressed in most tissues and possess a constitutive DNA-binding capability. NF1 proteins were originally identified from adenovirus as a DNA replication factor (20), and their binding sites have been subsequently found in a large number of gene-regulatory regions both in viral and cellular genes (8). The NF1 gene family contains four different but highly related genes: NF1A, NF1B, NF1C, and NF1X. Each of these genes encodes multiple proteins resulting from alternative polyadenylation sites, splicing, and promoter usage. The DNA-binding domain is located in the NH\(_2\) terminus of NF1 proteins and is highly conserved in all four NF1 proteins, whereas the COOH-terminal transactivation domain is variable (8).

In the present study, we identified the minimal functional promoter of the hNaPi-IIb cotransporter gene and examined the roles of NF1 proteins in its basal promoter activation. Functional analysis of promoter activity in Caco-2 cells demonstrated that NF1 protein(s) is essential for the activation of hNaPi-IIb gene transcription. This finding will likely have significant future implications in our understanding of the molecular mechanisms of hNaPi-IIb gene regulation.

MATERIALS AND METHODS

Cell culture. Human intestinal epithelial cells (Caco-2) were purchased from the American Type Culture Collection (Rockville, MD).

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DNA oligonucleotides were end labeled with [-32P]ATP, and 5 μg of nuclear extract was incubated with 1 ng of labeled probe in gel mobility shift assay (GMSA) binding buffer containing 10 mM HEPES (pH 7.5), 1 mM EDTA, 50 mM NaCl, 1 mM dithiothreitol, and 50 μg/ml poly(dI-dC). After incubation at room temperature for 20–30 min, the mixture was electrophoresed on a 6% polyacrylamide Tris-boric acid-EDTA buffer. Gels were subsequently dried and exposed to X-ray film. For competition experiments, 100– to 500-fold molar excess of unlabeled oligos was added to the reaction mixture before adding labeled oligo probes. For supershift assays, 4 μg of a rabbit polyclonal antibody raised against a recombinant protein corresponding to amino acids 1–300 from the NH2-terminal amino acid residues 1–300 of the human NF1 protein (at the NH2–terminal amino acid residues 1–300) was used to detect all NF1 subfamilies. Then, for NF1A protein detection, NF1A antibody raised against the COOH terminus at amino acid residues 478 – 492 of the human NF1A protein was used. For NF1B protein detection, NF1B antibody raised against the COOH terminus at amino acid residues 327–341 of the human NF1B2 protein was used. All NF1-related antibodies were purchased from Santa Cruz Biotechnology. A 1:5,000 dilution of these NF1 antibodies was used in these experiments. Western blot detection was performed with the BM chemiluminescence Western blotting kit (mouse/rabbit) (Roche Diagnostics).

Statistical analysis. ANOVA post hoc tests (StatView 5.0.1; SAS Institute, Cary, NC) were used to compare values of the experimental data. P values of <0.05 were considered significant.

RESULTS

Identification of minimal promoter and basal cis-elements of the hNaPi-IIb cotransporter gene. Our previous studies have shown that the functional promoter of hNaPi-IIb gene contains 181 bp upstream of the transcriptional initiation site (34). In the present study, we made further deletion constructs to determine the minimal promoter region necessary to drive hNaPi-IIb gene transcription. Three constructs were made containing various lengths of the hNaPi-IIb promoter upstream of the transcriptional initiation site. The constructs were then transfected into Caco-2 cells, which endogenously express hNaPi-IIb (34). The construct pGL3/58bp, which contains 58 bp of the hNaPi-IIb promoter sequence, showed promoter activity similar to both the pGL3/181bp and pGL3/119bp constructs (Fig. 1). However, the shortest construct, pGL3/19bp, which includes only 19 bp of the promoter sequences and 15 bp within the transcriptional unit, was inactive. These results suggest that the promoter sequences between bp −58 and −19 are critical for trans-activation of the hNaPi-IIb gene.

Identification of the DNA-protein interaction region on the basal promoter of hNaPi-IIb gene. To determine where DNA-protein interactions occur on the minimal functional promoter.

Table 1. PCR primers for detecting nuclear factor 1 (NF1) family gene expression in Caco-2 cells

<table>
<thead>
<tr>
<th>NF1 Gene</th>
<th>Forward, 5’ to 3’</th>
<th>Reverse, 5’ to 3’</th>
<th>GenBank Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>CCAGCAATGTTAATGATTCTC</td>
<td>CCGCTTCCCGGTAGTGGAA</td>
<td>XM_046827</td>
</tr>
<tr>
<td>1B</td>
<td>CTCAGGAAAGTGGGTTT</td>
<td>GAACGAGCTAGGCCCAGG</td>
<td>NM_005596</td>
</tr>
<tr>
<td>1C</td>
<td>GCCGGGAGTTATCATGGAGGT</td>
<td>GSSATTTCTCTCGGCAGAT</td>
<td>NM_005597</td>
</tr>
<tr>
<td>1X</td>
<td>ATGACTCCCCCTCTACTCTCT</td>
<td>GGACCTGAGACTGCTGCTGCG</td>
<td>L31881</td>
</tr>
</tbody>
</table>
region of the hNaPi-IIb gene, GMSA experiments were performed using oligos covering various regions. Three oligos (A, B, and C) homologous to the promoter region from −58 bp to −13 bp were designed. Oligo A covered the promoter region from bp −37 to −13, whereas oligos B and C covered the promoter region from bp −50 to −26 and −58 to −40, respectively. As shown in Fig. 2, strong DNA-protein interactions were detected with radiolabeled oligos A and B. In the presence of excess unlabeled oligos, these bands were significantly reduced or eliminated.

Identification of the DNA-protein interaction region on oligo A. To further elucidate the specific DNA sequences involved in the DNA-protein interaction within oligo A, three mutants were designed. GMSAs with radiolabeled oligo A were performed in the presence of excess amounts of mutant oligos A1, A2, or A3. As shown in Fig. 3, DNA-protein interactions are detected with radiolabeled oligo A. However, in the presence of unlabeled oligo A, A2, or A3, the DNA-protein interaction was abolished. These results indicate that these mutant oligos are also capable of interacting with nuclear protein. Furthermore, in the presence of unlabeled oligo A1, the DNA-protein interaction could not be competed, as illustrated by the shifted bands seen in Fig. 3 (lane A1). This suggests that the altered sequences in oligo A1 are the site of nuclear protein binding. To further identify the precise DNA sequences important for protein binding within oligo A, mutant oligos A4 and A5 were produced. Both mutants altered wild-type DNA sequence CC to positions −25 to −24 to either TT or AA, respectively. GMSA using mutants A4 and A5 showed the same pattern that was detected with mutant A1. These results suggest that the essential sequences for nuclear protein binding on the hNaPi-Ilb basal promoter region A is CC at position −25 to −24 bps.

Identification of the DNA-protein interaction region on oligo B. To further identify specific DNA sequences involved in the DNA-protein interaction within oligo B, three mutants were designed to cover different regions of oligo B. GMSAs with radiolabeled oligo B were performed in the presence of unlabeled mutant oligos B1, B2, or B3. As shown in Fig. 4, DNA-protein interactions are detected with radiolabeled oligo B. In the presence of unlabeled oligo B and B1, the DNA-protein interaction was abolished. These results indicate that the mutant B1 is still capable of interacting with nuclear protein. However, in the presence of unlabeled oligo B3, the DNA-protein interaction could not be competed, as illustrated by the shifted bands (lane B3). In addition, the DNA-protein interaction was decreased in the presence of unlabeled B2 (lane B2). This suggests that the mutation on oligo B3 removed...
sequences necessary for nuclear protein binding. To further identify the precise binding sequences within oligo B, mutant oligo B4 was produced. This mutant altered wild-type sequence GG to TT at position -41/-40 bp. GMSA using mutant B4 showed the same pattern that was detected with mutant B3. This result suggests that the essential sequences for nuclear protein binding on the hNaPi-IIb basal promoter region B is GG at position -41/-40 bp.

Functional characterization of the involvement of the DNA-protein interaction regions on hNaPi-IIb gene promoter activation. To verify the functional necessity of the nuclear protein-binding regions, mutant constructs (pGL3/A4 and pGL3/B4) and deletion constructs (pGL3/Del-A and pGL3/Del-B) were transfected into Caco-2 cells. Mutant construct pGL3/A4 altered wild-type sequence CC to TT at position -25/-24 bp, whereas mutant construct pGL3/B4 altered wild-type sequence GG to TT at position -41/-40 bp. Deletion construct pGL3/Del-A removed wild-type sequence GCC at position -26/-24 bp, and deletion construct pGL3/Del-B removed wild-type sequence GGG at position -41/-39 bp. Mutant construct pGL3/A4 effectively reduced promoter activity to background levels as seen in pGL3/19bp, whereas mutant construct pGL3/B4 was unable to alter promoter activity and displayed activity similar to pGL3/58bp. Similar results were also observed when deletion mutant constructs pGL3/Del-A and pGL3/Del-B were used (Fig. 5).

Identification of the nuclear protein involved in promoter activation. GMSAs and functional promoter studies revealed that the basal promoter region -26/-24 bp is necessary for activating hNaPi-IIb gene expression. The MatInspector program (http://www.genomatix.de) was used to search for potential transcriptional factor binding motifs in the basal promoter region of the hNaPi-IIb gene. This search yielded a potential binding site for NF1 at this site. Thus supershifts with NF1 antibodies were performed to determine whether the protein binding to this region belonged to the NF1 superfamily. Competition studies using NF1 consensus oligos (TTTGGATT-GAAAGCCAATATGATAA; bold indicates core sequences for NF1 protein binding) were also done to confirm the interaction between Caco-2 nuclear protein(s) and consensus NF1 binding sequences. In the presence of Caco-2 nuclear protein and radiolabeled oligo A, DNA-protein interactions were detected. This interaction could be diminished by unlabeled oligo A but not by the unlabeled oligo A4 (which had the -26/-24 bp region mutated). NF1 antiserum (a blocking antibody that recognizes all NF1 family members) blocked the DNA-protein interaction, whereas control IgG had no effect on the DNA-protein interaction (Fig. 6A). Furthermore, in the presence of unlabeled NF1 consensus oligos, the DNA-protein interaction was significantly reduced (Fig. 6B). These results suggested
that the *trans*-acting factor involved in the interaction with the hNaPi-IIb basal promoter belongs to the NF1 family.

Expression of NF1 family proteins in Caco-2 cells by PCR and Western blot. GMSAs and functional data indicated that hNaPi-IIb basal promoter activation requires an NF1 factor(s); thus we sought to characterize expression of NF1 genes in Caco-2 cells by RT-PCR using NF1 subfamily-specific primers. RT-PCR results indicated that all four NF1 genes (NF1A, NF1B, NF1C, and NF1X) were expressed in Caco-2 cells (Fig. 7A). Western blot analyses using both a nonspecific NF1 antibody (NF1) and subfamily-specific NF1 antibodies (NF1A, NF1B) confirmed expression of multiple NF1 proteins in Caco-2 cells (Fig. 7B).

Confirmation of the protein-DNA interaction by DNase I footprinting. To further confirm the DNA-protein interaction region on the basal promoter of the hNaPi-IIb gene, DNase I footprinting experiments were conducted. As shown in Fig. 8, three regions on the hNaPi-IIb promoter were protected by nuclear proteins isolated from Caco-2 cells. The first region, identified as the GCCA region (−26/−23 bp), correlated with the region A identified by GMSAs using radiolabeled oligo A. The second region, identified as the GGCG region (−41/−38 bp), correlated with the region B identified by GMSAs using radiolabeled oligo B. The third region might correlate with the region observed in oligo C.

**DISCUSSION**

Previous work from our laboratory has shown that the NaPi-IIb cotransporter is highly expressed in small intestine (33), and its gene expression is regulated by various physiological factors. Our studies have shown that EGF (34), vitamin D3 (32), and estrogen (36) can regulate NaPi-IIb gene expression at the transcriptional level. These studies have also identified potential promoter regions that respond to these factors to increase or decrease the expression of the NaPi-IIb gene. Thus unlike the NaPi-IIa gene, which is expressed predominantly in kidney and regulated mainly by protein trafficking, transcriptional regulation of the NaPi-IIb gene is important.

In the present study, we analyzed the basal transcriptional activation of the hNaPi-IIb gene in Caco-2 cells under normal growth conditions. Studies using various lengths of the hNaPi-IIb promoter indicated that the minimal promoter required for activation of gene expression is contained within 58 bp upstream of the transcriptional start site. GMSAs identified the presence of DNA-protein interactions among the promoter regions A and B at positions −25/−20 bp and −45/−36 bp, respectively. This observation was also supported by DNase I footprinting assays in which the same regions were protected from DNase I digestion by nuclear proteins isolated from Caco-2 cells. The additional protected region (region C) identified by DNase I footprinting assays might correlate with the observation from GMSAs with labeled oligo C. We do not, however, believe that this region is important for basal promoter function, because only mutations in region A completely abolished promoter activity.

Transfection studies with mutant promoter constructs demonstrated that DNA sequences at position −26/−25 bp of the hNaPi-IIb gene promoter are critical for basal gene transcription in Caco-2 cells, whereas DNA sequences at promoter region −41/−40 bp are not required. Additional transfection studies implementing deletion mutations in regions A and B confirmed these observations and showed that only deletions in the −26/−24 bp region were able to abolish promoter activity. Together, these results suggested that DNA sequences (GCC) within region A of the hNaPi-IIb promoter at position −26/
−24 bp are critical for promoter activation of the hNaPi-IIb gene under normal growth conditions.

A transcriptional factor binding motif search using the minimal promoter region of the hNaPi-IIb gene suggested a potential NF1 binding site at the promoter region −26/−23 bps. Supershifts with an anti-NF1 family blocking antibody diminished the DNA-protein interaction within this promoter region. Moreover, unlabeled NF1 consensus sequences mixed with labeled oligo A were able to compete for nuclear protein binding. These results suggest that there is an NF1 binding site involved in basal promoter activity of the hNaPi-IIb gene.

NF1 factors were initially discovered as part of an adenovirus DNA replication complex but have recently been implicated in the transcriptional regulation of various cellular and viral genes (8, 20). Four NF1 genes (NF1A, NF1B, NF1C, and NF1X) have been identified in chicken (18, 23), hamster (7), mouse (5, 10, 13), rat (37), and human (2, 22). The NF1 proteins have been found to play essential roles in mammalian development, and therefore loss of these proteins has severe consequences. NF1A gene knockout mice show severe neuroanatomic defects and die within 24 h of birth (24). NF1B gene knockout mice display similar lethal defects, most likely due to loss of lung development (9). Lastly, NF1C gene knockout mice present with severe postnatal tooth development defects that eventually lead to premature death of the animal (26).

Because multiple NF1 proteins are expressed in mammalian cells, we determined the NF1 expression pattern in Caco-2 cells by RT-PCR with NF1 subfamily cDNA-specific primers. Our data demonstrated that the four family members of the NF1 cDNA could be detected in Caco-2 cells. Western blot analysis with an NF1 antibody also confirmed that multiple NF1 proteins are expressed in Caco-2 cells. These data indicate the presence of multiple NF1 isoforms in intestinal epithelial cells. This agrees with earlier observations that NF1 proteins are ubiquitously expressed in most tissue types, including epithelial cells (1, 8).

Although there are four genes encoding NF1 proteins, the NH2-terminal region of the NF1 proteins is highly conserved. This NH2-terminal region is responsible for DNA binding, dimerization, and adenovirus replication. All NF1 proteins recognize the core binding sequence GCCA (8). NF1 proteins regulate gene expression via stimulating and/or repressing gene transcription. Several genes have been shown to be regulated by NF1 proteins, including the myelin basic protein (13), the liver-specific serum albumin, and α-fetoprotein genes (4, 29), the CYP2A3 gene (31), the IDHC gene (14), and the androgen receptor gene (25). In our present work, we found not only the presence of an NF1 binding site in the hNaPi-IIb gene basal promoter region but also that NF1 protein(s) is required to activate the basal transcription of the hNaPi-IIb gene in Caco-2 cells.

In summary, our studies show that the upstream 58 bp DNA sequences are required for activation of hNaPi-IIb gene expression in Caco-2 cells. In addition, NF1 protein interaction with the basal promoter region is critical for activation the hNaPi-IIb gene. This work has thus identified the hNaPi-IIb gene as a new target gene directly regulated by NF1 protein in the intestine.

GRANTS

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REFERENCES


