Regulation of the human sodium-phosphate cotransporter NaPi-IIb gene promoter by epidermal growth factor

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Received 18 July 2000; accepted in final form 11 October 2000

Xu, Hua, James F. Collins, Liqun Bai, Pawel R. Kiela, and Fayez K. Ghishan. Regulation of the human sodium-phosphate cotransporter NaPi-IIb gene promoter by epidermal growth factor. Am J Physiol Cell Physiol 280: C628–C636, 2001.—The intestinal sodium-phosphate cotransporter (NaPi-IIb) plays a major role in intestinal Pi absorption. Epidermal growth factor (EGF) is involved in the regulation of Pi homeostasis. However, the role of EGF in intestinal NaPi-IIb regulation is not clear. The current studies showed that EGF decreased NaPi-IIb mRNA abundance by 40–50% in both rat intestine and Caco-2 cells. To understand the mechanism of this regulation, we cloned the human NaPi-IIb gene and promoter region and studied the effect of EGF on NaPi-IIb gene transcription. The human NaPi-IIb gene has 12 exons and 11 introns. Two transcription initiation sites were identified by primer extension. Additionally, 2.8 kb of the 5′-flanking region of the gene was characterized as a functional promoter in human intestinal (Caco-2) and human lung (A549) cells. Additional studies showed that EGF inhibited promoter activity by 40–50% in Caco-2 cells and that actinomycin D treatment abolished this inhibition. EGF had no effect on promoter activity in lung (A549) cells. We conclude that the human NaPi-IIb gene promoter is functional in Caco-2 and A549 cells and that the gene is responsive to EGF by a transcriptionally mediated mechanism in intestinal cells.

Caco-2 cells; A549 cells; human intestine; sodium-phosphate cotransporter; transcriptional regulation

PHOSPHATE (Pi) plays a major role in growth, development, bone formation, and cellular metabolism. The kidney and small intestine are important sites that regulate body phosphate homeostasis. In both organs, sodium-coupled phosphate transport is the major form of Pi absorption. The human and mouse intestinal sodium-dependent phosphate cotransporter (NaPi-IIb) cDNAs were cloned recently (9, 17, 34), and the human NaPi-IIb cotransporter gene (SCLA34A2) was mapped solely to indicate this fact.

These observations suggest that EGF plays an important role in Pi homeostasis by regulating renal and possibly intestinal Pi absorption. As reported in the current communication, we initially detected a significant decrease in intestinal NaPi-IIb mRNA abundance in EGF-treated rats and in human intestinal cells (Caco-2). These results suggested a possible role for EGF in transcriptional regulation of the NaPi-IIb gene. Therefore, to further understand the role of EGF in the regulation of intestinal Pi homeostasis, we cloned the human NaPi-IIb gene and characterized in vitro regulation of the promoter by EGF.
MATERIALS AND METHODS

Animals. Suckling Sprague-Dawley rats (16 days old) received subcutaneous injections of human recombinant EGF (1 µg/g body wt; Austral Biological, San Ramon, CA) or saline twice a day for 3 days. Fifteen hours after the last injection, rats were killed, and jejunal mucosa was harvested and used for mRNA purification. All animal work was approved by the University of Arizona Institutional Animal Care and Use Committee.

Cell culture. Human lung cells (A549) and human intestinal cells (Caco-2) were purchased from American Type Culture Collection (ATCC) and cultured according to ATCC guidelines. Cells were cultured at 37°C in a 95% air–5% CO2 atmosphere and passaged every 72 h. Media and other reagents used for cell culture were purchased from Irvine Scientific (Irvine, CA).

RNA purification and Northern blot analyses. mRNA was isolated from A549 cells, Caco-2 cells, and rat jejunal mucosa with the FastTrack mRNA purification kit (Invitrogen, Carlsbad, CA). Part of the rat NaPi-IIb cDNA was amplified utilizing standard PCR conditions from rat jejunal cDNA, with highly conserved primers previously utilized for cloning of the human intestinal NaPi-IIb cDNA (34). This rat cDNA fragment was subcloned and sequenced on both strands. Ten micrograms of mRNA were utilized for Northern blot analysis with rat or human NaPi-IIb cDNA-specific probes under high stringency washing conditions (6). Blots were exposed to a phosphorimaging screen, and band intensities were determined with Quantity One software (FX Molecular Imager; Bio-Rad, Hercules, CA).

Semiquantitative RT-PCR analysis. mRNA was purified from Caco-2 cells treated with standard or EGF-containing medium (100 ng/ml). The primers used for detecting NaPi-IIb expression were the same as those previously described for cloning of the human intestinal NaPi-IIb cDNA (34). The primers used for detecting β-actin and RT-PCR conditions were described previously (3). Subsaturation levels of cDNA templates that were needed to produce a dose-dependent amount of PCR products were defined in initial experiments by testing a range of template concentrations. Subsequent PCR was carried out with subsaturation levels of RT reactions with identical parameters.

Isolation and characterization of genomic clones. A human genomic bacterial artificial chromosome (BAC) DNA library was screened by Research Genetics (Huntsville, AL) using a 32P-labeled human intestinal NaPi-IIb cDNA fragment (nt 175–350). Library screening resulted in three putative clones, and one was selected for further analysis. (200 fmol) was annealed to 50 ng mRNA isolated from Caco-2 or A549 cells in a 12-µl reaction by heating at 90°C for 2 min and then cooling to 58°C at 1°C/min. The annealing reaction was then held at 58°C for 30 min and snap chilled on ice. Annealed primers were extended at 42°C for 2 h by adding 200 units of SuperScript II Reverse Transcriptase (Life Technologies, Bethesda, MD), 1 µl RNasin, 1 µl 10 mM DNTPS, and 4 µl 5X first-strand reaction buffer in a 20-µl reaction volume. The reaction was terminated by adding 3 µl 0.2 M EDTA (pH 8), and the DNA was degraded by adding 0.9 µg DNase-free RNase A followed by incubation at 37°C for 30 min. The primer extension product was then EtOH precipitated, and the pellet was resuspended in 5 µl loading dye/10 mM Tris-1 mM EDTA buffer, pH 8.0 (1:1). Samples were heated at 75°C for 10 min before loading on a sequencing gel. The gel was subsequently dried and exposed to Kodak Xomat Blue XB-1 film overnight at −70°C.

A plasmid construct containing the human NaPi-IIb promoter region plus exons I and II (but missing intron 1) was used as a sequence template to indicate the size of primer extension products. The primer used for sequencing was 5′-CGATCTTGGCTCAGTTGCAA-3′ (note that the Eco47III/XhoI-digested human NaPi-IIb cDNA fragment (726 bp) was then inserted into the Eco47III/XhoI-digested pZero-4.5 vector (note that the Eco47III site is in exon I and the XhoI site is in the vector). Both plasmids were sequenced on both strands. The primer for the sequence reaction was the same one used for the primer extension reaction. The transcription initiation site was determined +1, nucleotides upstream (i.e., 5′) were numbered negatively. Downstream nucleotides (i.e., 3′) were numbered positively.

Construction of reporter plasmids. Luciferase reporter plasmids used in this study were derived from pGL3 Basic (pGL3b; Promega). A, −1,103/+15 promoter reporter construct (pGL3−1,103) was made by subcloning a SacI/Xmal-digested pZero-4.5 fragment into pGL3b. A, −181/+15 promoter reporter construct (pGL3−181) was made by digesting pGL3−1,103 construct with SacI/ApaI and then blunting both ends with Klenow fragment of DNA polymerase I and ligating them together. A, −380/+15 promoter reporter construct (pGL3−380) was made by inserting a SacI/Xmal-digested PCR product, amplified with forward primer 5′-TTATAGCTCTTGGACCGTCTACAGAG-3′ at nt −361 to −380 (underlined nucleotides represent SacI site and overhanging bases) and reverse primer 5′-TATGATCTCCGGAGTGGCTGT-3′ in intron 1 (underlined nucleotides indicate overhanging bases originally inserted for BglII subcloning not used for this construct), into pGL3b vector (note that the Xmal site is in exon I). A, −2,783/+15 promoter reporter construct (pGL3−2,783) was made by inserting a NotI/SacI-digested fragment (−1,7 kb) into the pGL3−1,103 construct. This 1.7-kb fragment was originally amplified from BAC DNA with a high fidelity thermostable DNA polymerase using forward primer 5′-CGATCTTGGCTCAGTTGCAA-3′ (nt−2,783 to −2,764) and reverse primer 5′-TTATGATCTCCGGAGTGGCTGT-3′ (nt −873 to −864) and cloned into the pTarget vector (Promega). In this strategy, SacI cut the human NaPi-IIb 5′-flanking region, and NotI cut the vectors. All constructs were sequenced to confirm the splice sites.

Transient transfection and functional promoter analysis. A549 and Caco-2 cells were cultured in 24-well plates. When cell density reached 60–70%, liposome-mediated transfection was performed as indicated. Promoter construct DNA (0.5
μg), 30 ng pRL-CMV (Renilla luciferase reporter construct used as an internal standard; Promega), and 5 μl Lipofectamine (GIBCO BRL, Grand Island, NY) were mixed with 200 μl OptiMEM medium (GIBCO BRL) for 30 min at room temperature. The mixture was then added to the cells, and they were incubated for 5 h, followed by the addition of an equal volume of medium that contained 20% fetal bovine serum (FBS). The next day, the medium was removed and replaced with standard medium with 10% FBS. After 24 h, the cells were harvested for reporter gene assays. For EGF treatment, 100 ng/ml human recombinant EGF (Austral Biological) were added for 8 h before harvesting cells. To study the effect of actinomycin D on NaP\textsubscript{i-IIb} promoter activity, transiently transfected cells were pretreated with actinomycin D (5 μg/ml) for 2 h before EGF treatment for 8 h in the presence of actinomycin D. Promoter reporter assays were performed using a dual luciferase assay kit according to the manufacturer’s instructions (Promega).

Statistical analysis. The Student’s t-test was used to compare values of the experimental data. P values of <0.05 were considered significant.

RESULTS

Effect of EGF treatment on NaP\textsubscript{i-IIb} mRNA levels in rat jejunum. Northern blot analysis of mRNA isolated from rat jejunal mucosa is shown in Fig. 1. Hybridization with a 757-bp rat intestinal NaP\textsubscript{i-IIb} cDNA probe clearly showed that NaP\textsubscript{i-IIb} mRNA abundance was decreased 50% by EGF administration (n = 3; P = 0.008). However, no change was observed for 1B15 mRNA abundance, which was used as an internal standard to normalize the NaP\textsubscript{i-IIb} signal. Furthermore, the rat NaP\textsubscript{i-IIb} cDNA fragment (GenBank accession no. AF247725) exhibited 93% nucleotide sequence homology with the mouse intestinal NaP\textsubscript{i-IIb} cDNA.

Effect of EGF treatment on NaP\textsubscript{i-IIb} mRNA levels in Caco-2 cells. Northern blot analysis of mRNA isolated from Caco-2 cells was performed by loading 10 μg mRNA per gel lane. Hybridization with radiolabeled, 757-bp human intestinal NaP\textsubscript{i-IIb} cDNA-specific probes showed no signal from untreated or treated cells. However, the 1B15 hybridization signal was readily apparent (data not shown, n = 3). These results suggested that the NaP\textsubscript{i-IIb} message was expressed below the Northern blot detection limits in Caco-2 cells under these culture conditions.

RT-PCR analysis of cells. Endogenous expression of human NaP\textsubscript{i-IIb} mRNA in human intestinal cells (Caco-2) and human lung cells (A549) was confirmed by RT-PCR using human intestinal NaP\textsubscript{i-IIb} primers (Fig. 2). These results showed that an ~760-bp band was amplified from both cells. The PCR product was subcloned and sequenced, and it was 100% identical to the human NaP\textsubscript{i-IIb} cDNA. This indicated that these cells endogenously express the NaP\textsubscript{i-IIb} gene.

Effect of EGF treatment on NaP\textsubscript{i-IIb} mRNA levels in Caco-2 cells. The expression of human NaP\textsubscript{i-IIb} mRNA in Caco-2 cells after exposure to standard or EGF-containing medium was assessed by semiquantitative RT-PCR using human intestinal NaP\textsubscript{i-IIb} and β-actin primers (Fig. 3). Data showed that human NaP\textsubscript{i-IIb} gene expression was significantly reduced by 40% in EGF-treated Caco-2 cells,
compared with untreated cells ($n = 3; P = 0.02$). This observation is in agreement with the in vivo observation that showed a ~50% decrease in NaPi-IIb mRNA abundance in EGF-treated rat intestine.

Genomic organization of the human NaP$_{i}$-Iib gene. We focused our studies on one BAC clone. Direct sequencing of this BAC DNA identified intron 1 and intron 2. PCR amplification with different sets of primers identified introns 3–11 (Fig. 4). The introns ranged in size from 91 to ~6,800 bp, whereas the exons ranged in size from 97 to 613 bp. The translation start site was located in exon II, and the translation stop site was located in exon XII. The human NaP$_{i}$-Iib gene spans 24 kb and has 11 introns and 12 exons. DNA sequences at the intron/exon boundaries conformed to the general GT/AG rule for intron donor and acceptor splice sites. The intronic sequences determined on both strands have been deposited in GenBank with accession nos. AF234236–234245.

Mapping of the transcription initiation site of the human NaP$_{i}$-Iib gene. The transcription initiation site was determined by primer extension with a reverse primer at cDNA nt 43–62. Figure 5A shows the primer extension results with mRNA isolated from both human lung cells (A549) and human intestinal cells (Caco-2). Two primer extension products were detected from both mRNA samples that aligned 106 bp and 119 bp upstream of the translation start site of human NaPi-IIb cDNA. This experiment was repeated three times, and identical results were obtained.

**Fig. 3.** The effect of EGF on NaPi-IIb mRNA levels in human intestinal cells. **A:** mRNA isolated from Caco-2 cells grown in normal (−EGF) or EGF-containing (+EGF) medium was used for first-strand cDNA synthesis. Subsequent PCR was performed with human NaPi-IIb primers or β-actin primers in separate reactions. Equal volumes of PCR products for NaPi-IIb and β-actin were loaded on the same gel and visualized with ethidium bromide. **B:** optical density analysis of RT-PCR results in Caco-2 cells. Data were presented by the ratio of NaPi-IIb mRNA over β-actin mRNA amplified by RT-PCR. Results are means ± SE. *$P = 0.02$, $n = 3$.*

**Fig. 4.** Human NaP$_{i}$-Iib gene structure. **A:** the locations of introns and donor/acceptor sites determined for the human NaP$_{i}$-Iib gene are shown. The intron location is based on the nucleotide number in the human intestinal NaP$_{i}$-Iib cDNA. Exonic sequences are in uppercase letters, and intronic sequences are in lowercase letters. **B:** human NaP$_{i}$-Iib gene organization is depicted with introns shown as triangles and numbered 1 to 11. The size of each intron (bp) is shown under each triangle. Exons are numbered I to XII, with numbers over the line indicating the location of introns in the human intestinal NaP$_{i}$-Iib cDNA.
Sequence of the 5'-flanking region of the human NaPi-IIb gene. The primer walking technique was used to directly sequence about 2,800 bp of BAC DNA upstream of exon I. Figure 5B shows ~500 bp from the 5'-flanking region of the human NaPi-IIb gene. Additionally, 1.1 kb of the proximal 5'-flanking region was examined for the presence of typical eukaryotic promoter elements by use of Omiga sequence analysis software (version 2.0; Oxford Molecular, Oxford, UK) and TRANSFAC promoter analysis software (32). Search results showed that there was no TATA or CAAT box in the first 500 bp upstream of the transcription initiation site of the human NaPi-IIb gene. However, several other putative cis elements were identified, including transcriptional factors AP1, AP2, AP4, C/EBP, GATA1, and Sp1 binding sites. Furthermore, a putative glucocorticoid receptor binding site was identified further upstream (~1,082 bp).
Promoter characterization of the human NaP$_{i-IIb}$ gene. To determine whether the 5' flanking region of the human NaP$_{i-IIb}$ gene contained a functional promoter, four constructs (pGL3-181/+15, -380/+15, -1,103/+15, and -2,783/+15) were transfected into A549 and Caco-2 cells. Reporter gene assays were performed 48 h after transfection (Fig. 6). The promoter assay data showed that all the promoter constructs were functional in both cell lines. Compared with the negative control transfections, these promoter constructs resulted in 10- to 17-fold stimulation of reporter gene activity ($n = 4–10; P < 0.002$).

Effect of EGF on human NaP$_{i-IIb}$ gene promoter activity. To test the EGF effect on human NaP$_{i-IIb}$ gene promoter activity, A549 and Caco-2 cells were first transfected with promoter constructs and then treated with 100 ng/ml EGF before harvesting cells. Promoter activity was 40–50% decreased in Caco-2 cells after 8 h of EGF treatment ($n = 4–10; P < 0.02$), but no change was seen in A549 cells ($n = 4–10$) (Fig. 7).

Fig. 6. Expression of human NaP$_{i-IIb}$ gene promoter constructs in transfected A549 cells (A) and Caco-2 cells (B). Cells were transfected with 0.5 μg pGL3 Basic (pGL3b) or 0.5 μg NaP$_{i-IIb}$ promoter constructs. Reporter gene assays were performed 48 h after transfection. To control for transfection efficiency, cells were cotransfected with 30 ng pRL-CMV. Data are presented as relative luciferase activity (firefly luciferase activity driven by NaP$_{i-IIb}$ gene promoter over Renilla luciferase activity driven by the CMV promoter). Results are means ± SE. *$P < 0.002$ vs. all promoter constructs; $n = 4–10$.

Fig. 7. EGF reduces human NaP$_{i-IIb}$ gene promoter activity in transfected A549 (A) and Caco-2 cells (B). Cells were cotransfected with pGL3b or promoter constructs plus pRL-CMV. EGF was applied 8 h before harvesting cells. Fold reduction is shown as the ratio of luciferase activity in EGF-treated cells over luciferase activity in untreated cells. Results are means ± SE. *$P < 0.02$ vs. pGL3b, $n = 4–10$.

Effect of actinomycin D on human NaP$_{i-IIb}$ gene promoter activity induced by EGF. To determine whether the EGF effect on human NaP$_{i-IIb}$ gene promoter activity is due to transcriptional regulation, Caco-2 cells were first transfected with promoter constructs and then treated with 5 μg/ml actinomycin D for 2 h followed by treatment with 100 ng/ml EGF in the presence of actinomycin D. Results showed that the downregulation of NaP$_{i-IIb}$ promoter activity by EGF treatment in the -1,103 bp construct transfected cells was blocked by actinomycin D ($n = 4; P < 0.03$) (Fig. 8). Furthermore, there was no effect of either EGF or EGF/actinomycin D treatment in pGL3b or the -118 bp construct transfected cells ($n = 4$).

DISCUSSION

EGF plays an important role in many physiological and pathophysiological processes, such as cell growth and recovery from injury. Additionally, it has been shown that the plasma P$_i$ level and EGF levels were increased with intestinal injury (10, 19, 30). Thus EGF may play a role in regulation of P$_i$ homeostasis in response to intestinal injury. However, the precise
relationship between EGF and intestinal Pi absorption is not clear. In this study, we demonstrated that EGF treatment reduced intestinal NaPi-IIb mRNA abundance by 40–50% in rat and human intestinal cells (Caco-2). To decipher the molecular mechanism, we first cloned the human NaPi-IIb gene and promoter. We then characterized the gene promoter function and studied EGF regulation of promoter activity in transfected human lung and intestinal epithelial cells. Our data suggest for the first time that EGF reduces NaPi-IIb mRNA abundance via a gene transcription-mediated mechanism.

Although EGF is clearly involved in regulating renal Pi absorption, the role of EGF in modulating intestinal Pi absorption is unknown. To understand the relationship between EGF and intestinal Pi absorption, we cloned the complete human NaPi-IIb gene. Interestingly, the human NaPi-IIb gene showed structural differences when compared with the human NaPi-IIa gene (15). The human NaPi-IIb gene is larger than the human NaPi-IIa gene (24 kb vs. 16 kb), although it has fewer exons and introns (12 exons/11 introns in NaPi-IIb vs. 13 exons/12 introns in NaPi-IIa). Also, in the human NaPi-IIb gene, intron 1 is the largest (~6,800 bp), whereas in NaPi-IIa, intron 8 is the largest (~5,000 bp). These differences may be the result of evolutionary divergence.

Furthermore, the human NaPi-IIb and NaPi-IIa genes showed differences in the 5′-flanking regions. Unlike the human NaPi-IIa gene promoter (15, 18), the typical TATA box was absent in the proximal region of the human NaPi-IIb gene. However, in the TATA-less human NaPi-IIb gene promoter, several GATA1 binding sites are predicted. Previous studies showed that GATA transcription factors are involved in the regulation of cell type and tissue-specific gene expression (14, 20, 23, 31). This finding is in agreement with the observation that this gene is expressed in only a few tissues [i.e., in human intestine, lung, and several glands (9, 34)].

Because the NaPi-IIb gene was highly expressed in human intestine and lung, we chose a human intestinal cell line (Caco-2) and a human lung cell line (A549) for promoter characterization. RT-PCR studies demonstrated that NaPi-IIb mRNA was endogenously expressed in both Caco-2 and A549 cell lines, which suggested that they were appropriate in vitro models for promoter analyses. All four human NaPi-IIb gene promoter constructs (as described in MATERIALS AND METHODS) significantly stimulated reporter gene expression in both cell lines. This finding suggested that the basal promoter region of the gene is within the first 181 bp upstream of the transcription initiation site, since the shortest construct (~181/+15) was active.

In vivo and in vitro studies showed that EGF treatment decreased intestinal NaPi-IIb mRNA abundance by ~50%, suggesting possible transcriptional regulation. In transient transfected Caco-2 cells, EGF also decreased NaPi-IIb gene promoter activity by ~40%. This promoter activity decrease could be abolished by actinomycin D, a transcriptional inhibitor. These data indicated that the effect of EGF on intestinal NaPi-IIb mRNA expression was due, at least in part, to reduced gene transcription. This reduction in promoter activity induced by EGF treatment was observed only in intestinal cells (Caco-2), but not in lung cells (A549), which suggested that the EGF effect on human NaPi-IIb gene expression was tissue specific.

Furthermore, the two larger promoter constructs (~1,103/+15 and −2,783/+15) were responsive to EGF treatment, but the two smaller ones (~181/+15 and −380/+15) were not responsive. These data suggested that the putative EGF response element(s) was located between 380 and 1,103 bp upstream of the transcriptional unit. This observation also suggests that EGF regulation of this gene is not mediated by the basal transcriptional machinery, which is likely located within the first 200 bp upstream of transcription initiation site.

Several EGF responsive elements have been previously identified from the c-fos gene (11), the rat preprothyrotropin-release hormone gene (26), the rat prolactin gene (8), and the human gastrin gene (12, 13, 22). These EGF responsive elements include a serum-response element and AP1 binding sequences in the c-fos gene and Sp1 binding sequences in the rat preprothyrotropin-release hormone gene and the human gastrin gene. We searched the human NaPi-IIb gene promoter region (~1,103 bp to −380 bp) for these known EGF response elements from these other genes, and we found two sequences (~792 bp GGAGGAGG −786 bp and −479 bp GGGGCAG −474 bp) that have high homology with the EGF response element of rat preprothyrotropin-release hormone gene. These sequences may be responsible for EGF regulation in the
human NaPi-IIb gene, although further experiments will be required to make this determination.

In summary, we cloned the complete human NaPi-IIb gene from a human genomic BAC DNA library. This gene contains 12 exons and 11 introns. Approximately 2.8 kb of the 5′-flanking region of the human NaPi-IIb gene was sequenced and confirmed to be a functional promoter. EGF treatment decreased NaPi-IIb mRNA abundance in rat intestine and in human intestinal promoter. EGF treatment decreased NaPi-IIb mRNA gene was sequenced and confirmed to be a functional transcription regulation. These novel findings suggest that transcriptional mechanisms are involved in EGF regulation of intestinal NaPi-IIb cotransporter gene expression. Further studies will focus on identification of the EGF responsive element(s) and the transcription factors involved in EFG regulation of the human NaPi-IIb gene.

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant R01-DK-33209–17 and the W. M. Keck Foundation.

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