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SUBCLONING AND REGULATION OF A HUMAN INTESTINAL SODIUM-PHOSPHATE COTRANSPORTER GENE

by

Hua Xu

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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Hua Xu

entitled Subcloning and Regulation of a Human Intestinal Sodium-Phosphate Cotransporter Gene

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy

Fayez Ghishan, MD	10/30/01 Date
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Ronald Lynch, Ph.D.	10/30/01 Date
William Dantzler, MD, Ph.D.	10/30/01 Date
Andrea Yool, Ph.D.	Oct 30, 2001 Date

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Dissertation Director	10/30/01 Date

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ABSTRACT

Phosphate plays a critical role in the body as a constituent of bone and tooth for body development, and as a urinary buffer for pH in body acid-base balance regulation. The phosphorus level in blood in human is between 3.0 to 4.5 mg/dl. When the blood phosphate concentration is lower than 2.5 mg/dl, the person develops hypophosphatemia. When the blood phosphate concentration is higher than 4.5 mg/dl, the person develops hyperphosphatemia. It is critically important for the body to control the phosphate level in blood and maintain the phosphate homeostasis. The kidney and the intestine are the important sites to regulate phosphate homeostasis.

This dissertation research was to explore the transporter gene(s) involved in the intestinal sodium-dependent phosphate absorption in human and to investigate the role of sodium-phosphate (NaPi) transporters in phosphate homeostatic regulation. The research was performed to test the hypothesis that the sodium-dependent phosphate (NaPi) cotransporter is involved in the phosphate absorption in intestine and various physiological regulators modulate the activity of this transporter. A cDNA encoding a novel human small intestinal Na^+-P_i transporter was isolated from a human intestinal cDNA library. This cDNA encodes a 689 amino acid polypeptide which is different from the renal NaPi cotransporters. This human intestinal NaPi cotransporter gene was mapped to human chromosome 4p15.1-p15.3 by the Fluorescence *In-Situ* Hybridization (FISH) method. The human intestinal NaPi cotransporter gene structure was studied by screening a human genomic DNA library. This gene contains 12 exons and 11 introns. There were two transcription initiation sites identified by primer extension. *In vivo* and *in vitro*

studies showed that the intestinal NaPi cotransporter gene expression is regulated by EGF and vitamin D_3. EGF inhibits NaPi-cotransporter gene expression, while vitamin D_3 stimulates NaPi-cotransporter gene expression. From these studies, I concluded that the intestinal sodium-dependent phosphate absorption is mainly mediated by intestinal NaPi cotransporters (NaPi-IIb), and this transporter is modulated by various physiological regulators in order to maintain the phosphate homeostasis.

CHAPTER 1

INTRODUCTION

Phosphate content in the body

Phosphorous is one of the most important minerals in the body. Phosphorous presents in the form of phosphate (one phosphorous and four oxygen atoms) in the body system. Phosphate plays a critical role in the body as a constituent of bone and tooth for body development, and as a urinary buffer for pH in body acid-base balance regulation. Phosphate is localized primarily in bone matrix (85%), and the reminder of the body phosphate is divided between ICF (intracellular fluids) (15%) and ECF (extracellular fluids) (<0.5%). In ICF, phosphate is a component of nucleotides (DNA and RNA), high energy molecules (e.g., ATP), and metabolic intermediates. In ECF, phosphate is present in its inorganic form and serves as a buffer for H^+. About 90% of the phosphate in plasma is free of binding, and only 10% of phosphate in plasma is protein bound. The phosphorus level in blood in human is between 3.0 to 4.5 mg/dl.

The daily requirement for phosphate in adult is about 0.9 grams. It will be required more for young children, as they need more phosphate to support their growth. Phosphate mainly comes from foods, such as milk, dairy products, most peas and beans, green leafy vegetables, nuts, chocolate and dark-colored soft drinks. Most of the body's phosphate is contained in bone. Phosphate is absorbed in intestine and secreted in the urine and stool.

When the blood phosphate concentration is lower than 2.5 mg/dl, the person

develops hypophosphatemia. Chronic hypophosphatemia occurs in hyperparathyroidism (excess parathyroid hormone production), hypothyroidism (an underactive thyroid gland), poor kidney function, and long-term use of diuretics. Toxic amounts of the drug theophylline (a bronchodilation drug, used for the treatment of asthma) can reduce the amount of phosphate in the body. Taking large amounts of aluminum hydroxide antacids for a long time can also deplete the body's phosphate, especially in people undergoing kidney dialysis. Phosphate stores are depleted in people with severe malnutrition, diabetic ketoacidosis, severe alcohol intoxication, or severe burns. As people with these conditions recover, the blood phosphate level can quickly fall dangerously low because the body uses large amounts of phosphate. Hypophosphatemia can cause muscle and bone weakness, blood cell disorders, growth retardation and abnormal bone development. An extremely low phosphate blood level (less than 1.5 mg/dl) can be very serious, leading to progressive muscle weakness, stupor, coma, and even death.

When the blood phosphate concentration is higher than 4.5 mg/dl, the person develops hyperphosphatemia. Hyperphosphatemia occurs in people with severe kidney dysfunction. In people with kidney failure, hyperphospahtemia is a problem as dialysis is not effective at removing phosphate. When the blood phosphate concentration is elevated in dialysis patients, the blood calcium level becomes low. This stimulates the parathyroid glands to produce parathyroid hormone, which in turn raises the blood calcium level by mobilizing calcium from the bone. Sustained hyperphosphatemia will cause progressive bone weakness, resulting in pain and fractures from minimal trauma. Crystallization of calcium and phosphate in blood vessels and heart will cause severe arteriosclerosis and

leading to stroke, heart attacks, and poor circulation.

It is critically important for the body to control the phosphate level in blood and maintain the phosphate homeostasis. To understand the phosphate homeostasis, it is important to understand how the body organs handle the phosphate.

Phosphate handling in kidney

Kidney is an important organ to handle phosphate. It plays a critical role in regulating the blood phosphate concentration. The renal handling of phosphate is illustrated in Figure 1-1.

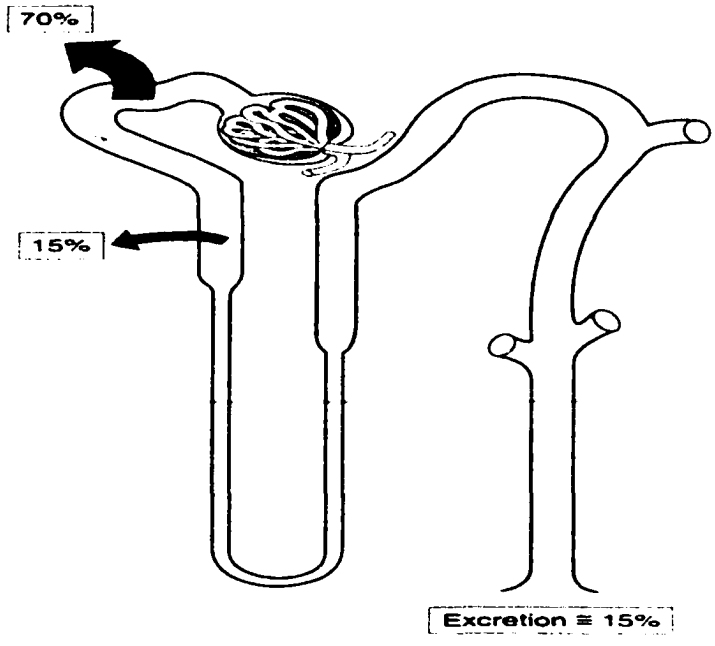

Figure 1-1. Phosphate Handling in Kidney

90% of the phosphate is not bound to plasma proteins. The unbound phosphate will be filtered across renal glomerular capillaries. Subsequently, about 70% of the filtered load will be reabsorbed in the proximal convoluted tubule, and 15% of the filtered load will be reabsorbed in the proximal straight tubule. At the cellular level, phosphate reabsorption is accomplished by a sodium-dependent phosphate (NaPi) cotransporter in the luminal membrane of the proximal tubular cells (Figure 1-2). Phosphate reabsorption through NaPi cotransporter is saturable and is a rate limit process. When the maximal transport rate is reached from NaPi cotransporter, any phosphate that is not reabsorbed will be excreted. The protein involved in the renal phosphate reabsorption process is mainly the NaPi-IIa cotransporter, which has been identified and well studied from several species in the past decade (112, 123, 124).

Figure 1-2. Cellular Mechanism of Pi Reabsorption in Kidney

The level of phosphate reabsorption is regulated by many factors, including dietary phosphate content and endocrinal factors. Higher phosphate diet will inhibit renal

phosphate reabsorption (91, 114), while low phosphate diet will enhance renal phosphate reabsorption (22, 45, 59, 83, 91, 96, 113, 115). The hormones involved in the inhibition of renal phosphate reabsorption include parathyriod hormone (PTH) (92), epidermal growth factor (EGF) (2, 4), and glucocorticiods (3, 69, 94). The hormones involved in the stimulation of renal phosphate reabsorption include 1,25-dihydroxyvitamin D_3 (9, 65, 70), thyroid hormone (T_3) (106), growth hormone (GH) (118), insulin (1), and insulin-like growth factor (IGF) (19).

Phosphate absorption in intestine

The small intestine is an important site for phosphate absorption from diet (Figure 1-3). Early studies showed that the transport of P_i through the apical membrane of small

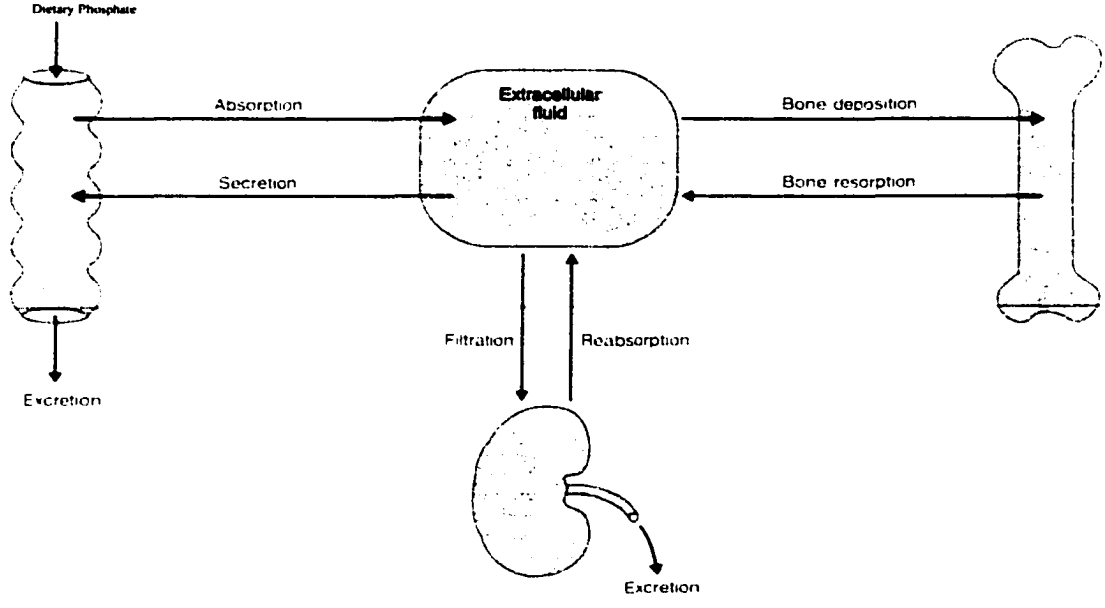

Figure 1-3. Phosphate Handling in Intestine

intestinal epithelial cells is coupled with sodium (8, 14, 26, 48, 82, 95, 99-101, 105, 112). Like the phosphate reabsorption in kidney, P_i absorption in small intestine is also modulated by many physiological factors, including hormonal and dietary (8). Endocrinal factors, such as glucocorticiods and 1,25-dihydroxyvitamin D_3 elicit their effect on intestinal phosphate absorption through modulating the sodium-dependent phosphate absorption process (13, 26-29, 31, 32, 67, 77, 85, 87, 90, 95, 99, 107, 119). Estrogen also plays a possible role in regulating intestinal P_i absorption (93). As a nutritional factor, phosphate content of the diet regulates intestinal P_i absorption through altering the sodium-dependent phosphate absorptive pathway (8, 32, 76, 83, 95, 99, 101). The protein(s) involving in the intestinal sodium-dependent phosphate absorption remained unidentified until late 1998.

Hypothesis to be tested

The absorption of phosphate in the intestine is mediated by a sodium-dependent phosphate cotransporting system. The protein(s) involved in the intestinal phosphate absorption process is different from the one identified in the kidney. Like the renal NaPi cotransporter, this intestinal NaPi cotransporter is also regulated by various hormonal factors, such as epidermal growth factor (EGF) and 1,25-dihydroxyvitamin D_3.

Research plans and strategies

- Design the primers from the region which is highly conserved in renal NaPi cotransporter (type IIa) from several species (human, rat, mouse and flounder) and intestinal NaPi cotransporter (type IIb) from mouse;
- Amplify the expected size products from human intestinal mRNA, sequence the products and compare the homology with these known NaPi cotransporters;
- Subclone the human intestinal NaPi cotransporter cDNA by screening a human small intestinal cDNA library with the probes generated from above RT-PCR;
- Characterize the transporter function by using oocyte expression system;
- Explore the expression pattern of this transporter in different human tissues by northern blotting;
- Localize the chromosomal locus of this gene in the human chromosome using Fluorescence *In Situ* Hybridization (FISH) method;
- Iddentify the gene structure and subclone the promoter region of the human intestinal NaPi cotransporter gene by screening a human genomic library;
- Characterize the promoter function by using liposome mediated transfection system in mammalian cells;
- Study the effect of Epidermal Growth Factor (EGF) on the intestinal NaPi gene expression;
- Study the effect of 1,25-dihydroxyvitamin D3 on the intestinal NaPi gene expression.

CHAPTER 2

SUBCLONING THE HUMAN SMALL INTESTINAL SODIUM-PHOSPHATE COTRANSPORTER[1,2]

Phosphate plays a crucial role in cellular metabolism, and its homeostatic regulation in intestinal and renal epithelia is critical. Apically expressed sodium-phosphate (Na^+-P_i) transporters play a critical role in this regulation. We have isolated a cDNA encoding a novel human small intestinal Na^+-P_i transporter. The cDNA is shown to be 4135 bp in length with an open reading frame that predicts a 689 amino acid polypeptide. The putative protein has 76% homology to mouse intestinal type II Na^+-P_i transporter (NaPi-IIb) and lower homologies with renal type II Na^+-P_i transporters (NaPi-IIa). Northern blots showed a singular transcript of 5.0 kb in human lung, small intestine, and kidney. Computer analysis suggests a protein with 11 transmembrane domains and several potential post-translational modification sites. Functional characterization in *Xenopus laevis* occytes showed this cDNA encodes a functional Na^+-P_i transporter. Furthermore, the gene encoding this cDNA was mapped to human chromosome 4p15.1-p15.3 by the Fluorescence *In* Situ Hybridization (FISH) method.

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[1] Sequence data for this article has been deposited in the GenBank with Accession # AF146796.

[2] Published as "Molecular cloning, functional characterization, tissue distribution, and chromosomal localization of a human small intestinal sodium-phosphate (Na^+-P_i) transporter (SLC34A2)" (*Genomics* 62: 281-4, 1999)

Introduction

Phosphate (P_i) plays a major role in growth, development, bone formation and cellular metabolism. The kidney and the small intestine are important regulatory sites which maintain extracellular P_i concentrations. Sodium-coupled phosphate transport is the major form of P_i absorption in both kidney and intestine. Phosphate uptake by renal and intestinal brush-border membrane vesicles (BBMV) has been studied previously in human (12), rat (43), rabbit (14) and mouse (83). The molecular basis of P_i uptake in kidney has been identified (sodium-phosphate [Na^+-P_i] transporters types I and II) and well characterized. Type II Na^+-P_i transporter is the major transport pathway of P_i reabsorption in kidney (23, 71). However, little is know about P_i absorption in the intestine. In order to understand the phosphate absorption regulation in the intestine, we conducted this study to subclone the intestinal sodium-dependent phosphate cotransporter from human.

Materials and methods

PCR amplification of the mouse intestinal NaPi-IIb cDNA homology sequence from human small intestinal mRNA: First-strand cDNA was subjected to PCR amplification from human small intestinal mRNA (Clontech; Palo Alto, CA), with oligonucleotide primers corresponding to a highly conserved region in the human renal NaPi-IIa, mouse renal NaPi-IIa, rabbit renal NaPi-IIa, flounder renal and intestinal NaPi and mouse intestinal NaPi IIb cotransporters: forward primer at 686-705bp and reverse primer at 1423-1442bp relatively in mouse intestinal NaPi-IIb cDNA sequence. PCR

products of the expected size (~760 bp) were gel purified and subcloned into the pGEM-T vector (Promega, CA). This DNA fragment was then sequenced at both strands by the Biotechnology Resource Facility at the University of Arizona, utilizing sequence-specific oligonucleotide primers. A cycle-sequencing protocol using *Taq* FS DNA polymerase and fluorescent, dideoxy chain termination was followed. The resulting DNA fragments were electrophoresed and analyzed using an automated Applied Biosystems 373A Stretch DNA Sequencer. The sequence was assembled and analyzed, and sequence comparisons were run using Omiga software (version 1.1.3) and GenBank Blast searches.

Human intestinal cDNA library screening: A human small intestinal cDNA library was constructed into pEAK8 vector. This human small intestinal cDNA library was transformed into E.coli (Edge Biosystems; Gaithersburg, MD). Bacterial colonies were screened by the colony hybridization method described by Muller et al. (80). Bacteria were grown on solid media at all times during library screening. Replicas of each master plate were made onto nitrocellulose membranes (S&S BA85), and the colonies were allowed to grow overnight. The bacteria grown on nitrocellulose membranes were lysed with 10mM NaOH - 1% SDS and neutralized with sodium acetate solution. These filters were screened by radioactive probes generated from the 760 bp PCR products under high stringency screening conditions. The positive colonies on the filters were located on the master plates. The DNAs were purified from these bacteria and sequenced on both strands.

Functional characterization of the human intestinal NaPi-IIb transporter by oocyte over-expression system: Initially, *Xenopus laevis* frogs (NASCO) were anesthetized in ice water with benzocaine for 15 min. Then oocytes were dissected from the frogs and defolliculated, as described by Bai et al. (7). The oocytes were injected with 50 ng of human intestinal NaPi-IIb cRNA 1 day following isolation. The oocytes were maintained at 18°C in Barth's solution containing 50 mg/ml gentamicin sulfate, 2.5 mM sodium pyruvate, and 5% heat-inactivated horse serum. Transport of $KH_2{}^{32}PO_4$ (DuPont-NEN) was measured 3 days after injection as described (7). Oocyte uptake buffers consisted of: 100 mM NaCl (Na buffer) or 100 mM choline chloride (choline buffer), with 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES-Tris, pH 7.5. For the transport assays, groups of five oocytes were washed briefly in choline buffer to remove serum and then incubated in 0.4 ml of the uptake buffer. After the indicated time period, uptake was stopped with four 4-ml washes of ice-cold choline buffer. Individual oocytes were transferred to scintillation vials and dissolved in 0.5 ml 10% SDS, and the radioactivity was measured by scintillation counting. Counts in control, uninjected oocytes were subtracted from counts in cRNA-injected oocytes. All uptake experiments were repeated three times, and statistical analysis (*t*-test or analysis of variance) was also done using the SigmaPlot program.

Northern blot analysis: A human multiple-tissue Northern blot and a Human Multiple Tissue Expression Array (Clontech) were purchased from Clontech (Palo Alto, CA). These blots were hybridized with [^{32}P]dCTP-labeled human intestinal NaPi-IIb

cDNA-specific probes generated by PCR. The blots were hybridized overnight 42°C and washed with high stringency conditions following the instructions from the blots.

Chromosomal localization of the human intestinal NaPi-IIb gene: PCR amplified fragment was used to localize the gene position by performing the Fluorescence *In Situ* Hybridization (FISH) mapping technique in lymphocytes isolated from human blood (SeeDNA Biotech Inc.; Windsor, Ontario, Canada). The 760bp human intestinal NaPi-IIb cDNA probe was biotinylated with dATP (53). FISH detection was performed as the procedure described by Heng et al. (54). FISH signals and the 4',6-diamidino-2-phenylindole (DAPI) banding pattern were recorded separately, and the assignment of the FISH mapping data with chromosomal bands was achieved by superimposing FISH signals with DAPI banded chromosome.

Results and discussion

In order to further define the role of the small intestinal Na^+-P_i transporter in body P_i homeostasis, we isolated a novel cDNA from a human small intestinal cDNA library by using the probes amplified from the human intestinal RT reaction with PCR primers homologous to the mouse intestinal Na/Pi-IIb cDNA. PCR products of the predicted size (760bp) were obtained from human small intestinal cDNA. Sequencing of the amplified fragment revealed 82% nucleotide sequence identity with the mouse intestinal Na/Pi-IIb. This suggested that this cDNA likely represents a type II Na^+-P_i transporter in human small intestine.

Using the PCR product to generate radioactive probes, we isolated a cDNA clone from an enriched, human small intestinal cDNA library utilizing high stringency screening conditions. Sequence data indicated that this human intestinal cDNA has 4135 bp and encodes a putative protein of 689 amino acids (open reading frame 36 to 2102 bp) [Figure 2-1]. Hydrophathy analysis (Omiga 1.1.3 software, Oxford Molecular LTD; Oxford, England) predicts 11 transmembrane domains. The putative protein has many potential post-translational modification sites. We compared this cDNA with other identified Na^+-P_i transporter cDNAs, which showed over 60% nucleotide sequence identity and over 75% amino acid sequence identity with bovine renal type II Na^+-P_i transporter (52), mouse intestinal type II Na^+-P_i transporter (56) and an unpublished human NPT (GenBank accession #AF111856). Recently, another type I Na^+-P_i transporter cDNA was isolated from a human intestinal cDNA library (103), however, the sequence similarity with our cDNA was very low (<20%). Overall, these findings suggest that our newly identified human cDNA clone encodes a protein that belongs to the intestinal type II Na^+-P_i transporter gene family.

mRNA expression of this cDNA clone was analyzed with a 76 human tissue mRNA blot (Human Multiple Tissue Expression Array). Hybridization utilizing the 760 bp PCR fragment as a template to generate radiolabelled probes showed that this gene is expressed in many tissues (Figure 2-2. upper panel). The highest expression was seen in lung, small intestine and kidney. Furthermore, Northern blot analysis from an 11-human tissue mRNA blot showed a single transcript at approximately 5.0kb in human lung, small intestine, kidney, liver and placenta (Figure 2-2. lower panel). Interestingly,

previous studies showed that human renal NaPi cotransporter mRNA transcripts were detected at 2.0 kb (type I Na^+-P_i transporter (79)) and at 2.7 kb (type II Na^+-P_i transporter (71)). This suggests that this 5 kb transcript detected in human kidney likely represents an unidentified type II Na^+-P_i transporter isoform. Also, since this gene is highly expressed in adult and fetal lung, it seems probable that this newly identified human NaP_i transporter has an important physiological function in lung (possibly involved in the production of surfactant by the alveoli).

To characterize the function of the protein encoded by this cDNA, we produced cRNA, injected it into *Xenopus laevis* oocytes and measured radiolabelled P_i influx in the presence or absence of Na^+. Compared with uninjected oocytes, the Na^+-P_i transporter cRNA injected oocytes exhibited approximately 55-fold increase of P_i transport ($p<0.0001$) (Figure 2-3), which suggested that this cDNA does indeed encode a functional Na^+-P_i transporter.

To identify the locus of our Na^+-P_i transporter gene, a PCR amplified 760 bp cDNA fragment was used to localize the gene position by performing the FISH mapping technique in lymphocytes isolated from human blood. Results showed that hybridization signal was only detected on chromosome 4p15.1-p15.3 (Figure 2-4). Previous investigations showed that human renal NaP_i transporters are found on chromosomes 5 and 6 (5q35 (64, 73)), 6p21.3 (unpublished; GenBank accession #s U90544 and U90545) and 6p21.1-p23 (21, 64). Also, type I NaP_i transporter cDNA from human intestinal mucosa was localized to 6p21.3-p23 (103). These data indicate that Na^+-P_i transporter genes are widely distributed in the genome.

Conclusion

In summary, we isolated a 4135 bp type II Na^+-P_i transporter cDNA from a human small intestinal cDNA library. This cDNA encodes a 689 amino acid protein, which shares high sequence homology with intestinal and renal type II Na^+-P_i cDNAs from various mammalian species. This gene encodes Na^+-P_i transporter which is highly expressed in human lung, small intestine and kidney, and was localized to chromosome 4p15.1-p15.3.

```
MAPWPELGDAQPNPDKYLEGAAGQQPTAPDKSKETNKNNTEAPVTKIELL  50
PSYSTATLIDEPTEVDDPWNLPTLQDSGIKWSERDTKGKILCFFQGIGRL 100
          TM1                                   TM2
ILLLGFLYFFVCSLDILSSAFQLVGGKMAGQFFSNSSIMSNPLLGLVIGV 150
                                          TM3
LVTVLVQSSSTSTSIVVSMVSSSLLTVRAAIPIIMGANIGTSITNTIVAL 200
                                 TM4
MQVGDRSEFRRAFAGATVHDFFNWLSLLVLLPVEVATHYLEIITQLIVES 250
FHFKNGEDAPDLLKVITKPFTKLIVQLDKKVISQIAMNDEKAKNKSLVKI 300
WCKTFTNKTQINVTVPSTANCTSPSLCWTDGIQNWTMKNVTYKENIAKCQ 350
                      TM5
HIFVNFHLPDLAVGTILLILSLLVLCGCLIMIVKILGSVLKGQVATVIKK 400
               TM6                        TM7
TINTDFPFPFAWLTGYLAILVGAGMTFIVQSSSVFTSALTPLIGIGVITI 450
             TM8                          TM9
ERAYPLTLGSNIGTTTTAILAALASPGNALRSSLQIALCHFFFNISGILL 500
                                        TM10
WYPIPFTRLPIRMAKGLGNISAKYRWFAVFYLIIFFFLIPLTVFGLSLAG 550
           TM11
WRVLVGVGVPVVFIIILVLCLRLLQSRCPRVLPKKLQNWNFLPLWMRSLK 600
PWDAVVSKFTGCFQMRCCCCCRVCCRACCLLCGCPKCCRCSKCCEDLEEA 650
QEGQDVPVKAPETFDNITISREAQGEVPASDSKTECTAL             689
```

Figure 2-1. Predicted Amino Acid Sequence of Human Small Intestinal NaPi-IIb Cotransporter. Underlined amino acid sequences represent potential transmembrane domain regions, which are numbered sequentially. Bold amino acids present putative N-glycosylation sites. Nucleotide sequence can be found in GenBank with accession # AF146796.

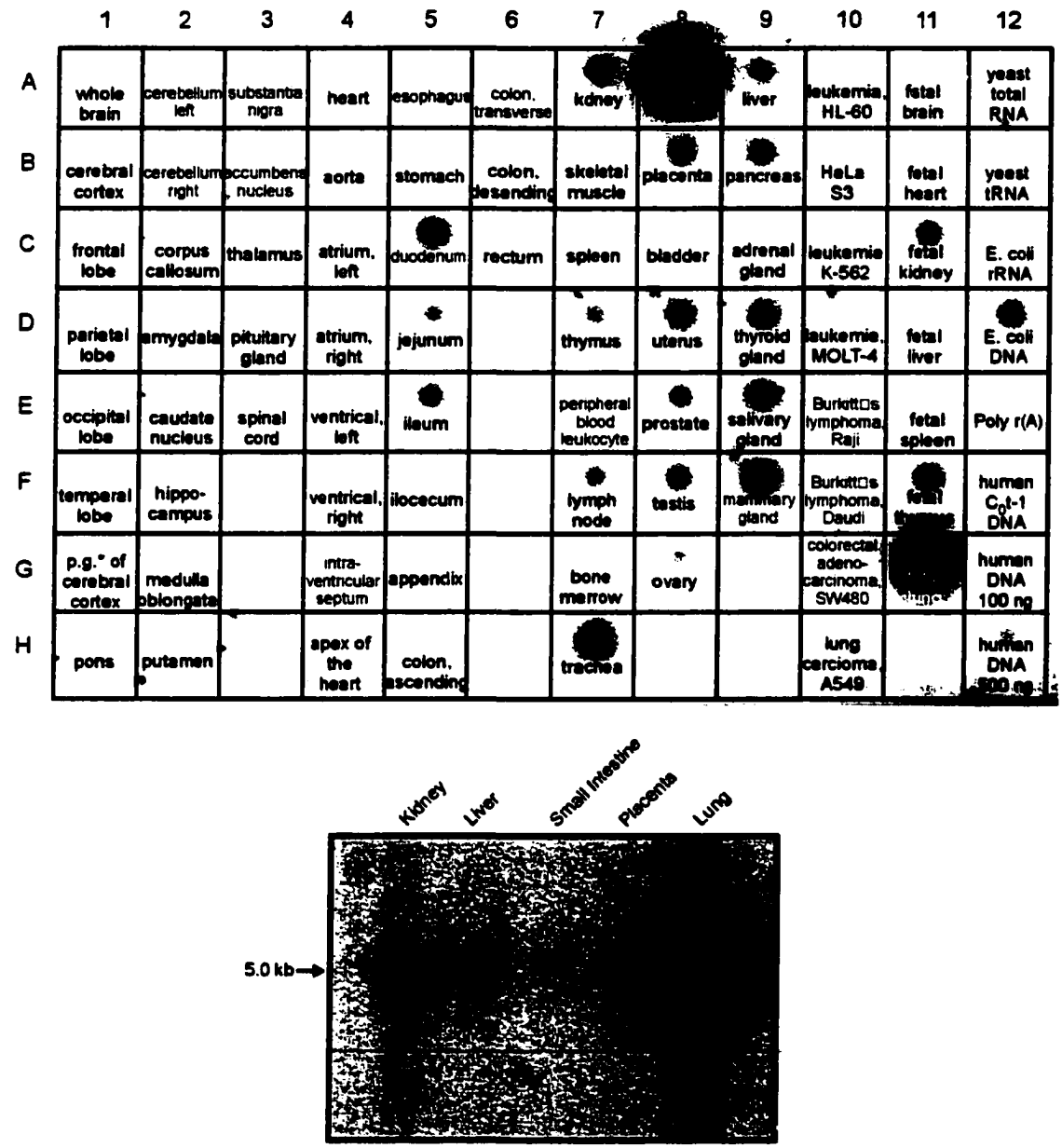

Figure 2-2. mRNA Expression of Human Intestinal NaPi-IIb Cotransporter. mRNA from 76 different human tissues was loaded on the nylon membrane (mRNA levels were normalized versus 8 different gene markers [Clontech manual], so this blot is quantitative) [Upper panel]. Blot was hybridized with cDNA-specific probes under high stringency conditions and exposed to film. Also, shown is Northern blot analysis of human small intestinal NaPi transporter (Lower panel). Blot was hybridized with cDNA-specific probes under high stringency conditions and exposed to film. A 5 kb transcript was detected in several tissues. Blot is not quantitative, as mRNA loading was not normalized.

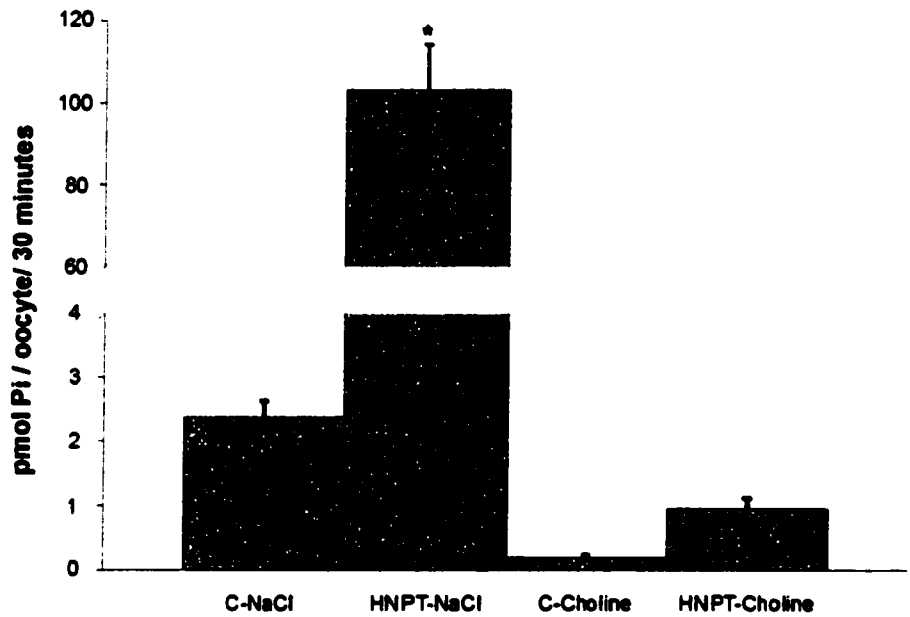

Figure 2-3. Characterization of Human Intestinal Type II NaPi-IIb Cotransporter in Oocytes Injected With cRNA. Isotope (P_i) influx measurements were performed in the presence of NaCl or choline-Cl. An approximate 55-fold increase of P_i uptake was seen in cRNA injected oocytes. C: uninjected oocytes. HNPT: cRNA injected oocytes. (Mean $\pm$ SEM of 5 oocytes per group; n=4, * $p<0.0001$ for HNPT versus all other groups)

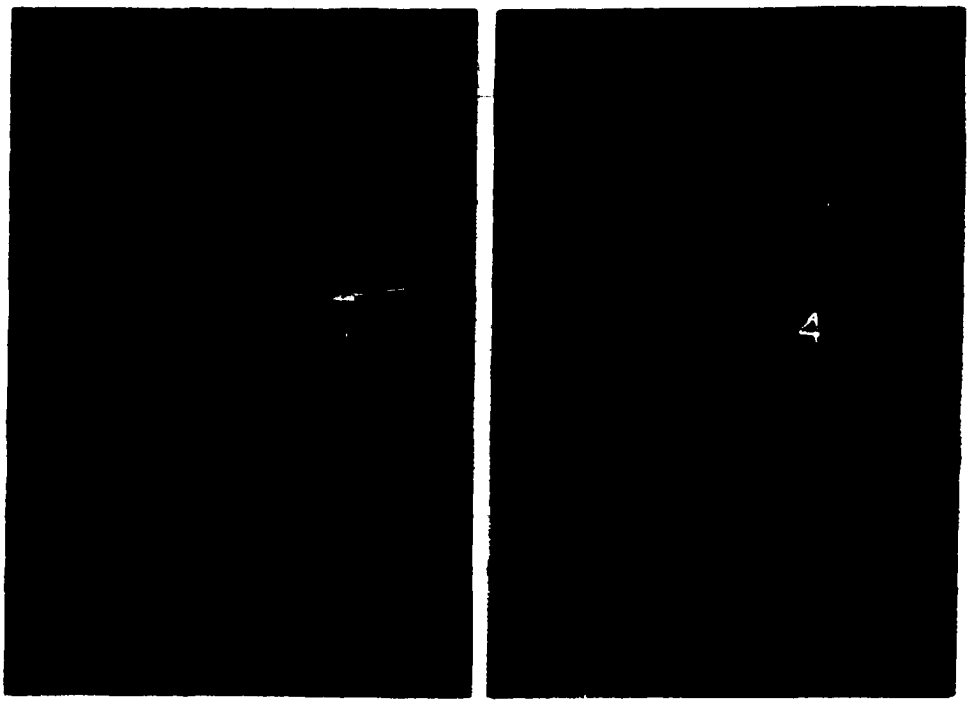

Figure 2-4. Chromosomal Localization of the Human Small Intestinal NaPi-IIb Cotransporter Gene to Chromosome 4p15.1-p15.3.
Left panel, as depicted by arrow, shows the FISH signals on human chromosome hybridized with the 760 bp probe.
Right panel shows the same mitotic chromosome stained with DAPI to identify human chromosome 4.

CHAPTER 3

SUBCLONING AND CHARACTERIZATION OF THE HUMAN SODIUM-PHOSPHATE COTRANSPORTER GENE PROMOTER[3, 4]

The intestinal sodium-phosphate cotransporter (NaPi-IIb) plays a major role in intestinal Pi absorption. The intestinal phosphate absorption process is regulated by many factors, including dietary factors and hormonal factors. To understand the mechanisms of these regulations, we cloned the human NaPi-IIb gene and promoter region. 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. We conclude that the human NaPi-IIb gene promoter is functional in Caco-2 and A549 cells.

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[3] Sequence data in this article has been deposited in GenBank with Accession Nos. AF234236-234245.

[4] Published as "Regulation of the human sodium-phosphate cotransporter NaPi-IIb gene promoter by epidermal growth factor" (*Am J Physiol* 280:C628-36, 2001)

Introduction

Phosphate (P_i) 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 type II intestinal sodium-dependent phosphate cotransporter (NaPi-IIb) cDNAs were cloned recently (36, 56, 127), and the human NaPi cotransporter (hNaPi-IIb) gene (SCLA34A2) was mapped to human chromosome 4p15.1-15.3 (126, 127).

Intestinal sodium-dependent phosphate absorption occurs predominantly through the NaPi cotransport system, while renal sodium-dependent phosphate absorption is mainly mediated by another closely related isoform, NaPi-IIa. In both tissues, Pi absorption across the brush-border membrane through NaPi cotransporters is the rate-limiting step. Furthermore, Pi absorption is tightly regulated in order to maintain body Pi homeostasis. Many nutritional and endocrine factors regulate Pi absorption in the intestine and kidney. For instance, low phosphate diet stimulates renal NaPi-IIa (22, 91, 111, 113) and intestinal NaPi-IIb cotransporter activity (51). Also, vitamin D_3 and glucocorticoids affect renal and intestinal Pi absorption (26, 109).

To be able to study the regulation of NaPi-IIb gene expression and to understand the role of this transporter protein in the regulation of intestinal Pi homeostasis, we cloned the human NaPi-IIb gene, and characterized the promoter function in human intestinal cell line Caco-2 and human lung cell line A549.

Materials and methods

Cell Culture: Human lung cells (A549) and human intestinal cells (Caco-2) were purchased from ATCC, and cultured according to ATCC guidelines. Cells were cultured at 37° C in a 95% air/ 5% CO_2 atmosphere and passaged every 72 hours. 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 and Caco-2 cells, using the Fast-Track mRNA purification kit (Invitrogen; Carlsbad, CA). 10 μg of mRNA was utilized for Northern blot analyses with human NaPi-IIb cDNA-specific probes under high stringency washing conditions (25). Blots were exposed to a phosphorimaging screeen and band intensities were determined with Quantity One software (FX Molecular Imager; Biorad, Hercules, CA).

RT-PCR Analysis: mRNA was purified from Caco-2 cells and A549 cells. The primers used for detecting NaPi-IIb expression were the same as previously described for cloning of the human intestinal NaPi-IIb cDNA (127). The primers used for detecting β-actin and the RT-PCR conditions were described previously (6). 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 Bac DNA library was screened by Research Genetics (Huntsville, AL), using a ^{32}P-labelled human intestinal NaPi-IIb 5' cDNA fragment (nt 175-350). Library screening resulted in 3 putative positive clones, and one was selected for further analysis. Two strategies were undertaken to determine the human NaPi-IIb gene structure: 1- PCR amplification of Bac DNA with cDNA specific primer pairs and 2- direct sequencing of Bac DNA via primer walking. PCR products were then subcloned into pGEM-T vector (Promega; Madison, WI) and sequenced on both strands. For primer walking, Bac DNA was isolated from bacterial cultures by using a standard plasmid DNA purification method. Bac DNA was treated with RNase A (Sigma; St. Louis, MO) and NotI restriction enzyme (Promega), and then extracted with phenol/chloroform, precipitated with EtOH and resuspended in water. The Bac DNA was sequenced on both strands.

Primer Extension: The transcription initiation site of the human NaPi-IIb gene was determined by primer extension. Briefly, a primer designed complementary to the human NaPi-IIb cDNA at nt 43-62 (in exon II) was end-labeled with γ-[^{32}P]-ATP and T_4 polynucleotide kinase. Then, 200 fmol of labeled primer 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, then cooling to 58° C at 1° C per 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 hours by adding 200 U of Superscript II Reverse Transcriptase (Life Technologies; Bethesda, MD), 1 µl RNasin, 1 µl 10 mM dNTPS, and 4 µl 5X first stand reaction buffer in a 20 µl

reaction volume. The reaction was terminated by adding 3 µl 0.2 M EDTA (pH 8), and the RNA 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/TE buffer (1:1). Samples were heated at 75° C for 10 min prior to 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. This plasmid was constructed as follows. Initially, pZero-4.5 plasmid was built by inserting a SacI digested Bac DNA fragment (~4.5 kb) into pZero vector (Invitrogen; Carlsbad, CA). This 4.5kb SacI fragment contained the 5' flanking region, exon I, and part of intron 1 from the human NaPi-IIb gene. Then, an Eco47III/XhoI digested human NaPi-IIb cDNA fragment (726bp) was 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 designated +1, and nucleotides upstream (i.e. 5') were numbered negatively and downstream nucleotides (i.e. 3') were numbered positively.

Construction of Reporter Plasmids: Luciferase reporter plasmids used in this study were derived from pGL3-Basic (Promega). A -1103/+15 promoter reporter construct (pGL3-1103) was made by subcloning a SacI/XmaI digested pZero-4.5

fragment into pGL3-Basic. A -181/+15 promoter reporter construct (pGL3-181) was made by digesting pGL3-1103 construct with SacI/ApaI, then blunting both ends with Klenow DNA polymerase and ligating them together. A -380/+15 promoter reporter construct (pGL3-380) was made by inserting a SacI/XmaI digested PCR product, amplified with forward primer 5'-**TATGAGCTC**TTGAGCCTCACAGGAG-3' at nt -361 to -380 (bold nucleotides represent SacI site and overhanging bases) and reverse primer 5'-**TATAGA**TCTCCGCAGGTGCTGGGCT-3' in intron 1 (bold nucleotides indicate overhanging bases originally inserted for BglII subcloning not used for this construct), into pGL3 Basic vector (note that the XmaI site is in exon I). A -2783/+15 promoter reporter construct (pGL3-2783) was made by inserting a KpnI/SacI digested fragment (~1.7kb) into the pGL3-1103 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-2783 to -2764) and reverse primer 5'-TTGTCTCCAATGTGCACACG-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 KpnI 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. 0.5 μg promoter construct DNA, 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 Opti-MEM medium (Gibco/BRL) for 30 min at room temperature. The mixture was then added to the cells and they were incubated for 5 hours, followed by the addition of an equal volume of medium containing 20% fetal bovine serum (FBS). The next day, the medium was removed and replaced with standard medium with 10% FBS. After 24 hours, the cells were harvested for reporter gene assays. Promoter reporter assays were performed using the Dual Luciferase Assay Kit according to the manufacture's instruction (Promega).

Statistical Analysis: Student's T-test was used to compare values of the experimental data. *P* values of < 0.05 were considered significant.

Results

Northern Detection of NaPi-IIb mRNA in Caco-2 cells and A549 cells: Northern blot analysis of mRNA isolated from Caco-2 cells and A549 cells was performed by loading 10 μg mRNA per gel lane. Hybridization with radiolabelled, 757 bp human intestinal NaPi-IIb cDNA-specific probes showed no signal from untreated or treated cells. However, the β-actin hybridization signal was readily apparent (data not shown, n=3). These results suggested that the NaPi-IIb message was expressed below the Northern blot detection limits in both Caco-2 cells and A549 cells under these culture conditions.

RT-PCR Analysis of Cells: Endogenous expression of human NaPi-IIb mRNA in human intestinal cells (Caco-2) and human lung cells (A549) was confirmed by RT-PCR using human intestinal NaPi-IIb primers (Figure 3-1). These results showed that a ~760 bp band was amplified from both cells. The PCR product was subcloned and sequenced, and it was 100% identical to the human NaPi-IIb cDNA. This indicated that these cells endogenously express the NaPi-IIb gene.

Genomic Organization of the Human NaPi-IIb Gene: Library screening resulted in 3 putative positive clones. 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 (Figure 3-2). The introns ranged in size from 91 to ~6800 bp, while 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 NaPi-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 numbers AF234237-234245.

Mapping of the Transcription Initiation Site of the Human NaPi-IIb Gene: The transcription initiation site was determined by primer extension with a reverse primer at cDNA nt 43-62. Figure 3-3 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 3 times and identical results were obtained.

Sequence of the 5' Flanking Region of the Human NaPi-IIb Gene: The primer walking technique was used to directly sequence about 2800 bp of Bac DNA upstream of exon I. Figure 3-4 shows ~500bp 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 using Omiga sequence analysis software (version 2.0; Oxford Molecular, Oxford, England), and TRANSFAC promoter analysis software (125). 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 AP1, AP2, AP4, C/EBP, GATA1, and Sp1 binding sites. Furthermore, a putative glucocorticoid receptor-binding site was identified further upstream (-1082 bp).

Promoter Characterizaton of the Human NaPi-IIb Gene: To determine whether the 5' flanking region of the human NaPi-IIb gene contained a functional promoter, four constructs (pGL3 -181/+15, -380/+15, -1103/+15, -2783/+15) were transfected into A549 and Caco-2 cells. Promoter reporter gene assays were performed 48 hours after transfection (Figure 3-5). The promoter assay data showed that all the promoter constructs were functional in both cell lines. Compared to the negative control

transfections, these promoter constructs resulted in 10 to17-fold stimulation of reporter gene activity (n=4-10; $p<0.002$).

Discussion

Sodium-dependent phosphate cotransporter plays an important role in intestinal phosphate absorption. Many physiological factors regulate the intestinal phosphate absorption process. However, the precise relationship between these regulators and intestinal Pi absorption is not clear. Knowing the gene structure and the gene promoter of the human intestinal NaPi-IIb transporter is essential for studying the molecular mechanisms involving in the phosphate homeostatic regulation by physiological factors. In this study, we first cloned the human NaPi-IIb gene and promoter. Then, we mapped the transcriptional initiation site of the human NaPi-IIb cotransporter gene and characterized the gene promoter function in transiently transfected human lung and intestinal epithelial cells.

While intestinal phosphate regulation is clearly involved in Pi homeostasis, it is not clear how physiological factors regulate this process. In order to understand the relationship between NaPi-IIb gene expression 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 (49). 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 (~6800 bp), while in NaPi-IIa,

intron 8 is the largest (~5000bp). 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 (49, 57), 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 regulation of cell type and tissue-specific gene expression (46, 68, 78, 117). This finding is in agreement with the observation that this gene is only expressed in a few tissues [i.e. in human intestine, lung and several glands (36, 127).

Since 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 the 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 the method) 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.

Conclusion

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. The transcription initiation sites is located upstream of the translation site 106 and 110 bp position. About 2.8 kb of the 5' flanking region of the human NaPi-IIb gene was sequenced and confirmed to be a functional promoter. Therefore, further studies will be pursued to study the hormonal regulation on the intestinal NaPi-IIb gene expression to address the role of the NaPi-IIb cotransporter in the phosphate homeostatic regulation.

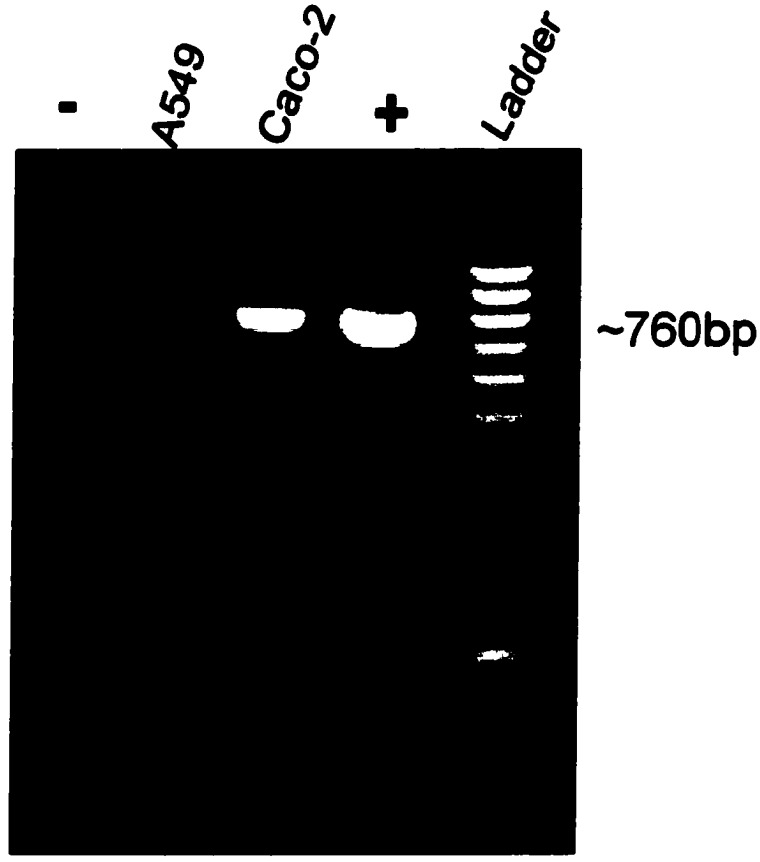

Figure 3-1. Detection of NaPi-IIb mRNA Expression in Human Intestinal and Lung Cells. mRNA prepared from Caco-2 cells and A549 cells was used for 1st-strand cDNA synthesis. Subsequent PCR was performed with human NaPi-IIb primers. PCR products were loaded on 1% agarose gel and visualized with ethidium bromide. (-) indicates PCR reaction negative control; where (+) indicates PCR reaction positive control.

A.

Intron	Location	Donor	Acceptor
1	32	GGGAGCGCTG/gtgagtaccg	tcatccacag/ACCATGGCTC
2	147	ACCAACAAAA/gtaagtgtcg	gtttcagcag/ATAACACTGA
3	282	AAGTGGTCAG/gtaaaagtga	ccccctgcag/AGAGAGACAC
4	411	CTGGTTGGAG/gtaagaatga	ttttttccag/GAAAAATGGC
5	555	TCCTCTTCAT/gtgagtcggg	catcctctag/TGCTCACTGT
6	863	CATTGTCCAG/gtaacttagc	gtttccacag/CTGGATAAAA
7	959	TACCAACAAG/gtacgtttcc	tgtcatccag/ACCCAGATTA
8	1080	ATCGCCAAAT/gtgagtggag	ggtcttttag/GCCAGCATAT
9	1248	ATCAACACTG/gtaggtacac	tgtcttccag/ATTTCCCCTT
10	1365	CCCCTGATTG/gtgagttaca	tttccccccag/GAATCGGCGT
11	1490	TTCACTCCAG/gtcaggactt	tgtgttgcag/ATCGCCCTGT

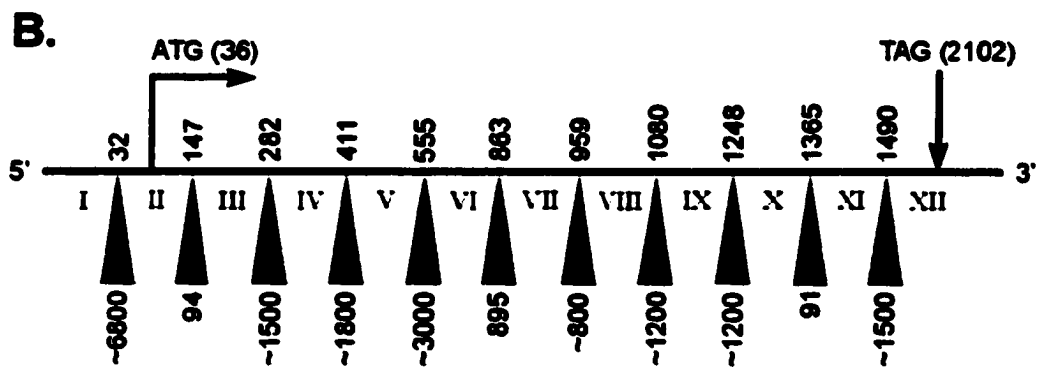

Figure 3-2. Human NaPi-IIb Gene Structure

Panel A. The locations of introns and donor/acceptor sites determined for the human NaPi-IIb gene are shown. The intron location is based on the nucleotide number in the human intestinal NaPi-IIb cDNA. Exonic sequences are in upper case letters and intronic sequences are in lowercase letters.

Panel B. Human NaPi-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 NaPi-IIb cDNA.

Figure 3-3. Transcription Initiation Site of the Human NaPi-IIb Gene. Mapping of the transcription initiation site of human NaPi-IIb gene is shown. mRNA from A549 and Caco-2 cells was hybridized with ^{32}P-labeled primer (nt 43-62 of the human NaPi-IIb cDNA), primer extension was performed and products were separated on 6% polyacylamide gels. A sequence ladder prepared with the same primer and a plasmid template was also loaded on the gel. The extended products were 119 bp and 106 bp upstream from the translation start site of human NaPi-IIb cDNA. Lane 1 shows the primer extension products from A549 cells and lane 2 shows the primer extension products from Caco-2 cells. The asterisks indicate alignment of the extension products with the DNA sequencing reaction.

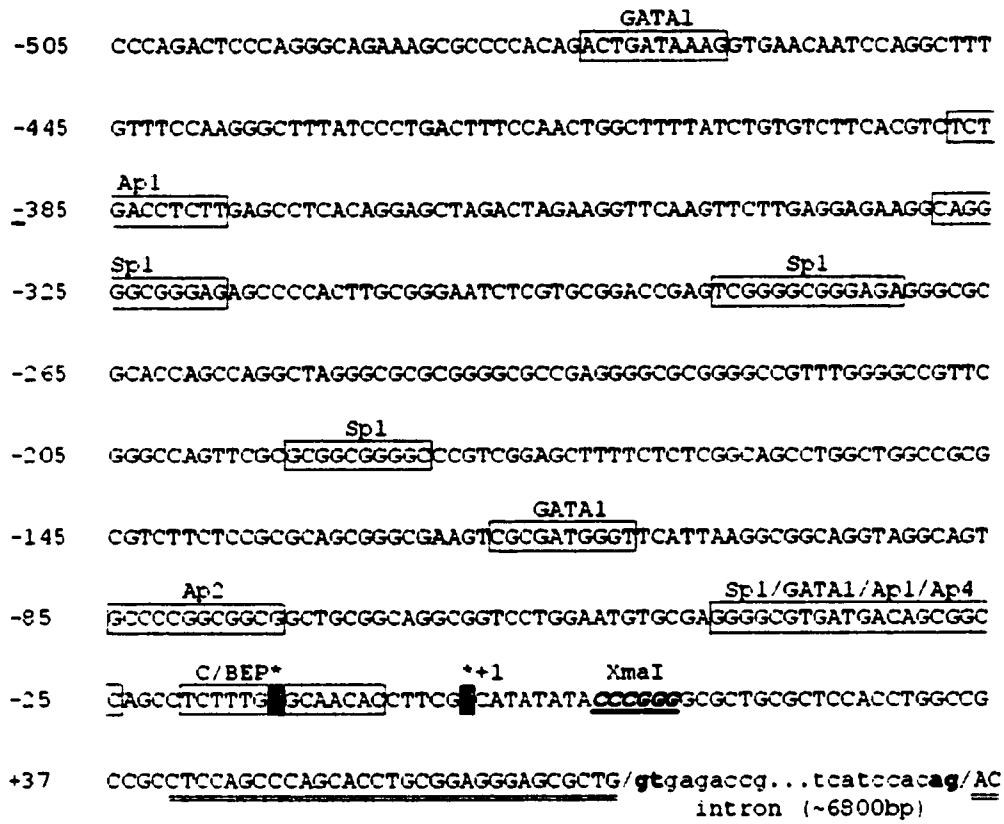

Figure 3-4. 5' Region of the Human NaPi-IIb Gene. Partial sequence of the 5' flanking region of human NaPi-IIb gene is shown. Nucleotide positions are numbered to the left with respect to the transcription initiation site at +1. Asterisks (*) and black boxes indicate the transcription initiation sites detected by primer extension. Open boxes indicate putative transcription factor recognition sequences. Double underlines show the previously identified human intestinal NaPi-IIb cDNA. Bold nucleotides in lower case indicate splice donor and acceptor sites, and slash marks indicate the splice sites. Only a few bases at both ends of the first intron (lower case) and 2 bases of exon II (upper case) are shown.

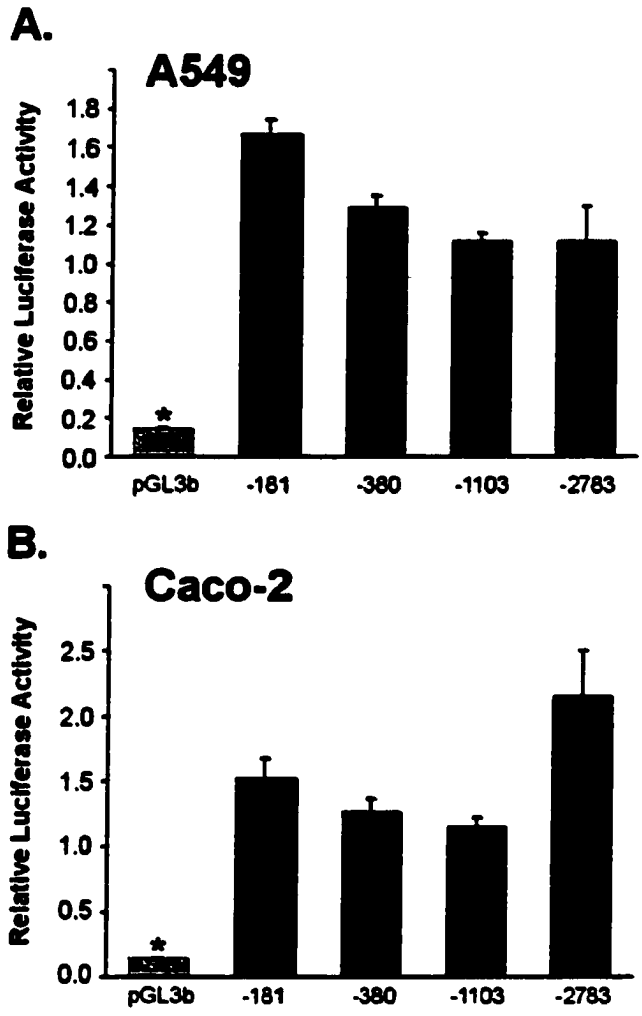

Figure 3-5. Expression of Human NaPi-IIb Gene Promoter Constructs in Transfected Cells. Cells were transfected with 0.5 μg pGL3 Basic (pGL3b), or 0.5 μg NaPi-IIb promoter constructs. Reporter gene assays were performed 48 hours after transfection. To control for transfection efficiency, cells were cotransfected with 30 ng pRL-CMV. Data are presented as relative luciferase activity (firefly luciferase activity driven by NaPi-IIb gene promoter over reneela luciferase activity driven by the CMV promoter). Results are mean ± S.E.M. * $p < 0.002$ vs. all promoter constructs; n=4-10.

CHAPTER 4

REGULATION OF THE HUMAN SODIUM-PHOSPHATE COTRANSPORTER GENE BY EPIDERMAL GROWTH FACTOR (EGF)[5, 6]

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 human intestinal Caco-2 cells. To understand the mechanism of this regulation, we studied the effect of EGF on NaPi-IIb gene transcription. The 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 is responsive to EGF treatment by a transcriptionally-mediated mechanism in intestinal cells.

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[5] Sequence data in this article has been deposited in GenBank with Accession number AF247725.

[6] Published as "Regulation of the human sodium-phosphate cotransporter NaPi-IIb gene promoter by epidermal growth factor" (*Am J Physiol* 280:C628-36, 2001)

Introduction

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 type II intestinal sodium-dependent phosphate cotransporter (NaPi-IIb) cDNAs were cloned recently (36, 56, 127), and the human NaPi cotransporter (hNaPi-IIb) gene (SCLA34A2) was mapped to human chromosome 4p15.1-15.3 (126, 127).

Intestinal sodium-dependent phosphate absorption occurs predominantly through the NaPi cotransport system, while renal sodium-dependent phosphate absorption is mainly mediated by another closely related isoform, NaPi-IIa. In both tissues, Pi absorption across the brush-border membrane through NaPi cotransporters is the rate-limiting step. Furthermore, Pi absorption is tightly regulated in order to maintain body Pi homeostasis. Many nutritional and endocrine factors regulate Pi absorption in the intestine and kidney. For instance, low phosphate diet stimulates renal NaPi-IIa (22, 91, 111, 113) and intestinal NaPi-IIb cotransporter activity (51). Also, vitamin D_3 and glucocorticoids affect renal and intestinal Pi absorption (26, 109).

An important physiological regulator of renal Pi absorption is epidermal growth factor (EGF), which acts through modulation of NaPi cotransporter activity (2, 4). EGF is a 53 amino acid polypeptide, which is secreted predominantly by salivary glands, with lower levels excreted by kidney and many other tissues. EGF receptors are expressed along the small intestinal tract (20), and EFG has broad effects on cell division, DNA

synthesis, tissue proliferation, cellular differentiation, electrolyte and nutrient absorption (88). More specifically, EGF was shown to inhibit the activity of several intestinal brush-border membrane proteins including sucrase, maltase, and alkaline phosphatase (74). Other studies showed that EGF inhibited renal Pi uptake by modulating NaPi-IIa cotransporter protein and mRNA levels (2, 4). Additionally, in some disease states, such as hyperphosphatemia induced by intestinal ischemia/injury, serum Pi levels are elevated (37, 60), and EGF production and utilization are increased (116). 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 studies, 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 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 (1μg/g body weight) [Austral Biological; San Ramon, CA] or saline, twice a day for three days. Fifteen hours after the last injection, rats were sacrificed, and jejunal mucosa was harvested and used for mRNA purification. All animal work has been approved by the University of Arizona Institutional Animal Care and Use Committee (IACUC).

Cell Culture: Human lung cells (A549) and human intestinal cells (Caco-2) were purchased from ATCC, and cultured according to ATCC guidelines. Cells were cultured at 37° C in a 95% air/ 5% CO_2 atmosphere and passaged every 72 hours. 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, using the Fast-Track 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 (127). This rat cDNA fragment was subcloned, and sequenced on both strands. 10 μg of mRNA was utilized for Northern blot analyses with rat or human NaPi-IIb cDNA-specific probes under high stringency washing conditions (25). 1B15 (encoding cyclophilin) was utilized as a constitutive probe (30). Blots were exposed to a phosphorimaging screeen and band intensities were determined with Quantity One software (FX Molecular Imager; Biorad, 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 previously described for cloning of the human intestinal NaPi-IIb cDNA (127). The primers used for detecting β-actin and the RT-PCR conditions were described previously (6). 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.

Construction of Reporter Plasmids: Luciferase reporter plasmids used in this study were derived from pGL3-Basic (Promega). A -1103/+15 promoter reporter construct (pGL3-1103) was made by subcloning a SacI/XmaI digested pZero-4.5 fragment into pGL3-Basic. A -181/+15 promoter reporter construct (pGL3-181) was made by digesting pGL3-1103 construct with SacI/ApaI, then blunting both ends with Klenow DNA polymerase and ligating them together. A -380/+15 promoter reporter construct (pGL3-380) was made by inserting a SacI/XmaI digested PCR product, amplified with forward primer 5'-**TATGAGCTC**TTGAGCCTCACAGGAG-3' at nt -361 to -380 (bold nucleotides represent SacI site and overhanging bases) and reverse primer 5'-**TATAGA**TCTCCGCAGGTGCTGGGCT-3' in intron 1 (bold nucleotides indicate overhanging bases originally inserted for BglII subcloning not used for this construct), into pGL3 Basic vector (note that the XmaI site is in exon I). A -2783/+15 promoter reporter construct (pGL3-2783) was made by inserting a KpnI/SacI digested fragment (~1.7kb) into the pGL3-1103 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-2783 to -2764) and reverse primer 5'-TTGTCTCCAATGTGCACACG-3' (nt -873 to -864), and cloned into the pTarget vector (Promega). In this strategy, SacI cut the human NaPi-IIb 5' flanking

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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. 0.5 μg promoter construct DNA, 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 Opti-MEM medium (Gibco/BRL) for 30 min at room temperature. The mixture was then added to the cells and they were incubated for 5 hours, followed by the addition of an equal volume of medium containing 20% fetal bovine serum (FBS). The next day, the medium was removed and replaced with standard medium with 10% FBS. After 24 hours, the cells were harvested for reporter gene assays. For EGF treatment, 100 ng/ml human recombinant EGF (Austral Biologicals; San Ramon, CA) was added for 8 hours before harvesting cells. To study the effect of actinomycin D on NaPi-IIb promoter activity, transiently transfected cells were pretreated with actinomycin D (5 μg/ml) for 2 hours prior to EGF treatment for 8 hours in the presence of actinomycin D. Promoter reporter assays were performed using the Dual Luciferase Assay Kit according to the manufacture's instruction (Promega).

Statistical Analysis: 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 NaPi-IIb mRNA Levels in Rat Jejunum: Northern blot analysis of mRNA isolated from rat jejunal mucosa is shown in Figure 4-1. Hybridization with a 757 bp rat intestinal NaPi-IIb cDNA probe clearly showed that NaPi-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 NaPi-IIb signal. Furthermore, the rat NaPi-IIb cDNA fragment (GenBank accession #AF247725) exhibits 93% nucleotide sequence homology with the mouse intestinal NaPi-IIb cDNA.

Effect of EGF Treatment on NaPi-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 radioaleblled, 757 bp human intestinal NaPi-IIb cDNA-specific probes showed no signal from untreated or treated cells. However, the β-actin hybridization signal was readily apparent (data not shown, n=3). These results suggested that the NaPi-IIb message was expressed below the Northern blot detection limits in Caco-2 cells under these culture conditions. Then, RT-PCR method was used to detect the NaPi-IIb gene expression in the cells. The expression of human intestinal NaPi-IIb mRNA in Caco-2 cells after exposure to standard or EGF containing medium, was assessed by semiquantitative RT-PCR using human intestinal NaPi-IIb and β-actin primers (Figure 4-2). Data showed that human NaPi-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, which showed a ~ 50% decrease in NaPi-IIb mRNA abundance in EGF treated rat intestine.

Effect of EGF on Human NaPi-IIb Gene Promoter Activity: To test the EGF effect on human NaPi-IIb gene promoter activity, A549 and Caco-2 cells were first transfected with promoter constructs, then treated with 100 ng/ml EGF before harvesting cells. Promoter activity was 40-50% decreased in Caco-2 cells after eight hours EGF treatment (n=4-10; $p<0.02$), but no change was seen in A549 cells (n=4-10) (Figure 4-3).

Effect of Actinomycin D on Human NaPi-IIb Gene Promoter Activity Induced by EGF: To determine if the EGF effect on human NaPi-IIb gene promoter activity is due to transcriptional regulation, Caco-2 cells were first transfected with promoter constructs, then treated with 5 μg/ml actinomycin D for two hours followed by treatment with 100 ng/ml EGF in the presence of actinomycin D. Results showed that the downregulation of NaPi-IIb promoter activity by EGF treatment in −1103 bp construct transfected cells was blocked by actinomycin D (n =4; $p<0.03$) (Figure 4-4). Furthermore, there was no effect of either EGF or EGF/actinomycin D treatment in pGL3b or the −118 bp construct transfected cells (n=4). Actinomycin D treatment did not affect the expression of reporter gene.

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 Pi level and EGF levels were increased with intestinal injury (37, 60, 116). Thus, EGF may play a role in regulation of Pi 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. Then, we 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.

While EGF is clearly involved in regulating renal Pi absorption, the role of EGF in modulating intestinal Pi absorption is unknown. In order to understand the relationship between EGF and intestinal Pi absorption, we choose human intestinal cells Caco-2 as the model to study the regulation of EGF on NaPi-Iib gene expression.

In vivo and *in vitro* studies showed that EGF treatment decreased intestinal NaPi-IIb mRNA abundance by about 50%, suggesting possible transcriptional regulation. In transient transfected Caco-2 cells, EGF also decreased NaPi-IIb gene promoter activity by about 40%. This promoter activity decrease could be abolished by 5 μg/ml 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 only observed 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 (-1103/+15 and -2783/+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 1103 bp upstream from 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 (38), the rat preprothyrotropin-release hormone gene (97), the rat prolactin gene (35) and the human gastrin gene (39, 44, 75). These EGF responsive elements (EREs) 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 (-1103 bp to –380 bp) for these known EGF response elements from these other genes, and we found two sequences (-792 bp GGGAAGG -786 bp and -479 bp GGGCGC -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.

Conclusion

In summary, EGF treatment decreased NaPi-IIb mRNA abundance in rat intestine and in human intestinal cells, and also reduced the NaPi-IIb gene promoter activity in transfected human intestinal cells. Actinomycin D treatment blocked the promoter activity decrease induced by EGF treatment, confirming transcriptional 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.

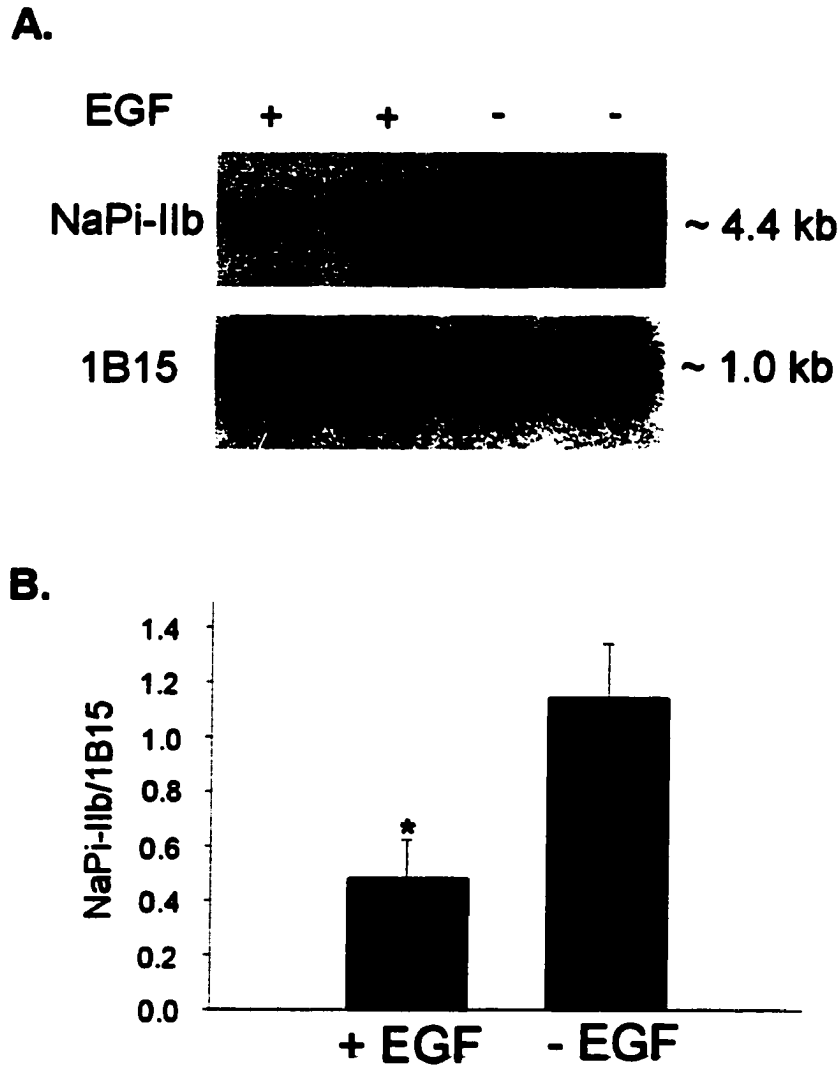

Figure 4-1. The Effect of EGF on Rat Intestinal NaPi-IIb mRNA Levels
Panel A. 10 μg mRNA isolated from rat jejunal mucosa was hybridized with rat NaPi-IIb cDNA and 1B15-specific probes. Blots were processed under high stringency conditions.
Panel B. Phosphorimage analysis of Northern blot data showed that EGF caused a 50% reduction of rat NaPi-IIb mRNA abundance. Results are mean ± S.E.M. *p=0.008, n=3

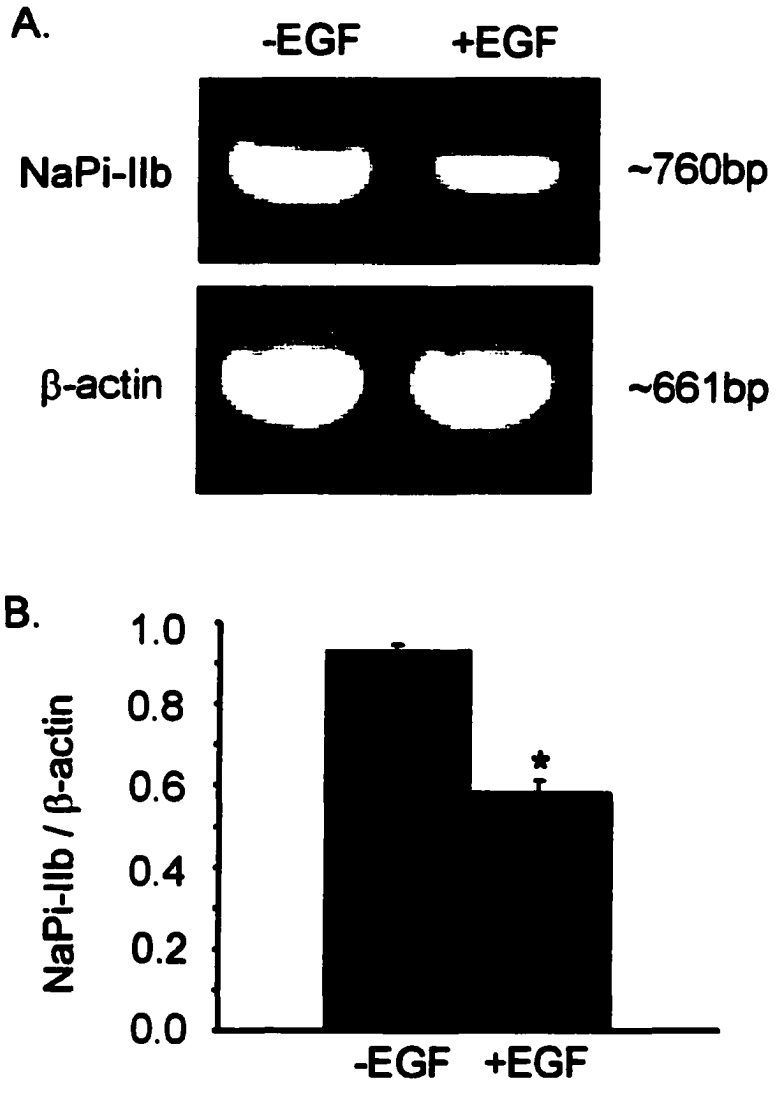

Figure 4-2. The Effect of EGF on NaPi-IIb mRNA Levels in Human Intestinal Cells

Panel A. mRNA isolated from Caco-2 cells grown in normal (-EGF) or EGF-containing (+EGF) medium was used for 1st-strand cDNA synthesis. Subsequent PCR was performed with human NaPi-IIb primers or β-actin primers in separate reactions. Equal volume of PCR products for NaPi-IIb and β-actin were loaded on the same gel and visualized with ethidium bromide.

Panel B. 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 mean ± S.E.M. *p=0.02, n=3.

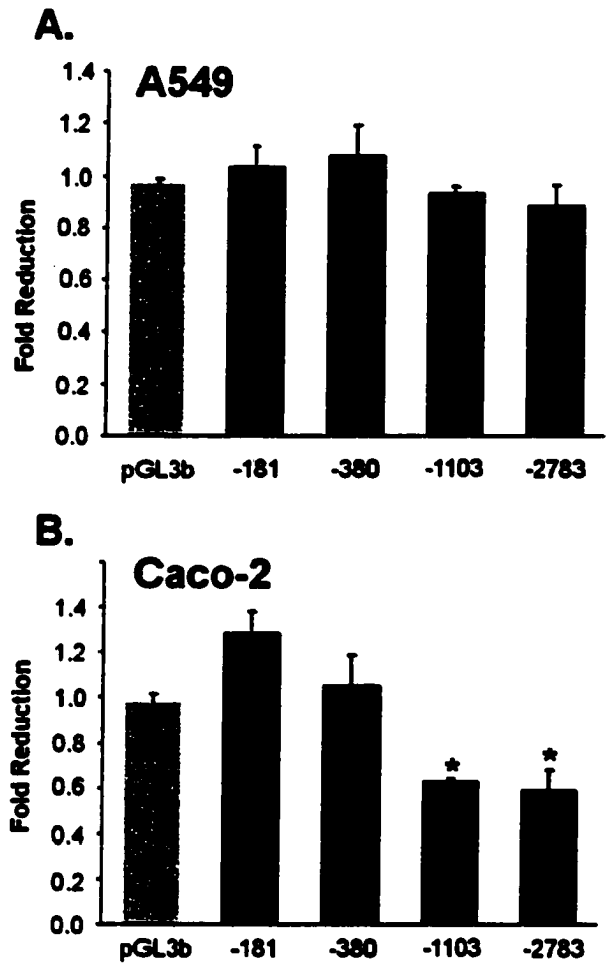

Figure 4-3. EGF Reduces Human NaPi-IIb Gene Promoter Activity in Transfected Cells.

Cells were cotransfected with 0.5 μg pGL3 Basic (pGL3b), or 0.5 μg NaPi-IIb promoter constructs. Reporter gene assays were performed 48 hours after transfection. To control for transfection efficiency, cells were cotransfected with 30 ng pRL-CMV. EGF was applied 8 hours 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 mean ± S.E.M._* $p < 0.02$ vs. pGL3b, n=4-10.

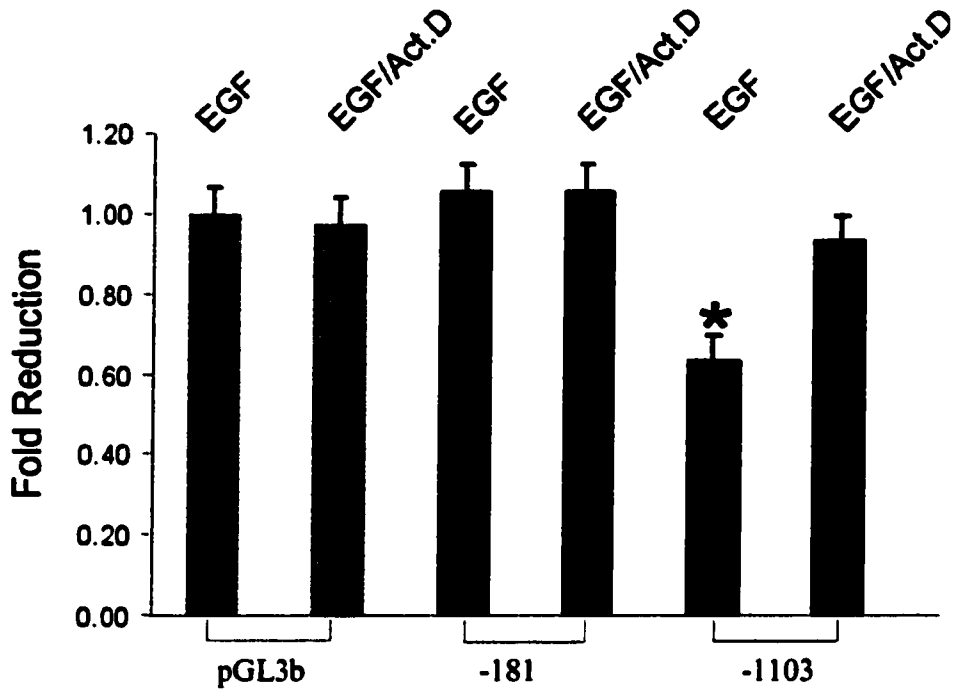

Figure 4-4. Effect of Actinomycin D on Human NaPi-IIb Gene Promoter Activity by EGF in Transfected Cells. Cells were cotransfected with pGL3 Basic (pGL3b) or promoter constructs plus pRL-CMV. Thirty-six hours after transfection, cells were treated with 5 μg/ml actinomycin D for 2 hours. Then EGF was applied in the presence of actinomycin D for 8 hours 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 mean ± S.E.M. * $p < 0.03$, n=4.

CHAPTER 5

REGULATION OF THE INTESTINAL SODIUM-PHOSPHATE COTRANSPORTER BY 1,25-$(OH)_2$ VITAMIN D_3[7]

1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2$ vitamin D_3) stimulates functional NaPi-IIb protein expression in adult rodent intestine, but the effect in younger animals is unclear. The current studies showed that NaPi-IIb mRNA expression decreases with age. 1,25-$(OH)_2$ vitamin D_3 treatment increased intestinal brush-border membrane vesicle (BBMV) Na/P_i absorption by ~2.5 in suckling rats and by ~2.1 fold in adult rats. 1,25-$(OH)_2$ vitamin D_3 treatment also increased NaPi-IIb mRNA abundance by ~ 2 fold in 14-day-old rats, but not in adults. In rat intestinal epithelial (RIE) cells, 1,25-$(OH)_2$ vitamin D_3 increased NaPi-IIb mRNA abundance, an effect that could be abolished by actinomycin D. Additionally, human NaPi-IIb promoter activity in transiently transfected RIE cells showed ~1.6-fold increase after 1,25-$(OH)_2$ vitamin D_3 treatment. In summary, we demonstrate that the age-related decrease in intestinal Na/P_i absorption correlates with decreased NaPi-IIb mRNA expression. Furthermore, 1,25-$(OH)_2$ vitamin D_3 stimulates intestinal Na/P_i absorption in adults and sucklings, but 1,25-$(OH)_2$ vitamin D_3-induced changes in NaPi-IIb mRNA expression are seen only in suckling rats. We conclude that the 1,25-$(OH)_2$ vitamin D_3 effect on NaPi-IIb gene expression is at least partially mediated by gene transcription.

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[7] Accepted as "Age-dependent regulation of rat intestinal sodium-phosphate cotransporter (NaPi-IIb) by 1,25$(OH)_2$ vitamin D_3" (*Am J Physiol. 2001*)

Introduction

Phosphate (P_i) plays important roles in growth, development, bone formation, acid-base regulation and cellular metabolism. The rate of growth is limited by the availability of P_i. A transport system capable of accumulating P_i against the electrochemical gradient is vital for normal development. The sodium-phosphate (NaPi) cotransporters are plasma membrane bound symporters that mediate the movement of extracellular P_i ions into cells coupled with Na^+ ions. There have been three families of sodium-phosphate cotransporters, called type I, type II and type III, identified from mammalian cells in recent years (81, 121, 122). These proteins play important roles in regulating phosphate absorption across cell membranes and in maintaining serum P_i levels.

The small intestine is an important site for phosphate absorption. Early studies showed that the transport of P_i through the apical membrane of small intestinal epithelial cells is coupled with sodium (11, 15, 26, 40, 58, 98, 104, 105). One transporter involved in intestinal P_i absorption is the type IIb sodium-coupled phosphate cotransporter (NaPi-IIb), which has been cloned from rodents and human (36, 50, 56, 127). P_i absorption is modulated by many physiological factors, including hormonal and dietary (8). Regarding hormonal regulation, glucocorticoids inhibit intestinal Na-dependent P_i (Na/P_i) absorption (13, 86), and EGF decreases intestinal Na/P_i absorption at least partially by inhibiting NaPi-IIb mRNA expression (128). Estrogen also plays a possible role in regulating intestinal P_i absorption (93). As a nutritional factor, phosphate content of the

diet also regulates intestinal P_i absorption. Furthermore, P_i deprivation stimulates intestinal Na-dependent P_i absorption process (18, 83, 95, 99, 107).

Vitamin D_3, a steroid hormone, plays a central role in modulating phosphate homeostasis and P_i uptake by the small intestine (5, 34). The active form of vitamin D_3 is 1,25-$(OH)_2$ vitamin D_3, which is mainly synthesized in kidney from 25-(OH) vitamin D_3. 1,25-$(OH)_2$ vitamin D_3 binds the vitamin D receptor (VDR), to elicit its effect on gene expression regulation. 1,25-$(OH)_2$ vitamin D_3 plays important roles in calcium and phosphate homeostasis, regulation of the parathyroid hormone system, inhibition of cell growth, and induction of cellular differentiation (17). Previous studies showed that 1,25-$(OH)_2$ vitamin D_3 increases intestinal P_i absorption through modulation of Na-dependent P_i absorption (28, 31, 32, 41, 42, 55, 61, 66, 72, 89, 90, 120). This increase is at least partially mediated by modulation of NaPi-IIb protein expression (51). However, there is lack of evidence demonstrating direct regulation of NaPi-IIb gene expression by 1,25-$(OH)_2$ vitamin D_3.

As reported in the current communication, we initially detected changes in NaPi-IIb gene expression in rats during post-natal development. We further show a significant increase in sodium-dependent phosphate uptake in intestinal brush-border membrane vesicles (BBMVs) in 1,25-$(OH)_2$ vitamin D_3 treated suckling and adult rats. We also demonstrate that NaPi-IIb mRNA abundance increases with 1,25-$(OH)_2$ vitamin D_3 treatment only in suckling rats. These results suggested a possible role for 1,25-$(OH)_2$ vitamin D_3 in transcriptional regulation of the NaPi-IIb gene in young animals. To further understand the role of 1,25-$(OH)_2$ vitamin D_3 in intestinal P_i absorption, we characterized

NaPi-IIb expression in rat intestinal epithelial (RIE) cells, and developed this cell line as an *in vitro* model to determine the molecular mechanism of gene regulation by 1,25-$(OH)_2$ vitamin D_3. These are the first studies which exemplify transcriptional regulation of NaPi-IIb gene expression by 1,25-$(OH)_2$ vitamin D_3.

Materials and methods

Animals: Sprague-Dawley rats of 2 weeks, 3 weeks, 6 weeks and 95-100 days of age were used for these studies. 2-week-old and adult (90-100days) rats were used for 1,25-$(OH)_2$ vitamin D_3 studies. Animals received subcutaneous injections of 1,25-$(OH)_2$ vitamin D_3 (6μg/kg body weight, one dose) [Sigma; St. Louis, MO] or vehicle [ethanol (1) / propylene glycol (4), v/v] alone. Sixteen hours after the injection, rats were sacrificed, and jejunal mucosa was harvested and used for mRNA and brush-border membrane vesicle (BBMV) purification. All animal work has been approved by the University of Arizona Institutional Animal Care and Use Committee (IACUC). The experiment was repeated 3 times with mRNA isolation from different groups of animals.

Cell Culture: Rat intestinal epithelial (RIE) cells were a gift from Dr. Raymond DuBois (Dept. of Medicine; Vanderbilt University, Nashville, Tennessee). RIE cells were cultured as previously described (129). Media and other reagents used for cell culture were purchased from Irvine Scientific (Irvine, CA). In 1,25-$(OH)_2$ vitamin D_3 treatment experiments, cells were incubated with 100 nM 1,25-$(OH)_2$ vitamin D_3 for 16 hours before harvesting cells. For transcriptional assays, cells were pretreated with actinomycin

D (100 nM) [Calbiochem-Novabiochem; San Diego, CA] for 2 hours and then treated with 100 nM 1,25-$(OH)_2$ vitamin D_3 for 16 hours in the presence of actinomycin D before harvesting cells.

RNA Purification and Northern Blot Analyses: mRNA was isolated from RIE cells and rat jejunal mucosa using the Fast-Track mRNA purification kit (Invitrogen; Carlsbad, CA). 10 μg of mRNA was utilized for Northern blot analyses with rat NaPi-IIb cDNA probes (128) under high stringency washing conditions as described previously (24). 1B15 [encoding rat cyclophilin] (30) cDNA specific probes were used as internal standards for quantitating NaPi-IIb gene expression. Blots were exposed to a phosphorimaging screen and band intensities were determined with Quantity One Software (FX Molecular Imager; Biorad; Hercules, CA). The experiment was repeated 3 times with mRNA isolation from different groups of animals.

Sodium-Dependent Phosphate Uptake Analysis in Brush-Border Membrane Vesicles: BBMVs were prepared from rat jejunal mucosa and sodium-dependent phosphate uptake was carried out as previously described (11, 47, 84). The contribution of sodium-dependent phosphate uptake was calculated by subtracting the sodium-independent uptake values observed in the absence of sodium from the uptake values in the presence of sodium. The experiment was repeated 3 times with BBMV preparation from different groups of animals.

PCR Analysis to Detect NaPi-IIb Expression in RIE Cells: mRNA was purified from RIE cells cultured in normal medium. RT-PCR conditions were identical to those described in a previous publication (128). The primers used to detect NaPi-IIb were designed from rat NaPi-IIb cDNA (GenBank accession #AF157026). The forward primer was at 1446 - 1465 bp (5'-AGCCCAGGCAACACATTGA-3'), and the reverse primer was at 1899 - 1917 bp (5'-ACACCATGCAGCAGACACG-3'). The expected amplification size from NaPi-IIb mRNA is 472 bp. The primers used to detect β-actin were purchased from Stratagene (Stratagene; La Jolla, CA). The size of the amplified product from the β-actin gene is 661 bp.

Semiquantitative RT-PCR Analysis of NaPi-IIb Gene Expression: mRNA was purified from RIE cells treated for 16 hours with vehicle (ethanol) or 1,25-$(OH)_2$ vitamin D_3 (100 nM). RT-PCR conditions were described previously (128). Subsaturation levels of cDNA templates that were needed to produce a dose-dependent amount of PCR product 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 amplification parameters.

Construction of Reporter Plasmids: Reporter plasmids used in this study were derived from pGL3-Basic (Promega), which contains the firefly luciferase reporter gene. The human NaPi-IIb promoter/reporter constructs pGL3/-2783bp, pGL3/-1103bp, pGL3/-181bp, were made by restriction enzyme digestion and PCR (128). The 3'-end of

all constructs ends at +15 bp of the human NaPi-IIb gene. All constructs were confirmed by sequencing on both strands.

Transient Transfection and Functional Promoter Analysis: RIE cells were cultured in 24-well plates. When cells reached 70-80% confluence, liposome-mediated transfection was performed as follows: 0.5 μg promoter construct DNA, 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 Opti-MEM medium (Gibco/BRL) for 30 min at room temperature. The mixture was then added to the cells and they were incubated for 5 hours, followed by the addition of an equal volume of DME medium containing 20% fetal bovine serum (FBS). The next day, the medium was removed and replaced with standard medium with 10% FBS. 24 hours later, cells were harvested for reporter gene assays. For 1,25-$(OH)_2$ vitamin D_3 treatment, 100 nM 1,25-$(OH)_2$ vitamin D_3 or vehicle (ethanol) was added for 16 hours before harvesting cells. Promoter reporter assays were performed using the Dual Luciferase Assay Kit according to the manufacturer's instructions (Promega).

Statistical Analysis: Student's T-test was used to compare values of the experimental data. *P* values of < 0.05 were considered significant.

Results

Effect of Age on NaPi-IIb Gene Expression in Rat Jejunum: Previous studies showed that apical sodium-dependent phosphate absorption decreases with age in rat (11), rabbit (14) and pig jejunum (100). To determine if NaPi-IIb gene expression follows the same pattern, we purified jejunal mRNA from 2w, 3w, 6w and adult rats, and quantitated NaPi-IIb mRNA abundance by Northern blot analyses (Figure 5-1). There were significant differences between ages on the expression level of NaPi-IIb mRNA ($p < 0.034$, $n = 3$). Data showed that the highest expression level was in 2-week-old-rats, and expression level gradually decreased 3-4 fold into adulthood.

Effect of 1,25-$(OH)_2$ Vitamin D_3 Treatment on BBMV Phosphate Absorption in Rat Jejunum: 2-week-old and adult rats were treated with 1,25-$(OH)_2$ vitamin D_3, BBMVs were purified from jejunum, and sodium-dependent phosphate absorption was measured by a membrane filtration method. Sodium-dependent phosphate uptake (in nmol P_i / mg protein / 10 second) in suckling rats was significantly higher than in adult rats (94.6 ± 4.7 in 2-week-old rats vs. 30.4 ± 2.3 in adult rats). Vitamin D_3 treatment increased sodium-dependent phosphate absorption in both suckling and adult rats (94.6 ± 4.7 for control vs. 232.5 ± 45.3 for treated in 2-week-old rats, 30.4 ± 2.3 for control vs. 67.2 ± 16.3 for treated in adult rats), with the fold inductions being similar (Fig. 5-2).

Effect of 1,25-$(OH)_2$ Vitamin D_3 Treatment on NaPi-IIb mRNA Levels in Rat Jejunum: 2-week-old-rats and adult rats were treated with 1,25-$(OH)_2$ vitamin D_3, mRNA

was purified from jejunal mucosa and Northern blots were performed with rat NaPi-IIb cDNA probes. Hybridization patterns clearly showed that intestinal NaPi-IIb mRNA abundance increased ~2 fold in 1,25-$(OH)_2$ vitamin D_3 treated 2-week-old-rats, but no change was detected in adult rats (Figure 5-3). Also, there was no change in intestinal 1B15 mRNA abundance with vehicle or 1,25-$(OH)_2$ vitamin D_3 treatment.

1,25-$(OH)_2$ Vitamin D_3 Treatment Increases NaPi-IIb mRNA Abundance in RIE cells: Preliminary results from RT-PCR indicated that RIE cells endogenously express the NaPi-IIb gene (data not shown). Subsequently, NaPi-IIb mRNA expression in RIE cells, after exposure to vehicle or 1,25-$(OH)_2$ vitamin D_3, was assessed by semiquantitative RT-PCR using rat NaPi-IIb and β-actin primers. Data showed that NaPi-IIb gene expression was increased by approximately 2 fold in 1,25-$(OH)_2$ vitamin D_3 treated RIE cells compared with untreated cells (Figure 5-4).

Actinomycin D Treatment Blocks the NaPi-IIb mRNA Increase Induced by 1,25-$(OH)_2$ Vitamin D_3 Treatment in RIE Cells: To test whether the effect of 1,25-$(OH)_2$ vitamin D_3 on NaPi-IIb gene expression is due to transcriptional regulation, RIE cells were first treated with actinomycin D, and then treated with 1,25-$(OH)_2$ vitamin D_3 in the presence of actinomycin D before harvesting cells. NaPi-IIb mRNA abundance was determined by semiquantitative RT-PCR using rat NaPi-IIb and β-actin primers. Results showed that the increase in NaPi-IIb mRNA abundance induced by 1,25-$(OH)_2$ vitamin D_3 treatment was abolished by actinomycin D treatment (Figure 5-5).

Human NaPi-IIb Gene Promoter Analysis in RIE Cells: To determine whether the 5' flanking region of the human NaPi-IIb gene contains a functional promoter in RIE cells, three constructs (pGL3/-2783bp, pGL3/-1103bp, and pGL3/-181bp) were transfected by lipofectamin into RIE cells (129). Promoter reporter gene assays were performed 48 hours after transfection. The promoter assay data showed that all promoter constructs were functional in RIE cells (Figure 5-6A).

To test the effect of 1,25-$(OH)_2$ vitamin D_3 on human NaPi-IIb gene promoter activity, RIE cells were first transfected with promoter constructs, then treated with 100 nM 1,25-$(OH)_2$ vitamin D_3 or vehicle for 16 hours before harvesting cells. 1,25-$(OH)_2$ vitamin D_3 treatment of transfected RIE cells did not affect the activity of the internal control construct, renilla luciferase driven by the CMV promoter. The data showed that human NaPi-IIb promoter activity increased ~1.6 fold with the pGL3/-2783 and pGL3/-1103 constructs in 1,25-$(OH)_2$ vitamin D_3 treated RIE cells, compared with control cells (Figure 5-6B). The pGL3/-181 construct showed no effect with 1,25-$(OH)_2$ vitamin D_3 treatment.

Discussion

Phosphate plays an important role in many physiological processes, such as skeletal development, and cellular metabolism. 1,25-$(OH)_2$ vitamin D_3 is a central regulator of calcium and phosphate homeostatic processes. Many previous studies showed that intestinal sodium-dependent phosphate absorption can be enhanced by 1,25-$(OH)_2$ vitamin D_3 treatment (27, 42, 55, 61, 62, 66, 89). Recent studies showed that 1,25-

$(OH)_2$ vitamin D_3 increases intestinal P_i absorption by increasing NaPi-IIb protein expression in adult mice (51), and type III NaPi cotransporter mRNA expression in adult rats (63), although the type III NaPi cotransporter is most unlikely involved in intestinal P_i absorption. Both studies concluded that NaPi-IIb gene expression in adult animals was not directly regulated by 1,25-$(OH)_2$ vitamin D_3. In the present study, we demonstrate that NaPi-IIb gene expression in suckling rats is highest, and it decreases into adulthood. 1,25-$(OH)_2$ vitamin D_3 treatment increases NaPi-IIb mRNA abundance only in suckling rats, but not in adults as previously documented. To decipher the molecular mechanism of vitamin D_3 regulation on NaPi-IIb gene expression, we developed the RIE cell-line as an *in vitro* model. Then, we characterized human NaPi-IIb gene promoter activity in transiently transfected RIE cells and studied 1,25-$(OH)_2$ vitamin D_3 regulation of the NaPi-IIb promoter in RIE cells. Our data suggest for the first time that 1,25-$(OH)_2$ vitamin D3 increases NaPi-IIb mRNA abundance and activity partially through a gene transcription-mediated mechanism in young animals.

Earlier studies indicated that intestinal sodium-dependent phosphate absorption declined with age in several mammalian species (11, 14, 100). These observations suggested that the expression of the transport protein(s), which is responsible for sodium-dependent phosphate absorption likely decreases with age. Our data demonstrate that NaPi-IIb gene expression decreases with age, and this observation correlates well with the functional studies. Thus, it seems likely that NaPi-IIb expression contributes to the ontogenic changes seen in intestinal P_i absorption.

Studies also showed that 1,25-$(OH)_2$ vitamin D_3 treatment stimulates intestinal sodium-dependent P_i absorption (27, 28, 31, 32, 41, 42, 55, 61, 66, 72, 89, 90, 120). More recently, two groups showed that the stimulation of intestinal P_i absorption by 1,25-$(OH)_2$ vitamin D_3 in adult rodents is not mediated by increases in NaPi-IIb gene expression (51, 63). Our result showed that 1,25-$(OH)_2$ vitamin D_3 treatment increased intestinal sodium-dependent phosphate uptake in adult rats, but not NaPi-IIb mRNA expression, which is comparable to these studies. The increase in Pi uptake is most likely due to increased apical NaPi-IIb protein expression in adult animals (51). Our data also showed that 1,25-$(OH)_2$ vitamin D_3 treatment increased intestinal sodium-dependent phosphate absorption and NaPi-IIb mRNA expression in suckling rats, which suggests that the effect of 1,25-$(OH)_2$ vitamin D_3 on NaPi-IIb gene expression is age specific.

In order to decipher the molecular mechanism of 1,25-$(OH)_2$ vitamin D_3 regulation of intestinal NaPi-IIb gene expression, we developed the rat intestinal epithelial (RIE) cell line as an *in vitro* model. RIE cells were originally isolated from rat small intestinal tissue and are epithelium-derived cells (10). Our results demonstrate that the NaPi-IIb gene is endogenously expressed in RIE cells, and that it is 1,25-$(OH)_2$ vitamin D_3 responsive. Furthermore, we performed NaPi cotransport studies in RIE cells with 1,25-$(OH)_2$ vitamin D_3 treatment, and found that activity was increased by about 25% and was blockable by actinomycin D treatment (data not shown). However, these data are difficult to interpret due to the fact that RIE cells likely contain other endogenous NaPi cotransporters including ubiquitously expressed type III NaPi cotransporters and possible other unidentified NaPi cotransporters. It is further possible that this other NaPi

cotransporter(s) may also be regulated by 1,25-$(OH)_2$ vitamin D_3 (as has been shown for the type III NaPi cotransporters (63)) and thus it is extremely difficult to assess the single contribution of NaPi-IIb cotransporters. Moreover, we could not selectively study the activity of NaPi-IIb in RIE cells, as no specific inhibitors are available at this time. Our intention was simply to demonstrate that RIE cells are a good *in vitro* model to study NaPi-IIb gene regulation by 1,25-$(OH)_2$ vitamin D_3, as exemplified by the facts that the cells endogenously express this gene and that the gene is 1,25-$(OH)_2$ vitamin D_3 responsive. However, these data suggest that other *in vitro* models would have to be developed to study post-transcriptional regulation of the NaPi-IIb gene.

In vivo studies in suckling rats and *in vitro* studies in RIE cells showed that 1,25-$(OH)_2$ vitamin D_3 treatment increases NaPi-IIb mRNA abundance by ~2 fold. Therefore, transcriptional regulation seems likely. Further studies showed that activation of NaPi-IIb gene expression by 1,25-$(OH)_2$ vitamin D_3 in RIE cells could be abolished by 100 nM actinomycin D, a transcriptional inhibitor. These results suggest that the increase in NaPi-IIb mRNA abundance induced by 1,25-$(OH)_2$ vitamin D_3 likely involves synthesis of new NaPi-IIb mRNA. Furthermore, transfection studies with human NaPi-IIb promoter constructs showed that 1,25-$(OH)_2$ vitamin D_3 increased NaPi-IIb gene promoter activity by ~2 fold in transiently transfected RIE cells. When considered together, these data indicate that the effect of 1,25-$(OH)_2$ vitamin D_3 on intestinal NaPi-IIb gene expression can be mediated by control of transcriptional initiation.

Transfection of cells with three NaPi-IIb gene promoter constructs (pGL3/-2783bp, pGL3/-1103bp, and pGL3/-181bp) resulted in significant reporter gene

expression. This finding suggests that the basal promoter region of NaPi-IIb gene is located within -181bp region in RIE cells, as was previously described in Caco-2 cells (57). Interestingly, the promoter construct pGL3/-2783bp showed lower activity in tranfected RIE cells, compared with transfected CaCo-2 cells (128). Furthermore, the two longer promoter constructs (pGL3/-2783bp and pGL3/-1103bp) were responsive to 1,25-$(OH)_2$ vitamin D_3 treatment, but the smaller one (pGL3/-181bp) was unresponsive. This observation suggests that the putative 1,25-$(OH)_2$ vitamin D_3 response element(s) is located between 181 and 1103 bp upstream of the transcriptional initiation site.

Vitamin D_3 responsive elements (VDRE) have been identified from many genes, including the human renal NaPi-IIa (NaPi-3) gene (108), the rat osteocalcin (OSC) gene, the mouse osteopontin (MOP) gene, the rat calbindin D-9k (CaBP) gene, and the human parathyroid hormone (PTH) gene (33, 102). By searching the human NaPi-IIb gene promoter region from -181 bp to -1103 bp for putative VDREs, no classical VDR binding sequences were identified. This may then classify the human NaPi-IIb gene into a group of genes which are responsive to 1,25-$(OH)_2$ vitamin D_3 treatment, but do not have classical VDRE sequences in their promoter regions (16, 102, 110). This data may also suggest that there might be a novel VDRE present in this gene, or alternatively, the 1,25-$(OH)_2$ vitamin D_3 response could be mediated by a trans-acting factor that acts independently of the VDR.

Conclusion

In summary, we showed that the decrease in sodium-dependent P_i absorption during development correlated with a decrease in NaPi-IIb gene expression in the intestinal mucosa. We also demonstrated that 1,25-$(OH)_2$ vitamin D_3 treatment increased NaPi-IIb mRNA abundance in suckling rats and RIE cells, and also increased NaPi-IIb gene promoter activity in transfected RIE cells. Since actinomycin D treatment blocked 1,25-$(OH)_2$ vitamin D_3-induced increases in NaPi-IIb mRNA expression in RIE cells, we hypothesize that a transcriptional mechanism is likely involved. Further studies will focus on identification of the responsive region in the promoter, and the trans-acting factors involved in regulation of the NaPi-IIb gene by 1,25-$(OH)_2$ vitamin D_3.

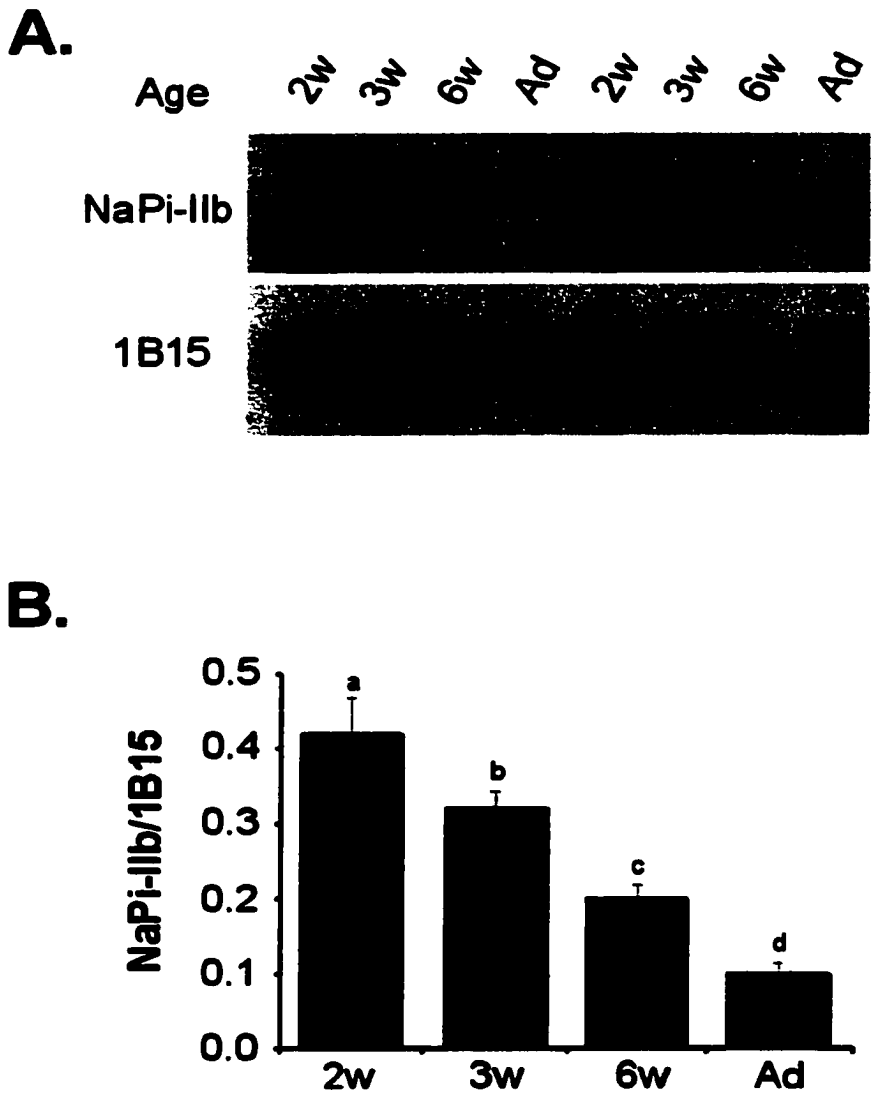


Figure 5-1: Expression of the Intestinal NaPi-IIb Gene in Different Aged Rats
Panel A. 10 μg mRNA isolated from rat jejunal mucosa was hybridized with rat NaPi-IIb cDNA and 1B15 probes. Blots were processed under high stringency conditions. NaPi-IIb probes recognize a hybridization signal at ~4.4 kb, and 1B15 probes recognize a hybridization signal at ~1.0 kb. Two of three experiments are shown.
Panel B. Phosphorimage analysis of Northern blot data showed that the abundance of rat NaPi-IIb mRNA decreases with age. Results are mean ± S.E.M. from three separate experiments. Different letters (a, b, c) indicate statistical significance at $p < 0.034$ between different ages.

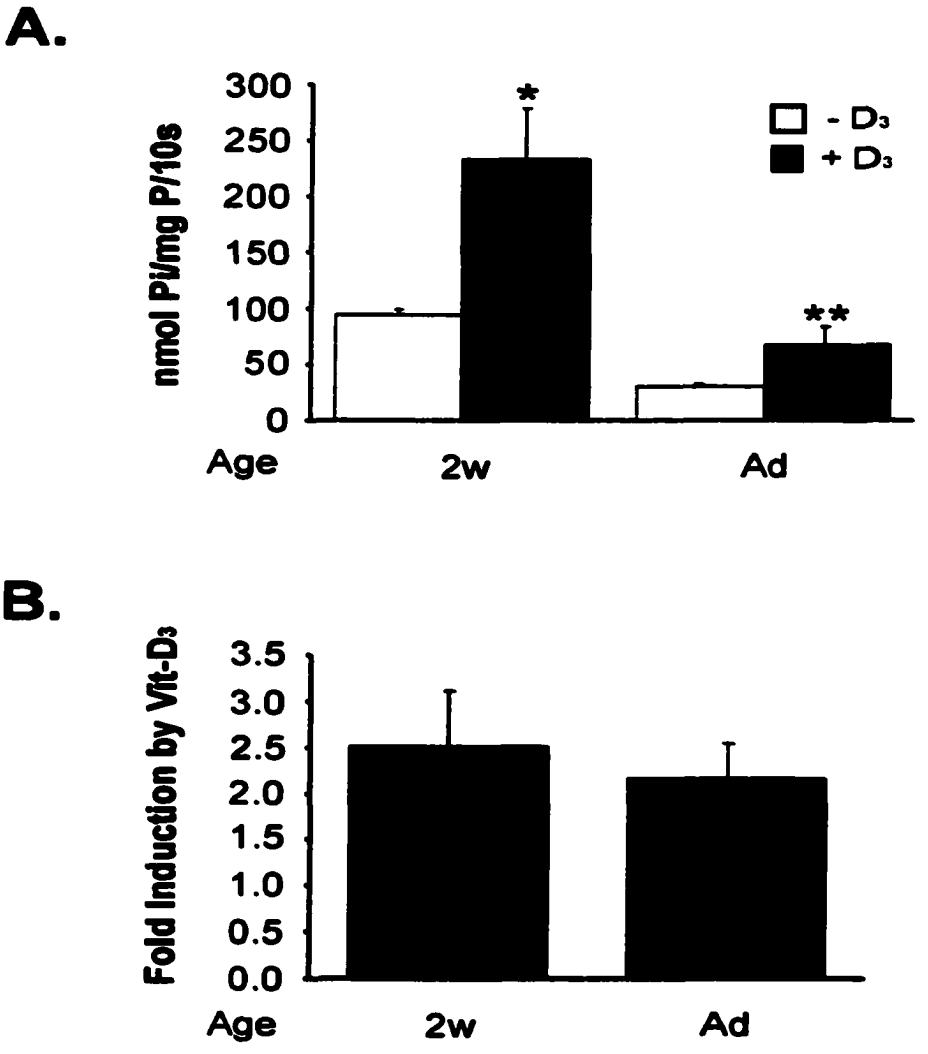


Figure 5-2: The Effect of 1,25-$(OH)_2$ Vitamin D_3 on Rat Intestinal Sodium-Dependent Phosphate Absorption

Panel A. Sodium-dependent phosphate uptake analysis of BBMVs isolated from rat jejunal mucosa treated with vehicle or 1,25-$(OH)_2$ vitamin D_3. Sodium-dependent phosphate uptake was measured in the presence of 100 mM sodium. Results are mean + S.E.M. from three separate experiments. * and ** indicate statistical significance at $p < 0.02$ for vehicle treatment vs. 1,25-$(OH)_2$ vitamin D_3 treatment in that age group.

Panel B. Fold induction of sodium-dependent phosphate uptake induced by 1,25-$(OH)_2$ vitamin D_3 at each age is depicted graphically. Results are mean + S.E.M. from three separate experiments.

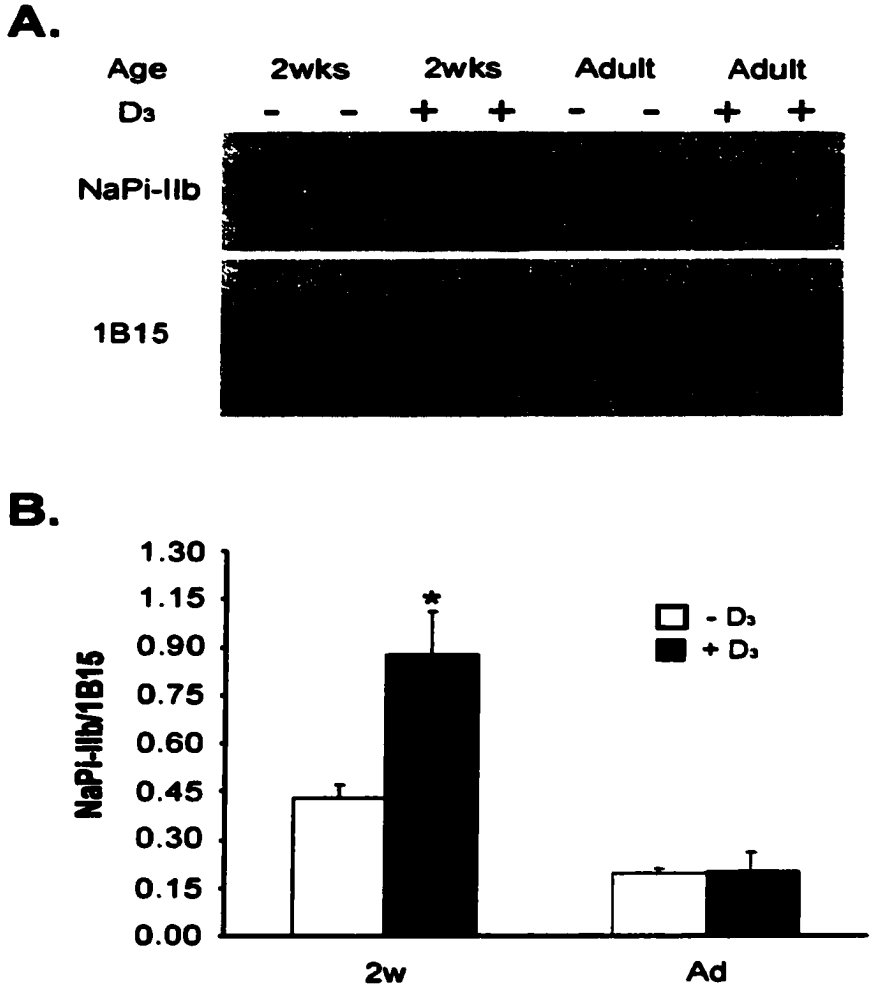


Figure 5-3: The Effect of 1,25-$(OH)_2$ Vitamin D_3 on Rat Intestinal NaPi-IIb mRNA Expression

Panel A. 10 μg mRNA isolated from rat jejunal mucosa were hybridized with rat NaPi-IIb cDNA and 1B15 cDNA probes. Blots were processed under high stringency conditions. NaPi-IIb probes recognize a hybridization signal at ~4.4 kb, and 1B15 probes recognize a hybridization signal at ~1.0 kb. Two of three experiments are shown.

Panel B. Phosphorimage analysis of Northern blot data showed that 1,25-$(OH)_2$ vitamin D_3 treatment caused ~2 fold induction of rat intestinal NaPi-IIb mRNA abundance in suckling animals. Results are mean ± S.E.M. from three separate experiments. *$p < 0.02$ for vehicle treatment vs. 1,25-$(OH)_2$ vitamin D_3 treatment in 2w old rats.

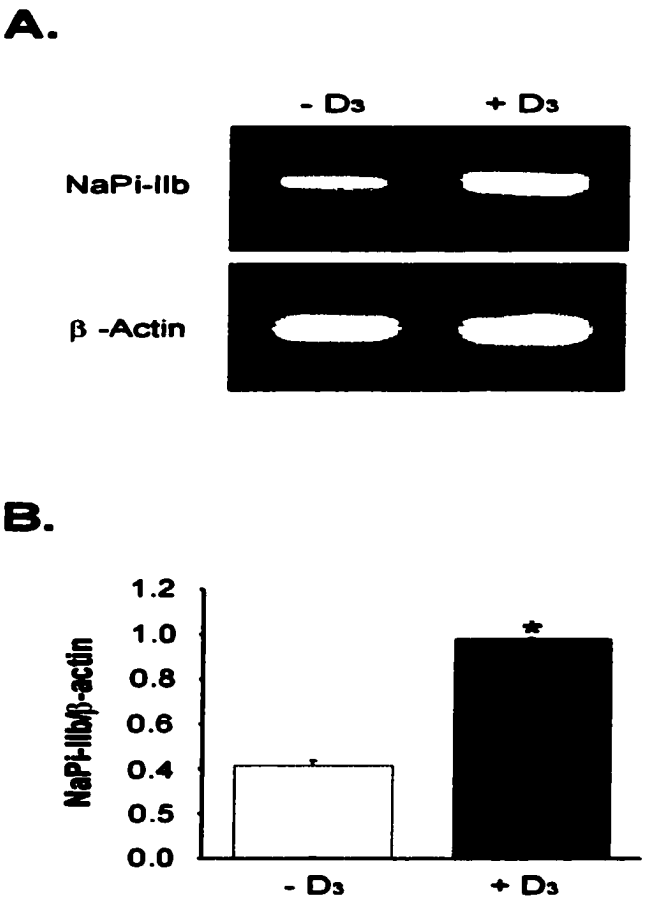


Figure 5-4: The Effect of 1,25-$(OH)_2$ Vitamin D_3 on NaPi-IIb mRNA Levels in Rat Intestinal Epithelial (RIE) Cells

Panel A. mRNA isolated from RIE cells grown in normal ($-D_3$) or 1,25-$(OH)_2$ vitamin D_3-containing ($+D_3$) medium was used for 1st-strand cDNA synthesis. Subsequent PCR was performed with rat NaPi-IIb or β-actin primers in separate reactions. Equal volume of NaPi-IIb and β-actin PCR reactions were loaded on the same gel and visualized with ethidium bromide.

Panel B. Optical density analysis of RT-PCR results in RIE cells. Data are presented as a ratio of NaPi-IIb to β-actin band intensities. Results are mean + S.E.M. from four separate experiments.

*$p < 0.03$ for vehicle treatment vs. 1,25-$(OH)_2$ vitamin D_3 treatment.

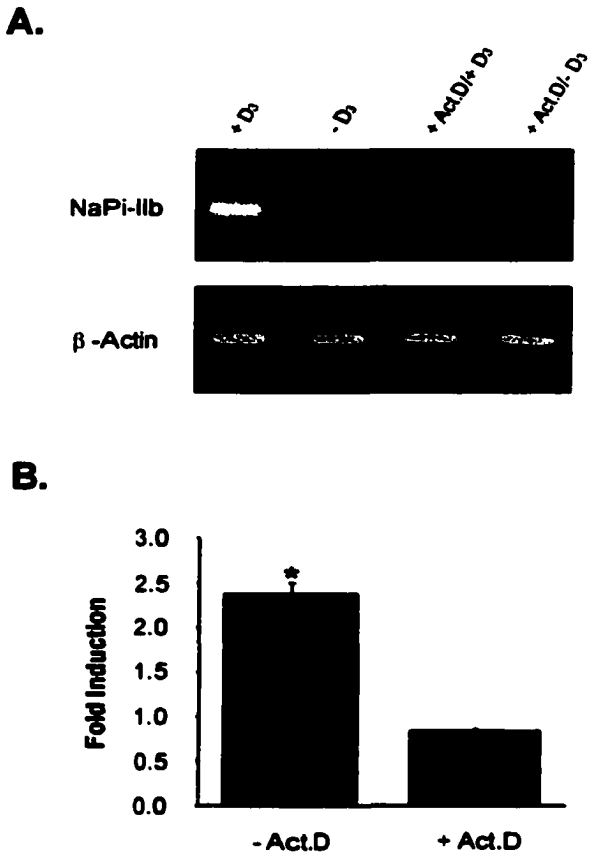


Figure 5-5: The Effect of Actinomycin D on NaPi-IIb mRNA Expression in 1,25-$(OH)_2$ Vitamin D_3 Treated RIE Cells.

Panel A. mRNA isolated from RIE cells treated under different conditions was used for first-strand cDNA synthesis. Subsequent PCR was performed with subsaturation levels of the RT reaction, and NaPi-IIb or β-actin primers were used in separate reactions. Equal volumes of PCR reactions for NaPi-IIb and β-actin were loaded on the same gel and visualized with ethidium bromide.

Panel B. Fold induction in NaPi-IIb mRNA expression induced by 1,25-$(OH)_2$ vitamin D_3 treatment in RIE cells in the presence or absence of actinomycin D. Data is calculated by comparing the ratio of NaPi-IIb mRNA/β-actin mRNA in 1,25-$(OH)_2$ vitamin D_3 treated cells over the ratio of NaPi-IIb mRNA/β-actin mRNA in vehicle treated cells. Results are mean $\pm$ S.E.M. from four separate experiments. * $p < 0.02$ for absence of actinomycin D vs. presence of actinomycin D.

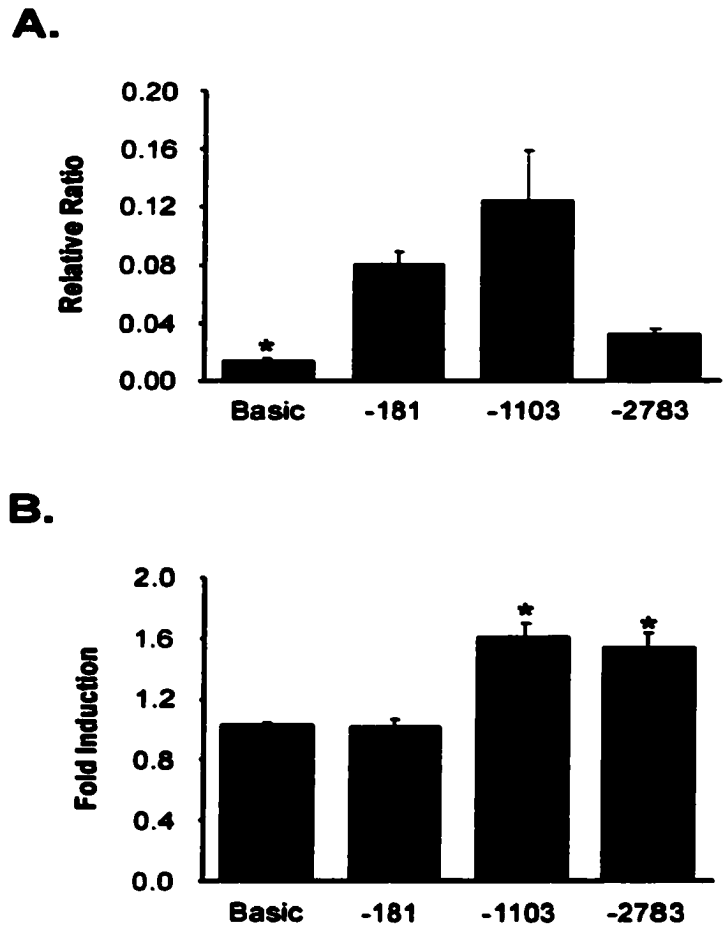


Figure 5-6: Activity of Human NaPi-IIb Gene Promoter Constructs in Transfected RIE Cells

Panel A. RIE cells were transiently transfected with 0.5 μg pGL3 Basic (pGL3b), or 0.5 μg human NaPi-IIb promoter constructs. To control for transfection efficiency, cells were cotransfected with 30 ng pRL-CMV. Reporter gene assays were performed 48 hours after transfection. Data are presented as relative luciferase activity (firefly luciferase activity driven by the human NaPi-IIb gene promoter over renilla luciferase activity driven by the CMV promoter). Results are mean ± S.E.M. from ten separate experiments. *$p < 0.003$, for pGL3b vs. other constructs.

Panel B. RIE cells were cotransfected with pGL3 Basic (pGL3b) or human NaPi-IIb promoter constructs plus pRL-CMV. 1,25-$(OH)_2$ vitamin D_3 was applied 16 hours before harvesting cells. Fold induction is shown as the ratio of luciferase activity in 1,25-$(OH)_2$ vitamin D_3 treated cells over luciferase activity in untreated cells. Results are mean ± S.E.M. from ten separate experiments. *$p < 0.0004$ for pGL3/-1103 and pGL3/-2783 vs. pGL3b and pGL3/-181.

CHAPTER 6

CONCLUSION

Phosphate plays a critical role in the body. Phosphate is absorbed in the intestine and reabsorbed in the kidney, and therefore these organs are the important sites to regulate phosphate homeostasis. Phosphate reabsorption in kidney has been studied for decades, and the molecular mechanism has been identified and well studied. Phosphate absorption in intestine has been known for long time, but the molecular mechanism remained unclear until late 1998.

The purpose of this dissertation research was to explore the molecular mechanism of phosphate absorption in intestine. From this work, a novel sodium-dependent phosphate (NaPi) cotransporter was identified and the regulation of gene expression was also studied.

This research identified an intestinal sodium-phosphate cotransporter (NaPi-IIb) from human small intestine, which is different from the one (NaPi-IIa) identified from the human kidney. The human NaPi-IIb cDNA encodes a 689 amino acid polypeptide, which functions as a sodium-dependent phosphate cotransporter. Tissue distribution analysis showed that the human NaPi-IIb gene is highly expressed in lung, small intestine, kidney and several glands. The chromosomal localization study indicated that the human NaPi-IIb gene was localized on the human chromosome $4p^{15.1\text{-}15.3}$. It should be mentioned that no other genes have been identified around this region, and no disease is linked to this region at this moment.

This human NaPi-IIb gene contains 12 exons and 11 introns. Two transcriptional initiation sites were identified in this human NaPi-IIb gene with a primer extension method. These transcriptional initiation sites are located upstream of the translation start site 106bp and 110bp position. About 2.8kb of the 5'- flanking region of the human NaPi-IIb gene was subcloned and confirmed to be a functional promoter. The human NaPi-IIb gene promoter does not contain a TATA-box at its proximal promoter region. Instead, it has several predicted GATA1 binding sites at that region. These GATA binding sites might be involved in tissue-specific gene expression regulation.

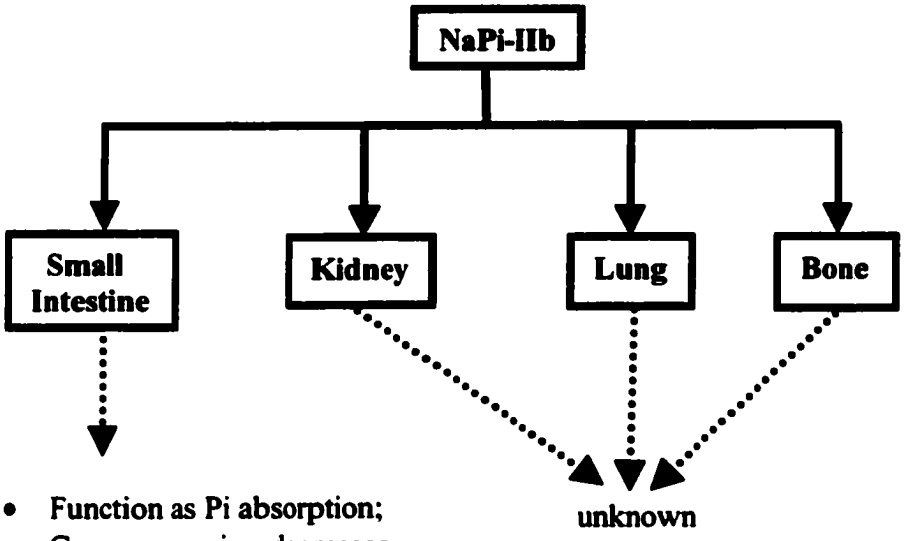
Epidermal Growth Factor (EGF) has been shown to inhibit renal phosphate absorption through inhibiting NaPi-IIa cotransporter expression. However, the effect of EGF on intestinal NaPi-IIb gene expression is not known. This study showed that EGF reduced the NaPi-IIb mRNA abundance by ~50% in suckling (16 days old) rats, as well as in human intestinal Caco-2 cells. EGF also inhibits the human NaPi-IIb gene promoter activity in transient transfected Caco-2 cells. This inhibition could be abolished by actinomycin D. These data suggest that the reduction induced by EGF treatment on the NaPi-IIb mRNA abundance is partially due to the reduced NaPi-IIb gene promoter activity. This finding has significant impact on phosphate homeostatic regulation under pathophysiological conditions, such as hyperphosphotemia induced by intestinal ischemia/injury and kidney damage. In hyperphosphotemia, the serum phosphate level rises, and EGF secretion increases. The physiological importance of EGF release at this point is to repair injured tissue, and suppress phosphate absorption in intestine and

phosphate reabsorption in kidney to bring the serum phosphate level back to normal. It also suggests the potential use of EGF on the treatment of hyperphosphatemia.

It is a well known fact that 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2$ vitamin D_3) stimulates intestinal phosphate absorption. Recent studies showed that 1,25-$(OH)_2$ vitamin D_3 stimulates functional NaPi-IIb protein expression in adult rodent intestine, but the effect in younger animals is unclear. This investigation showed that NaPi-IIb mRNA expression decreases with age. 1,25-$(OH)_2$ vitamin D_3 treatment increased intestinal brush-border membrane vesicle (BBMV) sodium-dependent phosphate absorption in both suckling and adult rats. 1,25-$(OH)_2$ vitamin D_3 treatment also increased NaPi-IIb mRNA abundance by ~ 2 fold in 14-day-old rats, but not in adults. In rat intestinal epithelial (RIE) cells, 1,25-$(OH)_2$ vitamin D_3 increased NaPi-IIb mRNA abundance, an effect that could be abolished by actinomycin D. Additionally, human NaPi-IIb promoter activity in transiently transfected RIE cells showed ~1.6-fold increase after 1,25-$(OH)_2$ vitamin D_3 treatment. From this study, it is concluded that the 1,25-$(OH)_2$ vitamin D_3 effect on NaPi-IIb gene expression is at least partially mediated by gene transcription in young animals. This finding has a significant physiological and pathophysiological impact that could lead to dietary and therapeutic changes in normal healthy children and in children with rickets. For example, dietary supplements of 1,25-$(OH)_2$ vitamin D_3 should benefit the large requirement of Pi in normal rapidly growing children, and could have a significant therapeutic effect in children with rickets to improve their Pi absorption.

Continuing studies immediately after this dissertation will mainly focus on: 1) the mechanisms that underline the regulation of NaPi-IIb by EGF; 2) the mechanisms underline the regulation of NaPi-IIb by vitamin D_3.

As the human NaPi-IIb gene is a newly identified gene, there are many studies that can be pursued in this area. 1) Studies on regulation of the gene, including basal expression regulation in intestinal cells, and tissue-specific expression regulation in cells from different tissues. These studies will give insight on which transcriptional factor(s) is (are) involved in the initiation of tissue-specific gene transcription. 2) Studies of age-related gene expression regulation in animals. This study will explore the effect of aging on gene expression and might lead to new treatments for the improvement of Pi uptake in older adults. 3) Study the roles of this gene in the lung by gene knockout techniques. This study will explore the importance of this gene in the functional development and maintenance of the normal lung. 4) Study the effects of hormones (estrogen, progesterone, testosterone) on NaPi-IIb gene expression. These studies may help to understand the role of this gene on the development of osteoporosis in older adults and therefore find information for treatment of bone loss. 6) Study the functional regulation of this gene. This study will explore the potential inhibitors for Pi uptake via NaPi-IIb transporter, which will lead to the development of new drugs used for hyperphosphatemia.



- Function as Pi absorption;
- Gene expression decreases with age;
- EGF down-regulates its expression in young animals;
- Vitamin D_3 up-regulates its expression in young animals.

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