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Gene structure and functional analysis of the human Na^{+}/phosphate co-transporter

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Three λ phage clones encompassing the Na^{+}/phosphate co-transporter (NaPi-3) gene and its 5′ flanking region were isolated from a human genomic DNA library. The gene comprises 13 exons and 12 introns and spans approx. 14 kb. All exon–intron junctions conform to the GT/AG rule. The major transcription-initiation site was determined by primer-extension analysis and is an adenosine residue 57 bp upstream of the 3′ end of the first exon. There is a typical TATA box 28 bp upstream of the major transcription-initiation site and various *cis*-acting elements, including a cAMP-responsive element, AP-1, AP-2 and SP-1 sites in the 5′ flanking region. This region also contains three direct-repeat-like sequences that resemble the consensus binding sequence for members of the steroid–thyroid hormone receptor superfamily, including vitamin D. Deletion analysis suggests that the region from nt −2409 to nt −1259 in the 5′ flanking region may be involved in kidney-specific gene expression. Vitamin D responsiveness of the NaPi-3 promoter was also detected in COS-7 cells co-transfected with a human vitamin D receptor expression vector. The presence of the three vitamin D receptor-responsive elements in the NaPi-3 promoter may be important in mediating the enhanced expression of the gene by 1,25-dihydroxyvitamin D_3.

INTRODUCTION

Reabsorption of P_i in the kidney is mediated largely by a Na^{+}-dependent phosphate (Na^{+}/P_i) co-transporter in the brush-border membrane of the proximal tubule, and is regulated by a variety of hormones, such as parathyroid hormone, 1α,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$], growth hormone, and dietary P_i [1,2]. Recently, cDNAs corresponding to kidney brush-border membrane Na^{+}/P_i co-transporters in rabbit (NaPi-1 [3] and NaPi-6 [4]), rat (RNaPi-1 [5] and NaPi-2 [6]), human (NaPi-3 [6] and NPT-1 [7,8]), mouse (NaPi-7 [9] and Npt-1 [10]) and opossum kidney (NaPi-4 [11]) have been identified. The encoded proteins have been divided into two types of renal apical Na^{+}/P_i co-transporters on the basis of their predicted amino acid sequence [12,13]: type I, represented by NaPi-1, RNaPi-1, NPT-1 and Npt-1; and type II, represented by NaPi-2, NaPi-3, NaPi-4, NaPi-6 and NaPi-7. Type-II transporter mRNA is increased by dietary P_i depletion [4,14,15], while the level of type-I transporter mRNA remains unchanged in these conditions [4]. Thus type-II transporters are hypothesized to play an important role in maintenance of P_i homoeostasis in the kidney.

Defective reabsorption of P_i in the proximal tubule is responsible for X-linked hypophosphataemia (XLH) as well as the autosomally hereditary hypophosphataemic rickets with hypercalciuria (HHRH) and hypophosphataemic bone disease [16]. Decreased levels of type-II transporter mRNA have recently been found to be associated with XLH [17]. Furthermore, the PEX (phosphate-regulating gene with homologies to endopeptidases, on the X chromosome) gene has been identified as a candidate gene for XLH [18]. However, it remains unclear how a mutation in PEX causes a decrease in type-II transporter.

In order to understand better the genetic basis of both these diseases as well as the regulation of renal P_i transporter gene expression, we have characterized the exon–intron organization and 5′ flanking region of the NaPi-3 gene. In addition, we have studied the mechanisms of transcription of the 5′ flanking region of the gene.

A portion of this work was presented at the 17th annual meeting of the American Society for Bone and Mineral Research in Baltimore, MD, U.S.A., September 9–13, 1995 [19].

MATERIALS AND METHODS

Preparation of DNA probes for screening

To isolate genomic DNA encoding NaPi-3, we synthesized oligonucleotiode primers specific for the NaPi-3 cDNA sequence described by Magagnin et al. [6]. The sequences of the upstream and downstream primers were 5′-CTCATAGTGGGTGCCC-AGGATG-3′ (nucleotide position −19 to +3, relative to the translation-start site) and 5′-GAGGTGCTGGAGCTCTGCA-CCAG-3′ (nucleotide position +475 to +497) respectively. Human genomic DNA was prepared from leucocytes from a normal donor as described [20] and was subjected to PCR amplification with the two primers and *Taq* DNA polymerase (Takara, Kyoto, Japan). The PCR product was subcloned into pBluescript II SK(+) (Stratagene, LaJolla, CA, U.S.A.) using the pGEM-T Vector system (Promega, Madison, WI, U.S.A.). The plasmid was digested with *Pst*I and *Apa*I, and the released DNA fragment was labelled with [α-^{32}P]dCTP (110 TBq/mmol)(ICN) by the Megaprime DNA labelling system (Amersham International, Amersham, Bucks., U.K.).

Abbreviations used: XLH, X-linked hypophosphataemia; NaPi-3, human type-II Na^{+}-dependent phosphate co-transporter; 1,25-$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; VDRE, vitamin D-responsive element; VDR, vitamin D receptor; RACE, rapid amplification of cDNA ends; DMEM, Dulbecco's modified Eagle's medium; HHRH, hereditary hypophosphataemic rickets with hypercalciuria.

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The nucleotide sequence of the 5′ flanking region of the NaPi-3 gene has been deposited in the EMBL/GenBank/DDBJ Nucleotide Sequence Databases under the accession number D89927.

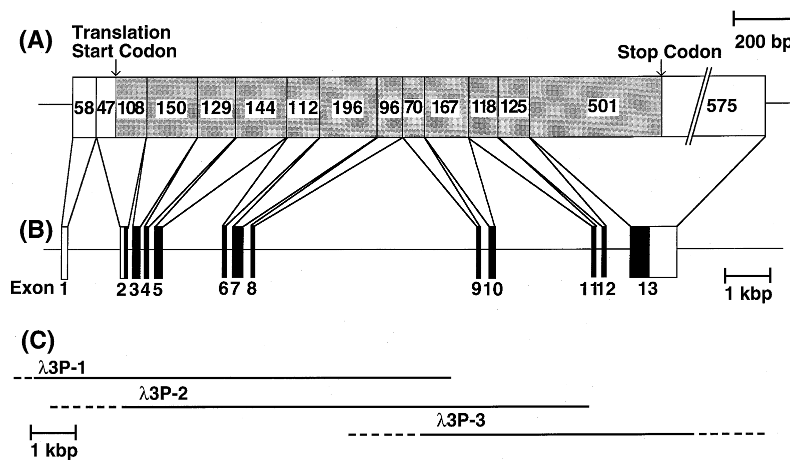

Figure 1 Organization of the human renal Na^+/P_i co-transporter (NaPi-3) gene

(**A**) The filled portions of the exons identify the protein-coding sequences, and the size of each exon is indicated in bp. The translation-initiation site is present in exon 2. (**B**) Genomic organization of the human NaPi-3 gene. The horizontal line indicates gene introns, and exons are shown by boxes. Open boxes indicate the non-coding region of the mRNA, and closed boxes designate coding regions. (**C**) The localization of the genomic clones λ3P-1, λ3P-2 and λ3P-3 is indicated.

Isolation of the 5′ promoter region of the NaPi-3 gene

We screened a genomic DNA library (Clontech, Palo Alto, CA, U.S.A.) constructed in λEMBL3 from fragments of human placental DNA generated by *Sau*3AI digestion. Plaques (1×10^8) were transferred to a nitrocellulose membrane (Hybond-C extra; Amersham), and hybridization and washing were performed as described previously [21]. Positive clones were purified, and phage DNA was extracted from large-scale liquid cultures. The human DNA fragments were excised by *Bam*HI or *Sac*I digestion and subcloned into pBluescript II SK(+). Sequence analysis was performed with a T7 DNA polymerase sequence kit (Pharmacia, Uppsala, Sweden) for manual sequencing or the SequiTherm sequence kit for autosequencing (LI-COR, Lincoln, NE, U.S.A.).

Primer-extension analysis

Primer-extension analysis was carried out essentially as previously reported [21]. The oligonucleotide 5′-CTTAGAATTC-TGTGTTTCAGCTTC-3′, complementary to bases −73 to −47 (located at the 3′ end of exon 1, counted from the translation-start codon) of the NaPi-3 gene, was labelled at its 5′ terminus with [γ-^{32}P]ATP (167 TBq/mmol) (ICN) by T4 polynucleotide kinase (New England Biolabs, Beverly, MA, U.S.A.). The labelled primer (100000 c.p.m.) was hybridized to total RNA (10 μg) from human kidney cortex at 42 °C for 1 h in a reaction mixture (20 μl) containing 10 mM Tris/HCl, pH 8.3, 250 mM KCl and 1 mM EDTA. Reverse transcription was performed for 1 h at 37 °C with 10 units of reverse transcriptase (RAV-2; Takara), in a reaction mixture (50 μl) containing 75 mM KCl, 10 mM $MgCl_2$, 20 mM Tris/HCl, pH 8.3, 10 mM dithiothreitol and 0.25 mM dNTPs. The products were subjected to electrophoresis in 8% polyacrylamide gels containing urea, and the sequence was determined using the same primer.

5′ Rapid amplification of the NaPi-3 cDNA

The 5′ rapid amplification of cDNA ends (RACE) ready cDNA kit was purchased from Clontech. The 5′ non-coding region was amplified by PCR with the R1 primer (5′-GTACATATTGTC-GTTAGAACGCG-3′; corresponding to the anchor region of the cDNA library) and the Pi-1 primer (5′-CAGCCAGTT-AAAGCAGTCATGCAC-3′; corresponding to +665 to +689 relative to the translation-initiation site) [6]. To amplify the target DNA specifically, a second PCR was performed on the first amplified product with the R2 primer (5′-TAATACG-ACTCACTATAGGGAGA-3′; corresponding to the anchor region of the 5′-RACE ready cDNA library), and either the Pi-2 primer (5′-GAGGTGCTGGAGCTCTGCACCAG-3′; corresponding to +476 to +498 relative to the translation-initiation site) or the Pi-3 primer (5′-TCCTGGGCACCCACTATGAGG-3′; corresponding to −20 to +1) [6]. PCR products were subcloned into the pBluescript II SK(+) vector using the TA-cloning method [22]. DNA sequencing was performed as described above.

Reporter plasmid construction

A 2462 bp *Bam*HI–*Eco*RI DNA fragment (nucleotide position −2409 to +53, relative to the transcription-initiation site) containing the 5′ flanking region of the NaPi-3 gene was subcloned into pBluescript II SK(+). A *Bam*HI–*Hin*dIII fragment from the resulting plasmid was then subcloned upstream of the coding region of the luciferase gene in the pGL-2 vector (Promega) to generate the reporter plasmid p3P2400. The p3P600 (nt−617 to +53) and p3P200 (nt−200 to +53) plasmids were cleared from p3P2400 by digestion with appropriate restriction enzymes. The p3P1260 (nt−1259 to +53) plasmid was a deletion mutant generated by digestion with exonuclease III. The internal control vector pCMV-β (Clontech), which expresses β-galactosidase, was used to normalize for luciferase activity. The pGL-2 control vector containing the simian virus 40 (SV40) promoter was used as a positive control in the luciferase assay. Each plasmid was purified with a QIAGEN plasmid kit (QIAGEN).

Cell culture and transient transfection

COS-7 and HeLa cells (Riken Cell Bank, Tokyo, Japan) were cultured at 37 °C under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) (Gibco–BRL) with 10% (v/v) fetal bovine

Table 1 Exon–intron structure of the human Na^{+}/P_{i} co-transporter (NaPi-3) gene

Nucleotide sequences at the intron (lower-case letters) and exon (upper-case letters) boundaries are shown. Numbers shown above the nucleotide sequence are counted from the first ATG codon in the cDNA, and the deduced amino acids are indicated below the sequence. Intron sizes are indicated in parentheses. The sequences that agree with GT/AG consensus at the 5′- and 3′-splice junctions are indicated by bold letters. The putative sequences surrounding the lariat branch site for the RNA-splicing reaction are underlined.

Intron number	Exon	Intron (size)	Exon
1	−47 AAT TCT AAG	(bp) **gt**gagcccaggac·(1.3 k) . . . agtgtcccggacacagctattgtcattc**ag**	CGT TGC TGA
2	+109 AGC CCT CAG G S P Q V	**gt**aagtgctgctc·(136) . . . . ctggggctcctgaccaggctccctccct**ag**	TC CTA CAC AGG L H R
3	+259 CAG AAG CCA G Q K P E	**gt**gggcctgggct·(84) . . . . . cccactatgctcatggcttcccccatcc**ag**	AG TCC AGG CTG S R L
4	+388 CTG GCT GGA G L A G G	**gt**agggcccgggt·(73) . . . . . ccttggacaacgctggctcatgctcccc**ag**	GG AAG GTG GCT K V A
5	+532 TCC TCT GGC T S S G L	**gt**gagttggccca·(1.3 k) . . . aattcattaggacgtcttctcttctacc**ag**	TG CTG GAG GTG L E V
6	+694 GAC TTC CGG CG D F R R	**gt**gagggggctg·(120) . . . . cctgccttgcaatgtggcctccctgccc**ag**	G GCC TTC GCG A F A
7	+840 ATC ATC CAG I I Q	**gt**gacagcaggg·(87) . . . ccttcactcccccctgcccacatcttgcccac**ag**	CTG GAC GAG L D E
8	+936 TCC TTA CAG S L Q	**gt**gagtcccaggc·(5.1 k) . . . tttcactaagtcaccctcctcctgatct**ag**	GCT CCC ACC A P T
9	+1006 ATG GAG AAA T M E K C	**gt**aagtgcctgca·(264) . . . . gtcagctgtcaggagctccaccccctgc**ag**	GC AAC CAC ATC N H I
10	+1174 ATC AAT ACG G I N T D	**gt**gagctgcga·(2.2 k) ctctgaccagcctgctgggatgcggtttccttgc**ag**	AC TTC CCT GCC F P A
11	+1291 CCA CTC ATC G P L I G	**gt**gagtgcccatg·(100) . . . . gggtcccacttcctctccctctgtcccc**ag**	GT CTT GGT GTG L G V
12	+1416 GCT TTC CAG A F Q	**gt**gcgctgggagt·(708) . . . . atctcagcccctctgcctcatcccctgc**ag**	ATT GCC CTC I A L
	Consensus:	**gt** . . . . **ag**	

serum (Equitech-Bio). OK cells (American Type Cell Collection: CRL1840) were maintained in F12/DMEM (1:1, v/v) (Gibco–BRL) containing 10% fetal bovine serum. Cells were transfected using the lipofectamine reagent (Life Technologies) with 0.5 μg of the NaPi-3 gene promoter–luciferase reporter plasmid, and 0.5 μg of pCMV-β per 5×10^{5} cells as described previously [23]. After transfection, cells were incubated under standard conditions for 36 h. In experiments where the human vitamin D receptor (VDR) was co-expressed in COS-7 cells, an expression vector was constructed by subcloning an *Eco*RI DNA fragment containing the full-length receptor cDNA into pcDL-SRα-296 [24] (kindly provided by N. Arai, DNAX, CA, U.S.A.). After transfection, cells were incubated with DMEM containing 10% charcoal-treated fetal bovine serum for 24 h and then incubated with the medium containing 1,25-$(OH)_2D_3$ (kindly provided by Chugai Pharmaceutical Co., Tokyo, Japan) or ethanol as control. Cells were then harvested in cell lysis buffer and the lysate was assayed for luciferase activity, β-galactosidase activity and protein concentrations [23].

RESULTS

Characterization of the nucleotide sequence and splice junctions of the NaPi-3 gene

We screened approx. 2.0×10^{6} plaques from a human genomic DNA library and detected three positive clones. These clones were purified and are referred to as λ3P-1, λ3P-2 and λ3P-3. DNA from the three clones was further analysed by restriction enzyme mapping, partial sequence analysis and Southern-blot hybridization. Sequence data were obtained by analysing the DNA inserts of all clones, and PCR products were obtained using primers constructed on the basis of the cDNA sequence. Intron sizes were estimated by sequencing and restriction enzyme digestion of PCR products. The NaPi-3 gene was found to be organized into 13 exons that span approx. 14 kb (Figure 1). The translation-initiation codon is located in exon 2. Sequences around the exon–intron junctions conformed to the GT/AG rule [25] (Table 1). Introns also contained the consensus sequence for RNA splicing, including an adenosine residue which could

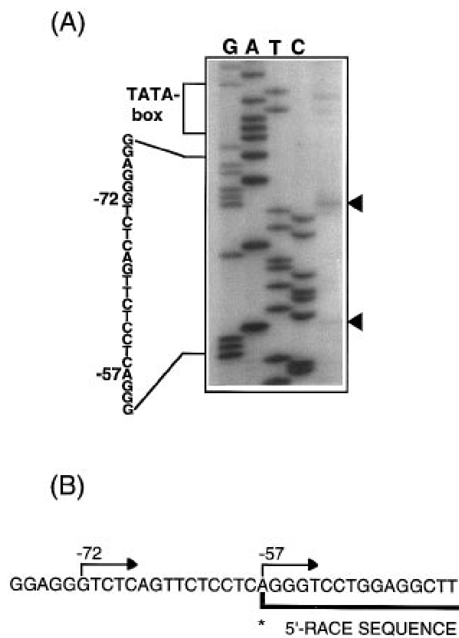

Figure 2 Primer-extension analysis and 5′-RACE with human kidney RNA

(**A**) Primer-extension analysis. A ^{32}P-labelled NaPi-3 antisense oligonucleotide primer was hybridized to 10 μg of total RNA from the human kidney cortex. The products were analysed by urea–agarose gel electrophoresis with reference to sequence ladders obtained with the same oligonucleotide primer. Bands in the far right lane corresponding to transcription initiation at nucleotides −72 and −57 relative to the 3′ end of exon 1 are indicated (by arrowheads). A TATA box upstream of this position is also indicated. (**B**) The sequence of the 5′-region of the 5′-RACE product. The 5′-RACE procedure was performed as described in the Materials and methods section.

function as a potential lariat acceptor site 8 to 36 bases upstream of the 3′ splice junctions [26].

Transcription-initiation site and nucleotide sequence of the 5′ flanking region

The transcription-initiation site of the NaPi-3 gene was determined by primer-extension analysis and by the 5′-RACE method. A ^{32}P-labelled antisense oligonucleotide based on the NaPi-3 cDNA sequence was hybridized to total RNA of human kidney cortex and extended by reverse transcriptase. We detected two transcripts starting at the adenosine and guanosine nucleotides at −57 and −72 relative to the 3′ end of exon 1 respectively (Figure 2A), by comparison with sequence ladders obtained with the same primer. The major transcription-initiation site was tentatively identified as the adenosine residue at position −57, because the TATA box is 28 bp upstream of this site and is generally located approx. 25–35 bp upstream of the transcription-initiation site in many genes [25]. We confirmed that this residue was indeed the major transcription-initiation site by the 5′-RACE method. The results from sequence analysis of PCR products demonstrated that the 5′-end nucleotide of the mRNA is the adenosine residue, at −57 bp upstream of the 3′ end of exon 1 (Figure 2B). This result was consistent with data obtained by primer extension.

The sequence of the 5′ flanking region of the NaPi-3 gene is shown in Figure 3. A typical TATA box is present 28 bp upstream of the transcription-initiation site as described above. Several well-characterized *cis*-acting elements, including a cAMP-responsive element at position −777 to −773, SP-1 sites at positions −2184 to −2179 and −195 to −190, and AP-1 sites at positions −2345 to −2339 and −875 to −867, were detected in the 5′ flanking region. Furthermore three direct-repeat-like sequences similar to consensus binding sites for members of the steroid–thyroid hormone receptor superfamily were found in this region [27].

Structures of putative vitamin D-response elements (VDREs) in the NaPi-3 promoter

Table 2 lists the VDREs in genes whose message levels are known to be sensitive to 1,25-$(OH)_2D_3$. The VDRE sequence bears strong resemblance to that of other steroid-responsive element half-palindromes, particularly those sequences lying proximal to a hypothetical 3 bp gap region [27]. The first such sequence in the NaPi-3 promoter (named DR-A), 5′-**GGGGGA** CA **GAGGGA**-3′ (positions −83 to −70), has a two-nucleotide spacer, therefore it resembles a retinoid X receptor element. The second sequence in the promoter (named DR-B), 5′-**GGGGAC** CCT **GGGAAC**-3′ (positions −244 to −230), has a three-nucleotide spacer and is similar to a VDRE. The third sequence (named DR-C), 5′-**GGGCAG** CAA **GGGCAG**-3′ (positions −1976 to −1962), is a perfect direct repeat with a three-nucleotide spacer and is also similar to a VDRE.

Transcriptional activity of the 5′ flanking region in OK cells

Reporter constructs were made in which the 5′ upstream sequence of the NaPi-3 gene was fused to a luciferase reporter gene, and transfected into OK cells (kidney epithelial cells of opossum), COS-7 cells (fibroblasts from monkey kidney) or HeLa cells (cervix carcinoma). When transfected into OK cells, a construct containing sequences −2409 to +53 from the NaPi-3 gene (p3P2400) was able to direct the highest luciferase expression. Progressive deletion to positions −1259, −617 and −200 resulted in decreases in activity ($P < 0.05$). In particular, deletion from −2409 to −1259 resulted in much reduction of luciferase activity, suggesting the loss of positive regulatory elements in this region in OK cells. In contrast, luciferase activity driven from the NaPi-3 promoter was not significantly increased in HeLa and COS-7 cells, as compared with the pGL-2 basic vector (Figure 4).

Transactivation by VDR and 1,25-$(OH)_2D_3$

To study the potential transcriptional activity of the NaPi-3 promoter in response to 1,25-$(OH)_2D_3$, the reporter gene construct containing the region −2409 to +53 was utilized (p3P2400). When co-transfected with the reporter vector and VDR expression vector into COS-7 cells, the increase in luciferase activity was approx. 14-fold higher than with activation of p3P2400 alone. In the presence of 0.5 nM and 50 nM 1,25-$(OH)_2D_3$, the promoter activity was increased 1.5-fold and 2.5-fold respectively (Table 3). Thus the NaPi-3 gene promoter was responsive to VDR and 1,25-$(OH)_2D_3$.

DISCUSSION

In this paper, we isolated the human NaPi-3 gene and characterized its genomic structure. Hartmann et al. [28] also reported on the exon–intron junction of the NaPi-3 gene. Our study demonstrating the exon–intron junctions and genomic organization reveals some differences from the data reported by Hartmann et al. [28]. We have therefore analysed the differences

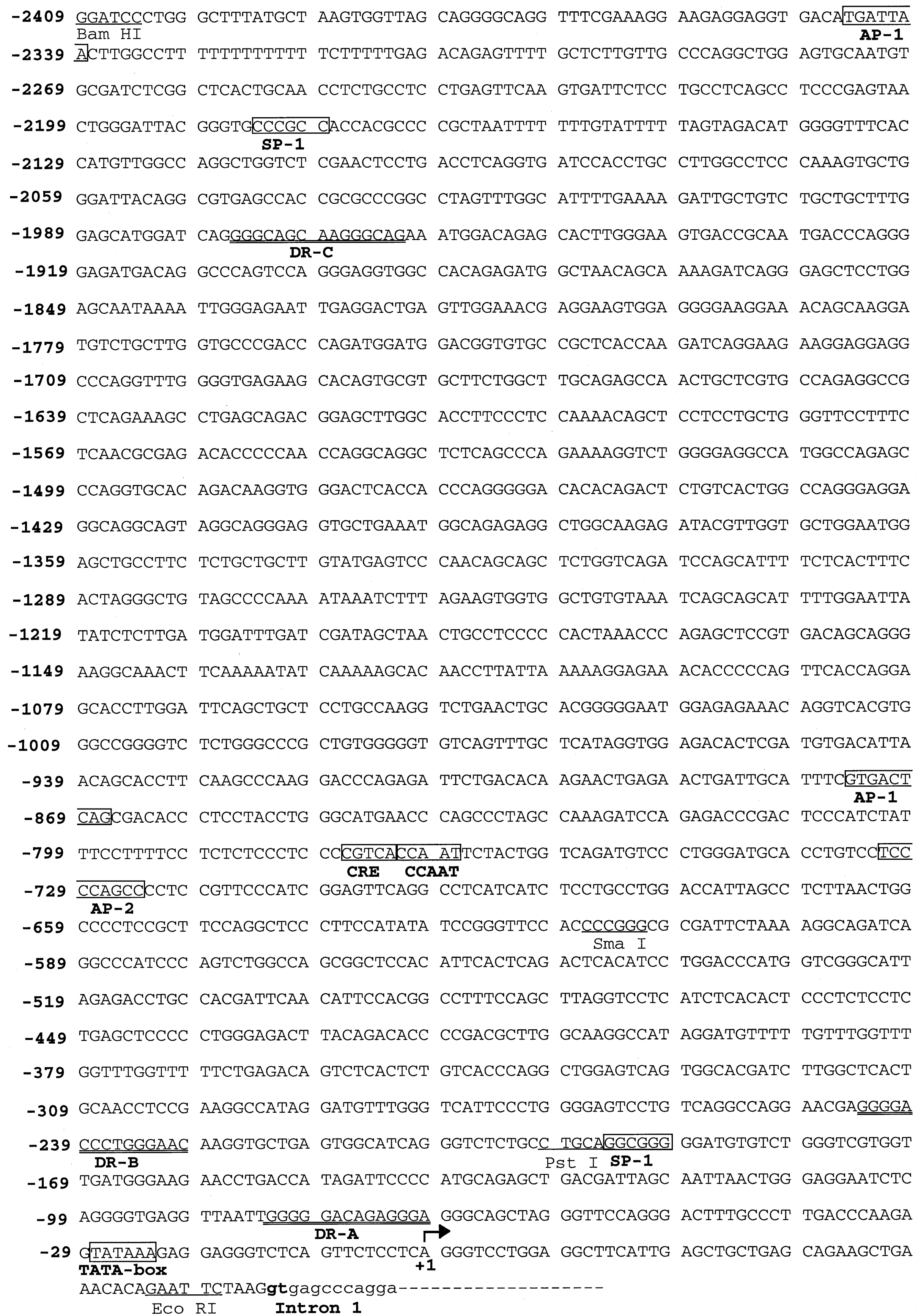

Figure 3 Nucleotide sequence of the 5′ flanking region and exon 1 of the NaPi-3 gene

Nucleotides are numbered, with the major transcription-initiation site designated as +1. Various well-established *cis*-acting elements are indicated by boxes. Major restriction endonuclease-digestion sites are underlined, and three direct-repeat regions are double underlined.

Table 2 Sequences of VDRE present in vitamin D-regulated genes and the direct-repeat sequence found in the 5′ flanking region of the NaPi-3 gene

Gene product	VDRE sequence	Sequence position (relative to promoter)
Rat 24-hydroxylase	AGGTGA gtg AGGGCG	(−151 to −137)
	CGCACC cgc TGAACC	(−259 to −245)
Mouse osteopontin	GGTTCA cga GGTTCA	(−757 to −743)
Human osteocalcin	GGGTCA acg GGGGCA	(−499 to −485)
Rat osteocalcin	GGGTGA atg AGGACA	(−455 to −441)
Mouse calbindin $D_{28\ k}$	GGGGGA tgtg AGGAGA	(−198 to −183)
Rat calbindin D_{9k}	GGGTGT cgg AAGCCC	(−489 to −475)
Avian integrin β_3	GAGGCA gaa GGGAGA	(−151 to −137)
Human NaPi-3 DR-A	GGGGGA ca GAGGGA	(−83 to −70)
Human NaPi-3 DR-B	GGGGAC cct GGGAAC	(−244 to −230)
Human NaPi-3 DR-C	GGGCAG cca GGGCAG	(−1976 to −1962)

Table 3 Regulation of the human NaPi-3 promoter by VDR and $1,25(OH)_2D_3$

A vector containing the NaPi-3 promoter, p3P2400 (0.5 μg), was transfected into COS-7 cells with or without VDR expression vector (0.05 μg) and $1,25(OH)_2D_3$ (10^{-10} or 10^{-8}M). Cells were harvested and assayed for luciferase activity 36 h after transfection. The p3P2400 activity was assessed as described in Figure 4. Data are represented as fold induction in COS-7 cells as compared with p3P2400 alone. Luciferase activity was corrected for differences in transfection efficiency between experiments by normalizing to β-galactosidase activity. Data are means ± S.E.M. from triplicate determinations and the results are representative of two separate experiments.

	Relative luciferase activity (-fold)			
VDR ...	None	+	+	+
$1,25\text{-}(OH)_2D_3$ ...	None	None	0.5 nM	50 nM
	1.00 ± 0.306	13.9 ± 0.670	21.5 ± 3.83	37.8 ± 17.2

very precisely. In particular, the positions of the exon 10/intron 10 junctions are different from those proposed by Hartmann et al. [28]. In addition, the 5′ sequence of intron 5 is not gtgagtt, but gtgttag. Minor differences are also detected at intron 1, intron 6 and intron 10. We have identified distinct transcription-initiation sites by 5′-RACE and primer-extension analysis. Although Hartmann et al. [28] also reported that the transcription-initiation site was a cytosine residue at position −34 relative to the 3′ end of exon 1 by primer extension analysis, this position is located 46 bases downstream of the TATA box. Furthermore they did not include the figures for primer-extension analysis and did not describe the RNA source. As a general rule for the position of TATA boxes in eukaryotic promoters, our result showing initiation at position −57 (28 bp upstream of the initiation site), more readily conforms to the general structure of transcription-start sites. The discrepancy may result from the different source of RNA or multiple transcription-start sites [28]. In addition, this is the first report characterizing the promoter region and assessing the transcriptional activation of the promoter.

The structure of the NaPi-3 gene is not homologous with other Na^+-dependent transporter family genes. Eukaryotic homologues of the human NaPi-3 protein include the Na^+/P_i co-transporter in rat, rabbit, mouse and opossum [4,6,9]. However, Na^+/P_i co-transporter genes are not homologous with members of the Na^+/glucose transporter (SGLT) family [29], such as the canine Na^+/*myo*-inositol co-transporter [30] or the rabbit Na^+/nucleoside co-transporter [31]. Other Na^+ co-transporters that are not homologous with the SGLT1 family include an hepatic Na^+/bile co-transporter and three Na^+/P_i co-transporters,

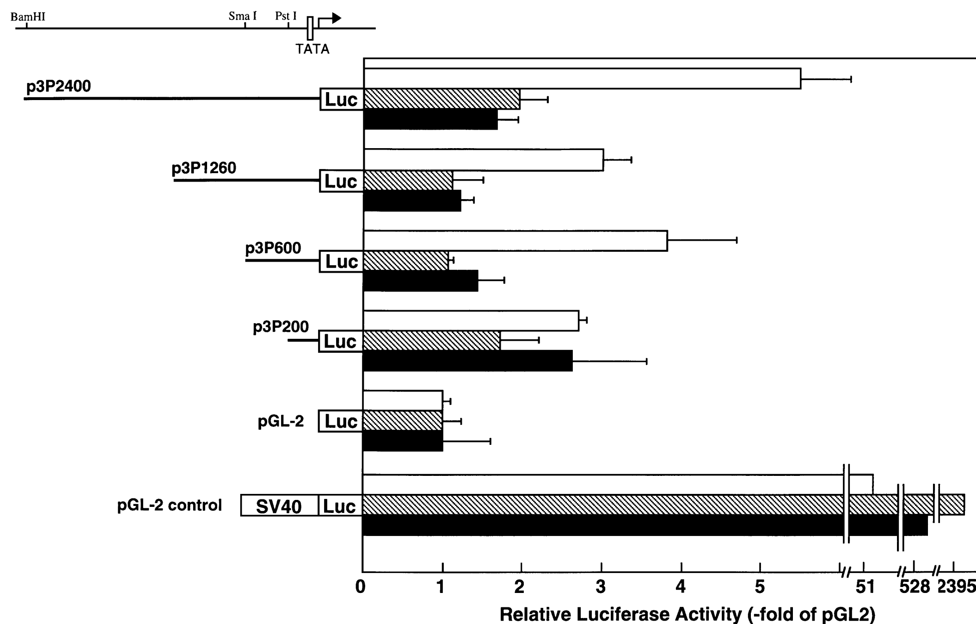

Figure 4 Transcriptional activity of the NaPi-3 promoter region

OK (□), COS-7 (▨) and HeLa (■) cells were transiently transfected with 0.5 μg of reporter vector containing the luciferase gene under the control of the NaPi-3 gene promoter and 0.5 μg of the pCMV-β control plasmid as described in the Materials and methods section. Transfected cells were harvested after 36 h and assayed for luciferase activity. Data are shown relative to activity observed with the pGL-2 vector (promoterless vector). The pGL-2 control vector was used as positive control. Luciferase activity was normalized to β-galactosidase activity in order to normalize for differences in transfection efficiency between experiments. Data are means ± S.E.M. from triplicate determinations, and the results are representative of three separate experiments.

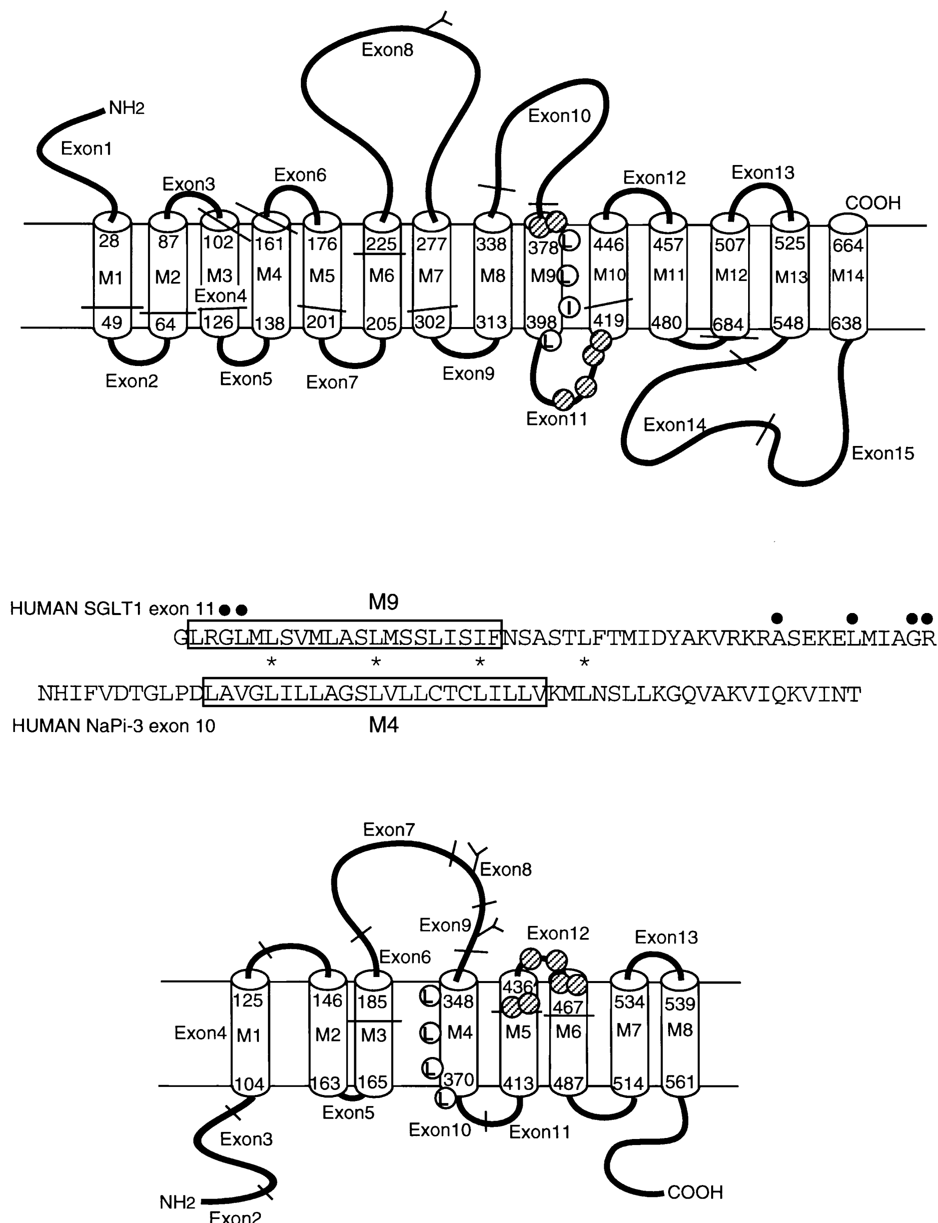

Figure 5 Comparison of exon positions with membrane-spanning secondary structures of the Na^+/P_i co-transporter (NaPi-3) and Na^+/glucose co-transporter (SGLT1)

The upper and lower panels indicate the predicted secondary structures of the human SGLT1 [32] and NaPi-3 [33] proteins respectively. Numbers show the position of the amino acids at the start and end of each transmembrane domain. The exon junctions are indicated by a thin line. Encircled letters show the amino acids comprising the leucine zipper motif. Hatched circles indicate the SOB motif. A comparison of sequences in the exon containing the leucine zipper motif in the SGLT1 and NaPi-3 genes is shown in the central panel, and the transmembrane domain is indicated by a box.

each of which is predicted to possess six transmembrane domains [29]. In a recent study Turk et al. [32] reported that the new membrane topology of the SGLT1 family consists of 14 transmembrane domains, an extracellular N-terminus and a large intracellular loop at the C-terminus (Figure 5). They reported that the SGLT1 topology is similar to that of the bacterial Na^+/proline and Na^+/pantothenate transporter [32]. The locations of the exon–intron junctions and the predicted eight membrane-spanning domains of NaPi-3 on the basis of a previous study [33] (Figure 5) suggest that it does not share a common ancestral six-transmembrane-span protein with SGLT1 family members. However, the leucine zipper motif near the Na^+-binding motif (SOB motif) is encoded by one exon in both genes (exon 11 in the human SGLT1 gene and exon 10 in the human NaPi-3 gene as indicated in Figure 5). Thus the leucine zipper motif and SOB motif may be evolutionarily conserved in the human NaPi-3 and SGLT-1 genes.

In the 5′ flanking region of the NaPi-3 gene, we demonstrated that the sequence between −2409 and −1259 may play an important role in kidney-specific gene expression, because this promoter was only active in OK cells, and not in HeLa or COS-7 cells (Figure 4). We could not identify a known *cis*-acting

element associated with kidney-specific gene expression in this region. Three direct-repeat-like sequences similar to steroid–thyroid hormone-responsive elements were found in the promoter region. The DR-B and DR-C sequences, each with a three-nucleotide spacer, resemble previously reported VDREs (Table 2) [34]. Vitamin D has been shown to increase or decrease P_i excretion, depending on experimental conditions such as length of exposure, dose and prior vitamin D and parathyroid hormone status [2]. Evidence indicates that short-term administration of $1,25\text{-}(OH)_2D_3$ increases P_i reabsorption in vitamin D-deprived animals [35,36]. Two mechanisms are involved in the increase in phosphate reabsorption stimulated by $1,25\text{-}(OH)_2D_3$. The first involves protein-synthesis-independent regulation, whereby vitamin D can stimulate Na^+-dependent phosphate-transport activity by changing membrane fluidity and/or membrane composition [37–39]. This mechanism is thought to be important in the acute response [39]. The second is mediated through transactivation of the gene promoter. In this study, VDR co-expression in the presence of $1,25\text{-}(OH)_2D_3$ in COS-7 cells significantly increased the activity of the NaPi-3 promoter. This explains results from an *in vivo* study in vitamin D-deficient rats where NaPi-2 mRNA expression was strongly induced by the presence of $1,25\text{-}(OH)_2D_3$ (K. Miyamoto, unpublished work). Thus further study of the VDRE locus in the promoter region of the human NaPi-3 gene is warranted.

XLH is a dominantly inherited disorder of P_i homoeostasis [1,13]. Studies in mice harbouring the homologous X-linked *hyp* mutation revealed a specific defect in Na^+-dependent P_i transport in the renal brush-border membrane, which contributes to hypophosphataemia and bone disease. Moreover, the deficit in Na^+/P_i co-transport is associated with a decrease in transport maximum velocity that is proportional to the decrease in the relative abundance of Na^+/P_i co-transporter mRNA and immunoreactive protein [14]. Vitamin D metabolism is also abnormal in the renal epithelial cells of these *hyp* mice [17]. The PEX gene has been recently isolated as a candidate for XLH [20], and thus its function must be closely related to a deficiency in the Na/P_i co-transporter and abnormal vitamin D metabolism. The two clinical states are XLH and HHRH [1]. HHRH is an autosomal recessive disorder characterized by a decrease in renal phosphate transport activity. We have suggested that the NaPi-3 gene may be a candidate gene for the HHRH syndrome [40]. Genetic analysis of the NaPi-3 gene in patients with HHRH is under way. Finally, our determination and study of the promoter sequence of a type-II transporter gene may lead to the elucidation of the molecular mechanisms of XLH and HHRH.

It should be noted that Hartmann et al. commented that our abstract about the structure of the NaPi-3 gene [19] had some discrepancies with their data [28]. Our results were preliminary at that time. Our observations in the present report essentially agree with their report.

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