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Cloning and expression of cDNA for a Na/P_i cotransport system of kidney cortex

(proximal tubule/phosphate/transport/*Xenopus laevis* oocytes)

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ABSTRACT **A cDNA library from rabbit kidney cortex was screened for expression of Na-dependent transport of phosphate (P_i) using *Xenopus laevis* oocytes as an expression system. A single clone was eventually isolated (designated NaPi-1) that stimulated expression of Na/P_i cotransport ≈700-fold compared to total mRNA. The predicted sequence of the Na/P_i cotransporter consists of 465 amino acids (relative molecular mass, 51,797); hydropathy profile predictions suggest six (possibly eight) membrane-spanning segments. *In vitro* translation of NaPi-1/complementary RNA in the presence of pancreatic microsomes indicated NaPi-1 to be a glycosylated protein; four potential N-glycosylation sites are present in the amino acid sequence. Northern blot analysis demonstrated the presence of NaPi-1/mRNA in kidney cortex and liver; no hybridization signal was obtained with mRNA from other tissues (including small intestine). Kinetic analysis of Na/P_i cotransport expressed by NaPi-1/complementary RNA demonstrated characteristics (sodium interaction) similar to those observed in cortical apical membranes. The alignment of 5 amino acid residues (Gly^{342}/Ala^{381}-Xaa-Xaa-Xaa-Xaa-Leu^{386}-Xaa-Xaa-Xaa-Pro^{390}-Arg^{391}) is consistent with a motif proposed for Na-dependent transport systems. We conclude that we have cloned a cDNA for a Na/P_i cotransport system present in rabbit kidney cortex.**

Reabsorption of phosphate (P_i) in the proximal tubule of the kidney contributes essentially to maintenance of the body P_i homeostasis (1). Influx of P_i at the brush border membrane of epithelial cells is mediated by a Na/P_i cotransporter and is driven by the transmembrane electrochemical potential gradient of sodium (2). Thereafter, P_i moves to the blood across the basolateral membrane, most likely via an anion-exchange mechanism and/or another Na-dependent P_i transport system. This transepithelial transport of P_i is controlled in a complex manner by various hormonal (e.g., parathyroid hormone) and nonhormonal (e.g., dietary P_i/P_i demand) factors (2–4).

By using different experimental systems such as isolated tubules, isolated brush border membranes, and established cell cultures, it has been demonstrated that regulation of proximal tubular P_i reabsorption is accomplished mainly by modulation of the apically localized Na/P_i cotransport system (2). Thus, this Na/P_i cotransport system is a central target within the complex control of P_i homeostasis. Studies with established cell lines (mainly opossum kidney cells) demonstrated that inhibition of the Na/P_i cotransport (by, for example, parathyroid hormone) is mediated by activation of protein kinase C and/or A followed by an internalization step (endocytosis) of the transport system. On the other hand, stimulation of Na/P_i cotransport by, for example, reduction of the concentration of extracellular P_i has been demonstrated to be dependent on *de novo* protein synthesis (2, 5).

Despite the detailed knowledge of kinetic and regulatory properties of the renal (proximal tubular) Na/P_i cotransport system and despite several biochemical attempts (e.g., labeling procedures; refs. 6–8) the structure of this transport system has not been elucidated. Information about the structure of the renal Na/P_i cotransporter would offer tools for future work related to the understanding of proximal tubular P_i reabsorption on a cellular and/or molecular level.

Successful cloning by expression of a membrane transport system using *Xenopus laevis* oocytes has been demonstrated for the small intestinal Na/glucose cotransport system (9) and also for various other membrane transport systems and receptors (10). Based on the recently reported expression of Na/P_i cotransport in *X. laevis* oocytes after injection of mRNA isolated from kidney cortex (11), we now have screened a rabbit kidney cortex cDNA library for expression of Na/P_i cotransport in oocytes. Here we describe the isolation and characterization of a cDNA clone coding for the putative Na/P_i cotransport system of the apical membrane of the rabbit proximal tubule.§

METHODS

***X. laevis* Oocytes.** All techniques and methods concerning the handling of the oocytes as well as the assay for transport of phosphate and sulfate have been described in detail in ref. 11.

cDNA Cloning. cDNA from total rabbit renal cortex mRNA (isolated as described in ref. 12) was synthesized following the methods described by Gubler and Hoffman (13). However, the oligo(dT) primer was extended on its 5′ end with a *Xho* I recognition site and a protecting $(GA)_{15}$ sequence, and, for the first strand synthesis, dCTP was substituted by its 5-methyl analogue. *Eco*RI adapters were added after second strand synthesis. cDNA was digested with *Xho* I to obtain cDNA with cohesive ends. The material was then separated on a 1% low-melting agarose gel and appropriate full-length cDNA was unidirectionally ligated into Bluescript SK+ (Stratagene) cut with *Eco*RI and *Xho* I. After transformation of *Escherichia coli* DH-5 about 25,000 clones were obtained, of which 10,000 have been screened. Plasmid DNA was extracted using an alkaline lysis method (14) and banded on a CsCl gradient. The plasmids were then linearized with *Xho* I and used for *in vitro* transcription and capping (15). Complementary RNA (cRNA) was dissolved in water at concentrations of 0.01–0.5 μg/μl and injected (50 nl) into 6–10 oocytes, which were assayed for transport after 1–3 days. Initially seven pools of about 500–1500 clones were tested. The only positive group was subdivided until a single clone

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Abbreviation: cRNA, complementary RNA.
‡To whom reprint requests should be addressed.
§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M76466).

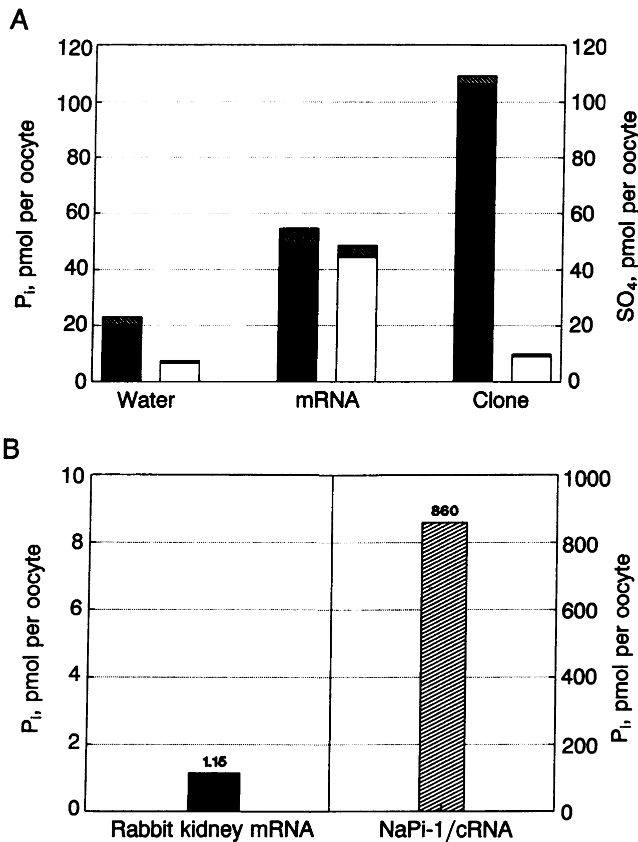

FIG. 1. (*A*) Stimulation of sodium-dependent phosphate and sulfate transport in *X. laevis* oocytes injected with total renal cortex poly(A)$^+$ RNA (25 ng per oocyte) and saturable amounts of cloned NaPi-1/cRNA (0.1–1 ng per oocyte). Water-injected oocytes were used as a control. After injection, the oocytes were kept in modified Barth's solution at 18°C and assayed for phosphate transport after 24 hr (filled bars) and sulfate after 48 hr (open bars) as described (9). Data are presented as means ± SD (striped bars) of six oocytes each. (*B*) Relative comparison of expression of Na/P$_i$ cotransport by total renal cortex poly(A)$^+$ RNA (left) and NaPi-1/cRNA (right). The data presented in *A* have been normalized to 1 ng of RNA injected.

(designated NaPi-1) stimulating Na/P$_i$ cotransport activity was obtained.

NaPi-1/cDNA was digested with *Alu* I, *Bam*HI, *Pal* I, *Sau*3A, *Sma* I, or *Xho* I and the fragments were subcloned into M13mp18 or M13mp19. Sequencing was carried out by the Sanger chain-termination method (16) using a T7 polymerase sequencing kit (Pharmacia). Primer extension was carried out as described by Boorstein and Craig (17).

***In Vitro* Translation.** Reticulocyte lysate and microsomes were from Promega and the supplier's protocol was followed. The reactions without microsomes were run in the presence of 0.5% (wt/vol) Triton X-100. Before loading onto the gel, the samples were denatured for 10 min at 50°C. SDS gel electrophoresis and autoradiography were performed following standard protocols.

Northern Blot Analysis. RNA was extracted from various rabbit tissues using a urea/LiCl method (12). Five micrograms of RNA was separated on a 1% formaldehyde/agarose gel and vacuum blotted onto a GeneScreen nylon membrane (NEN). Hybridization was carried out overnight at 37°C in 5× standard saline phosphate EDTA (SSPE) buffer containing 10× Denhardt's solution, 200 μg of herring sperm DNA per ml, and 40% formamide. DNA excised from clone NaPi-1 and labeled by random priming served as probe. The blot was then washed four times with cold buffer (1× SSPE/0.1% SDS) followed by two washes at 60°C in the same solution.

RESULTS AND DISCUSSION

As demonstrated recently (11), injection of poly(A)$^+$ RNA isolated from rabbit kidney cortex into *X. laevis* oocytes leads to expression of Na/P$_i$ cotransport, which, on a kinetic basis (Na affinity), could be distinguished from the intrinsic Na/P$_i$ cotransport activity present in water-injected oocytes. This observation provided the basis for cloning the corresponding cDNA: cDNA was synthesized from poly(A)$^+$ RNA from rabbit kidney cortex and unidirectionally cloned into the transcription vector pBluescript SK+. The library was screened by measuring stimulation of net Na-dependent P$_i$ transport after injection of cRNA synthesized *in vitro* from different sizes of pools of clones. A single cDNA clone, NaPi-1, was eventually isolated.

Injection of a saturating amount (>0.1 ng; data not shown) of cloned NaPi-1/cRNA into oocytes stimulated Na/P$_i$ cotransport 4- to 6-fold when assayed after 24 hr (Fig. 1*A*). Injection of larger amounts of cRNA (>2 ng) or incubation of

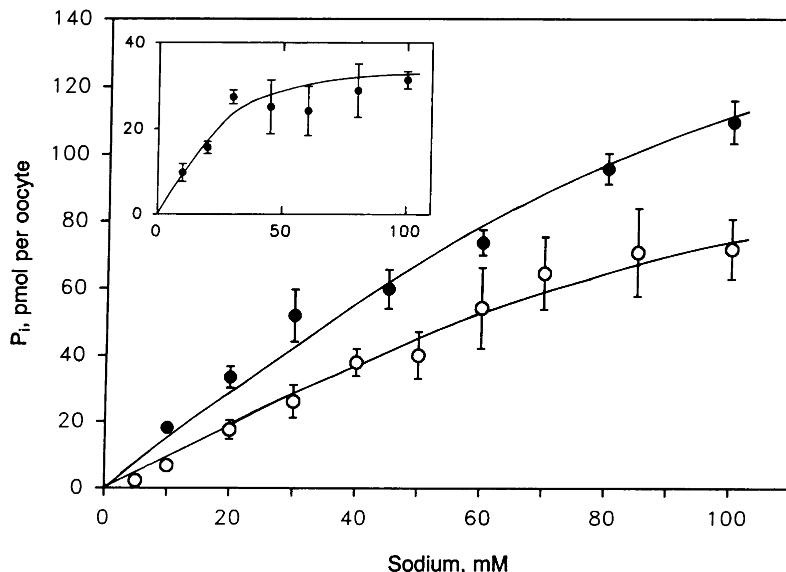

FIG. 2. Dependence of Na/P$_i$ cotransport on the sodium concentration. Oocytes were injected with rabbit kidney cortex mRNA (○, 25 ng per oocyte), cloned NaPi-1/cRNA (●, 1 ng per oocyte), or water (*Inset*). The oocytes were incubated in modified Barth's solution for 24 hr (cRNA and water) or 4 days (total mRNA). The sodium concentration during the phosphate uptake was varied and choline was added to maintain constant ionic strength.

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                                                             ▼
   1        gtaaagttgcaagaaagtcttccgagagaagaaggaatgcctgttcggtccTGAA

  56        TCAGTGGATGGAGACCAAACCTCTCAGCAAGACAAAGAGCAAAGTCAGCCTCGTAAGCAA

 116        ATGGATAACCAGTTCCCTTCCAGAAAAGGTCCATGTTTCTGTTCTTTCCGATATGTCTTG
         1  MetAspAsnGlnPheProSerArgLysGlyProCysPheCysSerPheArgTyrValLeu

 176        GCACTCTTTATGCATTTTTGTAACATTGTGATAATCGCACAGCGCATGTGTCTGAGCCTC
        21  AlaLeuPheMetHisPheCysAsnIleValIleIleAlaGlnArgMetCysLeuSerLeu

 236        ACCATGGTAGCCATGGTGAACAACACGAATCTACATGGTTCGCCCAACACCTCCGCAGAG
        41  ThrMetValAlaMetValAsnAsnThrAsnLeuHisGlySerProAsnThrSerAlaGlu

 296        AAGCGCCTGGATAATACAAAGAACCCCGTCTATAATTGGAGCCCTGATGTCCAGGGTATC
        61  LysArgLeuAspAsnThrLysAsnProValTyrAsnTrpSerProAspValGlnGlyIle

 356        ATCTTTAGCTCCATCTTCTATGGCGCTTTTCTCATTCAGATTCCTGTTGGATACATCTCT
        81  IlePheSerSerIlePheTyrGlyAlaPheLeuIleGlnIleProValGlyTyrIleSer

 416        GGAATATACTCTATAAAGAAATTGATTGGCTTTGCATTATTTCTGAGTTCTCTGGTCAGC
       101  GlyIleTyrSerIleLysLysLeuIleGlyPheAlaLeuPheLeuSerSerLeuValSer

 476        ATATTCATCCCGCAAGCTGCTGCAGTCGGAGAAACTTGGATCATTGTATGTAGAGTAGTC
       121  IlePheIleProGlnAlaAlaAlaValGlyGluThrTrpIleIleValCysArgValVal

 536        CAAGGAATAACTCAGGGGACAGTCACAACAGCCCAGCATGAGATATGGGTCAAATGGGCT
       141  GlnGlyIleThrGlnGlyThrValThrThrAlaGlnHisGluIleTrpValLysTrpAla

 596        CCTCCCTTGGAGCGAGGCCGCCTTACCTCTATGAGTCTATCAGGGTTTCTGCTGGGTCCG
       161  ProProLeuGluArgGlyArgLeuThrSerMetSerLeuSerGlyPheLeuLeuGlyPro

 656        TTCATCGTCCTGCTTGTAACGGGTATCATTTGTGAATCTCTGGGCTGGCCCATGGTCTTC
       181  PheIleValLeuLeuValThrGlyIleIleCysGluSerLeuGlyTrpProMetValPhe

 716        TACATTTTTGGTGCTTGTGGCTGCGCTGTGTGTCTTCTCTGGTTCGTTCTGTATTATGAT
       201  TyrIlePheGlyAlaCysGlyCysAlaValCysLeuLeuTrpPheValLeuTyrTyrAsp

 776        GATCCCAAGGACCACCCATGCGTGAGCCTGCATGAGAAAGAATACATCACGTCCTCCCTC
       221  AspProLysAspHisProCysValSerLeuHisGluLysGluTyrIleThrSerSerLeu

 836        ATCCAGCAGGGCAGCTCAACAAGACAATCTCTGCCCATCAAGGCTATGATTAAGTCTCTT
       241  IleGlnGlnGlySerSerThrArgGlnSerLeuProIleLysAlaMetIleLysSerLeu

 896        CCACTCTGGGCTATTTCTTTCTGCTGTTTTGCTTATTTATGGACATACAGCAGGTTGATT
       261  ProLeuTrpAlaIleSerPheCysCysPheAlaTyrLeuTrpThrTyrSerArgLeuIle

 956        GTATACACCCCAACGTTGATCAACTCCATGCTTCATGTTGACATAAGAGAGAACGGGCTG
       281  ValTyrThrProThrLeuIleAsnSerMetLeuHisValAspIleArgGluAsnGlyLeu

1016        CTGTCCAGCCTCCCCTACTTGTTTGCCTGGATCTGTGGTGTCATAGCAGGTCACACAGCA
       301  LeuSerSerLeuProTyrLeuPheAlaTrpIleCysGlyValIleAlaGlyHisThrAla

1076        GACTTCCTCATGTCCAGGAACATGCTCAGCCTAACTGCTATTCGGAAACTCTTCACGGCG
       321  AspPheLeuMetSerArgAsnMetLeuSerLeuThrAlaIleArgLysLeuPheThrAla

1136        ATAGGACTTCTCTTGCCTATAGTCTTCAGCATGTGCCTGCTCTACCTGAGTTCCGGCTTC
       341  IleGlyLeuLeuLeuProIleValPheSerMetCysLeuLeuTyrLeuSerSerGlyPhe

1196        TACAGCACCATCACTTTTCTCATACTTGCTAATGCAAGCAGCAGCTTCTGTTTGGGAGGA
       361  TyrSerThrIleThrPheLeuIleLeuAlaAsnAlaSerSerSerPheCysLeuGlyGly

1256        GCACTTATCAATGCCTTGGATCTTGCTCCCAGGTATTATGTATTTATTAAAGGAGTTACA
       381  AlaLeuIleAsnAlaLeuAspLeuAlaProArgTyrTyrValPheIleLysGlyValThr

1316        ACTTTAATTGGGATGACAGGAGGAATGACTTCTTCCACCGTGGCTGGATTGTTCCTTAGT
       401  ThrLeuIleGlyMetThrGlyGlyMetThrSerSerThrValAlaGlyLeuPheLeuSer

1376        CAGGATCCAGAATCTTCCTGGTTTAAAATCTTCCTCCTGATGTCCATCATTAATGTGATA
       421  GlnAspProGluSerSerTrpPheLysIlePheLeuLeuMetSerIleIleAsnValIle

1436        AGCGTGATTTTCTACCTTATATTTGCTAAAGCAGAAATCCAGGACTGGGCTAAGGAAAAA
       441  SerValIlePheTyrLeuIlePheAlaLysAlaGluIleGlnAspTrpAlaLysGluLys

1496        CAACATACACGGCTCTGAAGCATTGCAGTCAGAGGGGCCAGACCACTCCAAACACGGAAA
       461  GlnHisThrArgLeu*** 465

1556        GCTAACACCACAAGAAAGCAGTCTGAGTCAGACCTTCGCTACCTAAGCTGAACTTTTCTC

1616        ATTGCAACACTAAATTCTCCTCTTAGAGGAGATTGCCACCATTTTCGGAACACACAATGT

1676        ATGACAAAGCAGGAAGTTGTATGTTGCTTATGTTGTTTGAGTAAGAAGGGATCCCCCCTC

1736        CCTTCTCTTTTGCTACCCCTAACCCTTGGCATAAAACCCATAATTGTTTCTCAAGAGGTA

1796        CTTCCTCTTCTGGAGCTGTGTCTTCCCTCTGTCTTCTTTAAATAAAGGTTTCCTCTGCGC
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FIG. 3. Nucleotide sequence of the NaPi-1/cDNA and the deduced amino acid sequence of the putative Na/P_i cotransport system (uppercase letters). Lowercase letters indicate the nucleotides at the 5′ end obtained by primer extension and from a supplementary clone. The possible poly(A) signal is underlined; NaPi-1/cDNA contained a poly(A) tail of 60 A residues.

the oocytes for longer times (>36 hr) led to a decrease of P_i uptake (data not shown), suggesting that overexpression of the cognate protein is not tolerated by the oocyte. Therefore, to compare the specific activity of the isolated clone with that of total poly(A)$^+$ RNA, Na/P_i cotransport rates were normalized on the basis of the amount of RNA injected (Fig. 1*B*). This normalization suggests that the specific expression activity of the cloned cRNA is about 750-fold higher than that of total poly(A)$^+$ RNA.

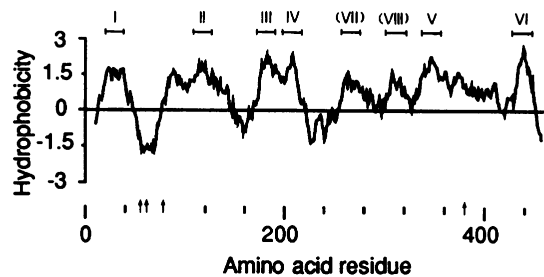

FIG. 4. Hydrophobicity plot of the putative Na/P_i cotransport system deduced from the NaPi-1/cDNA. The Kyte–Doolittle method (19) with a window of 19 amino acids was used. Four potential N-glycosylation sites at positions 47, 56, 72, and 371 are indicated by arrows. Bars indicate possible membrane-spanning segments.

Specificity of the cloned cDNA with regard to the expression of Na/P_i cotransport is shown by the observation that after injection of cloned cRNA, no stimulation of Na/sulfate cotransport was observed; in contrast, injection of total mRNA led to expression of both cotransport activities (Fig. 1*A*). This confirms our earlier observation made with total poly(A)$^+$ RNA (11) that these two anion transport systems of the apical membrane of the proximal tubular cell represent different molecular entities.

Based on a kinetic analysis of the sodium interaction, Na/P_i cotransport expressed by the cloned cRNA could be distinguished from the intrinsic Na/P_i cotransport of the oocytes, which exhibits an approximate K_m per sodium of 10–20 mM (Fig. 2). The approximate K_m for sodium after injection of cRNA was found to be around 50–60 mM, which is in good agreement with the data reported recently for injection of total poly(A)$^+$ RNA (ref. 11 and Fig. 2). A similar characteristic has also been described for Na/P_i cotransport in isolated rabbit renal brush border membrane vesicles (18).

The complete nucleotide sequence of the cloned NaPi-1/cDNA and the deduced amino acid sequence are presented in

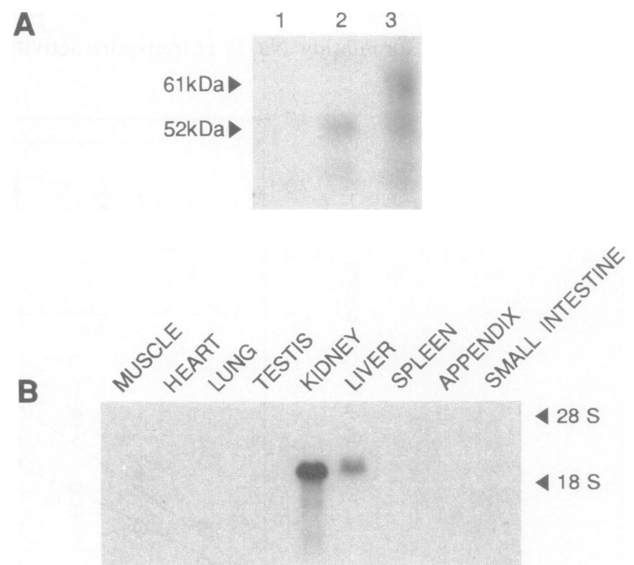

FIG. 5. (*A*) *In vitro* translation of NaPi-1/cRNA. The reaction was performed in the absence (lane 2) or presence (lane 3) of canine pancreatic microsomes. The glycosylated form (61 kDa) and the nonglycosylated form (52 kDa) of the NaPi-1/RNA translation product are indicated by arrowheads. Lane 1 represents the control reaction without cRNA. (*B*) Northern blot analysis of RNA extracted from various rabbit tissues.

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E. coli, Na/glutamate  :  40 AGGLL 44   -----  78 IGLNANIASLRAGGRVV 94
E. coli, Na/proline    : 326 IAGIL 330  ----- 362 LRKHASQKELVWVGRVM 378
Rabbit,  Na/glucose    : 378 LRGLM 382  ----- 413 IRKKASEKELMIAGRLF 429
Human,   Na/glucose    : 378 LRGLM 382  ----- 413 VRKRASEKELMIAGRLF 429
Rabbit,  Na/phosphate  : 340 AIGLL 344  ----- 377 CLGGALINALDLAPRYY 393
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FIG. 6. Comparison of the amino acid sequence deduced from NaPi-1 with the consensus sequence for Na-dependent transport systems as proposed by Deguchi *et al.* (23). Residues in boxes are identical among the Na/P_i cotransport system and the Na/glutamate (23) and Na/proline (24) transporter of *E. coli* and the small intestinal Na-dependent glucose transporter of the rabbit (9) and human (25).

Fig. 3. Beginning at the ATG codon at position 116, a long open reading frame codes for a protein of 465 amino acids (relative molecular mass, 51,797). The possible importance of a short reading frame (positions 63–204) was investigated by constructing a derivative of NaPi-1 lacking sequences from the 5′ end to position 75 of the sequence in Fig. 3. Injection of such truncated cRNA showed the same expression of Na/P_i cotransport as that observed with untruncated cRNA (data not shown), demonstrating that ATG at position 63 is not necessary for expression of Na/P_i cotransport. Hydrophobicity analysis (19) of the deduced amino acid sequence suggests that the cloned cDNA codes for a membrane protein that exhibits at least six (perhaps eight) transmembrane segments (Fig. 4). If all four potential N-glycosylation sites found (located in hydrophilic regions, see Fig. 4) are indeed used, the N and the C termini would be located at the cytoplasmic surface of the membrane.

Primer extension suggested that the NaPi-1/cDNA is missing 40–50 nucleotides at its 5′ end (not shown). Therefore, a λ phage cDNA library was screened using a 220-nucleotide probe from the 5′ end of NaPi-1/cDNA. Thereby an additional clone was identified and isolated, which was 51 nucleotides longer than the original one. Sequencing of the 5′ end of this additional clone (given as lowercase letters in Fig. 3) confirmed not only the 5′ end sequence as obtained by primer extension but also the 5′ end of the NaPi-1/cDNA. Thus, as the results indicate (Fig. 1), the complete 5′ end is not needed to get functional expression of NaPi-1 in the oocytes.

In vitro translation of NaPi-1/cRNA in the presence of microsomes showed two protein bands with apparent molecular masses of 52 and 61 kDa (Fig. 5*A*, lane 3). In the absence of microsomes only one band was observed (Fig. 5*A*, lane 2). Treatment of the *in vitro* translated protein with endoglucosidase H resulted in the conversion of the 61-kDa band into the 52-kDa band (data not shown). We conclude that the Na/P_i cotransporter is glycosylated.

The tissue distribution of the NaPi-1/mRNA was examined by Northern blotting using equal amounts of total RNA isolated from various tissues of the rabbit (Fig. 5*B*). In addition to kidney cortex, liver was the only tissue in which NaPi-1/mRNA was found. Quantitative analysis of the data indicated that in liver the signal was ≈3-fold lower compared with the same amount of kidney cortex RNA. In agreement with this observation, injection of total poly(A)$^+$ RNA isolated from rabbit liver also led to expression of Na/P_i cotransport (data not shown). At present, however, there is no detailed information available on the location of such a Na/P_i cotransport activity in liver. No hybridization was observed with RNA isolated from the intestine. Since intestinal Na/P_i cotransport is regulated by vitamin D_3 (20), poly(A)$^+$ RNA isolated from vitamin D_3-treated rabbits was also tested; again, no indication for the presence of mRNA similar to NaPi-1 was observed (data not shown).

By comparing the amino acid sequence deduced from the NaPi-1/cDNA with current protein data banks, no sequence homology to other proteins was found. Notably, no sequence homology was found with other (cloned) P_i transport systems from mitochondria (21) or *E. coli* (22). The deduced amino acid sequence was also compared with the consensus sequence proposed recently by Deguchi *et al.* (23) for various Na-dependent transport systems (Fig. 6). In clone NaPi-1, G, A, L, and R residues (at positions between 340 and 393) are found to fit the proposed motif. The spacing between the G and the A residue is 39, consistent with the spacings found in the other sequences (40, 38, 37, and 37). The replacement of the "GR" homology with a "PR" pair as found in NaPi-1 at position 390/391 is a conservative change, as P and G are "turn-inducing" residues. Based on the high homology of NaPi-1 to the proposed motif of G, A, L, and R amino acid residues, it is concluded that the cloned Na/P_i cotransporter belongs to this family of Na-dependent transport systems.

Based on the presented data, we conclude that we have cloned a cDNA coding for a rabbit kidney cortex Na/P_i cotransporter. It now remains to be established whether clone NaPi-1 is the sole component of this Na/P_i cotransport system and whether it is the site of regulation of renal phosphate reabsorption by parathyroid hormone or dietary phosphate or other agents.

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