J. Membrane Biol. 156, 19–24 (1997)

The Journal of
Membrane
Biology



# Identification of a cDNA/Protein Leading to an Increased $P_i$-uptake in *Xenopus laevis* Oocytes

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Received: 31 July 1996/Revised: 16 October 1996

**Abstract.** In a previous report we documented an increased $Na^+$-dependent transport of inorganic phosphate ($P_i$) in *Xenopus laevis* oocytes injected with mRNA isolated from rabbit duodenum (Yagci et al., *Pfluegers Arch.* **422:**211–216, 1992; ref 24). In the present study we have used expression cloning in oocytes to search for the cDNA/mRNA involved in this effect. The identified cDNA (provisionally named PiUS; for $P_i$-uptake stimulator) lead to a 3-4-fold stimulation of $Na^+$-dependent $P_i$-uptake (10ng cRNA injected, 3–5 days of expression). $Na^+$-independent uptake of $P_i$ was also affected but transport of sulphate and L-arginine (in the presence or absence of sodium) remained unchanged. The apparent $K_m$-values for the induced $Na^+$-dependent uptake were 0.26 ± 0.04 mM for $P_i$ and 14.8 ± 3.0 mM for $Na^+$. The 1796 bp cDNA codes for a protein of 425 amino acids. Hydropathy analysis suggests a lack of transmembrane segments. In vitro translation resulted in a protein of 60 kDa and provided no evidence of glycosylation. In Northern blots a mRNA of ~2 kb was recognized in various tissues including different intestinal segments, kidney cortex, kidney medulla, liver and heart. Homology searches showed no similarity to proteins involved in membrane transport and its control. In conclusion, we have cloned from a rabbit small intestinal cDNA library a novel cDNA encoding a protein stimulating $P_i$-uptake into *Xenopus laevis* oocytes, but which is not a $P_i$-transporter itself.

**Key words:** $Na/P_i$-cotransport — Expression cloning — Duodenum — Brush border membrane — *Xenopus laevis*

## Introduction

Transcellular $P_i$-reabsorption in the small intestine is $Na^+$-dependent involving a brush border membrane $Na/P_i$-cotransporter [5, 7]. This transporter has been extensively characterized in studies on brush border membrane vesicles in terms of its functional properties [1, 7] and it could also be shown that its transport rate is increased in animals with increased 1.25 $(OH)_2$ vitamin $D_3$ levels [6, 9, 15].

The *Xenopus laevis* oocyte expression system has been successfully used to identify ('clone') renal brush border membrane $Na/P_i$-cotransporters [12, 21, 23]. More recently, two phosphate transporters (related among each other, but different from the renal transporters) have been identified as virus receptors which are widely distributed in different tissues [10]. Furthermore, a $Na/P_i$-cotransporter expressed in brain and showing a partial hormology to one of the renal transporters has been identified [16].

With the *Xenopus laevis* oocyte expression system, we could show that injection of poly $(A)^+$ RNA (mRNA) isolated from rabbit duodenum leads to an increase in Na-dependent $P_i$-uptake into oocytes and that this increase was magnified by injection of mRNA from animals with increased levels of 1.25 $(OH)_2$ vitamin $D_3$ [24]. In Northern blots made with duodenal mRNA of either control or 1.25 $(OH)_2$ vitamin $D_3$-treated animals, we didn't observe hybridization signals by using cDNA probes related to one of the renal transporters [12, 23, 24 and *data not shown*]. In the present paper, we describe experiments aiming at expression cloning of the intestinal $Na/P_i$-cotransporter. By screening a rabbit intestinal cDNA library we have identified a 1796 bp cDNA encoding a protein of 425 amino acids which led to a stimulation of Na-dependent (and Na-independent) $P_i$-uptake into *Xenopus laevis* oocytes (provisionally named PiUS). Secondary structure predictions and tissue distribution

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studies seem to exclude a 'direct' role of this PiUS cDNA/protein in rabbit duodenal Na/$P_i$-cotransport.

## Materials and Methods

### Animals and RNA isolation

Total RNA from different tissues was isolated from New Zealand male rabbits (6-8 weeks old; weighing approximately 1 kg) as previously described; poly $(A)^+$ RNA (mRNA) from different rabbit tissues was isolated by an oligo-dT column [3, 4].

### Xenopus laevis oocytes and Transport Assays

All methods and reagents used have been described earlier [2, 12, 22, 23]. Oocytes were injected with 50 nl water or 50 nl cRNA (10–50 ng/oocyte). Uptake measurements were performed 3–5 days after injection using different substrates (0.01–5 mM of $^{32}$P-phosphate; 0.5 mM $^{35}$S-sulphate, 0.05 mM $^3$H-L-arginine), in the presence (5–100 mM NaCl) or absence (100 mM choline chloride) of sodium. Tracers were used at 20–25 μCi/ml and were obtained from Dupont/NEN. $P_i$-saturation kinetics were fitted to a simple Michaels-Menten equation, $Na^+$-saturation curves were fitted to a generalized Hill equation, using nonlinear regression. All results were expressed as mean ± (SE) of 8–10 oocytes; each experiment was repeated at least twice.

### cDNA Library Construction and Screening

The cDNA library was constructed as described previously [3, 4, 12]. Briefly, size-fractionated small intestinal (duodenum and upper jejunum) mRNA from rabbits (2.2–5.0 kb mRNA sizes) was used as starting material to construct a cDNA library using the Super Script Plasmid System (GIBCO-BRL, Gaithersburg, MD) following the supplier's instructions. The library contained about $2 \times 10^5$ colonies, 40,000 of which were screened by a sib-selection procedure; initial pools contained about 2,000 colonies. Plasmid DNA was isolated using Wizard Miniprep columns (Promega, Madison, WI). The size of the inserts was analyzed in a 1% agarose minigel after digestion with Mlu I. Plasmids were linearized with Not I and used for in vitro transcription including capping, using T7-RNA polymerase (Promega). cRNA was dissolved in water for further analysis.

### cDNA sequencing

The cDNA isolated by above sib-selection procedure stimulating maximally oocyte $P_i$-uptake (PiUS) was sequenced by the dideoxy-chain termination method using 16-mer synthetic oligonucleotides as primers.

### Northern Blot Analysis

Poly $(A)^+$ RNA (mRNA) (~5 μg/lane) was denaturated, electrophoresed through a 1% agarose/formaldehyde gel and transferred to Gene Screen membranes (Dupont/NEN). cDNA probes of the PiUS cDNA (full length; Mlu I digested) and of mouse β-actin (1150 bp Pst I fragment) were labeled by random priming (Oligolabeling kit; Pharmacia) using α-$^{32}$P-dCTP (Dupont/NEN). Blots were hybridized and washed at high stringency. Northern blot signals were visualized using a Phosphoimager and quantified with ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

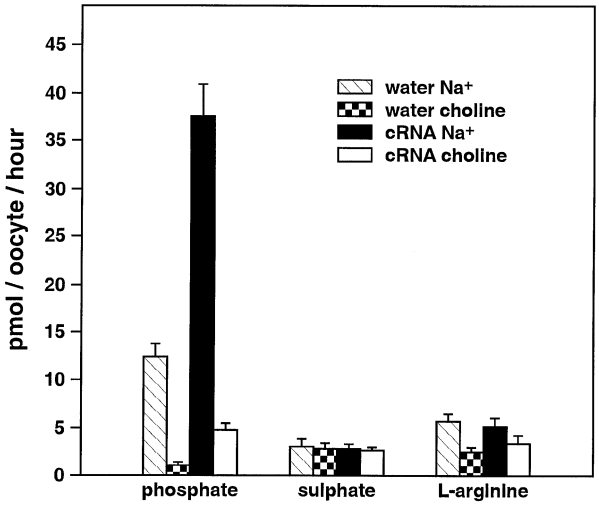


**Fig. 1.** Specificity of cRNA-induced transport in *Xenopus laevis* oocytes. Oocytes were injected with 50 nl of water or 50 nl of PiUS cRNA (10 ng/oocyte). Uptakes (60-min incubation) were performed 3–5 days postinjection with the following substrates: phosphate (0.5 mM), sulphate (0.5 mM), L-arginine (50 μM), in the presence (100 mM NaCl) or absence (100 mM choline chloride) of sodium.

### In vitro Translation

In vitro translation was performed with a rabbit reticulocyte lysate system in the absence and presence of canine pancreatic microsomes (Promega) [12]. After denaturation (60°C/10 min) in vitro translation products were analyzed by SDS-polyacrylamide gelelectrophoresis.

## Results

In previous studies, we have observed a stimulation of Na-dependent $P_i$-uptake in oocytes after injection of rabbit duodenal poly $(A)^+$ RNA (total and 2–3 kb size fraction) [24]. Based on this observation, we have screened by sib-selection and on the basis of stimulation of $Na^+$-dependent $P_i$-uptake a corresponding rabbit duodenal cDNA library. This library has been previously successfully used in our laboratory for the expression cloning of an $H^+$/dipeptide cotransporter [4].

As documented in Fig. 1, we could identify a cDNA (PiUS) which after cRNA-transcription and oocyte injection stimulated $P_i$-uptake (Fig. 1). After injection of 10 ng of PiUS cRNA and 3–5 days of expression usually a 3-4-fold stimulation of $P_i$-uptake in the presence of $Na^+$ could be observed. Among the different experiments performed we have never observed a stimulation higher than 8-fold using different amounts of cRNA injected and at different times of expression (*data not shown*). Injection of PiUS cRNA also led to a stimulation of

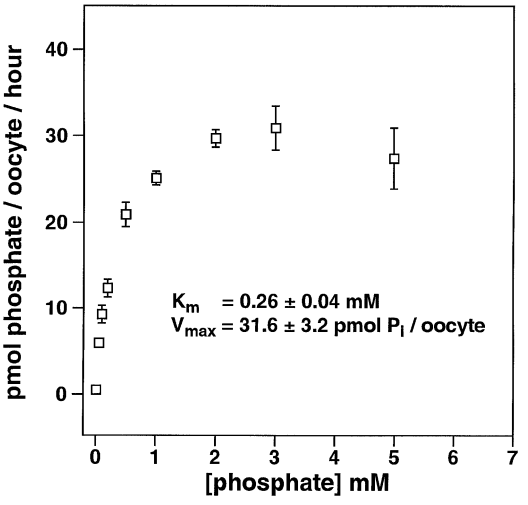


**Fig. 2.** $P_i$-concentration dependence. Oocytes were injected with 50 nl of water or 50 nl PiUS cRNA (10 ng per oocyte) and 3–5 days after injection transport (60-min incubation) was measured in the presence of 100 mM NaCl at different $P_i$ concentrations (0.01–5 mM). Data are shown as net cRNA induced $P_i$-uptake (values of water injected oocytes subtracted from cRNA-injected values, for each point), with means ± SE for 7–10 oocytes per condition, and are representative of three similar experiments. The curve was fitted to a Michaelis-Menten equation using nonlinear regression. Error bars not visible are smaller than the symbols.

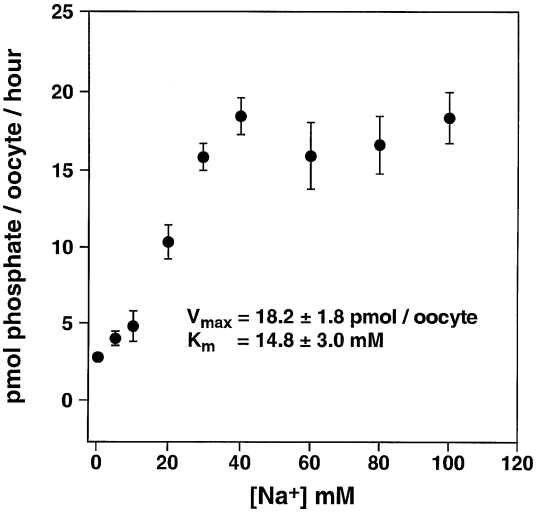


**Fig. 3.** $Na^+$-concentration dependence. Oocytes were injected with 50 nl of water or 50 nl PiUS cRNA (10 ng/oocyte) and transport (60 min of incubation) of 0.5 mM $P_i$ was measured in the presence of different $Na^+$ concentrations (0–100 mM NaCl, replaced by choline chloride) 3–5 days after injection. Data are shown as net cRNA-induced phosphate uptake (water values subtracted from cRNA values, for each point) with means ± SE for 7–10 oocytes per condition, and are representative of two similar experiments. The data for $Na^+$-dependent uptake were fitted to a generalized Hill equation using nonlinear regression. Error bars not visible are smaller than the symbols. The Hill coefficient was 2.0 ± 0.6.

$Na^+$-independent $P_i$-uptake which in absolute amounts was much smaller but percentagewise similar to the increase in $Na^+$-dependent uptake (3-4-fold in Fig. 1). Injection of cRNA had no influence on uptake of sulphate or L-arginine by the oocytes.

To characterized the PiUS cRNA-induced increase in $P_i$-uptake we have analyzed it as a function of different $P_i$-concentrations (Fig. 2) or as a function of different $Na^+$-concentrtions (Fig. 3). The apparent $K_m$ for expressed uptake was ~0.3 mM for $P_i$ (Fig. 2) and ~15 mM for $Na^+$ (Fig. 3). These values are similar to those found in our previous studies with total or size fractionated rabbit duodenal mRNA and are also similar to those observed in $H_2O$-injected oocytes [22, 24].

Sequencing of the PiUS cDNA-insert revealed a chain length of 1796 bp including a poly $(A)^+$ tail of 35 residues. The open reading frame encoded a protein of 425 amino acids (Fig. 4*A*). The derived protein sequence has multiple consenses sites: For N-glycosylations (3 sites), for cyclic nucleotide dependent kinases (2 sites), for casein kinase II (7 sites), for protein kinase C (9 sites), for tyrosine kinase (1 site) and for N-myristilation-sites (4 sites). A secondary structure prediction analysis based on hydropathy analysis [11] provided no obvious evidence for the existence of hydrophobic stretches of amino acids representing potential membrane spanning domains (Fig. 4*B*).

Figure 5 shows the product obtained after in vitro translation. The apparent molecular weight of the largest protein was ~60 kDa and was unaffected by the presence or absence of microsomes. These observations suggest that the PiUS protein is not glycosylated.

The tissue distribution of PiUS mRNA was studied with Northern blots (Fig. 6). An mRNA species of ~2 kb was observed in all tissues tested. Quantification of the signal intensities and normalization to GAPDH indicated a similar abundance of the PiUS mRNA in the different tissues (between 1 and 3.5 by taking the ratio in duodenum as 1).

## Discussion

On the basis of our previous studies showing a stimulation of oocyte Na-dependent $P_i$-uptake after injection of rabbit duodenal mRNA [24], we have applied an expression cloning strategy aiming at identifying a small intestinal brush border membrane Na/$P_i$-cotransporter. Such a strategy has in our laboratory successfully been applied for the identification of two different renal brush border membrane Na/$P_i$-cotransporters from different species [12, 13, 14, 21, 23]. In these previous studies we were unable to detect hybridization signals on Northern blots of small intestinal mRNA probed with cDNAs of the two different renal brush border Na/$P_i$-cotransporters sug-

**A**

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                                                        -216   5'-GCT
GGTACGCCTGCAGGTACCGGTCCGGAATTCCCGGGTCGACCCACGCGTCCGAACAATAGGACGGAAACGCC
GCCGGACCGGGCTGAGGCCGCAGAGCTCCTGGGCCTGCGCAAGCCAAGCAGCTGAGACCAAACAGAAGGAC
AGCAGACGGAGGACGTGCTGGCCGCAGGACCCCGCTGCCTCCCCCTTCTCCCTGCTGCCTGCGCCCGGAGG

ATG AGC CCA GCC TTC AGG GCC ATG GAC GTG GAG CCC CGC ACC AAG GGC ATC CTG 54
Met Ser Pro Ala Phe Arg Ala Met Asp Val Glu Pro Arg Thr Lys Gly Ile Leu 18

CTG GAG CCC TTT GTT CAC CAG GTT GGG GGG CAC TCG TGT GTG CTC CGC TTC AAT 108
Leu Glu Pro Phe Val His Gln Val Gly Gly His Ser Cys Val Leu Arg Phe Asn 36

GAG ACA ACC TTG TGC AAG CCT TTG ATC CCA AGG GAG CAT CAG TTC TAC GAG ACC 162
Glu Thr Thr Leu Cys Lys Pro Leu Ile Pro Arg Glu His Gln Phe Tyr Glu Thr 54

CTC CCA GCT GAG ATG CGT AAA TTC ACT CCC CAG TAC AAA GGT GTA GTA TCT GTG 216
Leu Pro Ala Glu Met Arg Lys Phe Thr Pro Gln Tyr Lys Gly Val Val Ser Val 72

TGT TTT GAA GAA GAT GAA GAC AGG AAT TTG TGT TTA ATA GCA TAT CCA TTA AAA 270
Cys Phe Glu Glu Asp Glu Asp Arg Asn Leu Cys Leu Ile Ala Tyr Pro Leu Lys 90

GGG GAC CAT GGA ACT GTG GAC CTT GTA GAC AAT TCA GAC TGT GAG CCA AAA AGT 324
Gly Asp His Gly Thr Val Asp Leu Val Asp Asn Ser Asp Cys Glu Pro Lys Ser 108

AAG GTC CTG AGG TGG ACA ACC AAA AAA CAC CAT GTT CTA GAA TCA GAA AAG ACT 378
Lys Val Leu Arg Trp Thr Thr Lys Lys His His Val Leu Glu Ser Glu Lys Thr 126

CCC AAG GAG TGG GTG CGC CAG CAC CGG AAA GAG GAG AAG ATG AAG AGC CAT AAG 432
Pro Lys Glu Trp Val Arg Gln His Arg Lys Glu Glu Lys Met Lys Ser His Lys 144

TTA GAA GAA GAA TTT GAG TGG CTA AAG AAA TCT GAA GTC TTG TAC TAC AGT GTA 486
Leu Glu Glu Glu Phe Glu Trp Leu Lys Lys Ser Glu Val Leu Tyr Tyr Ser Val 162

GAG AAA AAA GGG AAT GTA AGT TCC CAG CTT AAA CAC TAC AAC CCT TGG AGC ATG 540
Glu Lys Lys Gly Asn Val Ser Ser Gln Leu Lys His Tyr Asn Pro Trp Ser Met 180

AAA TGT CAT CAG CAG CAG TTA CAG AGA ATG AAG GAG AAC GCA AAG CAT CGG AAC 594
Lys Cys His Gln Gln Gln Leu Gln Arg Met Lys Glu Asn Ala Lys His Arg Asn 198

CAG TAC AAA TTC ATC TTA CTG GAA AAC CTG ACT TCC CGC TAC GAG GTG CCT TGT 648
Gln Tyr Lys Phe Ile Leu Leu Glu Asn Leu Thr Ser Arg Tyr Glu Val Pro Cys 216

GTC CTG GAC CTC AAG ATG GGC ACG CGC CAG CAT GGT GAT GAC GCG TCA GAG GAA 702
Val Leu Asp Leu Lys Met Gly Thr Arg Gln His Gly Asp Asp Ala Ser Glu Glu 234

AAG GCA GCC AAC CAG ATC CGA AAG TGT CAG CAG AGC ACA TCT GCG GTC ATT GGT 756
Lys Ala Ala Asn Gln Ile Arg Lys Cys Gln Gln Ser Thr Ser Ala Val Ile Gly 252

GTG CGT GTG TGT GGC ATG CAG GTG TAC CAG GCA GGC AGT GGG CAG CTC ATG TTC 810
Val Arg Val Cys Gly Met Gln Val Tyr Gln Ala Gly Ser Gly Gln Leu Met Phe 270

ATG AAC AAG TAC CAC GGG CGG AAG CTG TCG GTG CAG GGC TTC AAG GAG GCA CTT 864
Met Asn Lys Tyr His Gly Arg Lys Leu Ser Val Gln Gly Phe Lys Glu Ala Leu 288

TTC CAG TTC TTC CAC AAT GGG CGG TAC CTG CGC CGT GAG CTC CTG GGC CCT GTG 918
Phe Gln Phe Phe His Asn Gly Arg Tyr Leu Arg Arg Glu Leu Leu Gly Pro Val 306

CTC AAG AAG CTG GCA GAG CTC AAG GCA GTG TTG GAG CGA CAG GAG TCC TAC CGC 972
Leu Lys Lys Leu Ala Glu Leu Lys Ala Val Leu Glu Arg Gln Glu Ser Tyr Arg 324

TTC TAC TCC AGC TCC CTG CTA GTC ATA TAT GAT GGC AAG GAA TGG CCT GAA GTG 1026
Phe Tyr Ser Ser Ser Leu Leu Val Ile Tyr Asp Gly Lys Glu Trp Pro Glu Val 342

GCC CTG GAC TCA GAT GCT GAG GAC TTG GAG GAC CTG TCA GAG GAG TCG GCC GAT 1080
Ala Leu Asp Ser Asp Ala Glu Asp Leu Glu Asp Leu Ser Glu Glu Ser Ala Asp 360

GAA TCT GCT GGT GCC TAT GCC TAC AAA CCC ATT GGT GCC AGC TCC GTG GAC GTG 1134
Glu Ser Ala Gly Ala Tyr Ala Tyr Lys Pro Ile Gly Ala Ser Ser Val Asp Val 378

CGC ATG ATC GAC TTT GCA CAC ACC ACC TGC AGG CTG TAT GGC GAG GAC AGT GTG 1188
Arg Met Ile Asp Phe Ala His Thr Thr Cys Arg Leu Tyr Gly Glu Asp Ser Val 396

GTG CAC GAG GGC CAG GAC GCT GGC TAC ATC TTC GGG CTC CAG AGC CTG ATA GAC 1242
Val His Glu Gly Gln Asp Ala Gly Tyr Ile Phe Gly Leu Gln Ser Leu Ile Asp 414

ATT GTC ACA GAG ATC AGT GAG GAC AGT GGG GAG TGA 1278
Ile Val Thr Glu Ile Ser Glu Asp Ser Gly Glu *** 425

GCTCGCTGGCTGCTCAGTACCTGAGAGACGCTCTGTGTCCCAGGCACAGCTGTGCTGCGTCGGGGAGGAGG
CCAGTATGGCCAGGTGGTGGCCCCTGCAGCCTGGAGCTGATGTGCAGCGGCCTCCGAGCCCCAGCCTGAGC
CAGCCCCAGCTGCGCTTGGAGTCTTTTATTTATTTTAACTGTTTCTTCAACATTCCATATTTGATGATGCA
GATACCTCTTTCTTCCCTGGGTGTGATTGTTCTAATACACATCTCTTCGTTTATAAAAAAAAAAAAAAAAA
AAAAAAAAAAAAAAAAA-3'  1580
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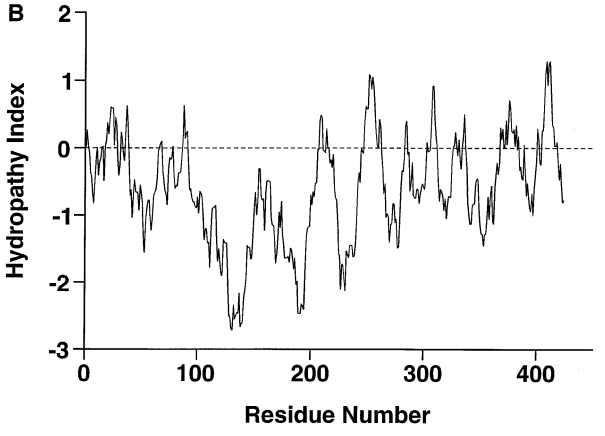


**Fig. 4.** (*A*) Nucleotide sequence and cDNA-derived amino acid sequence of PiUS. The cDNA-insert, 1796 bp in length, encoded a protein of 425 amino acids. The sequence is available at GenBank (accession-number: U74297). (*B*) Hydropathy analysis of PiUS. It was performed as described by Kyte and Doolittle [11]. As seen from the low hydropathy scores over the entire length of the deduced PiUS protein sequence there is no predicted transmembrane stretch.

gesting that in small intestine a different Na/$P_i$-cotransporter may exist. These observations and the recent identification of two additional Na/$P_i$-cotransproter molecules expressed in different nonepithelial tissues [10, 16] suggest the existence of more than four different membrane transport molecules mediating Na/$P_i$-cotransport in one mammalian species. Peerce and colleagues have isolated a protein proposed to be involved in small intestinal brush border membrane Na/$P_i$-cotransport. However its amino acid sequence has as yet not been reported [17, 18, 19, 20].

In the present study, we have identified by expression cloning a cDNA encoding a protein stimulating $P_i$-uptake into oocytes which we have provisionally named PiUS (for $P_i$-uptake stimulator). In principle this protein could be a transporter itself mediating $P_i$-influx into oo-

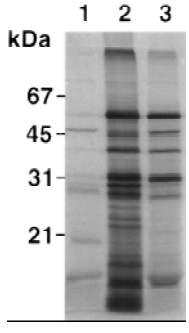


**Fig. 5.** In vitro translation. The reaction was performed in the absence or presence of microsomes. Lane 1: Control (no cRNA); Lane 2: PiUS cRNA without microsomes; Lane 3: PiUS cRNA with microsomes.

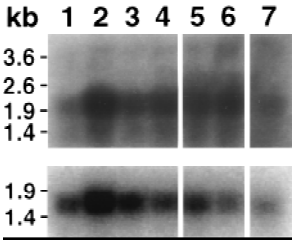


**Fig. 6.** Northern blot analysis of poly $(A)^+$ RNA extracted from different intestinal segments and from other tissues. Radioactive probes were prepared from the PiUS cDNA (upper panel) and from GAPDH cDNA (lower panel) and hybridized sequentially to the same Northern blot. The lanes (all originating from the same blot) correspond to following tissues: 1: duodenum; 2: jejunum; 3: ileum; 4: colon; 5: kidney cortex; 6: kidney medulla; 7: heart. The position and size (in kilobases) of RNA molecular weight markers is indicated (M). Note that the normalized ratio (to GAPDH signals), which was arbitrarily set to 1 for duodenum and tested in 3 Northern blots (for intestinal segments and kidney), was always above 1 and did not exceed a 3.5 maximum in kidney medulla.

cytes or a protein accelerating influx of $P_i$ into oocytes 'indirectly', e.g., being an 'activator' of intrinsic oocyte $P_i$-transporters or being an 'intracellular' protein leading, via an increase in intracellular '$P_i$-consumption' or $P_i$-binding, to an augmented oocyte $P_i$-uptake.

Based on sequence comparisons there is no significant homology of the PiUS protein to any of the known Na/$P_i$-cotransporters (e.g., 14% overall identity or 37% overall similarity to the rat type II Na/$P_i$ cotransporter with no particular molecular region showing a higher identity/similarity). Extensive homology searches suggest that the PiUS cDNA encodes a 'novel' protein. Highest similarity scores were obtained for proteins which to our knowledge have no function in cellular $P_i$-handling, such as an arginine metabolism regulatory protein of yeast (ARG III; Swiss Prot: P07250; ref 8).

We have also tested whether the PiUS-cDNA could be an 'activator' of one of the cloned renal Na/$P_i$-cotransporters (type I and/or type II; refs. 13 and 14). However, coinjection experiments did not show a PiUS-dependent alteration of type I and type II Na/$P_i$-cotransport functions (*data not shown*). It is also unlikely that the PiUS-protein might play a 'direct' role in 1.25 $(OH)_2$ Vit.$D_3$-dependent control of intestinal Na/$P_i$-cotransport activity. The main argument against such a role would be its ubiquitous expression as detected by Northern Blots. However, it cannot be excluded that it would interact rather specifically with the thus far unknown intestinal brush border membrane Na/$P_i$-cotransporter and would thus contribute 'indirectly' to 1.25 $(OH)_2$ Vit.$D_3$-dependent control of intestinal $P_i$-absorption.

Hydropathy analysis and in vitro translation experiments suggest that the PiUS cDNA encodes a soluble (cytosolic) unglycosylated protein rather than a membrane protein. What is the function of this identified, so far unknown PiUS protein? All what we know is that it stimulates oocyte $P_i$ influx/uptake without having an effect on uptake of other solutes e.g., sulfate or L-arginine. From the observation that both Na-dependent and Na-independent $P_i$-uptake into oocyte were stimulated similarly (Fig. 1) we conclude that this protein may not be a specific 'activator' of the intrinsic Na-dependent $P_i$-influx pathway but rather a protein affecting cellular $P_i$ metabolism and/or $P_i$ binding. This would then explain the stimulation of both Na-dependent and Na-independent oocyte $P_i$ uptake. Such a more 'general' role of the identified cDNA/protein would also be in agreement with its ubiquitous expression as detected in Northern blots.

The work was supported by the Swiss National Fonds (Grant No. 32.30785). The art work done by C. Gasser and secretarial help by N. Singer are acknowledged.

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