Translated anti-sense product of the Na/phosphate co-transporter (NaPi-II)

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The homeostasis of P_i in marine teleosts is maintained by renal P_i secretion as well as by P_i reabsorption. A Na/P_i co-transport system belonging to the NaPi-II protein family is instrumental in tightly controlled renal P_i handling in mammals and fish. We have isolated an NaPi-II related cDNA from winter flounder. It was cloned from a female gonad cDNA library and is 624 bp long. The transcript is expressed in female and male flounder gonads as well as in kidney and intestine, although at very low levels. RNase H digestion experiments revealed an opposite orientation of the transcript with regard to NaPi-II-related mRNA. The anti-sense orientation was confirmed by genomic sequence analysis and Southern blotting. Alluding to the sense transcript, the anti-sense transcript was denoted IPAN. The open reading frame of IPAN encodes a basic protein of 68 amino acid residues. Immunohistochemistry confined the anti-sense related protein, Ipan, to a submembranous compartment of immature oocytes, suggesting a role in oocyte development. In kidney and intestine Ipan is partly co-localized with the Na/P_i co-transporter, implying a regulatory function for the anti-sense protein. However, direct protein–protein interaction could not be established. The existence of a putative open reading frame in other species extends the biological significance of the novel protein.

INTRODUCTION

The homeostasis of P_i in vertebrates is maintained by tightly controlled renal excretion. The key protein involved is a Na/P_i co-transport system. cDNA species encoding this type of transport system, denoted NaPi-II, have been cloned and characterized from various species [1–5]. In mammals the transport protein is confined to the brush-border membrane of proximal tubules and mediates the reabsorption of the filtered tubular P_i.

In contrast with mammals, P_i excretion in teleosts such as the winter flounder involves tubular reabsorption and tubular P_i secretion [6]. Consequently the transport system can be directed to both the basolateral and apical membranes [7]. The direction of net P_i flux depends on hormones and physiological factors such as P_i availability and pH [6]. In winter flounder the same protein has been identified in intestinal brush-border membranes [8].

We report the identification of a novel transcript that was identified during screening for NaPi-II-related transporters. Interestingly, the mRNA is transcribed in the opposite orientation to NaPi-II gene and was therefore denoted IPAN. The gene product of the anti-sense transcript, Ipan, co-localizes with the Na/P_i co-transporter in kidney and intestine. Furthermore Ipan is expressed in male and female gonads. The present study combines aspects of membrane transport with anti-sense regulation.

EXPERIMENTAL

Animals

Winter flounder (*Pleuronectes americanus*) were obtained from Mount Desert Island Biological Laboratory (Salsbury Cove, ME, U.S.A.). The fish were caught in July or August and killed by decapitation. Different tissues of the animals were used for nucleic acid extraction and immunohistochemistry.

RNA preparation and Northern blot analysis

Total RNA from different tissues of winter flounder was isolated by the method of Chomczynski and Sacchi [9]. Poly(A)$^+$ RNA was purified from total RNA by using the Oligote-dT mRNA kit from Qiagen. It was separated on a 1.2% (w/v) agarose/formaldehyde gel and transferred to a GeneScreen Plus membrane (NEN). Hybridization was performed in SSPE buffer [0.15 M NaCl/10 mM sodium phosphate (pH 7.4)/1 mM EDTA] containing 50% (v/v) formamide at 42 °C overnight. For detection a ^{32}P-labelled 2.4 kb NaPi-II fragment was used. The filters were washed at low stringency (0.2 × SSC/0.1% SDS) at 50 °C.

cDNA library construction and screening

Size-fractionated gonad mRNA from a female winter flounder was used to construct a unidirectional cDNA library in *Escherichia coli* DH10 (Superscript system; Gibco BRL), of which 50000 clones were screened. Plasmid DNA from pools of 1000–1500 clones was extracted and analysed by dot–blot hybridization [50% (v/v) formamide, 42 °C]. A digoxigenin-labelled 0.5 kb fragment, consisting of the well-conserved nucleotides 1033–1543 of flounder NaPi-II cDNA, was used as probe. The membranes were washed at low stringency. The chemoluminescent detection of digoxigenin-labelled probes was performed in accordance with the suppliers' protocol (Boehringer-Mannheim).

Sequencing

DNA sequencing procedures were performed by the method of Sanger et al. [10], with the T7 Sequencing Kit from Pharmacia. Alternatively, amplified DNA fragments were purified (Qiaquick;

Abbreviations used: IPAN, anti-sense transcript of NaPi-II-related mRNA; RT–PCR, reverse transcriptase-mediated PCR.

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Qiagen, Hilden, Germany) and subjected to cycle sequencing (DyePrimer-*Taq* cycle sequencing kit, 373A DNA sequencer; Applied Biosystems, Weiterstadt, Germany).

RNase H digestion and reverse transcriptase-mediated PCR (RT–PCR)

Sense or anti-sense oligonucleotides (20 pmol) were annealed to 1–2 μg of total RNA in 5 μl of 100 mM KCl. After denaturation of the mixture at 70 °C for 10 min, it was cooled to room temperature within 10 min and placed on ice. A 20 μl sample of reaction mixture, containing 1 unit of RNase H (Gibco BRL), 50 units of RNasin (Promega, Madison, WI, U.S.A.), 5 μg of tRNA, 10 mM $MgCl_2$, 20 mM Tris/HCl, pH 7.5, 100 mM KCl and 1 mM dithiothreitol, was added. RNase H digestion was performed for 5 min at 37 °C, followed by phenol/chloroform extraction and ethanol precipitation. The pellets were dissolved in 20 μl of water; 1 μl of the solution was used for the RT–PCR. Oligo(dT)-primed RT was performed with the reverse transcription system from Promega. PCR was done in a Hybaid Omnigene Cycler (MWG-Biotech, Teddington, Surrey, U.K.). Primer sequences and cycling conditions are available from the authors on request.

Extraction of genomic DNA

Genomic DNA was extracted from erythropoietic tissue of winter flounder with the use of standard protocols [11]. Initially the erythropoietic tissue was separated from renal tubules by differential centrifugation, as described previously [12].

Genomic structure

The genomic sequence was elucidated by a PCR-based strategy. In a first step we amplified PCR fragments from flounder genomic DNA and the cDNA species in parallel. The primers were derived from NaPi-II or IPAN cDNA. Either *Taq* or r*Tth* DNA polymerase (Perkin Elmer, Weiterstadt, Germany) were used. Amplicons that showed an increased size when amplified from genomic DNA compared with cDNA were cloned and sequenced.

The 5′ promoter regions of both transcripts were determined by inverse PCR by the method of Kandpal et al. [13]. Genomic DNA (0.5 μg) was digested with *Hin*cII, *Pvu*II, *Ssp*I, *Dra*I, *Rsa*I or a suitable combination of the six enzymes. The digested DNA was precipitated and ligated at a concentration of 3 ng/μl or less. A 5 μl sample of the ligation mix (15 ng of DNA) was used directly for PCR followed by a second reaction with a nested primer pair. The exact cycling conditions and the sequence of the cognate oligonucleotides as well as aliquots of the primers are available from the authors on request.

Fragment assembling and sequence analysis were done with the Genetics Computer Group software [14].

Genomic Southern blot

Each 5 μg of flounder genomic DNA was digested with the restriction endonucleases *Bam*HI, *Pst*I, *Pvu*II, *Sac*I and *Stu*I for 24 h at 37 °C. The fragments were separated on a 0.8 % agarose gel and transferred to a nylon membrane (NEN) by capillary blotting. Two digoxigenin-labelled probes (bp 320–840 and bp 5680–6280) were generated by PCR. Hybridization and chemoluminescent detection were performed in accordance with the supplier's protocols (Boehringer-Mannheim).

Transcription *in vitro*

Capped RNA for oocyte injection was prepared by a standard protocol from Pharmacia (Uppsala, Sweden) for transcription *in vitro*. The amount of synthesized cRNA was determined photometrically.

Expression in *Xenopus laevis* oocytes

The handling of *Xenopus laevis* oocytes as well as the assay for P_i transport activity are described elsewhere [1]. Oocytes were co-injected with either 3 ng of NaPi-II cRNA plus 0.8 or 8 ng of IPAN cRNA (1:1 or 1:10 molar ratio). Oocytes injected with water, 3 ng of NaPi-II cRNA and 0.8 ng of IPAN cRNA were used as controls. The injected volume was 50 nl. P_i uptake was measured 2 days after cRNA injection.

Immunohistochemistry

Immunohistochemistry was performed on serial sections (7 μm) of perfusion-fixed tissues derived from sexually mature flounder [15]. Tissue blocks were frozen in methylbutane at −80 °C for cryosections, or dehydrated and embedded in paraffin. We used polyclonal antibodies against NaP_i-II protein [8] and Ipan. A polyclonal rabbit antiserum was raised in rabbits against the C-terminal portion (NH_2-CPNTVRGKTKKHRTR-CO_2H) of the putative anti-sense protein (EUROGENTEC, Seraing, Belgium). Immunofluorescence procedures followed a standard protocol [8], with a 1:400 dilution of the primary antibody or preimmune serum. The secondary antibody, a goat (anti-rabbit IgG) conjugated to Cy3 (DIANOVA, Hamburg, Germany) was used in a 1:100 dilution.

RESULTS

Screening for NaPi-II-related transporters

Northern blot analysis at low stringency demonstrated that,

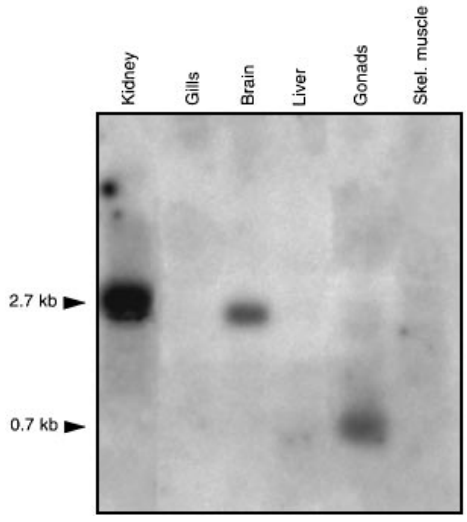

Figure 1 Expression of flounder NaPi-II mRNA and related transcripts

Northern blot analysis of different flounder tissues. mRNA (1–2 μg) was separated on a 1.2% (w/v) agarose/formaldehyde gel and transferred to a GeneScreen Plus membrane. Hybridization was performed in SSPE buffer containing 50% (v/v) formamide at 42 °C overnight. For detection a randomly ^{32}P-labelled 2.4 kb flounder NaPi-II fragment was used.

A

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        10                  30                  50
ttgtctcataaggtttgtcgtttcttgctgtcctcccctgctttctgagcttttgtgttc

        70                  90                  110
cctgtgcaccttgccactcaacttctgctcctctgtttcaccagagtaggcacctgtatt

       130                 150                 170
ttcgtcctcgtgtttttgccgcaccagagttttcacctcatttgcagcagcagcagcagt

       190                 210                 230
atttgccgcagaaccctaaggccgaggtcaccacagtgtcccaaggcgccATGGAATGCA
                                                  M  E  C  R

       250                 270                 290
GAGGTCGGGGCAAAAAGTCCCAGTCTTGTAGGACCTTGGGCAGGAAGCGGGGGCATCGAG
  G  R  G  K  K  S  Q  S  C  R  T  L  G  R  K  R  G  H  R  D
                   ↑

       310                 330                 350
ACTGCATCACGTTGACGACTATCACAAAGACAACCAACACAACGAATGGCACGCCGACTC
  C  I  T  L  T  T  I  T  K  T  T  N* T  T  N  G  T  P  T  P

       370                 390                 410
CAACGAGGACCTGCCAGCCAGCCATTGACAGCCCGAACACAGTCAGAGGGAAAACCAAGA
  T  R  T  C  Q  P  A  I  D  S  P  N  T  V  R  G  K  T  K  K
                                     ↑              ↑

       430                 450                 470
AGCACAGGACGAGGtagaggcccgcaaaccagcggtattttgctgtatggttgcccaacc
  H  R  T  R
       490                 510                 530
ctctggccaacctgatgggcacccgcgtgaaggggattggatagaacagtaagataccca

       550                 570                 590
tgacgttgaagaagaaatggcaaagtgcaatctggaagaaaataaaaacagataaatgac

       610
attaaaacaaggttataaaagctt
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B

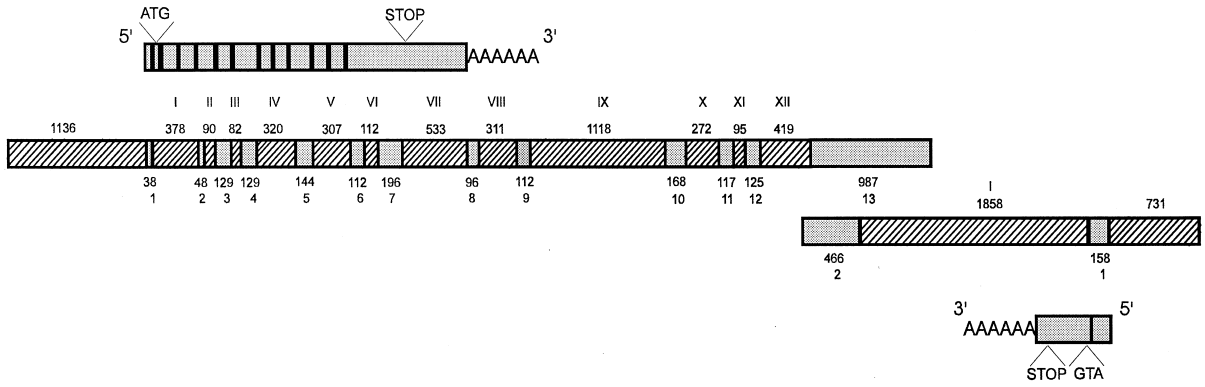

Figure 2 Organization and nucleotide sequence of IPAN and deduced amino acid sequence

(**A**) cDNA sequence of IPAN and deduced amino-acid sequence of the anti-sense transcript. The ATG codon at position 231 is in good agreement with Kozak's rules [31]. The potential polyadenylation signal is underlined. The star is placed at a potential N-glycosylation site. The potential phosphorylation sites for protein kinase C are marked by arrows. (**B**) Schematic representation of the transporter encoding gene and the deduced cDNA species, NaPi-II and IPAN. Exons are represented as light grey bars. Regions shown as hatched bars indicate promoter and intron sequences. The lengths of the exons/introns are indicated. The gene bank accession number of the flounder NaPi-II gene is U72067.

besides kidney and intestine, NaPi-II-related mRNA species are expressed in brain (2.6 kb) and gonads (0.7 kb) (Figure 1). Assuming that the transcript in gonads represented a 'mini' version of a P_i-transporting system, we focused our study on this gene product. A directional cDNA library from female gonad mRNA was constructed. Approx. 50000 clones were screened with a digoxigenin-labelled probe representing the conserved second hydrophobic domain of flounder NaPi-II. A single clone was isolated showing partial identity with flounder NaPi-II (Figure 2). The sequence of the cDNA indicated an anti-sense

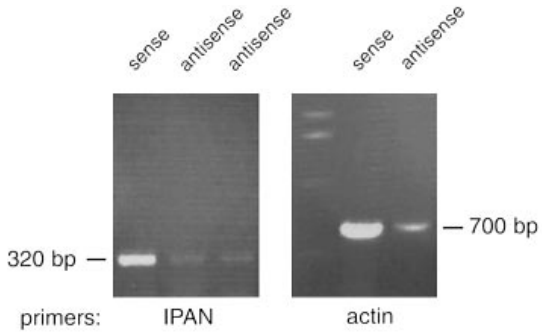

Figure 3 RNase H analysis of female flounder gonad mRNA

Left panel: mRNA was digested with IPAN-specific primers (lane 1, sense primer B7; lane 2, anti-sense primer B14; lane 3, anti-sense primers B14, B15 and B21). Right panel: mRNA was digested with actin-specific primers.

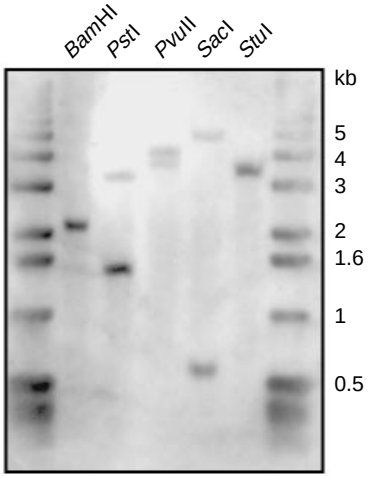

Figure 4 Southern blot analysis of flounder genomic DNA

The DNA was digested with the restriction endonucleases *Bam*HI, *Pst*I, *Pvu*II, *Sac*I and *Stu*I. The fragments were subjected to electrophoresis and transferred to a nylon membrane. The blot was probed simultaneously with two different digoxigenin-labelled PCR-generated probes (nt 320–840 and 5680–6280). A digoxigenin-labelled DNA 1 kb ladder was loaded for size calibration (outer left and right lanes).

orientation of the new clone with regard to the poly(A) tail and poly(A) addition site.

Anti-sense orientation

To verify the opposite orientation of the new clone, experiments with RNase H were performed. Sense oligonucleotides hybridizing to NaPi-II-related mRNA, or anti-sense oligonucleotides recognizing the potential anti-sense transcript, were annealed to gonad total RNA. The hybrids were subjected to digestion with RNase H and the specifically depleted samples were analysed by RT–PCR (Figure 3). A control with actin primers was included. Incubation with anti-sense primers resulted in the decrease of a gonad-specific PCR signal (Figure 3, left panel, lanes 2 and 3), compared with the reaction with the sense oligonucleotide (Figure 3, lane 1). RNA secondary structure and sensitivity of PCR might explain the faint bands seen in lanes 2 and 3. The combination of several specific anti-sense oligonucleotides and a prolongation of the RNase H digestion did not complete the reaction.

Further evidence for an opposite orientation of the new transcript was derived from genomic DNA sequencing and the deduced intron/exon structure of the NaPi-II gene.

Genomic organization of sense and anti-sense transcripts

We investigated the genomic structure of the NaPi-II gene by following a PCR-based strategy, including inverse PCR for the 3′ and 5′ untranscribed region [13].

The entire gene spanned 9.7 kb including the promoter sequences for the NaPi-II and the IPAN-related transcript. The transporter encoding part included 7.6 kb, comprising 13 exons and 12 introns. The anti-sense related part consisted of 3.2 kb with two exons interrupted by an intron of 1859 bp. The consensus 5′-GT and 3′-AG splice sites were conserved at the boundaries of all introns.

The first exon of IPAN was located 1300 bp downstream of the flounder NaPi-II polyadenylation signal; the second exon of IPAN overlapped with exon 13 of the sense transcript. The open reading frame of IPAN consisted of 204 bp and was located entirely within the second exon (Figure 2).

The identified promoter region comprised 1136 and 731 bp for NaPi-II and IPAN respectively. Sequence analysis revealed a TATA box at position −30 relative to the experimentally determined transcription initiation start of flounder NaPi-II cDNA (result not shown). For IPAN a TATA consensus sequence was detected at position −47 relative to the cloned cDNA. The promoter regions shared several putative binding sites for transcription factors such as the activator protein (AP-1), the zinc finger repressor CTCF and a cAMP-responsive element [16]. Their biological function remains to be established.

To exclude a second NaPi-II-related gene, Southern blot analysis was performed (Figure 4). Flounder genomic DNA was cleaved with different restriction endonucleases and blotted on a nylon membrane. The blot was hybridized with two different DNA probes representing either a near 3′ or 5′ region of the NaPi-II gene, both lacking recognition sites for the endonucleases used. If there was a single gene, two bands should be found in each lane. As shown in Figure 4, this was true for the samples *Pst*I (1.6 and 3.3 kb), *Pvu*II (3.8 and 4.6 kb) and *Sac*I (0.6 and 5.3 kb). The lanes *Bam*HI (2.3 kb) and *Stu*I (3.7 kb) only showed a single band, probably owing to the inefficient transfer of long DNA fragments to the nylon membrane. The size of the different fragments was in agreement with the restriction map of the genomic sequence. We concluded that a single gene encodes NaPi-II and the new transcript in flounder.

Characterization of IPAN

The cDNA clone consisted of 624 bp with an open reading frame starting at nt 231 (Figure 2). It encoded a highly basic protein (pI $\approx$ 12) with a calculated molecular mass of 7 kDa; 17 out of 68 amino acids were either Arg or Lys. The putative protein contained two potential N-glycosylation sites and three potential protein kinase C recognition sites. Furthermore Ipan contained two potential nuclear targeting sequences. Comparison of the predicted amino acid sequence with other proteins in the data bank revealed no similarity.

To investigate the biological relevance of IPAN a series of different experiments addressing its localization and function were performed.

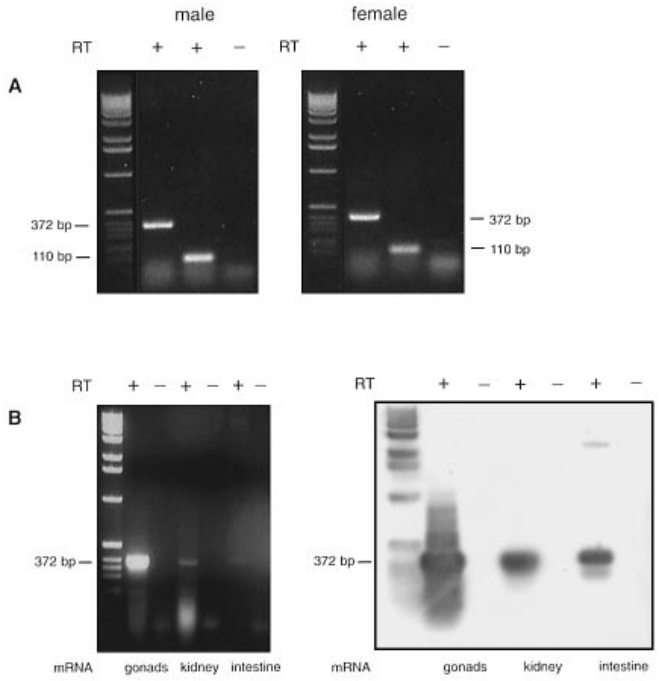

Figure 5 Expression of IPAN in different flounder tissues detected by RT–PCR

(**A**) Ethidium bromide-stained agarose gel showing RT–PCR products from male and female gonads, with two different sets of primers, B13 and B14 (lanes 2, 4, 6 and 8) and B12 and B7 (lanes 3 and 7). (**B**) Agarose gel of RT–PCR products amplified with primers B13 and B14 and a cognate Southern blot demonstrating the presence of IPAN-related transcripts in female gonads, kidney and intestine. Reactions without reverse transcriptase were performed as negative controls. The sequences of the primers used were as follows: B7, 5′-GTAGAGG-CCCGCAAACCA-3′; B12, 5′-TGCCATTTCTTCTTCAACG-3′; B13, 5′-CTGTGCACCTTGCCACTC-3′; B14, 5′-TGGTTTGCGGGCCTCTAC-3′.

Expression of IPAN in different flounder tissues

The tissue distribution of IPAN was investigated with RT–PCR because very low levels of mRNA were expected. As shown in Figure 5, IPAN was predominantly expressed in female and male gonads. Furthermore, it was detected in kidney and intestine, although at very low levels. Liver, skeletal muscle and gills were negative for IPAN (results not shown).

The co-expression of IPAN and NaPi-II implies a possible interaction between the anti-sense transcript and its sense counterpart. IPAN and NaPi-II could either interact on the RNA level or IPAN could be translated and act via protein–protein or protein–nucleic acid interactions.

Oocyte expression

The anti-sense transcript was complementary to NaPi-II over a stretch of 408 nt and could block the translation of the co-transporter. To assess this hypothesis we injected RNA synthesized *in vitro* (cRNA) into *Xenopus laevis* oocytes and measured Na^+-dependent P_i transport. The anti-sense RNA was present either in equimolar amounts or in a 10-fold excess. As illustrated in Table 1, the injection of NaPi-II-related cRNA increased Na/P_i co-transport activity 19-fold, whereas the injection of IPAN cRNA alone did not stimulate transport activity. Co-expression of both RNA species in equimolar amounts did not affect the NaPi-II-related stimulation of Na/P_i co-transport activity in a statistically significant manner. When a 10-fold excess of anti-sense RNA was injected, the enhanced transport activity decreased by 42 % (Table 1). Considering the low level of expression of IPAN in all the tissues tested, a direct RNA–RNA interaction seems unlikely.

Table 1 Functional expression of sense and anti-sense cRNA in *X. laevis* oocytes

Oocytes were co-injected with either 3 ng of NaPi-II cRNA plus 0.8 ng of IPAN cRNA (1:1 molar ratio) or sense cRNA plus 8 ng of anti-sense cRNA (1:10 molar ratio). Oocytes injected with water, 3 ng of NaPi-II cRNA and 0.8 ng of IPAN cRNA were used as controls. The injected volume was 50 nl. Values are means ± S.E.M. for six to eight oocytes per group in a representative experiment.

Injection into oocytes	Transport activity (pmol of P_i/h per oocyte)
Water	53.8 ± 6.0
NaPi-II	1016.4 ± 97.1
IPAN	53.1 ± 9.8
NaPi-II + IPAN (1:1)	1259.1 ± 94.4
NaPi-II + IPAN (1:10)	617.5 ± 32.1

Ipan

To study a putative translation product of IPAN we generated an antiserum against the C-terminal 18 amino acid residues of the deduced protein. The specificity of the antibody was demonstrated by immunoprecipitation of the ^{35}S-labelled *in vitro* translation product and by ELISA (data not shown). The antiserum was used for immunohistochemical analysis of perfusion-fixed cryostat sections of flounder gonads, intestine and kidney. As illustrated in Figure 6, Ipan was detected in a submembranous compartment in immature flounder oocytes: 20–30 % of the oocytes revealed a strong signal; 70–80 % showed either a moderate or weak staining, suggesting that the anti-sense gene product is transiently expressed throughout oocyte development. Incubation with the preimmune serum did not reveal any fluorescence (Figure 6b). Consistent with the RT–PCR results, an NaPi-II protein-specific staining could not be demonstrated for flounder oocytes (Figures 6c and 6d). A faint but non-specific staining occurred with both the NaPi-II antiserum and the preimmune serum. Preincubation of the NaPi-II antiserum with the cognate protein did not decrease the background staining. Figures 6(e)–6(h) show a fold of the thin portion of flounder small intestine. The epithelium consists of high and slender columnar enterocytes with intermingled mucocytes. The anti-sense protein and the Na/P_i co-transporter were predominantly confined to the apical membrane (Figures 6e and 6g). Strong staining with the Ipan-specific antibody also occurred in mucocytes. The significance of this signal is not clear. No specific binding was detected with the preimmune sera.

Figures 6(i)–6(l) show the two proximal tubular segments PI and PII, and the collecting duct. The tubular segment PI differs from segment PII by an extensive apical lysosomal apparatus. Furthermore the segments can be distinguished by lectin-specific staining [6]. As illustrated in Figure 6(i), Ipan-specific immunofluorescence was mainly localized to the apical compartment of the collecting duct. A faint staining also occcurred in the apical region of PII cells. Cells of the proximal tubule segment PI exhibited no binding of the fluorochrome. A marked binding of the fluorochrome was found in the apical and basolateral regions of collecting duct cells. In addition a faint staining was confined

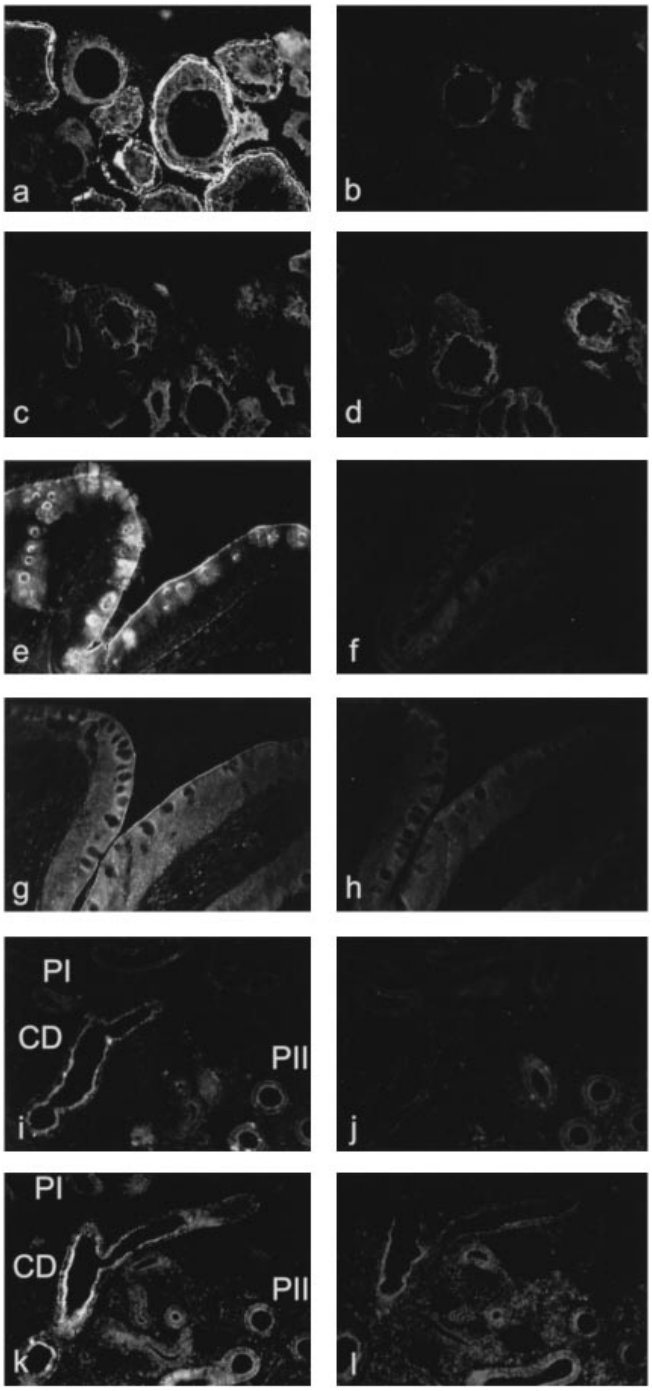

Figure 6 Immunohistochemical localization of Ipan and NaPi-II

Immunohistochemistry was performed on serial sections (7 μm) of perfusion-fixed sexually mature flounder [10]. Parallel sections of flounder ovary are shown at the top, of small intestine in the middle and of flounder kidney at the bottom. Proximal tubular segments PI and PII and collecting duct (CD) are indicated. Sections (**a**), (**e**) and (**i**) were incubated with anti-Ipan serum; sections (**c**), (**g**) and (**k**) were incubated with anti-NaPi-II protein serum. Panels on the right represent controls and were incubated either with the cognate preimmune serum (**b**, **d**, **f**, **h**, **j**) or with the peptide-protected antiserum (**l**). Magnification $\times$ 188.

to the apical and basolateral membranes of PII cells. In view of the co-localization of NaPi-II and Ipan in kidney and intestine in the apical compartment we assumed a direct interaction of the

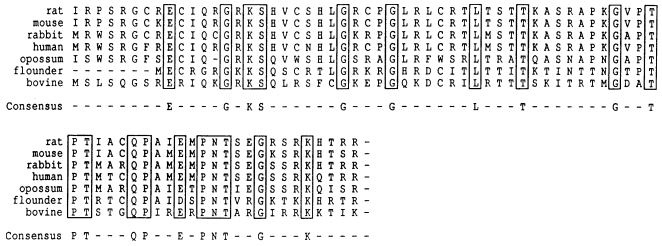

Figure 7 Putative IPAN expression in other vertebrates

Alignment of the candidate regions for a putative Ipan from rabbit, human, bovine, rat, mouse and opossum. Deduced amino acids from the opposite strand of the cognate cDNA species are shown; conserved residues are boxed.

two proteins. However, neither *in vitro* co-precipitation experiments of radioactively labelled Na/P_i co-transporter and Ipan nor pull-down assays with overexpressed fusion proteins indicated a direct association.

Anti-sense expression of IPAN in other vertebrates

The open reading frame of IPAN is located entirely within the flounder NaPi-II-encoding region. To test whether the expression of the anti-sense transcript is a phenomenon common to all species expressing NaPi-II, we searched for putative open reading frames within the relevant region of all known NaPi-II cDNA sequences. As demonstrated in Figure 7 the open reading frame was conserved in flounder and bovine as well as in rabbit and human. Rat, mouse and opossum contained no open reading frame. The putative ATG codon in these species was abolished by a single mutation (Met $\rightarrow$ Ile). No ATG could be found further upstream. The deduced amino acid sequences shared 40–57 % identity with the flounder protein. Structural amino acids (Pro and Gly) and amino acids with polar side chains (Lys, Glu, Gln and Thr) were conserved within the consensus sequence (Figure 7). All putative proteins were characterized by a pI between 12.1 and 12.4. It remains to be established whether a putative IPAN homologue is expressed in these species *in vivo*.

DISCUSSION

In a screen for NaPi-II related transporters we have isolated an anti-sense transcript (IPAN) partly identical with flounder NaPi-II. Southern blot analysis and sequencing of the entire flounder NaPi-II gene confirmed a common locus for NaPi-II and IPAN. The homologous genes from mouse and human share a comparable intron/exon structure with the flounder gene [17,18]. Only the human gene contains an open reading frame for a putative Ipan homologue. IPAN did not inhibit the translation of the Na/Pi-II-related RNA, as demonstrated by co-injection of the two cRNA species in equimolar amounts in *X. laevis* oocytes. In contrast with most other known anti-sense transcripts, IPAN is translated *in vivo*. Immunohistochemical data gave evidence for the co-ordinated expression of flounder Napi-II protein and Ipan. In flounder kidney and intestine both antisera, anti-Ipan and anti-Napi-II, identified co-localized structures in apical compartments. Furthermore Ipan was confined to a submembranous compartment in flounder oocytes. In view of the expression *in vivo* and the results obtained after co-expression in oocytes we propose that IPAN acts at the protein level rather than at the RNA level. We suggest that Ipan has a germ-cell-

specific function independent of NaPi-II expression, and a regulatory role directly related to the Na/P_i co-transport system.

Anti-sense transcripts have been described in prokaryotic systems as well as in eukaryotic systems [19]. In prokaryotes anti-sense RNA species are involved in the regulation of gene expression and DNA replication via hybridization of the complementary transcripts. In eukaryotes the biological relevance of anti-sense RNA as well as its mode of influence is more speculative. An increasing number of eukaryotic anti-sense transcripts have been reported [20–25], especially for genes involved in development. The translation of the basic fibroblast growth factor anti-sense RNA, GFG, has been demonstrated [26,27]; however, its cellular function is not clear.

The fact that IPAN is translated *in vivo* suggests that Ipan represents a so-called anti-sense peptide. These peptides, encoded by opposite DNA strands, are predicted to interact at the protein level [28–30]. However, this hypothesis seems unlikely because the two RNA species are translated in different reading frames.

To exclude Ipan from being a phenomenon restricted to winter flounder, we tested anti-Ipan serum on different fish species. Immunohistochemistry gave evidence for the existence of an Ipan-related protein in zebrafish ovaries (results not shown), emphasizing the significance of the new protein for oocyte development. A sequence search for a homologous vertebrate IPAN revealed an open reading frame within the relevant region in bovine, human and rabbit. It remains to be tested whether a putative IPAN homologue is expressed *in vivo*.

Among the hallmarks of flounder NaPi-II are the unique sorting behaviour and the presence of the anti-sense transcript. The flounder NaPi-II protein is sorted to either the apical membrane or the basolateral membrane of kidney and intestine. Mammalian NaPi-II is confined exclusively to the apical compartment in renal epithelium. Therefore it seems intriguing to assign Ipan a regulatory role in protein sorting, although co-precipitation experiments and pull-down assays of the Na/P_i co-transporter and Ipan *in vitro* did not show an interaction. Cofactors or auxiliary proteins might be important for the association. The Na/P_i co-transport system, NaPi-II, and the anti-sense transcript, IPAN, open up a new level of cellular regulation and provide a promising model system for the study of the biological role of a eukaryotic anti-sense RNA. However, additional genetic and biochemical approaches will be required to elucidate the cellular function of IPAN.

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