In vivo fractional Pi absorption and NaPi-II mRNA expression in rainbow trout are upregulated by dietary P restriction

Shozo H. Sugiuira, Nichole K. McDaniel, and Ronaldo P. Ferraris

Department of Pharmacology and Physiology, New Jersey Medical School, University of Medicine and Dentistry of New Jersey, Newark, New Jersey 07103

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Sugiura, Shozo H., Nichole K. McDaniel, and Ronaldo P. Ferraris. In vivo fractional Pi absorption and NaPi-II mRNA expression in rainbow trout are upregulated by dietary P restriction. Am J Physiol Regul Integr Comp Physiol 285: R770–R781, 2003.—Mammalian type II sodium-phosphate cotransporter (NaPi-II) and inorganic phosphate uptake stimulator (PiUS) genes are upregulated by dietary phosphorus (P) restriction to increase intestinal and renal P transport, but little is known about NaPi-II and PiUS regulation in other vertebrates. We studied 1) the tissue distribution and dietary regulation of NaPi-II, PiUS, and sodium-glucose cotransporter (SGLT1) mRNA and NaPi-II protein in juvenile rainbow trout (Oncorhynchus mykiss) and 2) effects of dietary P on intestinal Pi absorption in vivo. NaPi-II, PiUS, and SGLT1 mRNA were found in the proximal and distal intestine, pyloric ceca, and kidney. PiUS mRNA was also found in the heart, gill, blood, stomach, liver, skin, and muscle. Tissue distribution of NaPi-II protein correlated with that of NaPi-II mRNA except in gill ionocytes where NaPi-II antibodies recognized related epitopes. Chronic consumption of a low-P diet increased NaPi-II and PiUS but not SGLT1 mRNA abundance in the intestine and kidney. Unlike mammals, there was no detectable shift in tissue or cellular localization of NaPi-II protein in response to dietary P restriction. Regulation of NaPi-II and PiUS mRNA expression was observed only in fish grown under optimal aqueous oxygen concentrations. In vivo fractional absorption of Pi by the intestine decreased in fish fed high-P diets. Decreases in absorption were less pronounced in fish previously fed low-P diets, suggesting that diet history modulates acute regulation of P absorption. Regulation of dietary Pi absorption in vivo may involve a specific change in intestinal NaPi-II and PiUS gene expression.

fractional absorption; immunocytochemistry; phosphate uptake stimulator; sodium-glucose cotransporter; sodium-phosphate cotransporter

In mammals, phosphorus (P) homeostasis is accomplished mainly by inorganic phosphate (Pi) transporters in epithelial cells of the intestine and kidney. Intestinal absorption of Pi is mediated primarily via type II sodium-phosphate cotransporter (NaPi-II) in the brush-border membrane, whereas renal Pi reabsorption is mediated by the NaPi-IIa isoform located in the apical membrane of the renal proximal tubule (37). In fish, however, the intestine and kidney have only two closely related NaPi-II isoforms, named NaPi-IIb1 and NaPi-IIb2, respectively, and there appear to be multiple sites where NaPi-IIb2 is expressed in the kidney. In flounder (Pleuronectes americanus), NaPi-IIb is found in the basolateral membrane of cells in the second segment of the proximal tubule (PII; see Refs. 9 and 20), whereas in the zebrafish (Danio rerio), NaPi-IIb is located in the apical membrane of cells lining the PII region and the collecting tubule (9). In the intestine of these species, NaPi-IIb protein has been localized along the apical membrane of enterocytes (13, 20).

Proteins other than NaPi may also participate in the regulation of Pi balance. The Pi uptake stimulator (PiUS) or inositol hexakisphosphate kinase (34) has been shown to be involved in the absorption of dietary Pi. PiUS cRNA encodes a protein that markedly increased Na-dependent Pi-uptake when injected in Xenopus laevis oocytes (18, 26) but that is not a Pi transporter. PiUS mRNA has been isolated from rabbit (26) and rat (18) small intestine.

These findings have contributed to increases in our understanding of molecular mechanisms regulating intestinal and renal nutrient transport, especially in mammals. The rate of transport of many essential nutrients, including P in mammalian intestine and kidney, increases with decreasing dietary concentrations, and the adaptive mechanism is typically an increase in the number of transporters (11, 12). Dietary Pi restriction has been shown to upregulate Pi transport in rodents by translocation of renal NaPi-IIa protein to the apical surface of the epithelial cells independent of changes in NaPi-II transcription (reviewed in Ref. 24). Dietary Pi restriction also stimulates the recruitment of more distal regions of the rat renal tubules to participate in Pi reabsorption (32, 38).

In contrast, the adaptive patterns and mechanisms of transport of essential nutrients in other vertebrates are virtually unknown. Although we know that many genes are highly conserved among distantly related species, is regulation of those genes also highly conserved? This is an important question in regulatory biology. There have been advances in our knowledge regarding the evolutionary relationships of various
NaPi isoforms in many animals, including fish (37). However, our knowledge of mechanisms regulating P₁ transport in fish still remains rudimentary and lags far behind that of mammals. Moreover, dietary P is so poorly assimilated by fish that significant amounts eventually appear in effluents from fish farms and lead to severe pollution of nearby lakes and rivers, where P is the nutrient limiting primary productivity (5). These physiological, nutritional, and environmental roles of P have heightened the priority of P research in fish.

There has only been one study demonstrating dietary regulation of P₁ transport in fish. In rainbow trout (Oncorhynchus mykiss), dietary P restriction increases intestinal P₁ uptake (2). There have only been a few studies on the regulation of P₁ absorption in vivo. Moreover, the molecular mechanisms regulating P₁ transport have not been well studied in any fish species, except in an initial report indicating that trout renal and intestinal NaPi-IIb mRNA abundance increased under conditions of dietary P restriction and vitamin D excess (7). In rats, dietary P restriction increased intestinal PiUS mRNA abundance and P₁ uptake rate by twofold (18). However, nothing is currently known about the regulation of PiUS in other species.

To increase our understanding of regulation of P₁ transport, we studied the molecular mechanisms of P₁ transport in rainbow trout by identifying the tissue distribution and localization of NaPi-IIb, PiUS, and the sodium-glucose cotransporter (SGLT1; negative control) and characterizing their regulation by dietary P in the intestine and kidney. Because oxygen concentrations in the water affect dietary P retention in trout (23), we also evaluated the effect of aqueous oxygen concentrations on NaPi and PiUS expression. Finally, we studied the in vivo regulation of P₁ absorption by dietary P. We chose the rainbow trout as an animal model because it is one of the most important species in freshwater aquaculture and has been the subject of many studies on dietary P requirements and digestibility but unfortunately not on P₁ transport. It has a relatively short gastrointestinal tract typical of carnivores and a glomerular nephron similar to mammals (40).

MATERIALS AND METHODS

Fish, Diets, and Tissue Collection

Tissue distribution of NaPi-II, PiUS, and SGLT1 mRNA. A group of rainbow trout (mean body wt 221 g) was fed a low-P diet at 2% body wt/day for 1 wk. The purpose of the low-P diet was to stimulate NaPi-II and PiUS mRNA expression in all tissues. To make a low-P diet, 50% commercial low-P trout feed was mixed with 5% spray-dried egg white, 5% wheat gluten, 25% wheat flour, 10% soy oil, and 5% vitamin and mineral premixture. Preliminary digestibility measurements by Cr₂O₃ determined that the diet contained 0.35% available P, which is much less than the known dietary P requirement of 0.6%. On day 7, five fish were randomly sampled and analyzed for tissue distribution of NaPi-II, PiUS, and SGLT1 mRNA. Two fish were examined by Northern blot and the other three by RT-PCR for mRNA expression in the following tissues: heart (whole), skin (no scales), muscle (skeletal, white), stomach, gills (filaments only), scales (with mucus), blood (whole clotted blood), pyloric ceca (distal ½ from the intestinal-cecal junction), proximal intestine (distal to pyloric ceca, light-colored region without folds, roughly ½ of the total length of the intestine), distal intestine (dark-colored intestine lined with folds), kidney (posterior kidney), and liver (whole).

Immunolocalization of NaPi-II protein and regulation of NaPi-II, PiUS, and SGLT1 expression by dietary P and oxygen levels. Rainbow trout (initial mean body wt 188 ± 4.4 (SE) g) were randomly distributed in 12 tanks (1 m³) and pair fed either a low-P diet (0.46% available P; 0.60% total P) or high-P diet (0.74% available P; 0.90% total P) for 7 wk at two different oxygen levels (6 or 10 ppm). The moderately low-P diet and moderately high-P diet were chosen because these encompass the range of dietary P in commercial trout feeds, with the low-P diet expected to lead to moderate P deficiency and the high-P diet to P sufficiency. The modest differences in dietary P should not lead to marked changes in growth and therefore to nonspecific changes in P concentrations in the tissues. Moreover, severe hypophosphatemia is rarely encountered in cultured fish, whereas moderate hypophosphatemia is more prevalent.

Each treatment had triplicate tanks. Oxygen at 6 ppm was well above the lowest tolerance level for trout and similar to commercial trout ponds, whereas the high oxygen level was near saturation (31). Fish in all treatments were fed at 2% (1% at 8:00 AM and 1% at 2:00 PM) of their body weight daily. Diets were manufactured as dry pellets with the following composition (%): 18 soybean meal, 17.6 corn gluten, 12 soy oil, 10 blood meal, 10 fish meal, 6 fish protein hydrolysate, 2.4 pellet binder, 22.1 wheat flour, 0.4 L-lysine, 0.2 DL-methionine, 0.7 mineral mixture, and 0.6 vitamin mixture. The low-P diet and high-P diet had the same composition except that the high-P diet was supplemented with 1.35% NaH₂PO₄, replacing a portion of wheat flour in the low-P diet.

After 7 wk, 10 fish from each tank were randomly sampled for blood and carcass analysis. Tissues of the posterior kidney and the proximal intestine were sampled (2 fish/tank), pooled by tank, and kept in RNAlater solution (Ambion, Austin, TX) for mRNA analysis. Tissues collected for immunocytochemistry were rinsed in Kreb's Ringer bicarbonate buffer (KRB, in mM: 100 NaCl, 2 KCl, 2 CaCl₂, 1 MgSO₄, 1 NaHCO₃, and 0.5 NaH₂PO₄, pH 7.4) and placed in buffered formalin (10% formalin, 75 mM sodium phosphate buffer, pH 7.4). After 24–48 h of fixation, tissues were rinsed in KRB solution and then transferred to 70% ethanol and stored at 4°C for 10–30 days until further processing. Blood samples were collected from caudal vessels of each fish into heparinized syringes 24 h after the last feeding, and the plasma was separated (1,500 g) immediately after collection.

In vivo fractional P₁ absorption. Rainbow trout (initial mean body wt 129 ± 2.7 (SE) g) were initially fed either a low-P (0.18% total P) or high-P (0.92% total P) diet containing 0.5% Cr₂O₃ one time daily at 1% (dry basis) of their body weight for 32 days. Except the dietary P level, compositions of these diets were the same as those of diets used in the subsequent test period. After the 32-day period, fish were switched to one of 10 test diets of varied P concentrations for 12 days, and the feces were collected continuously for 24 h in an unstirred fecal collection tube attached to each tank (modified from Ref. 14) at 3, 6, 9, and 12 days after the switch in diet for P and Cr analyses. The unstirred collection tube minimized the leaching loss of P from feces after being voided.
by the fish. Each tank contained 14 fish. All diets contained (% dry diet): 0.18 (basal), 0.31, 0.44, 0.56, 0.69, 0.81, 0.92, 1.03, 1.14, and 1.25. The net Pi absorption of the diets were (%dry diet): 0.18 (basal), 0.31, 0.44, 0.56, 0.69, 0.81, 0.92, 1.03, 1.14, and 1.25. The net P; absorption of the basal diet was 64.8% on average. Diets were made low in calcium to minimize interaction (precipitation) of Pi in the intestine. Dietary inclusion of wheat gluten prevented fecal disintegration and leaking loss of P in the feces during collection. Compared with the other two (tissue distribution and regulation) experiments, the feeding rate was reduced to 1% daily because of the higher caloric content of the diet.

The initial fish size differed among the three experiments conducted over a 2-yr period, because trout spawn one time a year, meaning that it is possible to obtain fish of the same age only one time a year. However, trout grow extremely fast between 3 and 12 mo of age, and we estimate the age difference of fish used in this experiment (130-220 g) to be only ~3 mo. The rapid growth of trout at this stage makes the fish sensitive to small changes in dietary P concentrations. All fish were treated according to the guidelines of the Institutional Animal Care and Use Committee of the University of Medicine and Dentistry of New Jersey (animal care and use protocol no. 02038).

Analyses and Calculations

Northern blots and RT-PCR. Total RNA was extracted by Trizol reagent (Invitrogen, Carlsbad, CA) from tissues previously stored in RNAlater solution, and mRNA abundance of tissues was quantified by Northern blot analysis as described by Shu et al. (35). In the tissue distribution study, Northern membranes were probed with a mixture of intestinal and renal cDNA (equimolar basis) for NaPi, and SGLT1 since kidney and intestine sequences are different (64% homology for NaPi; 93% for SGLT1). For PiUS, intestinal cDNA was used for all tissues (PiUS sequences of other tissues were not studied). In addition to Northern analysis, semiquantitative RT-PCR was also performed to quantitate minor transcripts that were difficult to detect by Northern analysis. In the regulation study, the mRNA abundance (relative to 28S rRNA) of NaPi-II and PiUS in the intestine was compared with that of SGLT1. In the kidney, the abundance of SGLT1 mRNA was not quantified because its expression level was very low.

The cDNA probes for intestinal and renal NaPi-II, PiUS, and SGLT1 were generated by RT-PCR using oligo(dT)20 reverse-transcribed cDNA (Moloney murine leukemia virus RT; Promega, Madison, WI) as the starting template and degenerate primers designed from the consensus sequences of other animal species (Table 1). Several primers were designed and tested at various PCR conditions using a "touchdown" procedure. ClustalW 1.8 and BoxShade programs were used for sequence alignment. The PCR products were subcloned using plasmids (pDrive cloning vector; Qiagen, Valencia, CA) and competent cells (DH5α; Invitrogen). The identity of the insert was verified by DNA sequencing (Dye terminator, ABI 377) and the standard BLAST program of NCBI. The cDNA probes were made by PCR using purified, sequenced plasmids as the templates, followed by gel purification.

Immunocytochemistry. The distribution of NaPi-IIb in various tissues (proximal and distal intestine, pyloric ceca, kidney, gills, skin, scale, muscle, and stomach) was examined using a panel of three unique antibodies. TrIIb2 is a polyclonal rabbit antibody raised against a synthetic peptide (EDAPELLKVITEPVPT) corresponding to the E62-T76 of trout kidney NaPi (accession no. AA358800) linked to keyhole limpet hemocyanin (KLH), which was generated by Sigma Genosys. This peptide sequence comes from a highly conserved region of NaPi-IIb protein sequence and thus should recognize isoforms of NaPi-IIb other than those from which the original peptide sequence was derived. The corresponding intestinal peptide sequence has a homology of 64%, which when calculated including conservative amino acid substitutions increases to 93%. Polyclonal antibodies raised in rabbit against KLH-linked peptide sequences from zebrafish NaPi-IIb1 and NaPi-IIb2 isoforms were kindly supplied by Dr. Andreas Werner and have been characterized previously (13).

TrIIb2 serum was affinity purified by passing it through a column generated by covalently linking the NaPi peptide to beads (Aminolink; Pierce Endogen, Rockford, IL). After being washed thoroughly to remove weakly associated antibodies, those that specifically bound to the peptide were eluted in 1-ml fractions with glycine (100 mM, pH 2.5), neutralized (100 ml of 1 M Tris; pH 7.5/ml), and measured spectrophotometrically for protein concentration. Fractions with the highest concentration of protein were combined, dialyzed against PBS, and concentrated (Slide-A-Lyzer Concentrating Solution; Pierce Endogen). We then determined that the flow-through serum (that portion that did not bind to the peptide on the column beads) showed virtually no specific immunoreactivity in all tissues studied. In contrast, the eluted fraction (containing specifically bound antibodies) showed very clear staining patterns.

Zebrafish antisera were not affinity purified because of insufficient volume but were still used for the renal immunocytochemistry since they produced the most reproducibly clear staining pattern. Preliminary work indicated that non-

Table 1. Primers for cDNA probes

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<th>Primers position</th>
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<tr>
<td>NaPi-II (I)-fwd</td>
<td>5'-GCCGGGACATCTTCCAGG-3'</td>
</tr>
<tr>
<td>NaPi-II (I)-rev</td>
<td>5'-AAGTCRASARIGCAATCTG-3'</td>
</tr>
<tr>
<td>NaPi-II (K)-fwd</td>
<td>5'-AGAACG1CTTTTCGGGCGY-3'</td>
</tr>
<tr>
<td>NaPi-II (K)-rev</td>
<td>5'-GTRGCGUGUGATTTGGAAGCC-3'</td>
</tr>
<tr>
<td>PiUS (I, K)-fwd</td>
<td>5'-ATTCACCCCMRSSAATGAGG-3'</td>
</tr>
<tr>
<td>PiUS (I, K)-rev</td>
<td>5'-CTGTCACAGACATCAGATAG-3'</td>
</tr>
<tr>
<td>SGLT1 (I, K)-fwd</td>
<td>5'-GGIGCCCTTCYSTTCTCCGAG-3'</td>
</tr>
<tr>
<td>SGLT1 (I, K)-rev</td>
<td>5'-CCTGCTICITRCACTCCAGAC-3'</td>
</tr>
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</table>

NaPi-II, type II sodium-phosphate cotransporter; PiUS, inorganic phosphate uptake stimulator; SGLT1, sodium-glucose cotransporter 1; I, intestine; K, kidney; fwd, forward; rev, reverse. * In flounder NaPi-II sequence (gi: 11633138). † In rabbit PiUS sequence (gi: 1872497). ‡ In mouse SGLT1 sequence (gi: 6563311)
The in vivo fractional absorption data were analyzed for the effect of P status and the dietary P level using the following equation assuming that the fractional P absorption follows a standard sigmoid dose-response curve: 
\[ Y = \text{bottom plateau} + \left( \text{top plateau} - \text{bottom plateau} \right) \times \left( 1 + \frac{1}{1 + \left( \frac{X - \text{EC}_{50}}{\text{HillSlope}} \right)^2} \right) \]
where \( X \) is available dietary P, and \( Y \) is fractional P absorption. \( \text{EC}_{50} \) is the estimated X value when fractional absorption is halfway between the bottom and top plateaus, and the HillSlope describes the steepness of the curve. The two lines of low-P vs. high-P fish were compared by two-tailed paired \( t \)-test for each sampling day. Statistical calculations were performed with GraphPad Prism, version 3.03 (GraphPad Software, San Diego, CA). Treatment effects were considered significant at \( P < 0.05 \).

RESULTS

Tissue Distribution of NaPi-IIb, P,US, and SGLT1

NaPi-IIb mRNA. Subcloned trout intestinal and renal NaPi-IIb cDNAs had >99% sequence homology with known trout NaPi-IIb sequences (GenBank accession nos. AF297184 and AF297186). Tissue distribution of NaPi-IIb mRNA was determined by Northern analysis (Fig. 1) and by semiquantitative RT-PCR (data not shown). NaPi-IIb mRNA was found in proximal small intestine (most abundant), distal small intestine, pyloric ceca, and kidney but not in heart, skin, skeletal muscle, stomach, gills, scales, blood, or liver. Renal NaPi-IIb mRNA appears to be slightly larger in size than intestinal NaPi-II mRNA.

NaPi-II protein. In the kidney of fish fed a low-P diet, NaPi-IIb expression was intense along the brush-border margin of cells lining the first segment of the proximal tubule (P) and was faint or absent in the other regions of the nephron (Fig. 2, A-C). All three antisera produced similar staining patterns in the tubular kidney, suggesting overlapping affinities for the transporter, but the zebrafish NaPi-IIb2 antibody showed substantially better definition and intensity. Blood cells also stained with NaPi-IIb2 (Fig. 2C). Sections incubated with preimmune serum were completely devoid of NaPi-IIb2 signal (Fig. 2D). The distribution of NaPi-IIb2 within cells and along the nephron was independent of dietary P and of aqueous oxygen concentrations (data not shown). Moreover, the intensity of staining did not vary with dietary P or with aqueous oxygen concentrations.

Immunocytochemistry using affinity-purified TrIIb2 in the pyloric ceca (Fig. 3A) and proximal intestine (Fig. 3C) revealed a clear apical membrane stain in enterocytes along the entire villus. There was no clear difference in NaPi-IIb staining along the villus axis of the intestine and pyloric ceca. Localization and intensity of NaPi-IIb expression was intense along the entire villus. There was no clear difference in NaPi-IIb staining along the villus axis of the intestine and pyloric ceca. Localization and intensity of NaPi-IIb expression was intense along the entire villus. There was no clear difference in NaPi-IIb staining along the villus axis of the intestine and pyloric ceca. Localization and intensity of NaPi-IIb expression was intense along the entire villus. There was no clear difference in NaPi-IIb staining along the villus axis of the intestine and pyloric ceca. Localization and intensity of NaPi-IIb expression was intense along the entire villus. There was no clear difference in NaPi-IIb staining along the villus axis of the intestine and pyloric ceca. Localization and intensity of NaPi-IIb expression was intense along the entire villus. There was no clear difference in NaPi-IIb staining along the villus axis of the intestine and pyloric ceca. Localization and intensity of NaPi-IIb expression was intense along the entire villus.
oxygen concentrations (data not shown). The intensity of staining also did not apparently vary with dietary P and with aqueous oxygen concentrations.

Because Pi homeostasis is tightly associated with that of calcium, and because fish absorb calcium via gill epithelium, we also examined gill tissue for the presence and distribution of NaPi-IIb. Sections showed strong staining along the apical margin of the ionocytes or mitochondria-rich cells in the gill epithelium (Fig. 3F). All sections incubated with preimmune se-

Fig. 1. Tissue distribution of type II sodium-phosphate cotransporter (NaPi-IIb), inorganic phosphate uptake stimulator (PiUS), and sodium-glucose cotransporter (SGLT1) mRNA in rainbow trout determined by Northern analysis. Fish (mean body wt 221 g) were fed a low-phosphorus (P) diet (0.35% available P) at 2% of body weight daily for 1 wk. On day 7, five fish were sampled and analyzed for NaPi-II, PiUS, and SGLT1 mRNA by either Northern blot (n = 2, representative blot shown) or semiquantitative RT-PCR (n = 3, data not shown) for selected tissues. Total RNA (30 μg) isolated from respective tissues was gel electrophoresed, membrane transferred, and hybridized with a mixture (1:1 wt/wt) of trout intestinal and renal NaPi-II cDNA, with trout intestinal PiUS cDNA, or with a mixture (1:1 wt/wt) of trout intestinal and renal SGLT1 cDNA. The long and short arrows on right indicate the positions of 28S and 18S rRNA, respectively. RT-PCR detected SGLT1 in the stomach (data not shown), which is only faintly seen in the Northern blot.

Fig. 2. Immunolocalization of NaPi-IIb in the kidney with zebrafish NaPi-IIb2 antibody. Representative images are shown from fish fed low-P diet, although two fish from each tank (n = 3 tanks/treatment [see MATERIALS AND METHODS and Table 2 for treatment details]) were examined. See text for description of specific cell types in each region. A: 1st (PI) and 2nd (PII) segments of the proximal tubule. Only the first segment shows specific labeling of the brush-border membrane. B: two PII tubules in which the brush-border membrane is clearly visible and unlabeled. C: unlabeled distal tubule (DT; note absence of brush border) adjacent to two PI tubules. Blood cells also stained with NaPi-IIb (arrows). D: unlabeled negative control (preimmune serum). Melanocytes, visible as dark spots in A–D, are not labeled but appear exactly the same in tissue sections stained with hematoxylin and eosin. Images are representative. Bar = 50 μm.
rum were completely blank for all tissues (Fig. 3, B, and G).

Trout skin, scale, muscle, and stomach were also examined for NaPi-IIb protein expression by immunocytochemistry (only skin shown, Fig. 3H). Staining in these tissues was entirely absent when probed with affinity-purified TrIIb2 serum. Hence, with the exception of discordant results in the gill, NaPi protein expression determined by immunocytochemistry in various tissues paralleled exactly mRNA expression determined by Northern blots and RT-PCR.

**PiUS mRNA.** Subcloned trout intestinal PiUS cDNA (GenBank accession no. AY210434) showed 68% sequence homology with rabbit and rat intestinal PiUS and 68% with human inositol hexaphosphate kinase 2 (IP6K2). The base sequence did not significantly match any other sequences from any species. The translated protein sequence had 69–70% identity with rabbit and rat PiUS and human IP6K2. The PiUS mRNA had similar distribution to that of NaPi-IIb mRNA, but PiUS mRNA was also found in the heart, skin, muscle, stomach, gill, blood, and liver where NaPi-IIb mRNA was not detectable (Fig. 1).

**SGLT1.** The subcloned trout intestinal SGLT1 cDNA (GenBank accession no. AY210435) had 70–72% sequence homology with human, equine, and bovine SGLT1. The translated protein sequence had 74–76% identity with rat, human, mouse, bovine, and chicken SGLT1. The homology to other SGLT families was also high but significantly lower than for SGLT1. SGLT1 mRNA was abundant in pyloric ceca and the proximal small intestine, much less abundant in distal intestine and kidney, barely detectable in the stomach, and not detectable in the other tissues examined (Fig. 1).

**Regulation of Renal and Intestinal NaPi-II, PiUS, and SGLT1 Expression by Dietary P and Ambient Oxygen Concentrations**

Fish P status indicates that the low-P diet (0.46% available P) did not induce hypophosphatemia even after 7 wk of feeding, and the carcass P levels only slightly (P = 0.05) differed between low-P and high-P fish (Table 2). In addition, the growth rate did not differ significantly between the low-P and high-P fish. These results indicate that fish fed the low-P diet were
only modestly, but not clinically, deficient in dietary P intake. Hematocrit values were not affected by dietary P but were markedly affected by ambient oxygen levels.

NaPi-IIb mRNA abundance was significantly higher in the low-P than high-P fish in both kidney (P = 0.04) and intestine (P = 0.03; Fig. 4). However, the effect of diet was observed only when ambient oxygen concentrations were high. In the low-oxygen group, where fish growth was decreased significantly, NaPi-II mRNA abundance in both intestine and kidney did not differ significantly between low-P and high-P fish. The mRNA abundance of renal PiUS was slightly but significantly higher (P = 0.04) in low-P than high-P fish in the high-oxygen group, whereas that of intestinal PiUS followed a similar pattern but was not significant (P = 0.08). There was no such difference in the low-oxygen group. Intestinal SGLT1 mRNA was similar among all treatments (P > 0.3). Northern analysis of renal SGLT1 was performed but not shown since its mRNA abundance was lower than the reasonable quantification limit.

**In Vivo Fractional Phosphate Absorption**

Fractional P absorption, which represents the absorption efficiency of NaH$_2$PO$_4$ at various dietary P levels, was close to 100% when the dietary level (intake) was near the requirement (0.6%; Fig. 5 and Table 3). Fish previously fed high P before the switch to the test diets had significantly lower fractional P absorption at 3 days after the switch than fish previously fed low P (P = 0.003). However, the diet history of the fish had no effect on fractional P absorption 6, 9, or 12 days after the switch in diet. The absorption efficiency gradually decreased at higher (>0.85%) dietary available P levels in all sampling days in both low-P and high-P fish. However, the total absorption, which represents the overall absorption (often called digestibility), remained mostly above 90% even at high dietary P intakes (data not shown).

**DISCUSSION**

**Tissue Distribution of NaPi-II, PiUS, and SGLT1**

NaPi-II mRNA was found in the intestine and kidney as expected, but it was also detected in pyloric ceca, where the abundance was relatively low. Pyloric ceca are fingerlike diverticula that appear immediately posterior to the pyloric sphincter of the stomach along the first one-third to one-half of the proximal intestine. On average, trout have 56 pyloric ceca, which account for roughly 70% of the surface area of the entire gut (4). NaPi-IIb protein was also detected in the pyloric ceca. Given the presence of NaPi-IIb protein in the ceca and the tremendous surface area, this is likely to be an important site of P absorption in trout. Little is known, however, about the roles of this organ on di-

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**Table 2. Effect of ambient oxygen and dietary P concentrations on body composition and growth of fish**

<table>
<thead>
<tr>
<th>Ambient oxygen condition:</th>
<th>High Oxygen</th>
<th>Low Oxygen</th>
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<tr>
<td>Diet:</td>
<td>High P</td>
<td>Low P</td>
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<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma P, mM</td>
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<td>4.18</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>39.2*</td>
<td>39.7*</td>
</tr>
<tr>
<td>Carcass P, % in wet carcass</td>
<td>0.409*</td>
<td>0.394†</td>
</tr>
<tr>
<td>Gain, %</td>
<td>42.7*</td>
<td>43.3*</td>
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Initial values of hematocrit and carcass phosphorus (P) were 40.2 ± 0.8 (SE, n = 20 fish) and 0.409 ± 0.006 (SE, n = 25 fish), respectively. Initial fish body weight was 188 ± 4.4 g (SE, n = 50). Fish were pair fed either a low-P diet (0.46% available P) or high-P diet (0.74% available P) at 2% of their body weight daily for 7 wk at 2 different oxygen levels (6 or 10 ppm). NS, not significant. Values in rows with different symbols are significantly different by two-way ANOVA.

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Fig. 4. Effects of dietary P and water oxygen levels on NaPi-II, PiUS, and SGLT1 mRNA abundance in the kidney (A) and intestine (B) of rainbow trout. HO, high oxygen; LO, low oxygen; HP, high P; LP, low P. Fish were pair fed either the low-P diet (0.46% available P) or high-P diet (0.74% available P) at 2% of their body weight daily for 7 wk at 2 different oxygen levels (6 or 10 ppm). Each column represents the mean ± SE (error bar) of 3 tanks. Two fish per tank (pooled) were analyzed. *Significant difference (P < 0.05) by 1-tailed t-test.
protein in the intestine was similar to that found in flounder and zebrafish (13, 20). Interestingly, expression appeared to diminish toward the distal end of the intestine. This is consistent with $P_i$ uptake measurements in trout (2) that showed significantly lower $P_i$ uptake from the distal intestine than from the proximal and with the present study that shows much higher abundance of NaPi-IIa mRNA in the proximal than distal region of the intestine (Fig. 1).

Rainbow trout have a glomerular nephron typical of freshwater fish, and the nephron consists of a Bowman’s capsule, glomerulus, short neck segment, two morphologically distinct sections of proximal tubule, an intermediate segment, a distal tubule, and a collecting duct terminating in the mesonephric duct (1, 40). As shown in Fig. 2, PI consists of tall columnar cells with basal nuclei and a prominent apical brush border. PII has even taller columnar cells with central nuclei, a dense apical brush border, and a smaller lumen that often appears occluded by the brush border. The distal tubule consists of shorter columnar cells with basal nuclei, no brush border, a small lumen (smaller than PI), and a smaller overall diameter (1, 40).

In the flounder kidney, NaPi-IIb protein has been localized to the basolateral membrane of PII cells (9, 20) as well as the apical membrane of the collecting tubule (9). In the freshwater zebrafish, NaPi-IIb is found in the apical membrane of PII and the collecting tubule (13). In trout, another freshwater species, NaPi-IIb is also found at the apical pole but only in the proximal tubule PI. Hence, the trout NaPi-IIb is also likely to be involved in reabsorbing and recycling $P_i$ secreted by the typically active glomerulus of freshwater fish, following the model presented by Graham et al. (13). However, the trout NaPi-IIb distribution along the nephron is different from that in zebrafish but similar to that in mammals fed normal dietary $P$ where NaPi-IIa is located only in the early proximal region of the kidney. After the NaPi-II evolutionary tree (37), it is interesting to note that trout renal NaPi-IIb is much more closely related to the mammalian renal NaPi-IIa located in the PI region than to the zebrafish renal NaPi-IIb located in the PII region and may reflect an intermediate shift from distal (more primitive) to proximal (mammalian) $P_i$ reabsorption (13). Although both live in freshwater habitats, differences in NaPi-IIb distribution along the nephron may be because of the fact that the zebrafish (order: Cypriniformes) and trout (order: Salmoniformes) are distantly related and diverged early in teleostean evolution.

The intensity of NaPi-IIb staining in trout kidney is less than that in the trout intestine. This finding is also supported by Northern blotting and RT-PCR results, suggesting that expression of NaPi-IIb, relative to other brush-border proteins, may be lower in the trout kidney.

$P_iUS$ mRNA was found in all tissues where NaPi-IIb mRNA was located. However, $P_iUS$ mRNA was also found in the gill, heart, blood, and other tissues where NaPi-IIb mRNA was absent. It is not clear why $P_iUS$ is present in those tissues where NaPi-IIb mRNA is not
Table 3. Fractional absorption of dietary P

<table>
<thead>
<tr>
<th>Initial Diet:</th>
<th>Top plateau</th>
<th>SE</th>
<th>( R^2 )</th>
<th>Dietary P at 95% plateau, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 3</td>
<td>Day 6</td>
<td>Day 9</td>
<td>Day 12</td>
</tr>
<tr>
<td>LP</td>
<td>96.9</td>
<td>96.5</td>
<td>96.1</td>
<td>97.5</td>
</tr>
<tr>
<td>HP</td>
<td>96.7</td>
<td>95.0</td>
<td>97.3</td>
<td>97.5</td>
</tr>
</tbody>
</table>

LP, fish fed low-P diet until day 0; HP, fish fed high-P diet until day 0. Days 3, 6, 9, and 12 are days after the switch in diet. Top plateau, the maximum fractional absorption in the dose-response curve (Fig. 5); SE, the standard error of the plateau; \( R^2 \), the correlation of the curve. Fish (initial mean body wt 125 g) were fed either low-P diet or high-P diet containing 0.5% Cr2O3 once daily at 1% (dry basis) of their body wt for 32 days. After this period, fish were fed one of 10 test diets of varied P concentrations but the same dietary composition and Cr2O3 concentration (0.5%) for 12 days, and the feces were collected for 24 h at days 3, 6, 9, and 12 after the switch in diet for P and Cr analyses. Total P levels of the test diets (%dry diet) ranged from 0.18 to 1.25.

detectable. In those tissues, there could be other P transporters that coordinate their function with P-US. Indeed, immunostaining of branchial ionocytes and blood cells with TrIIb2 strongly supports this possibility.

We examined gill tissue for NaPi because we had previously observed that P concentrations in inflow water (to fish tanks) were often higher than that in the outflow water when fish were fed low-P diets and because fish are known to absorb minerals, such as calcium, from water via gill epithelia (10). Functional studies measuring gill \[^{32}P\] uptake in vivo (29, 30, 41) did not show active uptake of \[^{32}P\]; however, these studies were conducted on fish with normal P status. It is possible that an inward-directed P transport exists in trout but is only active in fish with low-P status. Conversely, it is also possible that the transport of P is outward directed in fish with high-P status to assist the kidney in elimination of excess P. In the current study, NaPi-II staining was concentrated in the apical margin of ionocytes. Ionocytes are found in the gill epithelium and are involved with calcium uptake as well as ion and osmoregulation (for review, see Refs. 22 and 42). Gill ionocytes were identified based on their bulbous appearance and distribution along the gill filament of rainbow trout. There were also nombulbus cells at the base of the gill filament that stained strongly. The latter are also ionocytes because salmonids have two types, one bulbous along the arterial blood supply of the gill filament and the other (not bulbous) along the venous blood supply at the base of the filaments (27, 40). The possibility of a P transport mechanism in these cells is consistent with its many functions and is also intriguing since P transport has not been well studied in the gill.

Regulation of Intestinal and Renal NaPi-II, P-US, and SGLT1 Expression by Dietary P and Aqueous Oxygen Levels

Intestinal regulation. Moderate dietary P restriction for 6–8 wk causes a modest increase in abundance of NaPi-IIb mRNA and protein, as well as in rates of P uptake by juvenile goat jejunal (16). This observation is similar to our findings of diet-related changes in mRNA abundance (Fig. 4) and P uptake (2). In contrast, dietary P restriction had no effect on NaPi-IIb mRNA abundance in adult rat and mouse small intestine. In rats, P-US mRNA abundance and P uptake increased even though NaPi-II and -III mRNA levels did not change (18). In mice, dietary P restriction or calcitriol \([1,25-\text{OH}_2\text{D}_3\)] vitamin D) injection increased the abundance of NaPi-IIb protein and rates of P uptake in the brush-border membranes, independent of changes in mRNA abundance (15).

It was initially unclear to us why in rats and mice severe dietary P restriction accompanied with hypophosphatemia did not increase intestinal NaPi-IIb mRNA, whereas in trout, even when they were apparently not clinically P deficient, NaPi-IIb mRNA expression increased. It turns out that intestinal NaPi-II mRNA expression may be highly age dependent. Calcitriol treatment increased intestinal brush-border membrane vesicle P_{i} absorption by 2.5-fold in suckling rats and by 2.1-fold in adult rats, whereas the same treatment increased NaPi-IIb mRNA abundance by 2-fold in 14-day-old rats but had no effect on mRNA expression in adults (39). The studies reporting an absence of diet effects on intestinal NaPi-II mRNA abundance were conducted using adults, whereas those reporting the presence of diet effects were conducted using neonates or juveniles. For example, trout used in this study (body wt 188 g) were juvenile and very small compared with full-grown size (~3 kg). The apparent discrepancy of intestinal mRNA expression in dietary P restriction therefore might be dependent on the developmental stage of the animal or on unidentified growth-related mechanisms.

Unlike NaPi-II, the mRNA of trout P-US did not increase significantly in response to reduced dietary P intake. However, in rat small intestine, Katai et al. (18) reported approximately twofold increases of both P-US mRNA and maximal velocity (V_{max}; P uptake) after 7 days of dietary P restriction (0.02% dietary P). The apparent disagreement in the P-US response to dietary P restriction could be attributed to the degree of P deficiency. In our present study, dietary P was only moderately, but not severely, restricted; thus, the low-P fish had only slightly lower body P content than

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high-P fish. The plasma P level showed that fish fed a low-P diet were not hypophosphatemic. In most mammalian experiments, animals were fed nearly P-free rations and developed pronounced hypophosphatemia before studies on the expression of NaPi-IIa, PiUS, and other related genes. Thus direct comparison of our data with mammalian studies may be inappropriate.

Renal regulation. In rat kidney, changes of apical NaPi-IIa protein abundance and $V_{\text{max}}$ (within a few hours) precede the change of mRNA level (5–7 days), and the acute response is the result of the translocation of NaPi-IIa protein from the intracellular compartment to the apical membrane independent of NaPi-II transcription (21, 28, 32). In these studies, the renal NaPi-IIa mRNA indeed responded to dietary P levels but only in the chronic phase (5–7 days), and the chronic high-P rat still had one-half the level of NaPi-IIa mRNA compared with the chronic low-P rat. Coloso et al. (6) found that rainbow trout fed for 30 days with a 0.6% P diet had significantly higher renal NaPi-IIb mRNA abundance than those fed a 1% P diet. Our present results also showed upregulation of renal NaPi-IIb mRNA in response to dietary P restriction, which is in good agreement with mammalian “chronic” data and our previous data.

Dietary regulation of renal PiUS mRNA expression has not been studied in any species. Although trout renal PiUS mRNA was found to be upregulated in dietary P restriction, the difference was small compared with that of NaPi-IIb mRNA. Thus its functional role in renal P handling could be minor or indirect.

In rats and mice, severe dietary P restriction causes a drastic upregulation of NaPi-IIa protein expression in the early proximal tubule and also the appearance of NaPi-IIa protein in the middle and late proximal tubules where it was not previously visible (32, 38). This sort of change would be more difficult to see in the fish kidney because it lacks the three-dimensional organization that characterizes the mammalian kidney (3). However, we examined sections from areas throughout the kidney and identified the tubules that showed NaPi-II expression and those lacking expression, and we found no observable change with low dietary P.

Upregulation of NaPi-II and PiUS mRNA occurred only in fish reared in high-oxygen water and not in fish reared in waters with suboptimal oxygen concentrations. Because low oxygen resulted in a significant growth depression and because dietary P is required mainly for the growth of the skeletal system, the absence of upregulation in low-P fish could be attributed to the growth-related shift of dietary P requirement and the consequent relative sufficiency of dietary P intake for fish grown in low ambient oxygen concentrations. Nonetheless, a direct effect of hypoxia on NaPi expression has not been excluded.

In Vivo Fractional Phosphate Absorption

Dietary P restriction increases the efficiency of intestinal P$_i$ absorption and renal P$_i$ reabsorption (8, 24). Such an adaptive response has been shown in many mammals and in trout (2) by functional P$_i$ uptake assay using either brush-border membrane vesicles or intact excised tissues. These in vitro assays are, however, determined under artificial conditions, and there still remains a question whether or not intact live fish are regulating P$_i$ absorption as well. There have been few studies on the dietary regulation of P$_i$ uptake in vivo.

In carp, intestinal P$_i$ absorption in vivo increased linearly from 0.35% up to 3.14% dietary P (dietary requirement is ~0.6%), and the absorption efficiency was constant and did not decrease over this range (25). This demonstrated that intestinal P$_i$ absorption was not regulated in carp. In humans, the Institute of Medicine (17) expressed a similar view, as it wrote, “fractional phosphorus absorption is virtually constant across a broad range of intakes.” In the present study, however, the in vivo absorption efficiency of Na$_2$HPO$_4$ by trout is clearly regulated by diet, for two reasons. First, in low-P fish, fractional absorption remained close to 100% up to the known dietary requirement of 0.6%, but then decreased gradually to 70–90% at higher dietary P intakes, depending on the duration after the switch in diet. In high-P fish, absorption efficiency decreased markedly from 90–100% to 70–85% when dietary P increased from 0.6 to 1.2%. Second, at day 3 after the switch in diet, the maximum fractional absorption in vivo was 98.9% for low-P fish but 93.7% for high-P fish. Hence, diet history modulates acute regulation of intestinal P$_i$ absorption.

At all dietary levels, fractional absorption in vivo continued to take place with considerably high efficiency (70–100%). This observation may be partly explained by the potentially significant passive P$_i$ transport in pyloric ceca (36). Rodehutscord et al. (33) reported a much lower fractional absorption of Na$_2$HPO$_4$ (maximum 73%) in trout, which peaked well below the dietary requirement and decreased thereafter. The authors therefore suggested that the dietary P level, when testing dietary P availability, must be 2.5 g/kg dry diet or lower. This is less than one-half of the known minimum P requirement for fish. Conversely, our data showed the highest fractional P$_i$ absorption (~100%) at around the requirement level and the fractional absorption only gradually decreased at higher dietary P levels, which kept overall P$_i$ absorption nearly constant across a broad range of dietary P intakes.

Perspectives

Currently, very little is known in fish regarding the molecular and physiological mechanisms of dietary P regulation. We have identified and localized NaPi-II, P$_i$US, and SGLT1 in selected tissues of rainbow trout and have shown that renal and intestinal NaPi-II and P$_i$US, but not SGLT1, are upregulated in response to moderate, chronic dietary P restriction. Adaptive upregulation of NaPi-II and P$_i$US expression paralleled that of in vivo fractional P$_i$ absorption at low dietary P$_i$ intakes. However, the severity of dietary P restriction,
the time course of adaptive responses, the development- 
mental stage of the fish, and the interactions among these 
facors on NaPi-II and PiUS expression are largely 
unknown. Future studies should focus on these mecha-
nisms and on the involvement of other genes related 
to P transport.

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