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

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Sugiura, Shozo H., Nichole K. McDaniel, and Ronaldo P. Ferraris. In vivo fractional $P_i$ 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 $P$ restriction to increase intestinal and renal $P$ transport, but little is known about NaPi-II and PiUS regulation in other vertebrates. We studied the 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 $P_i$ uptake 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 $P_i$ 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 $P_i$ absorption. Regulation of dietary $P_i$ 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 ($P_i$) transporters in epithelial cells of the intestine and kidney. Intestinal absorption of $P_i$ is mediated primarily via type II sodium-phosphate cotransporter (NaPi-IIb) in the brush-border membrane, whereas renal $P_i$ 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-IIb 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-II 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 $P$ balance. The $P_i$ uptake stimulator (PiUS) or inositol hexakisphosphate kinase (34) has been shown to be involved in the absorption of dietary $P$. PiUS cRNA encodes a protein that markedly increased Na-dependent $P_i$-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 $P$ restriction has been shown to upregulate $P_i$ 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 $P$ restriction also stimulates the recruitment of more distal regions of the rat renal tubules to participate in $P_i$ 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...
Dietary regulation of phosphate transport in trout

MATERIALS AND METHODS

Fish, Diets, and Tissue Collection

Tissue distribution of NaPi-II, P,US, 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 P,US 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, 2% DL-methionine, 0.7 mineral mixture, and 0.6 vitamin mixture. 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, P,US, 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, P,US, 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 rate and therefore to nonspecific effects on tissue concentrations. 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, 6 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 RNA later 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 (1500 g) immediately after collection.

In vivo fractional P 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 kept 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) was 42.8% crude protein, 0.054% calcium, and 5,260 kcal/kg diet digestible energy. Total P levels of the test diets were (dry basis) was 42.8% crude protein, 0.054% calcium, and 5,260 kcal/kg diet digestible energy. Total P levels of the test 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 leaching loss of P in the feces during collection. Compared with the other two (tissue distribution study), the intake P was 35% higher in fish with that of SGLT1. In the kidney, the abundance of SGLT1 was 93% for SGLT1. For PiUS, intestinal cDNA was used for all tissues (PiUS sequences of other tissues were not studied). In the tissue distribution study, 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-II 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). Several primers were designed and tested at various PCR conditions using a “touch-down” 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. TrIb2 is a polyclonal rabbit antibody raised against a synthetic peptide (EDAPELLKVITEPV) corresponding to the E62-T76 of trout kidney NaPi (accession no. AA358000) 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-IIb and NaPi-IIb isoforms were kindly supplied by Dr. Andreas Werner and have been characterized previously (13).

TrIb2 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 immuno-reactivity in all tissues studied. In contrast, the eluted fraction (containing specifically bound antibodies) showed very clear staining patterns.

Zebrafish antiserum 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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<td>1403–1422*</td>
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<td>587–607*</td>
</tr>
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<td>1324–1344*</td>
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| 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: 1163138). † In rabbit PiUS sequence (gi: 1872497). ‡ In mouse SGLT1 sequence (gi: 6563311).
specific staining using unpurified zebrafish antisera in the intestine was restricted to mucous and goblet cells, and, since there are no mucous cells in the kidney, purification was considered unnecessary. Hence, zebrafish NaPi-IIb antisera were used in kidney sections, whereas affinity-purified trout antisera were used in all sections except the kidney. However, other than nonspecific staining in readily identifiable mucous and goblet cells, immunocytochemistry results between affinity-purified trout antisera and unpurified zebrafish antisera were qualitatively similar. Although goblet cell staining was virtually eliminated in sections incubated with affinity-purified antiserum, to rule out mucous as an epitope for the affinity-purified serum, we examined two tissues known to have mucous cells but that were unlikely to have NaPi, (skin and stomach). Preimmune serum was also not affinity purified since immunocytochemistry using this serum showed no staining, even at high concentrations.

Paraffin sections (8 μm) were rehydrated and placed in a humidified chamber at room temperature. Sections were incubated with blocking solution (BS, 5% goat serum, 0.1% Tween 20 in PBS, pH 7.4) for 45 min and then sequentially blocked with avidin and biotin each for 10 min (Vector Laboratories, Burlingame, CA). Sections were incubated with primary antibody (affinity-purified-TrIIb2 1:100, zebrafish antisera 1:1,000 in BS) for 2 h and with secondary antibody (biotinylated goat anti-rabbit IgG diluted to 5 μg/ml in BS; Vector Laboratories) for 1 h. Sections were washed two times with PBS and incubated for 10 min with 0.05% sodium azide and four drops of Vector H2O2 stock in 5 ml PBS to block endogenous peroxidase activity. Sections were then incubated with an avidin-biotin-horseradish peroxidase complex (Vector Laboratories) containing 0.2 M ω-methyl mannoside and 0.4 M NaCl to block lectin binding and ionic interaction, respectively. Slides were then washed and incubated with substrate (3,3’-diaminobenzidine containing Ni2+; Vector Laboratories), counterstained with Nuclear Fast Red, and mounted with Permush (Fisher Scientific, Suwanee, GA).

Plasma and carcass P analysis. For plasma P analysis, plasma from five or six fish/tank were pooled, whereas, for carcass P analysis, four fish from each tank were individually analyzed following the Fiske and Subbarow method (Sigma Diagnostics, St. Louis, MO). The tank average was used for statistical comparison.

Calculation of in vivo fractional absorption. Absorption of P, (NaH2PO4) was determined in both total and various fractions of dietary P, levels in low-P and high-P fish at days 3, 6, 9, and 12 according to the partial digestibility method (19). Calculations were as follows: fractional P absorption = (ΔI − ∆F) / ΔI, where ΔI is NaH2PO4 Xq (q = one incremental unit or fraction), and ∆F is the fecal excretion of the corresponding fraction. The ∆F was determined based on the standard digestibility protocol using Cr2O3 as an indicator. The F of the basal diet represented the background, i.e., undigested dietary P + endogenous P. Any increase of F (∆F) above the baseline (F of the basal diet) was assumed to be the nonabsorbed portion of supplemented P, (i.e., NaH2PO4).

Statistical Analysis

The experiment on dietary regulation was designed as a 2 × 2 factorial arrangement, and the data were analyzed by two-way ANOVA with dietary P and oxygen levels as independent variables. Each tank was considered as the experimental unit. When there was no significant difference (ANOVA P > 0.05), the data were reanalyzed by one-tailed t-test between low-P and high-P groups within each oxygen group. 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: 

\[ Y = \frac{bottom\ plateau + [(top\ plateau - bottom\ plateau)/(1 + [EC50 - X]/HillSlope)]} \]

where X = available dietary P, and Y = fractional P absorption. 

Log EC50 is the estimated X value when fractional absorption is halfway between the bottom and top plateaus, and the Hill-Slope 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 (PI) 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 in both tissues were very similar. However, NaPi-IIb staining in the distal intestine was noticeably less intense than in the other regions (Fig. 3E).

The distribution of NaPi-IIb within cells, among cells in the same villi, and between proximal and intestinal regions was independent of dietary P and of aqueous oxygen concentrations.
oxygen concentrations (data not shown). The intensity of staining also did not apparently vary with dietary P and with aqueous oxygen concentrations.

Because P 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 ionoocytes or mitochondria-rich cells in the gill epithelium (Fig. 3F). All sections incubated with preimmune se-

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**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.

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**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, D, 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, NaPit 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.

\( \text{NaPi-IIb mRNA abundance was significantly higher} \)

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, \( \text{NaPi-II mRNA abundance in both intestine and kidney did not differ} \)

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 (\( P > 0.3 \)).

\( \text{Northern analysis of renal SGLT1 was performed but not shown since its mRNA abundance was lower than the reasonable quantification limit.} \)

**In Vivo Fractional Phosphate Absorption**

Fractional P\(_i\) absorption, which represents the absorption efficiency of Na\( \text{H}_2\text{PO}_4\) 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\(_i\) 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\(_i\) 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\(_i\) 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**

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<td>42.7*</td>
<td>43.3*</td>
<td>40.6</td>
<td>37.7†</td>
</tr>
</tbody>
</table>

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.
etary nutrient absorption. Our recent study showed that trout pyloric ceca had much higher luminal Pi concentration than other regions of the intestine and that Pi absorption in pyloric ceca was largely a passive process at postprandial luminal Pi concentrations (36).

The trout intestine can be divided into two morphologically and histologically distinct regions of roughly equal lengths. The proximal intestine is thin and lighter colored and has typical villi projecting in the lumen, whereas the distal intestine is thick and darker colored with mucosal folds. Localization of NaPi-IIb 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 Pi uptake measurements in trout (2) that showed significantly lower Pi uptake from the distal intestine than from the proximal and with the present study that shows much higher abundance of NaPi-II 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 Pi 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) Pi 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.

PUS mRNA was found in all tissues where NaPi-IIb mRNA was located. However, PUS mRNA was also found in the gill, heart, blood, and other tissues where NaPi-IIb mRNA was absent. It is not clear why PUS is present in those tissues where NaPi-IIb mRNA is not
The maximum fractional absorption in the dose-response curve (Fig. 5); SE, the standard error of the plateau; Total P levels of the test diets (% dry diet) ranged from 0.18 to 1.25.

<table>
<thead>
<tr>
<th>Initial Diet:</th>
<th>Day 3</th>
<th>Day 6</th>
<th>Day 9</th>
<th>Day 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>LP</td>
<td>HP</td>
<td>LP</td>
<td>HP</td>
<td>LP</td>
</tr>
<tr>
<td>Top plateau</td>
<td>98.9</td>
<td>93.7</td>
<td>99.5</td>
<td>95.0</td>
</tr>
<tr>
<td>SE</td>
<td>1.03</td>
<td>3.88</td>
<td>1.85</td>
<td>3.20</td>
</tr>
<tr>
<td>$R^2$</td>
<td>0.63</td>
<td>0.57</td>
<td>0.90</td>
<td>0.23</td>
</tr>
<tr>
<td>Dietary P at 95% plateau, %</td>
<td>1.11</td>
<td>0.80</td>
<td>0.90</td>
<td>0.97</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; $R^2$ is the correlation of the curve.

Fish (initial mean body wt 129 g) were fed either low-P diet or high-P diet containing 0.5% Cr$_2$O$_3$ 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 Cr$_2$O$_3$ 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$_i$US. Indeed, immunostaining of branchial ionocytes and blood cells with TrIIb2 strongly supports this possibility.

We examined gill tissue for NaP$_i$ 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 T 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, NaP$_i$-II staining was concentrated in the apical margin of ionocytes. Ionocytes are found in the gill epithelium and are involved with 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 nonbulbous 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$_i$ transport mechanism in these cells is consistent with its many functions and is also intriguing since P$_i$ transport has not been well studied in the gill.

Regulation of Intestinal and Renal NaP$_i$-II, P$_i$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 NaP$_i$-Ib mRNA and protein, as well as in rates of P uptake by juvenile goat jejunum (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 NaP$_i$-Ib mRNA abundance in adult rat and mouse small intestine. In rats, P$_i$US mRNA abundance and P$_i$ uptake increased even though NaP$_i$-II and -III mRNA levels did not change (18). In mice, dietary P restriction or calcitriol [1,25-(OH)$_2$ vitamin D] injection increased the abundance of NaP$_i$-Ib protein and rates of P$_i$ 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 NaP$_i$-Ib mRNA, whereas in trout, even when they were apparently not clinically P deficient, NaP$_i$-Ib mRNA expression increased. It turns out that intestinal NaP$_i$-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 NaP$_i$-Ib 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 NaP$_i$-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 NaP$_i$-II, the mRNA of trout P$_i$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$_i$US mRNA and maximal velocity ($V_{max}$; P$_i$ uptake) after 7 days of dietary P restriction (0.02% dietary P). The apparent disagreement in the P$_i$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
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-II, 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_{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 NaH_{2}PO_{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, PiUS, and SGLT1 in selected tissues of rainbow trout and have shown that renal and intestinal NaPi-II and PiUS, but not SGLT1, are upregulated in response to moderate, chronic dietary P restriction. Adaptive upregulation of NaPi-II and PiUS 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 developmental stage of the fish, and the interactions among these factors on NaPi-II and PUS expression are largely unknown. Future studies should focus on these mechanisms and on the involvement of other genes related to P transport.

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DISCLOSURES

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