# Effects of *Npt2* gene ablation and low-phosphate diet on renal $Na^+$/phosphate cotransport and cotransporter gene expression

Hannah M. Hoag,[1] Josée Martel,[2] Claude Gauthier,[2] and Harriet S. Tenenhouse[1,2,3]

[1]Department of Biology,
[2]Department of Pediatrics, and
[3]Department of Human Genetics, McGill University–Montreal Children's Hospital Research Institute,
Montreal, Quebec H3H 1P3, Canada

Address correspondence to: Harriet S. Tenenhouse, Montreal Children's Hospital, 2300 Tupper Street, Montreal, Quebec H3H 1P3, Canada. Phone: (514) 934-4417; Fax: (514) 934-4329; E-mail: mhdt@www.debelle.mcgill.ca.

Received for publication April 15, 1999, and accepted in revised form August 2, 1999.

**The renal $Na^+$/phosphate (Pi) cotransporter *Npt2* is expressed in the brush border membrane (BBM) of proximal tubular cells. We examined the effect of *Npt2* gene knockout on age-dependent BBM $Na^+$/Pi cotransport, expression of $Na^+$/Pi cotransporter genes *Npt1*, *Glvr-1*, and *Ram-1*, and the adaptive response to chronic Pi deprivation. $Na^+$/Pi cotransport declines with age in wild-type mice ($Npt2^{+/+}$), but not in mice homozygous for the disrupted Npt2 allele ($Npt2^{-/-}$). At all ages, $Na^+$/Pi cotransport in $Npt2^{-/-}$ mice is approximately 15% of that in $Npt2^{+/+}$ littermates. Only Npt1 mRNA abundance increases with age in $Npt2^{+/+}$ mice, whereas Npt1, Glvr-1, and Ram-1 mRNAs show an age-dependent increase in $Npt2^{-/-}$ mice. Pi deprivation significantly increases $Na^+$/Pi cotransport, Npt2 protein, and mRNA in $Npt2^{+/+}$ mice. In contrast, Pi-deprived $Npt2^{-/-}$ mice fail to show the adaptive increase in transport despite exhibiting a fall in serum Pi. We conclude that (a) Npt2 is a major determinant of BBM $Na^+$/Pi cotransport; (b) the age-dependent increase in Npt1, Glvr-1, and Ram-1 mRNAs in $Npt2^{-/-}$ mice is insufficient to compensate for loss of Npt2; and (c) Npt2 is essential for the adaptive BBM $Na^+$/Pi cotransport response to Pi deprivation.**

*J. Clin. Inv.* **104**:679–686 (1999).

## Introduction

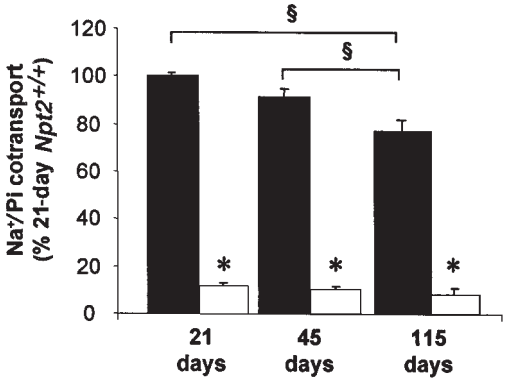
Phosphate (Pi) is a major constituent of bone mineral and plays a crucial role in cellular function. The kidney is an important determinant of Pi homeostasis and functions to reabsorb Pi to accommodate Pi need. In mammals, approximately 60–70% of the filtered Pi load is reabsorbed in the proximal tubule by $Na^+$-coupled Pi transport systems that mediate the translocation of Pi across the brush border membrane (BBM) from the lumen into the cell (1–3). The extent of Pi reabsorption in this segment of the nephron is subject to hormonal control and to regulation by dietary Pi content (4–8).

To date, 3 families of $Na^+$/Pi cotransporters have been identified in mammalian kidney. The type I (Npt1) (9–11) and type II (Npt2) (12–17) cotransporters are expressed predominantly in the kidney and have been localized to the BBM of proximal tubular cells, where the bulk of filtered Pi is reabsorbed (18–20). The type III cotransporters (Glvr-1 and Ram-1) are retroviral receptors that are ubiquitously expressed and may serve as housekeeping $Na^+$/Pi cotransporters (21).

We recently demonstrated that Npt1, Npt2, Glvr-1, and Ram-1 account for approximately 15%, 84%, 0.5%, and 0.5%, respectively, of total $Na^+$/Pi cotransporter mRNA in mouse kidney (22). Not only is Npt2 the most abundant of the renal $Na^+$/Pi cotransporters, it is also the major target for regulation by parathyroid hormone (PTH) and dietary Pi. PTH decreases $Na^+$/Pi cotransport through the endocytic retrieval of Npt2 protein from the BBM (23) and its subsequent lysosomal degradation (24). In contrast, both acute and chronic Pi deprivation elicit an adaptive increase in BBM $Na^+$/Pi cotransport that can be ascribed to microtubule-dependent recruitment of Npt2 protein to the apical surface (25). The increase in Npt2 protein in acute Pi restriction is not associated with an increase in Npt2 mRNA, whereas in chronic Pi deprivation, a corresponding augmentation in Npt2 transcript abundance might (26–28) or might not (22, 25) be apparent.

Npt2 protein also appears to be the target for ontogenic regulation of renal $Na^+$/Pi cotransport. Recent studies have demonstrated that BBM $Na^+$/Pi cotransport is significantly higher in 24-day-old weanling rats than in 12-day-old suckling rats, and that the increase in transport activity is accompanied by a corresponding increase in Npt2 protein and mRNA (29). In addition, studies in weanling and 3-month-old rats provided evidence for higher BBM $Na^+$/Pi cotransport in the weanlings and suggested that a unique growth-related $Na^+$/Pi cotransporter that is homologous to, but distinct from, Npt2 may be mediating this activity (30). Finally, Sorribas et al. (31) demonstrated that aged rats (12–16 months old) exhibit a 2-fold decrease in BBM $Na^+$/Pi cotransport when compared with young adult rats (3–4 months old), and that the decrease in transport is associated with a comparable decrease in the renal abundance of Npt2 protein and mRNA. Although age-related differences in Pi requirement likely play an important role in the ontogenic changes in $Na^+$/Pi cotransporter gene expression, the contribution of Npt1, Glvr-1, and

**Figure 1**
Effect of *Npt2* gene knockout and age on renal BBM $Na^+$/Pi cotransport. Renal BBM vesicles were prepared from $Npt2^{+/+}$ (filled bars) and $Npt2^{-/-}$ (open bars) mice at 21, 45, and 115 days of age. The BBM vesicle preparations were used for both transport studies and Western analysis (Figure 2) as described in Methods. One hundred percent activity represents the $Na^+$-dependent component of Pi transport ($Na^+/K^+$) in BBMs from 21-day-old $Npt^{+/+}$ mice (590 ± 30 pmol/mg protein per 6 seconds). Values (mean ± SEM) were derived from 3 experiments, each assayed in quadruplicate. *Effect of genotype, $P < 0.05$. §Effect of age, $P < 0.05$.




Ram-1 to this process is not known.

The crucial role of Npt2 protein to renal Pi reabsorption and the overall maintenance of Pi homeostasis was recently demonstrated in mice in which the Npt2 gene was inactivated by targeted mutagenesis (32). Mice homozygous for the disrupted gene ($Npt2^{-/-}$) exhibit increased urinary excretion of Pi, decreased BBM $Na^+$/Pi cotransport, hypophosphatemia, and an appropriate increase in the serum concentration of 1,25-dihydroxyvitamin D [1,25-$(OH)_2$D] with attendant hypercalcemia and hypercalciuria (32).

In the present study, we examined the effect of Npt2 gene ablation and age on renal BBM $Na^+$/Pi cotransport and the relative renal expression of Npt1, Glvr-1, and Ram-1. In addition, we sought to determine whether disruption of the Npt2 gene had an impact on the adaptive increase in BBM $Na^+$/Pi cotransport in response to Pi deprivation. We demonstrate that the age-dependent upregulation of Npt1, Glvr-1, and Ram-1 mRNAs is not associated with an increase in BBM $Na^+$/Pi cotransport in $Npt2^{-/-}$ mice and cannot compensate for the loss of Npt2 function in homozygous mutants. Furthermore, we show that Npt2 is essential for the BBM adaptive response to dietary Pi restriction.

## Methods

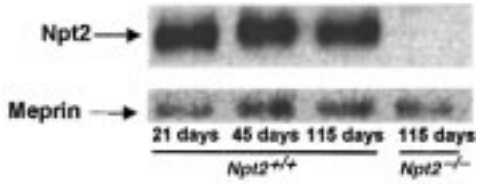
*Mice. Npt2* knockout mice were established in our laboratory by homologous recombination as described previously (32). Wild-type ($Npt2^{+/+}$) and homozygous mutant ($Npt2^{-/-}$) mice, generated by crossing heterozygous ($Npt2^{+/-}$) male and female mice, were maintained on a 0.6% Pi diet (5001, Purina Lab Chow; Purina Mills Inc., St. Louis, Missouri, USA) unless otherwise indicated. For Pi-deprivation experiments, 45-day-old $Npt2^{+/+}$ and $Npt2^{-/-}$ mice were fed either low-Pi (0.02% Pi) or control (1.0% Pi) diets for 5 days (test diets TD 86128 and TD 86129; Harlan Teklad, Madison, Wisconsin, USA). The test diets were otherwise identical. All animal studies were conducted in accordance with the guidelines of the Canadian Council on Animal Care.

*Genotyping of mice.* Mice were genotyped by PCR amplification of genomic DNA obtained from tail tissue, using *Taq* polymerase and 3 primers [sense primer 3F (5′-TGC CCA GGT TGG CAC GAA GC-3′) in exon 4 of *Npt2*; antisense primer 4R (5′-AGT CCT GTC CCC TGC CTG CA-3′) in exon 6 of *Npt2*; and antisense primer PGKR (5′-TGC TAC TTC CAT TTG TCA CGT CC-3′) in the $neo^r$ gene cassette] as described previously (32). Expected sizes of amplified fragments are 1.8 kb for the normal allele (primers 3F and 4R) and 1.4 kb for the disrupted allele (primers 3F and PGKR).

*BBM vesicle preparation, Western blot analysis, and transport studies.* Renal BBM vesicles were prepared from kidney cortex by the $MgCl_2$ precipitation method described previously (33) and were used for both transport studies and Western blot analysis. One kidney from each of 3–5 mice was used for each BBM vesicle preparation, and the remaining kidney was used for the ribonuclease protection analysis described later here. The uptakes of Pi (100 μM) and glucose (10 μM), each performed in quadruplicate, were measured at 6 seconds (initial rate) and 90 minutes (steady state) in medium containing either 100 mM NaCl or 100 mM KCl by the rapid filtration technique (33). BBM proteins (10–40 μg) were fractionated on 10% SDS-PAGE gels according to the method of Laemmli (34), transferred to supported nitrocellulose membranes (Hybond-C Extra; Amersham Pharmacia Biotech, Baie d'Urfé, Quebec, Canada), and probed either sequentially or simultaneously with a rabbit polyclonal antibody raised against a COOH-terminal peptide of rat Npt2 (gift of H. Murer, University of Zurich, Zurich, Switzerland) and an mAb raised against the α subunit of rat renal endopeptidase-24.18 (meprin) (kindly provided by P. Crine, University of Montreal, Montreal, Quebec, Canada) as described previously (28). Immunoblotting was also performed with 2 additional rabbit polyclonal antibodies — one raised against a COOH-terminal peptide of rabbit Npt1 (19) (gift of H. Murer, University of Zurich), and the other raised against a Glvr-1 peptide (35) (gift of R. Beliveau, University of Quebec at Montreal, Montreal, Quebec, Canada). Primary antibodies were viewed using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, and exposed to Kodak Biomax MR1 film (Eastman Kodak Co., Rochester, New York, USA). Npt2 and Npt1 protein abundance, relative to that of meprin, was estimated using PhosphorImager analysis of scanned images (Fuji Phospor Imager Bas 2000, Tokyo, Japan).

*Preparation of riboprobes.* Riboprobes for mouse Npt1, Npt2, Glvr-1, Ram-1, and β-actin were prepared by transcription of subcloned cDNA fragments for the respective $Na^+$/Pi cotransporters, using either T3 or T7 RNA

**Figure 2**
Effect of *Npt2* gene knockout and age on renal BBM Npt2 and meprin immunoreactive protein. BBM proteins, prepared from *Npt2*$^{+/+}$ mice at 21, 45, and 115 days of age and from 115-day-old *Npt2*$^{-/-}$ mice, were fractionated on 10% SDS-PAGE gels, transferred to nitrocellulose membranes, and probed with a rabbit polyclonal anti-rat Npt2 antibody and an mAb raised against the α subunit of rat meprin. Bands depicting Npt2 (83 kDa) and meprin (65 kDa) immunoreactive proteins are indicated by arrows. The figure depicts 1 of 3 BBM preparations per group.




polymerases and [α-$^{32}$P]UTP (800 Ci/mmol; ICN Biomedicals Inc., Mississauga, Ontario, Canada) as described previously (22). For the size and position of the subcloned $Na^+$/Pi cotransporter cDNA fragments, see Table 1 in ref. 22.

*Ribonuclease protection analysis.* The ribonuclease protection assay was performed as described previously (22, 36). Total RNA (5–20 μg), isolated from kidney with Trizol reagent (GIBCO BRL, Burlington, Ontario, Canada), was hybridized with the appropriate labeled riboprobes ($5 \times 10^5$ cpm) at 50°C for 18 hours and treated with 2 μg/mL RNase T1 for 1 hour at 30°C. The protected fragments were precipitated, heat-denatured, and electrophoresed on 6% denaturing polyacrylamide gels. The gels were dried and exposed to a PhosphorImager screen for quantification of radioactive signals under conditions where linearity is achieved, and to Kodak Biomax MR1 film for photography.

*Serum and urine parameters*. Serum Pi and urine Pi/creatinine were assayed using phosphorous and creatinine kits (Stanbio Laboratory Inc., San Antonio, Texas, USA) as described previously (37). The fractional excretion index for Pi ($FEI_{Pi}$) was calculated as follows: urine Pi/(urine creatinine × serum Pi).

*Statistical analysis.* Each group studied contained 5–8 mice. Statistical analysis was performed using 1-factor ANOVA and Student's *t* test. A probability of $P < 0.05$ was considered to be statistically significant.

## Results

*Effect of Npt2 gene ablation on renal BBM $Na^+$/Pi cotransport and renal expression of $Na^+$/Pi cotransporter mRNAs.* We first measured the initial rate of $Na^+$/Pi cotransport in BBM vesicles derived from *Npt2*$^{+/+}$ and *Npt2*$^{-/-}$ mice as a function of age. The data in Figure 1 demonstrate that at all ages examined, $Na^+$/Pi cotransport is significantly reduced in BBM vesicles derived from *Npt2*$^{-/-}$ mice when compared with *Npt2*$^{+/+}$ mice. Figure 1 also shows that $Na^+$/Pi cotransport decreases with age in *Npt2*$^{+/+}$ mice (activity at 115 days: 77 ± 8% and 84 ± 7% of that at 21 and 45 days, respectively; $P < 0.05$), but is not age-dependent in homozygous mutant mice. In agreement with previous findings (32), $Na^+$-dependent glucose transport is not significantly decreased in BBM from *Npt2*$^{-/-}$ mice when compared with age-matched *Npt2*$^{+/+}$ mice (at 21 days: 63 ± 17 vs. 53 ± 4 pmol/mg protein per 6 seconds for *Npt2*$^{+/+}$ vs. *Npt2*$^{-/-}$ mice; at 45 days: 59 ± 12 vs. 63 ± 11 pmol/mg protein per 6 seconds for *Npt2*$^{+/+}$ vs. *Npt2*$^{-/-}$ mice; and at 115 days: 58 ± 21 vs. 53 ± 5 pmol/mg protein per 6 seconds for *Npt2*$^{+/+}$ vs. *Npt2*$^{-/-}$ mice).

The effect of age on the abundance of Npt2 protein in renal BBMs derived from *Npt2*$^{+/+}$ mice was estimated by Western analysis (Figure 2). Quantitation of proteins bands revealed that the 13% decrease in abundance of Npt2 protein (83 kDa), relative to meprin (65 kDa), in mice at 115 days, compared with mice at 21 days of age ($n = 4$), was not statistically significant. Similarly, renal Npt2 mRNA abundance, relative to that of β-actin, does not appear to decrease significantly with age in *Npt2*$^{+/+}$ mice (Figure 3a). As reported previously (32), neither Npt2 immunoreactive protein (Figure 2) nor Npt2 mRNA (Figure 3b) is expressed in *Npt2*$^{-/-}$ mice.

We then compared the renal abundance of Npt1, Glvr-1, and Ram-1 mRNAs, relative to β-actin mRNA, in *Npt2*$^{+/+}$ and *Npt2*$^{-/-}$ mice as a function of age. Npt1

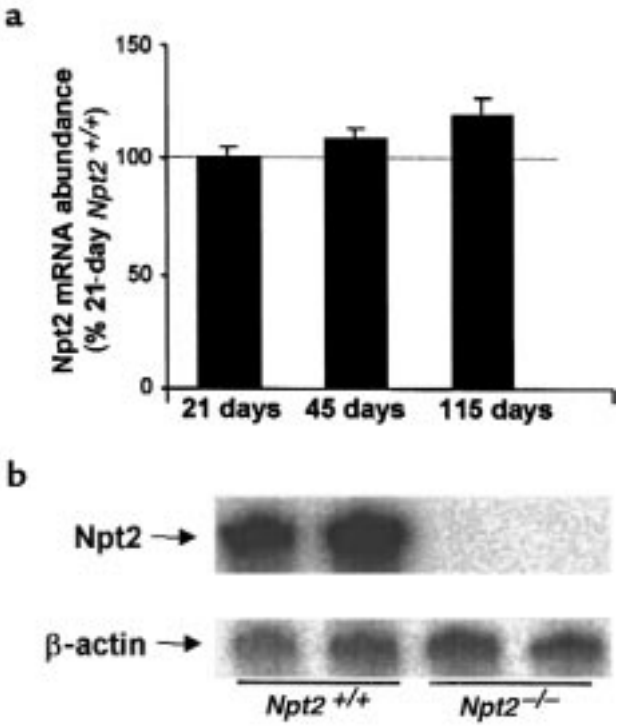



**Figure 3**
Effect of age on renal abundance of Npt2 mRNA in *Npt2*$^{+/+}$ mice (**a**) and ribonuclease protection assay of renal RNA from *Npt2*$^{+/+}$ and *Npt2*$^{-/-}$ mice (**b**). Renal RNA was prepared from *Npt2*$^{+/+}$ mice at 21, 45, and 115 days of age, and the abundance of Npt2 mRNA, relative to β-actin mRNA, was determined by ribonuclease protection assay as described in Methods. The values are mean ± SEM derived from 5–8 mice per group (**a**). Protected Npt2 and β-actin mRNA fragments are evident with renal RNA from 45-day-old *Npt2*$^{+/+}$ mice, whereas only a protected β-actin mRNA fragment is detected with RNA from age-matched *Npt2*$^{-/-}$ mice (**b**).

mRNA increases with age in both mouse strains, and, at both 21 and 45 days of age, Npt1 mRNA expression is significantly lower in the *Npt2*$^{-/-}$ mice relative to age-matched *Npt2*$^{+/+}$ mice (Figure 4a). Age has no effect on the renal abundance of Glvr-1 (Figure 4b) and Ram-1 (Figure 4c) mRNAs in *Npt2*$^{+/+}$ mice. However, in *Npt2*$^{-/-}$ mice, a significant age-related increase in the renal expression of both Glvr-1 (Figure 4b) and Ram-1 (Figure 4c) mRNAs is evident. In addition, although the abundance of both Glvr-1 and Ram-1 mRNAs is lower in 21-day-old *Npt2*$^{-/-}$ mice relative to *Npt2*$^{+/+}$ mice, it is not significantly different from *Npt2*$^{+/+}$ mice at 45 days of age (Figure 4, b and c). At 115 days of age, Glvr-1 mRNA abundance is greater in *Npt2*$^{-/-}$ mice than in *Npt2*$^{+/+}$ mice (Figure 4b), whereas Ram-1 mRNA abundance is similar in both genotypes (Figure 4c).

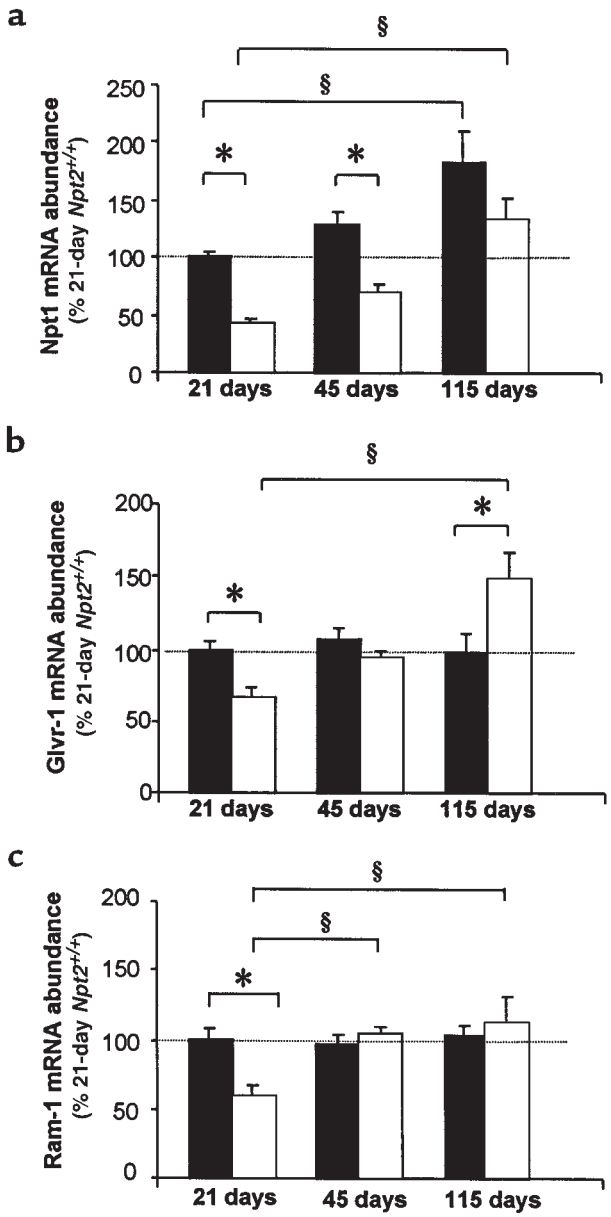



**Figure 4**
Effect of *Npt2* gene knockout and age on renal abundance of Npt1 (**a**), Glvr-1 (**b**), and Ram-1 (**c**) mRNAs. The abundance of each transcript, relative to β-actin mRNA, was determined in renal RNA samples derived from 21-, 45-, and 115-day-old *Npt2*$^{+/+}$ (filled bars) and *Npt2*$^{-/-}$ (open bars) mice by ribonuclease protection assay as described in Methods. The values depict the mean ± SEM derived from 5–8 mice per group. *Effect of genotype, $P < 0.05$. §Effect of age, $P < 0.05$.

*Effect of Npt2 gene ablation on the renal adaptive response to Pi deprivation*. Low-Pi diet (0.02%) elicits a significant fall in serum Pi in *Npt2*$^{+/+}$ and *Npt2*$^{-/-}$ mice when compared with counterparts fed the control diet (1% Pi) (Table 1). Moreover, although serum Pi levels are similar in *Npt2*$^{+/+}$ and *Npt2*$^{-/-}$ mice on the low-Pi diet, *Npt2*$^{-/-}$ mice are significantly hypophosphatemic, relative to *Npt2*$^{+/+}$ mice, on the control diet (1% Pi) (Table 1), in agreement with previous data reported in mice fed standard chow (0.6% Pi) (32). Pi deprivation also elicits a decrease in urine Pi/creatinine and fractional Pi excretion index ($FEI_{Pi}$) in *Npt2*$^{+/+}$ and *Npt2*$^{-/-}$ mice (Table 1). Under both dietary conditions, however, urine Pi/creatinine and $FEI_{Pi}$ are significantly elevated in *Npt2*$^{-/-}$ mice when compared with *Npt2*$^{+/+}$ mice.

BBM $Na^+$/Pi cotransport is increased 3.4-fold in *Npt2*$^{+/+}$ mice after Pi restriction (Figure 5a), consistent with earlier studies (28, 37). Moreover, as reported previously (28), the increase in $Na^+$/Pi cotransport in Pi-deprived *Npt2*$^{+/+}$ mice is associated with a corresponding increase (~3-fold) in Npt2 protein (Figure 5b) and a modest but significant increase in Npt2 mRNA (Figure 6a). In contrast, *Npt2*$^{-/-}$ mice, which are devoid of Npt2 protein (Figure 2 and Figure 5b) and mRNA (Figure 3b), fail to show an adaptive increase in BBM $Na^+$/Pi cotransport in response to the low-Pi diet (Figure 5a).

We also examined the effect of low-Pi diet on the renal expression of Npt1, Glvr-1, and Ram-1 mRNAs in normal and *Npt2*$^{-/-}$ mice. The abundance of Npt1 mRNA is significantly increased, by 1.3- and 1.4-fold, respectively, in both *Npt2*$^{+/+}$ and *Npt2*$^{-/-}$ mice fed the low-Pi diet when compared with counterparts on the control diet (Figure 6b). In contrast, Pi restriction failed to elicit a significant alteration in the renal abundance of Glvr-1 (Figure 6c) and Ram-1 (Figure 6d) mRNAs in either *Npt2*$^{+/+}$ or *Npt2*$^{-/-}$ mice.

*Effect of Npt2 gene ablation and low-Pi diet on renal BBM Npt1 protein abundance*. Western analysis revealed the presence of a 64-kDa Npt1 immunoreactive protein in renal BBMs derived from *Npt2*$^{+/+}$ and *Npt2*$^{-/-}$ mice fed control and low-Pi diets (Figure 7). The abundance of Npt1 protein, relative to meprin, was not increased by either Npt2 gene disruption or dietary Pi (Figure 7). The size of the detected Npt1 protein and the absence of regulation by dietary Pi are consistent with previous reports (14, 19).

## Discussion

In the present study, we examined the impact of *Npt2* gene ablation on age-dependent renal BBM $Na^+$/Pi cotransport; the expression of $Na^+$/Pi cotransporter genes *Npt1*, *Glvr-1*, and *Ram-1*; and the adaptive BBM $Na^+$/Pi cotransport response to Pi deprivation. We demonstrate that $Na^+$/Pi cotransport is not age-dependent and is significantly compromised (~15% of *Npt2*$^{+/+}$) in *Npt2*$^{-/-}$ mice. Moreover, we show that although the abundance of Npt1, Glvr-1, and

**Table 1**
Effect of *Npt2* gene knockout and low-Pi diet on serum Pi, urine Pi/creatinine, and $FEI_{Pi}$

| | Control diet | | Low-Pi diet | |
|---|---|---|---|---|
| | *Npt2*$^{+/+}$ | *Npt2*$^{-/-}$ | *Npt2*$^{+/+}$ | *Npt2*$^{-/-}$ |
| Serum Pi (mM) | 3.46 ± 0.41 | 2.24 ± 0.09$^{A}$ | 1.14 ± 0.07$^{B}$ | 1.14 ± 0.09$^{A,B}$ |
| Urine Pi/creatinine | 32.77 ± 4.1 | 61.97 ± 11.7$^{A}$ | 0.14 ± 0.05$^{B}$ | 1.36 ± 0.22$^{A,B}$ |
| $FEI_{Pi}$ | 11.82 ± 1.95 | 20.78 ± 1.95$^{A}$ | 0.13 ± 0.05$^{B}$ | 1.21 ± 0.24$^{A,B}$ |

Values depicted represent mean ± SEM for 5–8 mice per group. $^{A}$Effect of genotype, $P < 0.05$. $^{B}$Effect of diet, $P < 0.05$.

Ram-1 mRNAs increases with age in *Npt2*$^{-/-}$ mice, the augmentation in $Na^+$/Pi cotransporter gene expression is not sufficient to compensate for the loss of Npt2. We also show that *Npt2*$^{-/-}$ mice fail to exhibit an increase in BBM $Na^+$/Pi cotransport in response to Pi deprivation, thereby documenting the crucial role of Npt2 in the renal adaptive response to low Pi intake.

Previous studies have provided evidence for higher $Na^+$/Pi cotransport activity in BBMs from younger animals when compared with older animals, consistent with the notion that the requirement for Pi is increased during growth (30, 31, 38). Although it was suggested that the higher transport activity observed in weanling rats, relative to 3-month-old rats, is mediated by a $Na^+$/Pi cotransporter that is distinct from, but related to, Npt2 (30), this growth-related $Na^+$/Pi cotransporter has not yet been identified at the molecular level. In the present study, we also provide evidence for an age-dependent decrease in BBM $Na^+$/Pi cotransport in *Npt2*$^{+/+}$ mice. Moreover, the demonstration that BBM $Na^+$/Pi cotransport is not age-dependent in mice homozygous for the disrupted Npt2 gene supports the hypothesis that differences in the Npt2 protein abundance alone could account for the age-dependent decrease in transport in *Npt2*$^{+/+}$ mice.

In a previous study, it was reported that the decrease in BBM $Na^+$/Pi cotransport in aged (12- to 16-month-old) rats, relative to young (3- to 4-month-old) rats, could be ascribed to a proportional decrease in both Npt2 protein and mRNA (31). In the present study, however, a significant age-dependent decrease in Npt2 protein and mRNA was not observed. The discrepancy between the 2 studies may be explained by the fact that the ages and the species investigated were different, with the age of the older mice in the present study comparable to the younger rats in the earlier report (31), or by the magnitude of the age-related decrease in transport activity (~20% in the present study vs. a 2-fold decrease in ref. 31). In this regard, it is possible that the age-dependent reduction in BBM $Na^+$/Pi cotransport observed in *Npt2*$^{+/+}$ mice (Figure 1) may not have been of sufficient magnitude to detect corresponding changes in Npt2 protein and mRNA.

It was of interest to determine whether *Npt2* gene ablation elicits the upregulation of other $Na^+$/Pi cotransporter genes that are expressed in the kidney (22). We demonstrate that, with the exception of renal Glvr-1 mRNA expression in 115-day-old *Npt2*$^{-/-}$ mice, there is no evidence for an increase in the abundance of Npt1, Glvr-1, or Ram-1 mRNAs in 21-, 45-, and 115-day-old *Npt2*$^{-/-}$ mice, relative to age-matched *Npt2*$^{+/+}$ littermates. We also demonstrate that the abundance of Npt1, Glvr-1, and Ram-1 mRNAs is significantly increased with age in *Npt2*$^{-/-}$ mice. However, because $Na^+$/Pi cotransport continues to be markedly impaired and unchanged in *Npt2*$^{-/-}$ mice at all ages examined, it is clear that the increases in Npt1, Glvr-1, and Ram-1 mRNA abundance are insufficient to compensate for the loss of Npt2.

The changes in renal Npt1, Glvr-1, and Ram-1 mRNA levels in *Npt2*$^{-/-}$ mice may not reflect the actual abundance of functional cotransporter protein. We thus evaluated the effect of *Npt2* gene ablation on BBM Npt1 and Glvr-1 protein abundance. We demonstrate that Npt1 protein is not upregulated in *Npt2*$^{-/-}$ mice. However, the contribution of Npt1 to BBM $Na^+$/Pi cotransport is not entirely clear. Electrophysiological studies in cRNA-injected oocytes suggest that Npt1 can

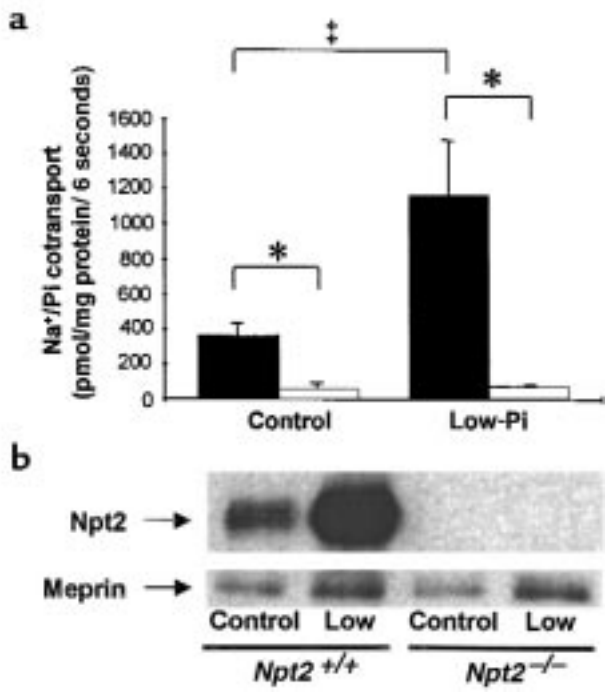
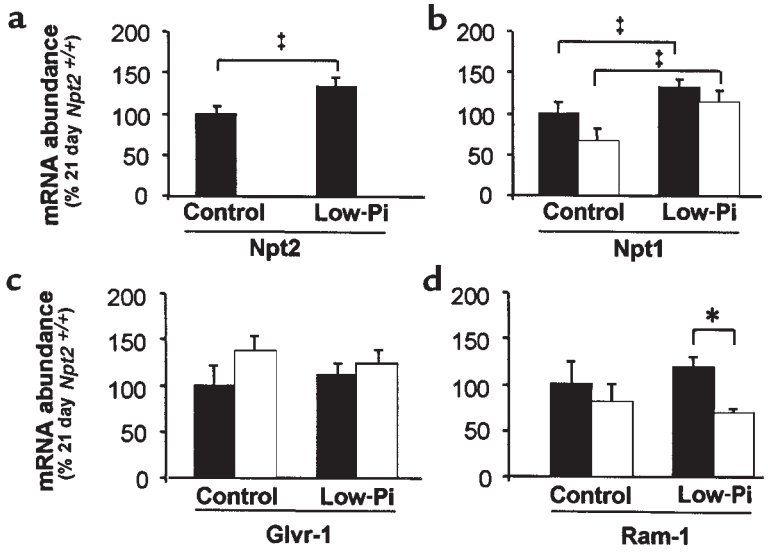



**Figure 5**
Effect of *Npt2* gene knockout and low-Pi diet on renal BBM $Na^+$/Pi cotransport (**a**) and Npt2 and meprin immunoreactive protein (**b**). Renal BBM vesicles were prepared from 45-day-old *Npt2*$^{+/+}$ (filled bars) and *Npt2*$^{-/-}$ (open bars) mice fed the control (1%) and low-Pi (0.02%) diets for 5 days. The BBM vesicle preparations were used for both transport studies (**a**) and Western analysis (**b**) as described in Methods and legends to Figures 1 and 2. The $Na^+$-dependent component of Pi transport (mean ± SEM of 2 typical experiments, each assayed in quadruplicate) is depicted. *Effect of genotype, $P < 0.05$. ‡Effect of diet, $P < 0.05$.

**Figure 6**
Effect of *Npt2* gene knockout and low-Pi diet on renal abundance of Npt2 (**a**), Npt1 (**b**), Glvr-1 (**c**), and Ram-1 (**d**) mRNAs. Renal RNA was prepared from 45-day-old $Npt2^{+/+}$ (filled bars) and $Npt2^{-/-}$ (open bars) mice fed the control and low-Pi diets for 5 days. The abundance of Npt1, Glvr-1, and Ram-1 mRNAs, relative to β-actin mRNA, was determined by ribonuclease protection assay as described in Methods. The values depict the mean ± SEM derived from 5–8 mice per group. *Effect of genotype, $P < 0.05$. ‡Effect of diet, $P < 0.05$.




function not only as a $Na^+$/Pi cotransporter, but also as a channel for $Cl^-$ and organic anions in the kidney (39, 40). Accordingly, the extent of the role of Npt1 in the maintenance of Pi homeostasis remains to be elucidated. To this end, we have cloned the murine *Npt1* gene and initiated studies to construct a targeting vector to generate mice in which the *Npt1* gene has been disrupted by homologous recombination (41).

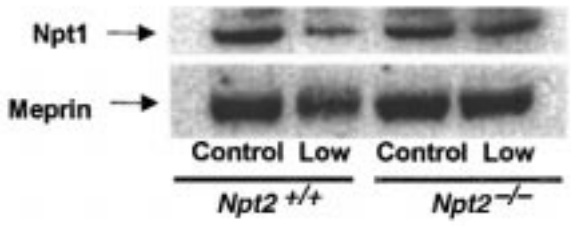
We were unable to detect Glvr-1 protein expression in BBMs from either $Npt2^{+/+}$ or $Npt2^{-/-}$ mice (data not shown). Using the same antibody, Boyer et al. provided evidence for an 85-kDa protein in crude membranes prepared from several mouse tissues (35), consistent with the ubiquitous expression of Glvr-1 (21). Our failure to detect Glvr-1 protein in mouse renal BBMs suggests that Glvr-1 may not be expressed at the apical surface. In this regard, Glvr-1 and Ram-1 have a higher affinity for Pi (10 μM) than does Npt2 (100 μM), and more closely resemble basolateral membrane–associated $Na^+$-dependent Pi transporters (42). Thus, these transporters are postulated to subserve cellular metabolic requirements for Pi under conditions in which inadequate quantities of Pi are available in the glomerular filtrate (43). In any case, the age-dependent upregulation of Glvr-1 and Ram-1 mRNAs in $Npt2^{-/-}$ mice may be a moot point. They only comprise about 1% of $Na^+$/Pi cotransporter mRNAs expressed in mouse kidney (22) and, thus, are not likely to contribute significantly to the BBM transport process.

There are several reports documenting the importance of Npt2 in the adaptive regulation of BBM $Na^+$/Pi cotransport by dietary Pi (25–28). Consistent with these data, we demonstrate a comparable adaptive increase in BBM $Na^+$/Pi cotransport and Npt2 protein in $Npt2^{+/+}$ mice fed the low-Pi diet. In contrast, Pi-deprived $Npt2^{-/-}$ mice fail to show the adaptive increase in BBM $Na^+$/Pi cotransport. On the basis of these findings, we conclude that *Npt2* gene expression is essential for the adaptive BBM $Na^+$/Pi cotransport response to Pi deprivation.

The contribution of Npt1, Glvr-1, and Ram-1 to the adaptive increase in BBM $Na^+$/Pi cotransport was also investigated in the present study. We demonstrate that Npt1 protein is not mRNA is increased in Pi-deprived $Npt2^{+/+}$ and $Npt2^{-/-}$ mice. These data indicate that Npt1 does not contribute to the adaptive increase in BBM $Na^+$/Pi cotransport in $Npt2^{+/+}$ mice, consistent with previous reports in Pi-deprived rabbits (14) and rats (44). Moreover, our findings demonstrate that Npt1 does not participate in an adaptive response in $Npt2^{-/-}$ mice. We also show that Glvr-1 and Ram-1 mRNAs are not increased by Pi deprivation in either $Npt2^{+/+}$ or $Npt2^{-/-}$ mice, in agreement with the report that the renal abundance of Glvr-1 and Ram-1 mRNAs is not increased in Pi-deprived normal and X-linked Hyp mice, relative to counterparts fed the control diet (22). In support of these findings is the recent demonstration that post-translational modification accounts for the regulation of Ram-1–mediated $Na^+$/Pi cotransport in response to low-Pi challenge (45).

It is of interest that serum Pi values are similar in Pi-deprived $Npt2^{+/+}$ and Pi-deprived $Npt2^{-/-}$ mice. Although

**Figure 7**
Effect of *Npt2* gene knockout and low-Pi diet on renal BBM Npt1 and meprin immunoreactive protein. Renal BBM vesicles were prepared from 55-day-old $Npt2^{+/+}$ and $Npt2^{-/-}$ mice fed the control (1%) and low-Pi (0.02%) diets for 5 days. The BBM proteins were fractionated on 10% SDS-PAGE gels, transferred to nitrocellulose membranes, and probed with a rabbit polyclonal anti-rabbit Npt1 antibody and subsequently with an mAb raised against the α subunit of rat meprin. Bands depicting Npt1 (64 kDa) and meprin (65 kDa) immunoreactive proteins are indicated by arrows. The figure depicts 1 of 3 BBM preparations per group.



the underlying mechanism for these findings is not clear, our studies in X-linked Hyp mice, which harbor a large deletion in the *Phex* gene (36), suggest a possible explanation. In contrast to Pi-deprived *Npt2*$^{-/-}$ mice, serum Pi in Pi-deprived Hyp mice is significantly lower than that in Pi-deprived normal mice (28, 37, 46). Moreover, Hyp mice exhibit abnormal regulation of both renal 1,25-$(OH)_2$D synthesis and catabolism by dietary Pi (46, 47), whereas *Npt2*$^{-/-}$ mice show the expected increase in serum 1,25-$(OH)_2$D levels in the face of hypophosphatemia (32). On the basis of these findings, it is possible that normal *Phex* function and appropriate regulation of renal vitamin D metabolism account for the similar serum Pi concentrations in Pi-deprived *Npt2*$^{+/+}$ and *Npt2*$^{-/-}$ mice. It is also of interest that Hyp mice that are significantly deficient in, but not devoid of, renal Npt2 mRNA and protein (33) retain the capacity to mount an adaptive increase in BBM $Na^+$/Pi cotransport in response to low-Pi challenge (28, 37). While the mechanism whereby loss of Phex function elicits the decrease in renal *Npt2* gene expression is not understood, it is clear that, in contrast to Npt2, Phex is not necessary for the adaptive $Na^+$/Pi cotransport response at the renal BBM.

Mice homozygous for the disrupted *Npt2* gene exhibit many of the biochemical features of patients with hereditary hypophosphatemic rickets with hypercalciuria, a rare Mendelian disorder of renal Pi reabsorption (48, 49). These include increased urinary excretion of Pi, hypophosphatemia, and an elevated serum concentration of 1,25-$(OH)_2$D with attendant hypercalciuria. To date, there is no clear association between *Npt2* and hereditary hypophosphatemic rickets with hypercalciuria. Additionally, it is not known whether patients with this disorder exhibit an adaptive increase in renal Pi reabsorption in response to low-Pi challenge.

In summary, we demonstrate that Npt2 is responsible for approximately 85% of $Na^+$/Pi cotransport at the renal BBM, and that homozygous disruption of the *Npt2* gene cannot be compensated for by the age-dependent increase in renal expression of Npt1, Glvr-1, and Ram-1 mRNAs. In addition, we show that Npt2 is essential for the adaptive increase in renal BBM $Na^+$/Pi cotransport in response to low-Pi challenge. Our results support the conclusion that Npt2 plays a fundamental role in the overall maintenance of Pi homeostasis.

## Acknowledgments

We thank Danielle Boulais for technical assistance, H. Murer and J. Biber for the Npt2 and Npt1 antibodies, P. Crine for the meprin antibody, and R. Beliveau for the Glvr-1 antibody. This work was supported by a grant from the Medical Research Council of Canada (Medical Research Council Group Grant in Medical Genetics to H.S. Tenenhouse). H.M. Hoag is the recipient of a Medical Research Council Studentship Award.

1. Mizgala, C.L., and Quamme, G.A. 1985. Renal handling of phosphate. *Physiol. Rev.* **65**:431–466.
2. Gmaj, P., and Murer, H. 1986. Cellular mechanisms of inorganic phosphate transport in kidney. *Physiol. Rev.* **66**:36–70.
3. Murer, H., Werner, A., Reshkin, S., Wuarin, R., and Biber, J. 1991. Cellular mechanisms in proximal tubular reabsorption of inorganic phosphate. *Am. J. Physiol.* **260**:C885–C899.
4. Berndt, T.J., and Knox, F.G. 1992. Renal regulation of phosphate excretion. In *The kidney: physiology and pathophysiology*. D.W. Seldin and G. Giebisch, editors. Raven Press. New York, NY. 2511–2532.
5. Levi, M., Kempson, S.A., Lotscher, M., Biber, J., and Murer, H. 1996. Molecular regulation of renal phosphate transport. *J. Membr. Biol.* **154**:1–9.
6. Tenenhouse, H.S. 1997. Cellular and molecular mechanisms of renal phosphate transport. *J. Bone Miner. Res.* **12**:159–164.
7. Murer, H., and Biber, J. 1997. A molecular view of proximal tubular inorganic phosphate (Pi) reabsorption and of its regulation. *Pflugers Arch.* **433**:379–389.
8. Murer, H., et al. 1998. Cellular/molecular control of renal Na/Pi-cotransport. *Kidney Int. Suppl.* **65**:S2–S10.
9. Werner, A., et al. 1991. Cloning and expression of cDNA for a Na/Pi cotransport system of kidney cortex. *Proc. Natl. Acad. Sci. USA.* **88**:9608–9612.
10. Chong, S.S., Kristjansson, K., Zoghbi, H.Y., and Hughes, M.R. 1993. Molecular cloning of the cDNA encoding a human renal sodium phosphate transport protein and its assignment to chromosome 6p21.3-p23. *Genomics.* **18**:355–359.
11. Chong, S.S., et al. 1995. Cloning, genetic mapping, and expression analysis of a mouse renal sodium-dependent phosphate cotransporter. *Am. J. Physiol.* **268**:F1038–F1045.
12. Magagnin, S., et al. 1993. Expression cloning of human and rat renal cortex Na/Pi cotransport. *Proc. Natl. Acad. Sci. USA.* **90**:5979–5983.
13. Sorribas, V., et al. 1994. Cloning of a Na/Pi cotransporter from opossum kidney cells. *J. Biol. Chem.* **269**:6615–6621.
14. Verri, T., et al. 1995. Cloning of a rabbit renal Na-Pi cotransporter, which is regulated by dietary phosphate. *Am. J. Physiol.* **268**:F626–F633.
15. Collins, J.F., and Ghishan, F.K. 1994. Molecular cloning, functional expression, tissue distribution, and in situ hybridization of the renal sodium phosphate ($Na^+$/Pi) transporter in the control and hypophosphatemic mouse. *FASEB J.* **8**:862–868.
16. Hartmann, C.M., et al. 1995. Transport characteristics of a murine renal Na/Pi-cotransporter. *Pflugers Arch.* **430**:830–836.
17. Helps, C., Murer, H., and McGivan, J. 1995. Cloning, sequence analysis and expression of the cDNA encoding a sodium-dependent phosphate transporter from the bovine renal epithelial cell line NBL-1. *Eur. J. Biochem.* **228**:927–930.
18. Custer, M., et al. 1993. Localization of NaPi-1, a Na-Pi-cotransporter, in rabbit kidney proximal tubules. I. mRNA-localization by RT-PCR. *Pflugers Arch.* **424**:203–209.
19. Biber, J., Custer, M., Werner, A., Kaissling, B., and Murer, H. 1993. Localization of NaPi-1, a Na/Pi cotransporter, in rabbit kidney proximal tubules. II. Localization by immunohistochemistry. *Pflugers Arch.* **424**:210–215.
20. Custer, M., Lötscher, M., Biber, J., Murer, H., and Kaissling, B. 1994. Expression of Na-Pi cotransport in rat kidney: localization by RT-PCR and immunohistochemistry. *Am. J. Physiol.* **266**:F767–F774.
21. Kavanaugh, M.P., et al. 1994. Cell-surface receptors for gibbon ape leukemia virus and amphotropic murine retrovirus are inducible sodium-phosphate symporters. *Proc. Natl. Acad. Sci. USA.* **91**:7071–7075.
22. Tenenhouse, H.S., Roy, S., Martel, J., and Gauthier, C. 1998. Differential expression, abundance and regulation of $Na^+$-phosphate cotransporter genes in murine kidney. *Am. J. Physiol.* **44**:F527–F534.
23. Kempson, S.A., et al. 1995. Parathyroid hormone action on phosphate transporter mRNA and protein in rat renal proximal tubules. *Am. J. Physiol.* **268**:F784–F791.
24. Pfister, M.K., et al. 1998. Parathyroid hormone leads to the lysosomal degradation of the renal type II Na/Pi cotransporter. *Proc. Natl. Acad. Sci. USA.* **95**:1909–1914.
25. Pfister, M.F., et al. 1998. Cellular mechanisms involved in the acute adaptation of OK cell Na/Pi-cotransport to high- or low-Pi medium. *Pflugers Arch.* **435**:713–719.
26. Werner, A., Kempson, S.A., Biber, J., and Murer, H. 1994. Increase of Na/Pi-cotransport encoding mRNA in response to low Pi diet in rat kidney cortex. *J. Biol. Chem.* **269**:6637–6639.
27. Levi, M., et al. 1994. Cellular mechanisms of acute and chronic adaptation of rat renal phosphate transporter to alterations in dietary phosphate. *Am. J. Physiol.* **267**:F900–F908.
28. Tenenhouse, H.S., Martel, J., Biber, J., and Murer, H. 1995. Effect of Pi restriction on renal $Na^+$-Pi cotransporter mRNA and immunoreactive protein in X-linked Hyp mice. *Am. J. Physiol.* **268**:F1062–F1069.
29. Arar, M., Zajicek, H.K., Elshihabi, I., and Levi, M. 1999. Epidermal growth factor inhibits Na-Pi cotransport in weaned and suckling rats. *Am. J. Physiol.* **276**:F72–F78.
30. Silverstein, D.M., Barac-Nieto, M., Murer, H., and Spitzer, A. 1997. A putative growth-related renal $Na^+$-Pi cotransporter. *Am. J. Physiol.* **273**:R928–R933.
31. Sorribas, V., et al. 1996. Cellular mechanisms for the age-related decrease in renal phosphate reabsorption. *Kidney Int.* **50**:855–863.
32. Beck, L., et al. 1998. Targeted inactivation of *Npt2* in mice leads to severe renal phosphate wasting, hypercalciuria and skeletal abnormalities. *Proc. Natl. Acad. Sci. USA.* **95**:5372–5377.

33. Tenenhouse, H.S., et al. 1994. Renal $Na^+$-phosphate cotransport in murine X-linked hypophosphatemic rickets: molecular characterization. *J. Clin. Invest.* **93**:671–676.
34. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227**:680–685.
35. Boyer, C.J.C., Baines, A.D., Beaulieu, E., and Beliveau, R. 1998. Immunodetection of a type III sodium-dependent phosphate cotransporter in tissues and OK cells. *Biochim. Biophys. Acta.* **1368**:73–83.
36. Beck, L., et al. 1997. *Pex/PEX* tissue distribution and evidence for a deletion in the 3′ region of the *Pex* gene in X-linked hypophosphatemic mice. *J. Clin. Invest.* **99**:1200–1209.
37. Tenenhouse, H.S., and Scriver, C.R. 1979. Renal brush border membrane adaptation to phosphorus deprivation in the Hyp/Y mouse. *Nature.* **281**:225–227.
38. Caverzasio, J., Murer, H., Fleisch, H., and Bonjour, J.-P. 1982. Phosphate transport in brush-border membrane vesicles isolated from renal cortex of young growing and adult rats. Comparison with whole kidney data. *Pflugers Arch.* **394**:217–221.
39. Busch, A.E., et al. 1996. Expression of a renal type I sodium/phosphate transporter (NaPi-1) induces a conductance in *Xenopus* oocytes permeable for organic and inorganic anions. *Proc. Natl. Acad. Sci. USA.* **93**:5347–5351.
40. Yabuuchi, H., et al. 1998. Hepatic sinusoidal membrane transport of anionic drugs mediated by anion transporter Npt1. *J. Pharmacol. Exp. Ther.* **286**:1391–1396.
41. Tenenhouse, H.S., and Soumounou, Y. 1998. Cloning and characterization of the mouse $Na^+$-phosphate cotransporter gene, Npt1, and construction of an Npt1 targeting vector. *J. Bone Miner. Res.* **23**:S465. (Abstr.)
42. Schwab, S.J., Klahr, S., and Hammerman, M.R. 1984. $Na^+$ gradient-dependent Pi uptake in basolateral membrane vesicles from dog kidney. *Am. J. Physiol.* **24**:F663–F669.
43. Hammerman, M.R. 1986. Phosphate transport across renal proximal tubular cell membranes. *Am. J. Physiol.* **251**:F385–F398.
44. Delisle, M.C., Boyer, C., Vachon, V., Giroux, S., and Béliveau, R. 1994. Immunodetection and characterization of proteins implicated in renal sodium phosphate cotransport. *Biochim. Biophys. Acta.* **1190**:289–296.
45. Rodrigues, P., and Heard, J.M. 1999. Modulation of phosphate uptake and amphotropic murine leukemia virus entry by posttranslational modifications of PIT-2. *J. Virol.* **73**:3789–3799.
46. Tenenhouse, H.S., and Jones, G. 1990. Abnormal regulation of renal vitamin D catabolism by dietary phosphate in murine X-linked hypophosphatemic rickets. *J. Clin. Invest.* **85**:1450–1455.
47. Yamaoka, K., et al. 1986. Abnormal relationship between serum phosphate concentration and renal 25-hydroxycholecalciferol-1-alpha-hydroxylase activity in X-linked hypophosphatemic mice. *Miner. Electrolyte Metab.* **12**:194–198.
48. Tieder, M., et al. 1985. Hereditary hypophosphatemic rickets with hypercalciuria. *N. Engl. J. Med.* **312**:611–617.
49. Rasmussen, H., and Tenenhouse, H.S. 1995. Mendelian hypophosphatemias. In *The metabolic and molecular bases of inherited disease*. C.R. Scriver, A.L. Beaudet, W.S. Sly, and D. Valle, editors. McGraw-Hill. New York, NY. 3717–3745.