Renal Na^+-Phosphate Cotransport in Murine X-linked Hypophosphatemic Rickets

Molecular Characterization

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Abstract

The X-linked *Hyp* mouse is characterized by a specific defect in proximal tubular phosphate (Pi) reabsorption that is associated with a decrease in V_{max} of the high affinity Na^+-Pi cotransport system in the renal brush border membrane. To understand the mechanism for V_{max} reduction, we examined the effect of the *Hyp* mutation on renal expression of Na^+-Pi cotransporter mRNA and protein. Northern hybridization of renal RNA with a rat, renal-specific Na^+-Pi cotransporter cDNA probe (NaPi-2) (Magagnin et al. 1993. *Proc. Natl. Acad. Sci. USA.* 90:5979–5983.) demonstrated a reduction in a 2.6-kb transcript in kidneys of *Hyp* mice relative to normal littermates (NaPi-2/β-actin mRNA = 57±6% of normal in *Hyp* mice, $n = 6$, $P < 0.01$). Na^+-Pi cotransport, but not Na^+-sulfate cotransport, was ~ 50% lower in Xenopus oocytes injected with renal mRNA extracted from *Hyp* mice when compared with that from normal mice. Hybrid depletion experiments documented that the mRNA-dependent expression of Na^+-Pi cotransport in oocytes was related to NaPi-2. Western analysis demonstrated that NaPi-2 protein is also significantly reduced in brush border membranes of *Hyp* mice when compared to normals. The present data demonstrate that the specific reduction in renal Na^+-Pi cotransport in brush border membranes of *Hyp* mice can be ascribed to a proportionate decrease in the abundance of Na^+-Pi cotransporter mRNA and protein. (*J. Clin. Invest.* 1994. 93:671–676.) Key words: X-linked hypophosphatemia • vitamin D resistant rickets • familial hypophosphatemic rickets • brush border membrane • proximal tubule • NaPi-1 • NaPi-2 • NaPi-3

Introduction

The X-linked *Hyp* mutation is a murine homologue of X-linked hypophosphatemia, the most prevalent form of inherited rickets in humans (1). Like their human counterparts, *Hyp* mice are characterized by rachitic bone disease, hypophosphatemia, and impaired renal reabsorption of filtered phosphate (Pi)[1] (1, 2). In both mouse (3) and humans (4), the mutation maps to one of five highly conserved regions on the X chromosome (5). Neither the gene nor its product have been identified.

The renal defect in Pi reabsorption in *Hyp* mice has been localized to the brush border membrane of the proximal tubule (6–8) and is not dependent on parathyroid hormone for its expression (9). Kinetic studies provided evidence for two distinct Na^+-Pi cotransport systems in renal brush border membrane vesicles of *Hyp* mice (10). While the low affinity, high capacity mechanism is intact in the mutant strain, the high affinity, low capacity process has half normal V_{max}, with no change in apparent affinity for Pi (10). These findings underscore the importance of the high affinity Pi transport system in the overall maintenance of Pi homeostasis, and they suggest that a partial loss of this function contributes to hypophosphatemia and bone disease in *Hyp* mice.

Several approaches have been taken to elucidate the mechanism for the decrease in V_{max} of the high affinity Na^+-Pi cotransport system in the renal brush border membrane of *Hyp* mice. These studies demonstrated that loss of transport function could not be ascribed to an impaired response of the transporter to the Na^+ gradient driving force, membrane potential or external pH (11), or to an alteration in the lipid composition of renal brush border membranes in *Hyp* mice (12). In addition, the in situ molecular size of the high affinity Na^+-Pi cotransporter, determined by radiation inactivation analysis, is similar in normal and *Hyp* mice (13).

Efforts to determine whether the number of high affinity Na^+-Pi cotransport sites is decreased in the renal brush border membrane of *Hyp* mice have been hampered by the lack of specific probes for the cotransporter. The demonstration that Na^+-dependent phosphonoformic acid (PFA) binding and the Pi-displaceable component of PFA binding are similar in renal brush border membranes of normal and *Hyp* mice suggested that the number of Pi transport sites is not reduced in the mutant strain (13). However, because PFA does not interact exclusively with the Na^+-Pi cotransporter (14), nonspecific PFA binding to the brush border membrane may have masked a genotype difference in Na^+-Pi cotransporter number.

Recently, cDNAs related to renal cortical brush border membrane Na^+-Pi cotransport have been identified by expression cloning in *Xenopus laevis* oocyte systems (15, 16). Na^+-Pi cotransporter cDNAs, cloned from rat (NaPi-2) and human (NaPi-3) kidney cortex, hybridize at high stringency with renal transcripts from a variety of mammalian species including rabbit and mouse, and are expressed only in the kidney (16). In the rat, NaPi-2 mRNA was shown to be present exclusively in the proximal tubule by reverse transcriptase-PCR of mRNA isolated from single microdissected nephron segments (17). In addition, NaPi-2–related protein was localized to the brush border membrane of the rat proximal tubule by immunocytochemistry (17). A similar approach was recently used by the same group to localize NaPi-1 mRNA (18) and protein (19) to

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1. *Abbreviations used in this paper:* NaPi-2, rat kidney Na^+-Pi cotransporter; Pi, phosphate; PFA, phosphonoformic acid.

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the proximal tubule and brush border membrane of rabbit kidney.

In the present study, we have used NaPi-2 cDNA and NaPi-2–specific antibody probes to investigate the basis for the specific reduction in Na^+-Pi cotransport in renal brush border membranes of *Hyp* mice. We provide evidence for a proportionate reduction in renal abundance of Na^+-Pi cotransporter mRNA and protein in the mutant strain.

Methods

Mice. Mutant *Hyp* mice and normal littermates were obtained by breeding C57Bl/6J *Hyp*/+ females with C57Bl/6J +/Y males; the original breeding pairs were purchased from Jackson Laboratory (Bar Harbor, ME). All mice were maintained on Wayne Rodent Blox from Teklad Premier Laboratory Diets (Madison, WI) containing 1.2% calcium and 0.99% phosphorus. Mice aged 6–12 mo were used in all experiments and were killed by decapitation.

Brush border membrane isolation and transport studies. Renal brush border membrane vesicles were prepared from kidney cortex by the $MgCl_2$ precipitation procedure as described previously (7). Enrichments of 8- to 10-fold with respect to alkaline phosphatase activity were routinely obtained. Transport of [^{32}P]phosphate (0.1 mM), [^{35}S]-sulfate (0.1 mM), and [^{3}H]glucose (0.01 mM) was measured in the presence of 100 mM mannitol, 10 mM Tris-Hepes, pH 7.4, 100 mM KCl or 100 mM NaCl (7). The Na^+-dependent component of transport was obtained by the difference in uptake in NaCl and KCl, respectively. Uptake in KCl comprised less than 10% of uptake in NaCl.

X. laevis oocytes and transport assay. Methods related to the handling of oocytes and the transport assay have been described earlier (15, 16, 20, 21). Oocytes were injected with either 50 nl H_2O or mRNA (5–25 ng/50 nl) and uptake of [^{32}P]phosphate and [^{35}S]sulfate measured 1–5 d after injection. Based on our previous studies of Na^+-Pi and Na^+-sulfate cotransport in oocytes injected with rat and rabbit renal mRNA, we assumed that, under the above conditions, transport rates would be proportional to the amount of mRNA injected (15, 16, 20, 21). Indeed, in a preliminary study of oocytes injected with renal mRNA from normal mice, this prediction was confirmed (data not shown). Furthermore, we routinely used two different concentrations of mouse renal mRNA and two different times after injection in all oocyte experiments and qualitatively similar results were obtained under these conditions (data not shown). The transport studies were performed in either 100 mM NaCl or 100 mM choline chloride at Pi and sulfate concentrations of 0.5 mM. The Na^+-dependent component of transport was obtained by the difference in uptake in NaCl and choline chloride, respectively. Uptake in choline chloride was < 20 and 30% of that in NaCl for Pi and sulfate, respectively (15, 16, 20, 21).

Hybrid depletion studies. RNAase H–mediated hybrid depletion, followed by functional expression in *Xenopus laevis* oocytes, was performed as described by Meyerhof et al. (22). Two complementary 16-mer oligonucleotides (GGCCTCTACCCTGGAC, antisense; GTCCAGGGTAGAGGCC, sense) at position 1,004–1,020 of NaPi-2 cDNA (16) were used. Renal mRNA (2 μg) from normal and *Hyp* mice was annealed to 0.8 pmol of either antisense or sense oligonucleotides in 5 μl of 100 mM KCl: after denaturing the mix for 5 min at 70°C, the tubes were cooled at room temperature for 10 min, and then kept on ice. 20 μl of RNase H digestion mix (25 mM Tris-HCl [pH 6.9], 115 mM KCl, 6 mM $MgCl_2$, 0.2 mM β NAD^+, 12.5 mM $[NH_4]_2SO_4$, 70 U RNase inhibitor [Biofinex], 2.5 μg transfer RNA, 0.25 U RNase H [Gibco BRL, Gaithersburg, MD]) was added and the reaction mixture incubated for 10 min at 37°C. The reaction mixture was then extracted with phenol/chloroform/isoamylalcohol (25:24:1) and precipitated with ethanol. The resulting RNA was dissolved in 3 μl of water; 1 μl was used for northern analysis and the rest used for functional expression in *Xenopus laevis* oocytes.

Isolation of total and mRNA. Total RNA was isolated by the method of Chirgwin et al. (23) from kidneys which had been quick frozen in liquid N_2. Poly(A)$^+$ RNA selection was achieved with oligo(dT)-cellulose (Boehringer Mannheim Corp., Indianapolis, IN) using the manufacturer's protocol.

Northern analysis. Total RNA (20 μg) or mRNA (0.5 μg) was denatured and subjected to electrophoresis on 1% agarose/formaldehyde gels (24). The RNA was visualized with ethidium bromide before transfer onto a nylon membrane by vacuum blotting. The membranes were exposed to ultraviolet light for 2 min and fixed at 80°C for 2 h. Full-length cDNA probes for NaPi-2 (16), NaSi-1 (21), and β-actin (25) were labeled by random priming using α[^{32}P]dCTP. Blots were prehybridized and hybridized in buffer containing 50% formamide, 5 × SSPE (0.75 M NaCl, 0.05 M NaH_2PO_4, 5 mM EDTA, pH 7.4), 25 mM KPO_4 (pH 7.4), 0.1% SDS, 10% dextran sulfate, 2.5× Denhardt's, 0.25 mg/ml salmon sperm DNA, and 0.5 mg/ml yeast transfer RNA. After hybridization, blots were washed to high stringency and exposed to Kodak XAR film. The relative intensities of the resulting autoradiographic images were assessed using the Ultroscan Laser Densitometer from LKB Instruments Inc. (Bromma, Sweden). The signal for NaPi-2 was related to that for β-actin. The latter was not influenced by the *Hyp* mutation.

Slot blots. 4–8 μg of total RNA were applied to a nylon membrane with the aid of slot blot manifold and fixed as described above. Membranes were hybridized and autoradiographic images quantitated as described above.

Western blot analysis. SDS-polyacrylamide gel electrophoresis was carried out according to Laemmli (26) using 10% acrylamide gels. Before electrophoresis, brush border membrane proteins were denatured at 95°C for 2 min in sample buffer (final concentrations: 2% SDS, 10% glycerol, 10 mM DTT, 0.5 mM EDTA, and 95 mM Tris/HCl, pH 6.8). Electrophoretic transfer onto nitrocellulose paper (BA 83; Schleicher & Schuell, Inc. (Keene, NH) was performed according to Towbin et al. (27). After blocking with 3% BSA (no. 7030; Sigma Immunochemicals, St. Louis, MO) in 150 mM NaCl, 20 mM Tris-HCl, pH 7.3, the blots were incubated with polyclonal antibodies raised against a synthetic COOH-terminal peptide of the recently cloned NaPi-2 of rat kidney cortex (16, 17) or with polyclonal antibodies raised against purified ecto 5′-nucleotidase from rat (kindly provided by Dr. B. Kaissling, University of Zurich) (28). Both antisera were used at a dilution of 1:5,000, either in the absence or in the presence of the antigenic peptide (100 μg/ml). Binding of the primary antibody was visualized using a goat anti–rabbit IgG conjugated to alkaline phosphatase (Bio-Rad Laboratories, Richmond, CA) and 5-bromo-4-chloro-3-indolylphosphate and nitro-blue-tetrazolium as chromogens. The staining intensities were quantitated by densitometry. Molecular weight standards (Bio-Rad Laboratories) were run in parallel and stained with India ink.

Statistics. Transport measurements were performed in quadruplicate and all experiments were repeated at least three times. Data are expressed as mean±SEM unless otherwise indicated. Where indicated, statistical significance was determined by Student's *t* test, paired or unpaired as appropriate.

Results

Initial rate Na^+-dependent Pi transport is significantly reduced in renal brush border membrane vesicles prepared from *Hyp* mice when compared to normal littermates (Table I). In contrast, Na^+-dependent sulfate and Na^+-dependent glucose uptake are not significantly different in normal and mutant mice (Table I). These data provide evidence for a specific Pi transport defect in the renal brush border membrane of *Hyp* mice and are consistent with previous findings (6, 7, 10, 29).

Northern analysis of total RNA isolated from kidneys of normal and *Hyp* is depicted in Fig. 1. Hybridization of the membrane with the NaPi-2 cDNA probe clearly demonstrates a 2.6-kb transcript that is significantly reduced in kidneys from *Hyp* mice when compared to normal littermates. Ethidium

Table I. Na^+-dependent Pi, Sulfate, and Glucose Uptake in Renal Brush Border Membrane Vesicles Prepared from Normal and Hyp *Mice*

	Uptake	
	Normal	*Hyp*
	pmol/6 s per mg protein	
Pi	382±27	209±21*
Sulfate	147±37	228±38
Glucose	22±3	22±4

Renal brush border membrane vesicle preparation and transport studies were performed as described in Methods. The Na^+-dependent component of uptake is depicted and was obtained by the difference in uptake measured in NaCl and in KCl. The latter, which comprised <10% of uptake in NaCl, was not significantly different in *Hyp* mice. Mean±SEM of three experiments performed in quadruplicate are shown. * $P < 0.01$ Normal vs *Hyp*.

bromide staining of the gel (not shown) and rehybridization of the Northern blot with a β-actin cDNA probe (Fig. 1) confirm that the reduction in NaPi-2 mRNA abundance in kidneys of *Hyp* mice cannot be ascribed to differences in RNA loading on the gels. The abundance of NaPi-2 mRNA relative to β-actin mRNA, determined by densitometric analysis of three Northern blots and three slot blots, each obtained with different renal RNA samples from normal and *Hyp* mice ($n = 6$), indicate that Na^+-Pi cotransporter mRNA is ~ 57% of normal in kidneys of *Hyp* mice (Fig. 1, $P < 0.01$).

Expression of Na^+-Pi and Na^+-sulfate cotransport in oocytes injected with mRNA extracted from kidneys of normal and *Hyp* mice are shown in Fig. 2. While Na^+-dependent Pi transport is significantly higher in mRNA-injected oocytes than in water-injected controls, renal mRNA from *Hyp* mice elicited a response that was ~ 50% of that seen with a similar amount of renal mRNA from normal littermates (Fig. 2 *A*). In contrast, Na^+-dependent sulfate transport was not significantly different in oocytes injected with renal mRNA from normal or *Hyp* mice but was markedly higher than that in water-injected controls (Fig. 2 *B*). These data suggest that the mRNA species encoding the Na^+-Pi cotransporter is significantly reduced in kidneys of *Hyp* mice.

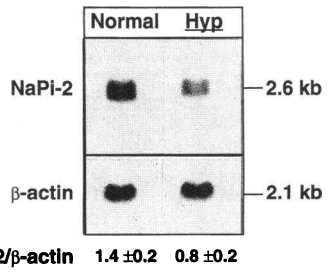

Figure 1. Northern blot analysis of total RNA extracted from kidneys of normal and *Hyp* mice. After electrophoresis on agarose/formaldehyde gels, RNA was transferred to nylon membranes and hybridized sequentially with ^{32}P-labeled NaPi-2 and β-actin cDNA probes as described in Methods. Na^+-Pi cotransporter and β-actin mRNAs were visualized by autoradiography. The NaPi-2/β-actin mRNA ratios (mean±SD, $n = 6$, $P < 0.01$, normal vs *Hyp*) were obtained by densitometric analyses of three Northern blots and three slot blots, each obtained with different renal RNA samples from normal and *Hyp* mice.

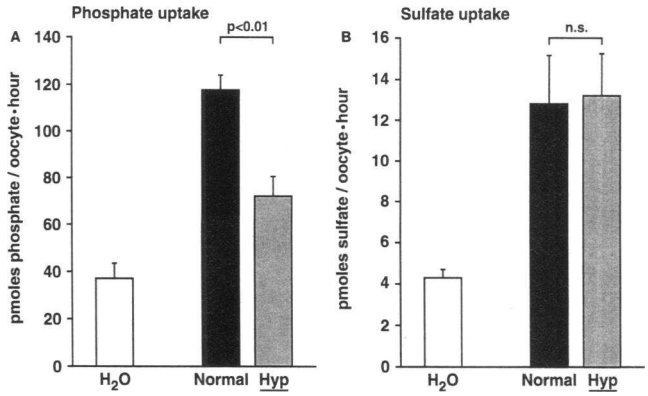

Figure 2. Expression of Na^+-Pi and Na^+-sulphate cotransport activity in *Xenopus laevis* oocytes. Na^+-dependent Pi uptake (*A*) and Na^+-dependent sulfate uptake (*B*) were assayed 3 d after injection of oocytes with either water or 15 ng mRNA from kidneys of normal or *Hyp* mice as described in Methods. Qualitatively similar results were obtained with different amounts of mRNA and at different times after injection. The values are mean±SEM (five to seven oocytes, representative experiment).

To determine whether the mRNA-induced oocyte expression of Na^+-Pi cotransport and the mutation-related difference in mRNA-stimulated activity, depicted in Fig. 2 *A*, are indeed a function of an mRNA species related to NaPi-2, mRNA from kidneys of normal and *Hyp* mice was hybridized with an antisense NaPi-2 oligonucleotide probe and treated with RNase H digestion before oocyte injection. As shown in Fig. 3 *A*, mRNA-dependent Na^+-Pi cotransport was abolished in oocytes injected with renal mRNA, from either normal or *Hyp* mice, after hybridization with the antisense NaPi-2 oligonucleotide. In contrast, application of the same protocol with a NaPi-2 sense oligonucleotide did not affect mRNA-stimulated Na^+-Pi cotransport and did not abolish the mutation-related decrement in Na^+-dependent Pi transport in injected oocytes (Fig. 3 *A*). Northern analysis of mRNA from normal and *Hyp* mice demonstrates the expected reduction in size of the NaPi-2 transcript after hybridization with antisense but not with sense NaPi-2 oligonucleotides (Fig. 3 *B*). Moreover, consistent with the data in Fig. 1, a reduced signal with the NaPi-2 cDNA probe was obtained with mRNA from kidneys of *Hyp* mice relative to normals after hybridization with either sense or antisense NaPi-2 oligonucleotides and treatment with RNase H (Fig. 3 *B*). In contrast, hybridization of renal mRNA from normal and *Hyp* mice with either antisense or sense NaPi-2 oligonucleotides had no effect on the size of the transcript detected with a NaSi-1 cDNA probe that encodes a Na^+-sulfate cotransporter cloned from rat kidney cortex (21) (Fig. 3 *C*). Moreover, hybridization of renal mRNA from normal and *Hyp* mice with NaPi-2 oligonucleotides (sense or antisense) had no effect on Na^+-dependent sulfate transport in mRNA-injected oocytes (data not shown). In addition, the abundance of Na^+-sulfate cotransporter mRNA is not decreased in kidneys of *Hyp* mice relative to normal littermates (Fig. 3 *C*).

Western analysis of brush border membrane proteins derived from kidneys of normal and *Hyp* mice was performed using an antiserum raised against a COOH terminal peptide of NaPi-2 protein (17). As illustrated in Fig. 4 *A*, the antiserum recognizes proteins with apparent molecular weights of 86, 82, 77, and 67 kD, in apical membranes of normal and mutant

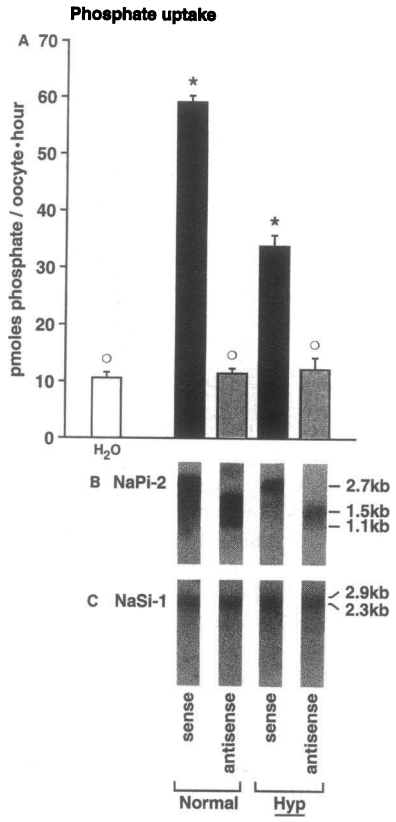

Figure 3. Hybrid depletion of NaPi-2 mRNA: effect on oocyte expression and mRNA content. mRNA from kidneys of normal and *Hyp* mice was hybridized with antisense or sense NaPi-2 oligonucleotide probes, treated with RNase H, and used for oocyte expression and Northern analysis as described in Methods. (*A*) Expression of Na^+-dependent Pi uptake in oocytes. Na^+-dependent Pi uptake was assayed 3 d after injection of oocytes with 15–20 ng mRNA that had been treated as described above. Values are mean±SEM (five to seven oocytes, representative experiment) $^*P < 0.01$, normal vs *Hyp*, sense condition. (○) Lack of significance between mRNA injected oocytes (antisense condition) and H_2O-injected oocytes. (*B* and *C*) Northern analysis of renal mRNA from normal and *Hyp* mice. 0.5 μg mRNA that had been treated as described above was subjected to electrophoresis and blotted as described in Methods. The blots were first hybridized with a NaPi-2 cDNA probe (*B*) and then rehybridized with a NaSi-1 cDNA probe (*C*) (21).

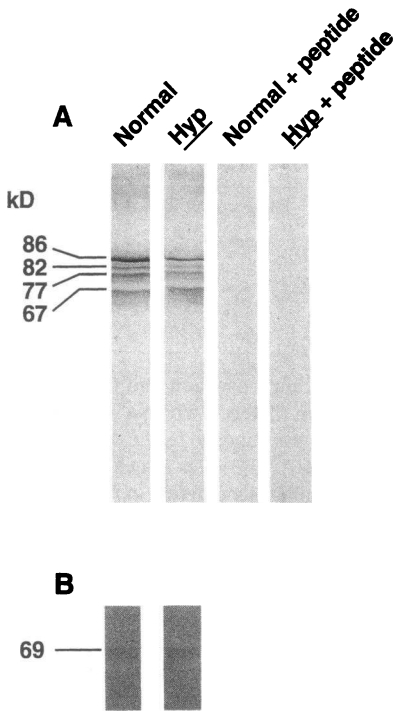

Figure 4. Western blot analysis of renal brush border membrane proteins from normal and *Hyp* mice. Brush border membrane proteins, isolated from renal cortex of normal and *Hyp* mice, were denatured for 2 min at 95°C in 2% SDS, 10% glycerol, 10 mM DTT, 0.5 mM EDTA, and 95 mM Tris-HCl, pH 6.8. The proteins (40 μg/lane) were separated on 10% SDS-polyacrylamide gels (26) and transferred onto nitrocellulose membranes (Schleicher & Schuell, Inc.) (27). Electroblotted proteins were probed with (*A*) a polyclonal antibody raised against a synthetic COOH-terminal peptide of the recently cloned rat renal brush border membrane NaPi-2 (16) or with (*B*) a polyclonal antibody raised against purified ecto-5′-nucleotidase (28). Both antisera were used at dilutions of 1:5,000. Binding of primary antibodies were visualized with anti-rabbit IgG conjugated to alkaline phosphatase (Bio-Rad Laboratories), and the staining intensities were quantitated by densitometry.

mice. The staining pattern is likely to be specific since the protein bands were not detectable upon the addition of excess antigenic peptide (Fig. 4 *A*). Except for the 67-kD protein, the proteins detected by the NaPi-2 antiserum were half as abundant in brush border membranes of *Hyp* mice when compared to normal littermates (Table II). We also show that a genotype difference in immunoreactive protein was not apparent using an antibody against ecto 5′-nucleotidase (Fig. 4 *B*, Table II), indicating that the reduction in immunoreactive Na^+-Pi cotransporter proteins evident in *Hyp* mice could not be attributed to differences in protein loading.

Discussion

Mutations in homologous genes on the human (HYP) and mouse (Hyp) X chromosomes are responsible for X-linked hypophosphatemia, a disorder in renal Pi reabsorption characterized by rachitic bone disease (1, 5). In the present study, we demonstrate that the specific defect in Na^+-dependent Pi transport in the renal brush border membrane of *Hyp* mice (6, 7, 10) is associated with a specific decrease in the renal abundance of Na^+-Pi cotransporter mRNA and protein. Moreover, the magnitude of the decrease in mRNA and protein is consistent with the 50% decrease in Na^+-dependent Pi transport function in the mutant strain (6, 7, 10). Our data thus provide evidence for a reduction in the number of high affinity Na^+-Pi cotransport sites in the renal brush border membrane of *Hyp* mice.

Xenopus laevis oocytes provide a useful model to study mRNA-mediated functional expression. Using this approach, it was reported that injection of mRNA extracted from kidneys of normal mice stimulated Na^+-dependent Pi transport in oocytes, while renal mRNA from *Hyp* mice failed to elicit a signal above that of water-injected controls (30). These data are difficult to reconcile in view of the well-documented finding that *Hyp* mice retain ~ 50% of Na^+-Pi cotransport function on the high affinity Pi transport system (6, 7, 10, 30, 31). In the present study, we show that Na^+-Pi cotransport is ~ 50% lower in oocytes injected with renal mRNA from *Hyp* mice when com-

Table II. Quantitation of Proteins on Western Blots

Protein	Densitometric units (*Hyp*/normal)
86 kD	0.6±0.2*
82 kD	0.5±0.1*
77 kD	0.5±0.2*
67 kD	1.1±0.2*
Ecto 5′-nucleotidase	1.3; 1.4‡

Solubilized renal brush border membrane proteins from normal and *Hyp* mice were analyzed by Western blotting as described in Methods and in the legend to Fig. 4. Binding of primary antibodies was visualized with anti-rabbit IgG conjugated to alkaline phosphatase. Staining intensities were quantified by densitometry. * Mean±SD of two different membrane preparations each run in duplicate. ‡ Values from two determinations.

pared with that from normals. Moreover, mRNA-stimulated Na^+-Pi cotransport in oocytes is abolished after RNase H digestion of renal mRNA from either normal or mutant mice that had been hybridized with antisense but not with sense NaPi-2 oligonucleotides. These findings, along with results from Northern analysis, support the hypothesis that the defect in renal Na^+-Pi cotransport in *Hyp* mice is associated with a specific reduction in renal abundance of Na^+-Pi cotransporter mRNA. Further study is required to establish whether the mechanism for this reduction involves a decrease in Na^+-Pi cotransporter gene transcription or mRNA stability.

In the present study, we used a polyclonal antibody raised against a COOH-terminal peptide of the NaPi-2 protein to quantitate the relative abundance of Na^+-Pi cotransporter protein in the renal brush border membrane of normal and *Hyp* mice. Recently, the same antibody was used to localize the expression of NaPi-2 protein in rat kidney by immunocytochemistry (17). These studies demonstrated that immunofluorescence was restricted to the brush border membrane of both the proximal convoluted tubule, where the signal was strongest, and the proximal straight tubule, where a weaker signal was obtained (17). Based on these findings, it is likely that the Na^+-Pi cotransporter protein that is reduced in *Hyp* mice is expressed in the brush border membrane of both proximal tubular segments, consistent with results of renal micropuncture studies in intact mice (8, 9) and Pi transport measurements in isolated renal brush border membrane vesicles (6, 7).

An important question that still remains unanswered is how the reduction in renal Na^+-Pi cotransporter mRNA and protein is related to the X-linked *Hyp* mutation. The gene product at the Hyp (HYP) locus on the mouse (human) X chromosome may encode either the renal Na^+-Pi cotransporter itself or a regulator thereof. Support for the latter hypothesis derives from physiological studies. Using parabiosis (32, 33) and renal transplantation (34) protocols it was demonstrated that the renal defect in brush border membrane Na^+-dependent Pi transport in *Hyp* mice is not intrinsic to the kidney but rather depends on a circulating humoral factor, which is not PTH (33), for its expression. Consistent with these findings is the demonstration that the Pi transport defect in the mutant strain is less prominent after culturing renal epithelial cells from normal and *Hyp* mice in a hormonally defined medium for several days (35). Further support for a humoral mechanism in *Hyp* mice derives from the failure to demonstrate the gene dose effect that is expected in X-linked traits. In the case of the *Hyp* mutation, metrical phenotypic traits are equivalently expressed in mutant heterozygous females (*Hyp*/+), homozygous females (*Hyp*/*Hyp*), and hemizygous males (*Hyp*/Y) (36).

Recently, the renal specific Na^+-Pi cotransporter gene was mapped to human chromosome 5q35 (37) by high resolution, fluorescence in situ hybridization using a full-length NaPi-3 cDNA probe which was cloned from human kidney cortex and is highly homologous to NaPi-2 (16). This map assignment excludes the renal specific Na^+-Pi cotransporter gene as a candidate gene for X-linked hypophosphatemia in mouse and humans (37). On the basis of the mapping studies and the physiological studies described above, we suggest that the gene at the Hyp (HYP) locus is involved in the regulation of renal Na^+-Pi cotransport, and that it acts by decreasing Na^+-Pi cotransporter gene transcription or increasing cotransporter mRNA turnover.

It is of interest that NaPi-2 cDNA (16), like the brush border membrane defect in Na^+-Pi cotransport in *Hyp* mice, is expressed only in the kidney and not in intestinal epithelial cells (30, 38). These data are consistent with the notion that the putative circulating factor, which is responsible for the Pi transport defect in *Hyp* mice, does not play a role in modulating intestinal Na^+-dependent Pi absorption. Thus far, attempts to identify the circulating phosphaturic factor in *Hyp* mice, by demonstrating differential effects of serum/plasma from normal and *Hyp* mice on Na^+-Pi cotransport in cultured opossum kidney cells, have met with little success (39).

In summary, we have demonstrated that the specific defect in Na^+-Pi cotransport at the renal brush border membrane of *Hyp* mice is associated with a proportionate reduction in cotransporter mRNA and protein. Further work is required to identify the gene at the X-linked Hyp (HYP) locus, which is responsible for reduced Na^+-Pi cotransporter gene expression in the mutant strain.

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