

NEPHROLITHIASIS AND OSTEOPOROSIS ASSOCIATED WITH HYPOPHOSPHATEMIA CAUSED BY MUTATIONS IN THE TYPE 2a SODIUM-PHOSPHATE COTRANSPORTER

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ABSTRACT

Background Epidemiologic studies suggest that genetic factors confer a predisposition to the formation of renal calcium stones or bone demineralization. Low serum phosphate concentrations due to a decrease in renal phosphate reabsorption have been reported in some patients with these conditions, suggesting that genetic factors leading to a decrease in renal phosphate reabsorption may contribute to them. We hypothesized that mutations in the gene coding for the main renal sodium-phosphate cotransporter (NPT2a) may be present in patients with these disorders.

Methods We studied 20 patients with urolithiasis or bone demineralization and persistent idiopathic hypophosphatemia associated with a decrease in maximal renal phosphate reabsorption. The coding region of the gene for NPT2a was sequenced in all patients. The functional consequences of the mutations identified were analyzed by expressing the mutated RNA in *Xenopus laevis* oocytes.

Results Two patients, one with recurrent urolithiasis and one with bone demineralization, were heterozygous for two distinct mutations. One mutation resulted in the substitution of phenylalanine for alanine at position 48, and the other in a substitution of methionine for valine at position 147. Phosphate-induced current and sodium-dependent phosphate uptake were impaired in oocytes expressing the mutant NPT2a. Coinjection of oocytes with wild-type and mutant RNA indicated that the mutant protein had altered function.

Conclusions Heterozygous mutations in the *NPT2a* gene may be responsible for hypophosphatemia and urinary phosphate loss in persons with urolithiasis or bone demineralization. (N Engl J Med 2002;347:983-91.)

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EPIDEMIOLOGIC studies have shown that both the formation of renal calcium stones¹⁻⁶ and bone demineralization⁷⁻¹¹ exhibit familial aggregation — findings that are compatible with the presence of genetic factors in these disorders. However, the search for gene variants that confer a predisposition to these common disorders has generally had negative results. Both disorders are likely

to be genetically heterogeneous, in which case a variety of biologic abnormalities may contribute to the clinical phenotype in individual patients. For example, low serum phosphate concentrations due to a decrease in renal phosphate reabsorption have been reported in some patients with urolithiasis¹²⁻¹⁷ or bone demineralization,^{14,15,18} suggesting that genetic factors leading to a decrease in renal phosphate reabsorption can contribute to these diseases.

Since an evaluation of candidate genes can be useful in identifying loci involved in a subgroup of patients, we have been using this approach to identify genetic abnormalities in patients who have urolithiasis or bone demineralization associated with hypophosphatemia and low renal phosphate reabsorption. The gene coding for the type 2a sodium-phosphate cotransporter (NPT2a), which resides in the apical membrane of renal proximal tubular cells, is a likely candidate for these diseases.^{19,20} Indeed, the kidney is principally responsible for phosphate homeostasis, and it regulates serum phosphate by modulating urinary phosphate excretion.¹⁹ Phosphate is filtered by the glomerulus and subsequently reabsorbed in the proximal tubule, in which the rate-limiting step is the uptake of phosphate through NPT2a.^{19,20}

METHODS

Patients

We evaluated 20 unrelated patients at Hôpital Bichat in Paris, using a protocol described previously.^{17,21} In accordance with the national guidelines for research on human subjects, written, informed consent was obtained from all patients. Specimens were obtained from the patients on two or more days. All the patients had persistent hypophosphatemia; low maximal renal phosphate reabsorption, normalized according to the glomerular filtration rate; normal ionized serum calcium concentrations; and normal plasma parathyroid hormone concentrations. Maximal renal phosphate reabsorption, normalized according to the glomerular filtration rate, was determined with the use of the nomogram described by Walton and Bijvoët.²²

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Detection of Mutations

We used intronic primers to amplify the 13 exons of the *NPT2a* gene. The 190-bp region upstream of exon 1 was also sequenced. Exons 3 and 5 were amplified with the use of the following primers: exon 3, 5'CCGCTGTTCCTCCCCGCCTC (upper primer), 5'CCATGAGCATAGTGGGCAGAG (lower primer); exon 5, 5'AGGACCTGGGAGGGGTTC (upper primer), 5'AAGCTCTTCCCACCCTGG (lower primer). Amplification was performed for 35 cycles at an annealing temperature of 60°C. The sequence of other primers is available as Supplementary Appendix 1 with the full text of this article at <http://www.nejm.org>. The PCR products were sequenced with the use of the BigDye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer) on an ABI PRISM 3100 sequencer (Applied Biosystems). We analyzed both strands of DNA using Sequencing Analysis (Applied Biosystems) and Clustal W (Infobiogen) software programs.

Expression of NPT2a in *Xenopus laevis* Oocytes

Wild-type complementary DNA (cDNA) encoding human NPT2a (kindly provided by Drs. J. Biber and H. Mürer, Physiology Institute, University of Zurich, Zurich) was introduced into the RNA expression vector pSP64T^{23,24} between the *XhoI* and *NotI* sites. Mutant cDNA was obtained from the wild-type construct with the use of the Quick Change Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's instructions. All constructs were verified by sequencing. RNA was synthesized with the use of the Riboprobe in Vitro Transcription System kit (Promega) with SP6 RNA polymerase. Three independent RNA preparations were obtained for each construct, each from a different plasmid preparation. Purified RNA was quantified by absorption at 260 nm, and the quality was verified by calculating the ratio of readings at 260 and 280 nm (A260/A280). The homogeneity and quantification of all preparations used for oocyte injection were controlled by agarose-gel electrophoresis.

Defolliculated oocytes (stage V or VI) from *X. laevis* were obtained with the use of standard procedures and injected 24 hours later with the indicated amount of wild-type or mutant RNA in 50 nl of water or with water alone. Oocytes were incubated at 18°C in modified Barth's solution (5 mM HEPES-sodium hydroxide [pH 7.5] containing 85 mM sodium chloride, 1 mM potassium chloride, 1 mM calcium chloride, 1 mM magnesium chloride, and penicillin-streptomycin). Phosphate uptake was measured and voltage-clamp analysis performed three days after the injection.

Oocytes were washed at room temperature in a sodium-free solution (sodium-free uptake solution without phosphorus-32) and incubated either with the sodium-containing uptake solution (10 mM HEPES-TRIS [pH 7.5] containing 96 mM sodium chloride, 2 mM potassium chloride, 1.8 mM calcium chloride, 1 mM magnesium chloride, 0.1 mM potassium phosphate, and 10 μ Ci of [³²P]orthophosphate per milliliter) or with the sodium-free uptake solution (with 100 mM choline chloride substituted for sodium chloride). After 30 minutes, the oocytes were washed five times with sodium-free solution and dissolved in 0.5 percent sodium dodecyl sulfate, and radioactivity was determined. Ten to 14 oocytes were studied in each experimental condition. Three independent experiments with the use of different preparations of RNA were performed.

Voltage-Clamp Experiments

Phosphate-induced currents were measured in two-electrode voltage-clamp experiments with the use of a current-voltage amplifier (Axoclamp-2A). Three days after the RNA injection, a -50 mV holding potential was imposed, during which current was recorded on a multi chart-recorder (Arc-en-Ciel). Oocytes were perfused with a sodium-containing solution (5 mM HEPES-sodium hydroxide [pH 7.5] containing 85 mM sodium chloride, 1 mM potassium chloride, 1 mM calcium chloride, and 1 mM magnesium chloride) before various concentrations of phosphate were added, as indicated.

Statistical Analysis

The results are presented as means \pm SD. Statistical analyses were performed with the use of two-way analysis of variance and a protected-least-significant-difference Fisher's test, when allowed by F values, or repeated-measures analysis of variance.

RESULTS

The 20 patients were 13 men and 7 women (mean age, 47 \pm 11 years) who had urolithiasis (14 patients, 10 of whom were men) or bone demineralization (6 patients, 3 of whom were men) associated with idiopathic hypophosphatemia (phosphate concentration, <2.48 mg per deciliter [0.80 mmol per liter]) and reduced maximal renal phosphate reabsorption, normalized according to the glomerular filtration rate (<0.70 mmol per liter). The glomerular filtration rate, determined by inulin clearance, was normal in all 20 patients (mean value, 90 \pm 14 ml per minute per 1.73 m² of body-surface area). None of the patients had had rickets during childhood, and none had abnormal height as adults.

To determine whether NPT2a has a role in renal phosphate leak, we sequenced the 13 exons of the *NPT2a* gene and 190 nucleotides within the promoter region. Two distinct mutations were identified in two patients (Fig. 1) in the heterozygous state.

Patient 1, a 34-year-old man, had recurrent urolithiasis, a phosphate concentration of 1.58 mg per deciliter (0.51 mmol per liter), and maximal renal phosphate reabsorption, normalized according to the glomerular filtration rate, of 0.47 mmol per liter. He was heterozygous for two nucleotide substitutions: G \rightarrow T at position 223 and C \rightarrow T at position 224 (Fig. 1A). The sequencing of cloned PCR products established that these substitutions corresponded to a two-base change on the same allele located in exon 3 and resulted in a substitution of phenylalanine for alanine at position 48 (A48F).

Patient 2 was a 64-year-old woman with idiopathic bone demineralization (bone mineral density at the lumbar spine, 0.639 g per square centimeter; at the femoral neck, 0.679 g per square centimeter); the phosphate concentration was 2.17 mg per deciliter (0.70 mmol per liter), and maximal renal phosphate reabsorption, normalized according to the glomerular filtration rate, was 0.58 mmol per liter. She had a single G \rightarrow A transition at position 520 in exon 5 that resulted in a substitution of methionine for valine at position 147 (V147M) (Fig. 1B). Her sole daughter had a spinal deformity and a history of arm fractures, with a low phosphate concentration (2.35 mg per deciliter [0.76 mmol per liter]) and low maximal renal phosphate reabsorption, normalized according to the glomerular filtration rate (0.67 mmol per liter). Her daughter had the same mutation. To exclude the possibility that the base changes identified in these pa-

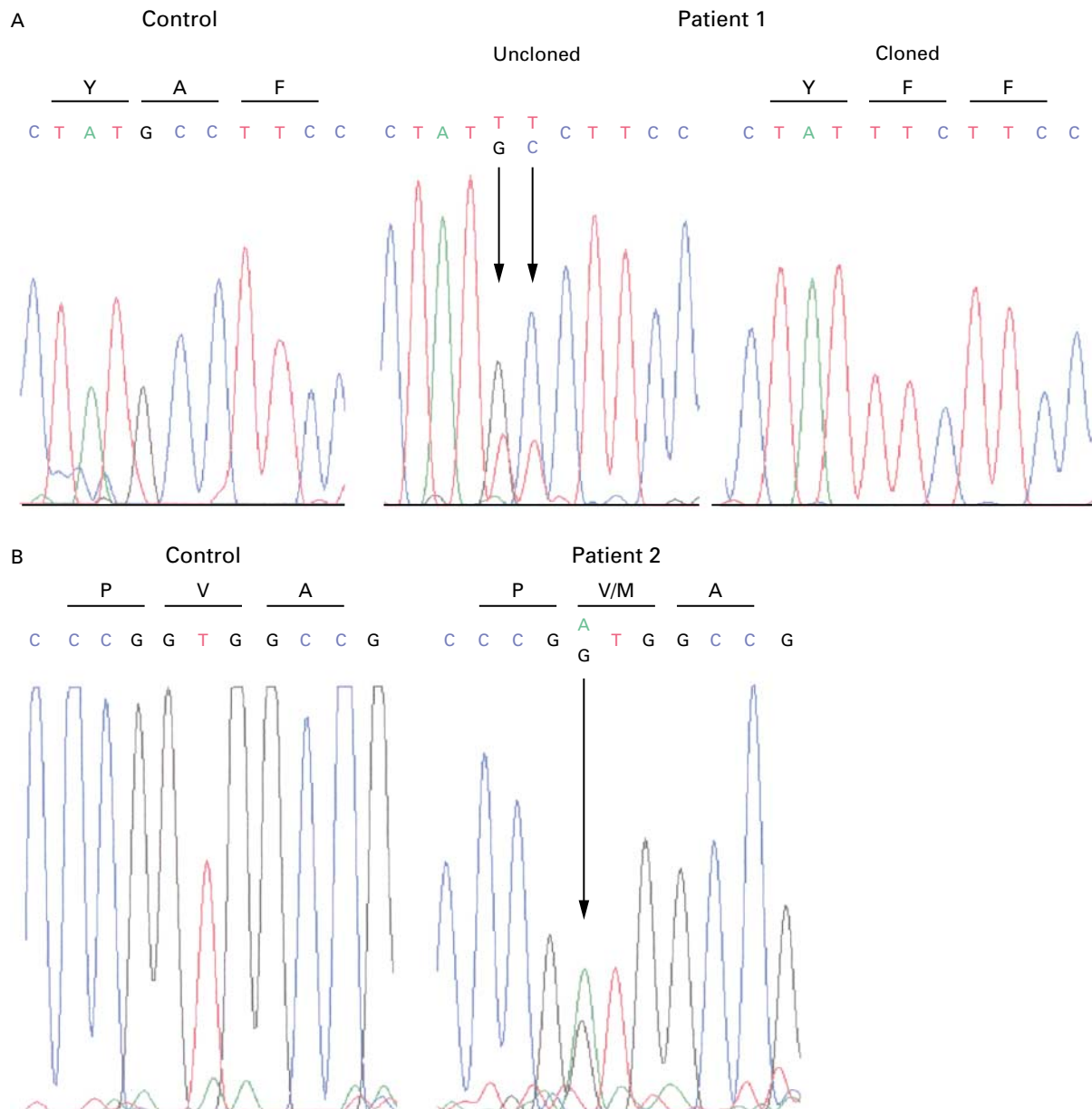


Figure 1. Sequence Analysis of DNA from Two Patients with Hypophosphatemia and Mutations in *NPT2a*.

Panel A shows a portion of the sequence of exon 3 from a control subject and from Patient 1. The arrows indicate the heterozygous G→T mutation at position 223 and C→T mutation at position 224. The two mutations are present in the same allele, as shown in a fragment of exon 3 that was cloned after amplification by a polymerase-chain-reaction assay, resulting in the A48F substitution. Panel B shows a portion of the sequence of exon 5 from a control subject and from Patient 2. The arrow indicates the heterozygous G→A mutation at position 520, which caused the V147M substitution. The same mutation was identified in the patient's daughter.

tients represented common polymorphisms, exons 3 and 5 were amplified and sequenced from the genomic DNA of 120 subjects with normal maximal renal phosphate reabsorption, normalized according to the glomerular filtration rate. No nucleotide variants were detected in these subjects, with the excep-

tion of two previously described single-nucleotide polymorphisms.^{25,26}

The *NPT2a* protein is thought to have an intracytoplasmic NH₂ tail and eight transmembrane segments.²⁰ The A48 residue is located in the NH₂ terminal portion of the *NPT2a* protein,²⁰ and the V147

residue is located in the second transmembrane segment.²⁰ Amino acids A48 and V147 are conserved in humans, rats, rabbits, mice, opossums, and flounder (Fig. 2). The conservation of A48 and V147 residues across species and the absence of variants in control subjects support the hypothesis that the identified mutations may cause the defect in renal phosphate reabsorption observed in these patients.

We tested this hypothesis further by comparing the function of wild-type and mutant NPT2a expressed in *X. laevis* oocytes. Injection of RNA into oocytes, as compared with cDNA transfection with the use of an expression vector, allows the expression of known amounts of RNA and the assessment of protein function with two independent and complementary methods: measurement of phosphate-induced currents by electrophysiological studies and quantification of phosphate uptake. NPT2a transports three sodium ions for one divalent phosphate ion.²⁷ Hence, the uptake of phosphate results in a net movement of positive charges into the oocytes that can be recorded as an inward current in oocytes injected with NPT2a RNA.²⁷ Accordingly, we injected oocytes with increasing concentrations of wild-type or mutant NPT2a RNA (Fig. 3A). The inward current was related to the amount of injected NPT2a RNA until it reached a plateau of 30 ng of wild-type or mutant RNA per oocyte. In the presence of a concentration of 1 mM phosphate, at all concentrations of RNA tested, the current induced by the mutant NPT2a was lower than that induced by the wild-type NPT2a (Fig. 3A). In oocytes injected with 30 ng of mutant RNA, the current was 47 per-

cent lower (in the case of V147M) and 65 percent lower (in the case of A48F) than the current in oocytes injected with 30 ng of wild-type RNA.

We also tested the effect of increasing extracellular phosphate concentrations on phosphate-induced current in oocytes injected with 10 ng of wild-type or mutant NPT2a RNA (Fig. 3B). The inward current increased with increasing concentrations of extracellular phosphate, reaching a plateau at 1 mM phosphate only in oocytes expressing wild-type NPT2a; the phosphate concentration at which the current was half the maximal value was 0.55 mM (Fig. 3B). In oocytes expressing the mutant NPT2a, the phosphate-induced current was lower at all phosphate concentrations tested than that in oocytes expressing the wild-type transporter (Fig. 3B). No plateau in phosphate-induced current was observed in oocytes expressing the mutant NPT2a over the range of phosphate concentrations tested, suggesting that the mutant transporters have a decreased affinity for phosphate.

The measurement of phosphate uptake in oocytes injected with 10 ng of wild-type or mutant RNA confirmed the impaired function of mutant NPT2a (Fig. 3C). As expected, sodium-dependent phosphate uptake was lower in oocytes injected with vehicle (water) alone than in those injected with wild-type NPT2a (Fig. 3C), and in the absence of sodium, phosphate uptake was low and was similar in oocytes whether they were injected with wild-type or mutant RNA or water (Fig. 3C). These results indicate that the phosphate uptake observed was due to the expression of NPT2a. Phosphate uptake was significantly lower in

A																							
Species	Accession No.	Amino Acid Sequence																					
NPT2 human	L13258	L	H	R	I	P	G	T	S	A	Y	A	F	P	S	L	G	P	V	A	L	A	E
NPT2 rat	L13257	L	H	R	I	P	G	T	T	T	Y	A	I	S	S	L	S	P	V	A	L	T	E
NPT2 rabbit	U20793	L	H	R	I	P	G	T	S	A	Y	A	F	P	S	L	S	P	V	A	L	T	E
NPT2 mouse	U22465	L	H	R	I	P	G	T	S	T	Y	A	I	S	S	L	S	P	V	T	L	T	E
NPT2 opossum	L26308	L	H	R	I	P	G	A	P	A	Y	A	F	P	T	M	G	P	G	S	L	P	E
NPT2 flounder	U13936	D	D	A	P	V	G	N	I	P	P	A	Y	S	T	L	D	L	V	S	D	D	P

B																							
Species	Accession No.	Amino Acid Sequence																					
NPT2 human	L13258	D	I	F	K	D	N	A	I	L	S	N	P	V	A	G	L	V	V	G	I		
NPT2 rat	L13257	D	I	F	K	D	N	A	I	L	S	N	P	V	A	G	L	V	V	G	I		
NPT2 rabbit	U20793	D	I	F	K	D	N	A	I	L	A	N	P	V	A	G	L	V	V	G	I		
NPT2 mouse	U22465	D	I	F	K	D	N	A	I	L	S	N	P	V	A	G	L	V	V	G	I		
NPT2 opossum	L26308	D	I	F	K	D	N	A	I	L	S	N	P	V	A	G	L	V	V	G	I		
NPT2 flounder	U13936	D	I	F	K	D	N	A	V	L	A	N	P	V	A	G	L	V	I	G	V		

Figure 2. Evolutionary Conservation of the A48 and V147 Residues.

Partial amino acid sequences encoded by exon 3 (Panel A) and exon 5 (Panel B) are shown. The residues that were mutated in Patients 1 and 2 are boxed. Bold letters indicate residues conserved in at least five species.

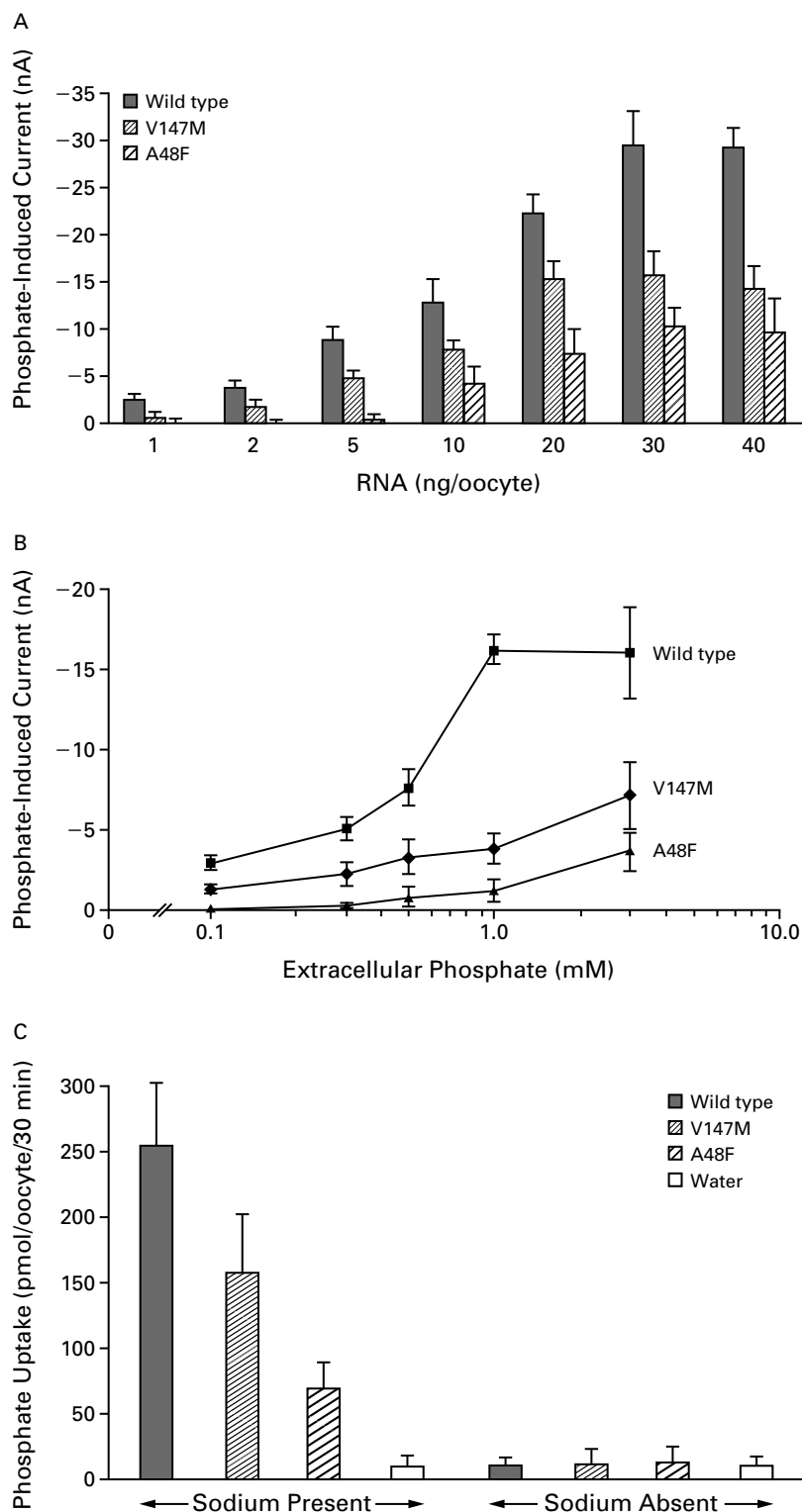


Figure 3. Phosphate-Induced Current and Phosphate Uptake in *Xenopus laevis* Oocytes Injected with Wild-Type or Mutant NPT2a RNA.

Panel A shows the relation between injected RNA and phosphate uptake. Phosphate-induced current was recorded in voltage-clamped *X. laevis* oocytes (holding potential, -50 mV) injected with various amounts of wild-type or mutant NPT2a RNA in the presence of 1 mM extracellular phosphate. The phosphate-induced current increased with increasing doses of RNA and reached a plateau at 30 ng of RNA per oocyte. In oocytes expressing a mutant transporter, the amplitude of the current was decreased at all doses of RNA ($P < 0.001$ by analysis of variance). Results are means \pm SD for 5 to 12 oocytes per dose of RNA.

Panel B shows the effect of the extracellular phosphate concentration on the amplitude of phosphate-induced current. The maximal phosphate-induced current was recorded in oocytes injected with 10 ng of RNA coding for NPT2a with the wild-type, V147M, or A48F sequence. Voltage-clamped oocytes (holding potential, -50 mV) were exposed to increasing phosphate concentrations while the induced change in current was continuously recorded. Data are presented as the mean (\pm SD) result of three independent experiments, each involving 10 to 14 oocytes. The mutant transporters were associated with a significant reduction in the amplitude of the current at all phosphate concentrations ($P < 0.001$ by analysis of variance for repeated measures).

Panel C shows phosphate uptake in oocytes. Phosphorus-32 uptake was measured in the presence or the absence of sodium in oocytes injected with 10 ng of RNA coding for the wild-type, A48F, or V147M sequence or injected with water alone. Sodium-dependent phosphate uptake was significantly lower in oocytes injected with A48F or V147M RNA than in oocytes injected with wild-type RNA and was also significantly lower in oocytes injected with A48F RNA than in those injected with V147M RNA ($P < 0.001$ for all three comparisons). In the absence of sodium, phosphate uptake did not differ significantly between oocytes injected with water and those injected with wild-type or mutant RNA. Data are presented as the mean (\pm SD) result of three independent experiments, each performed in 10 to 14 oocytes.

oocytes injected with the RNA coding for the V147M or A48F mutation than in oocytes injected with the wild-type RNA ($P < 0.001$).

In all experiments, both phosphate-induced current and sodium-dependent phosphate uptake were lower in oocytes expressing NPT2a with the A48F mutation than in those expressing the transporter with the V147M mutation (Fig. 3). Since both patients were heterozygous for the mutations but had low maximal renal phosphate reabsorption, normalized according to the glomerular filtration rate, we assessed whether the mutant proteins could interfere with the wild-type product and diminish wild-type protein function by a dominant negative effect. In oocytes coinjected with 10 ng of wild-type RNA and 10 ng of mutant RNA, the phosphate-induced current was either similar to that in oocytes expressing 10 ng of wild-type NPT2a RNA alone (in the case of the V147M mutation) or significantly lower (in the case of the A48F mutation) and was reduced by 50 percent (in the case of the V147M mutation) and 65 percent (in the case of the

A48F mutation) as compared with the current in oocytes injected with 20 ng of wild-type RNA alone (Fig. 4). These results indicate that the mutant proteins altered the function of the wild-type NPT2a through a dominant negative effect.

To determine whether biologic data could help identify patients with mutations in the *NPT2a* gene, we compared the findings in the 2 patients who had mutations in the gene with those in the 18 patients who did not have mutations (Fig. 5). None of the variables evaluated clearly distinguished patients with *NPT2a* mutations from those without mutations. The abnormalities observed for the patient with the A48F mutation were generally more pronounced than those observed for the patient with the V147M mutation.

DISCUSSION

Our findings suggest that *NPT2a* mutations identified in two patients caused an impairment in renal phosphate reabsorption, resulting in hypophosphatemia. A low serum phosphate concentration, in turn,

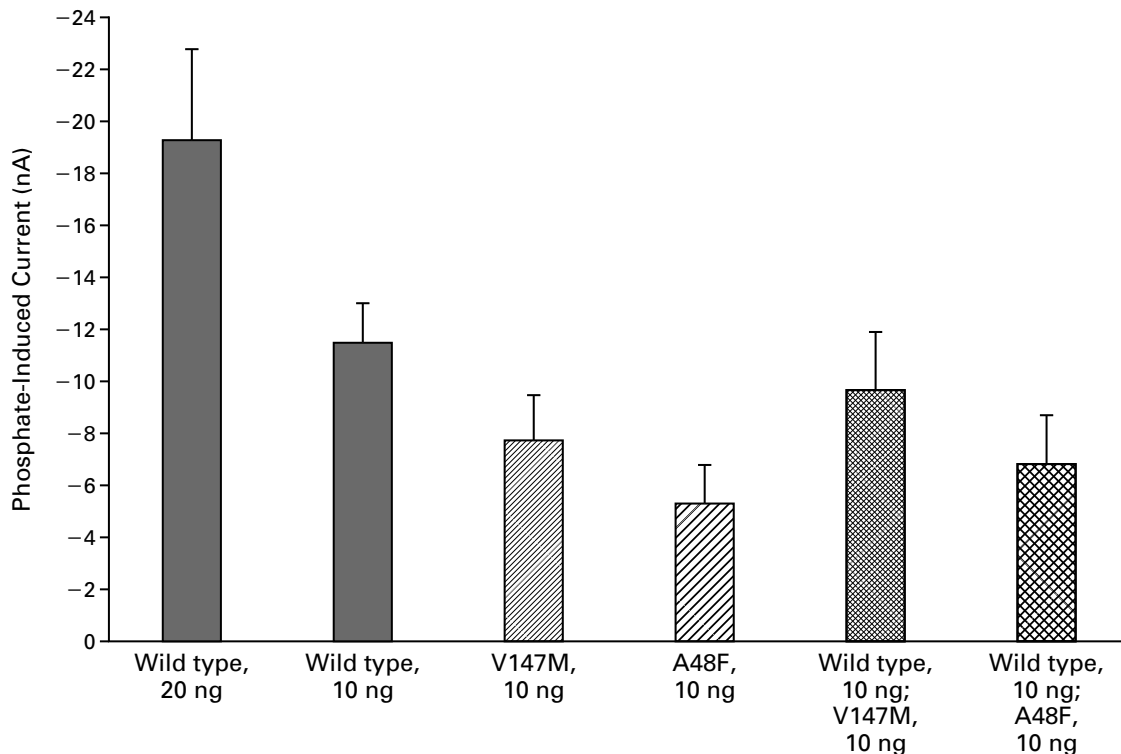


Figure 4. Effect of the Coexpression of Wild-Type and Mutant NPT2a RNA on Phosphate-Induced Current.

As compared with the injection of oocytes with 10 ng of wild-type RNA alone, injection with 10 ng of mutant RNA together with 10 ng of wild-type RNA resulted in a slight decrease in phosphate-induced current (in the case of the V147M mutation) or a significant decrease (in the case of the A48F mutation, $P < 0.001$). For both mutations, the phosphate-induced current was significantly lower than that associated with injection of 20 ng of wild-type RNA alone ($P < 0.001$ for both comparisons). Data are presented as the mean (\pm SD) result of two independent experiments, each involving 10 oocytes.

would be expected to increase 1,25-dihydroxyvitamin D production,^{28,29} leading to hypercalciuria,¹⁶ as observed. This phenotype resembles that of heterozygous Npt2a-deficient mice (Npt2a+/-), which have increased urinary phosphate and calcium excretion, elevated plasma concentrations of 1,25-dihydroxyvitamin D,³⁰ and urolithiasis.³¹ The functional

defect of NPT2a is more severe with the A48F mutation than with the V147M mutation; this observation is consistent with the phenotype in our two patients, since the serum phosphorus concentration and the maximal capacity of the kidney to reabsorb phosphate were lower in the patient with the A48F mutation.

The findings in the daughter of the patient with the

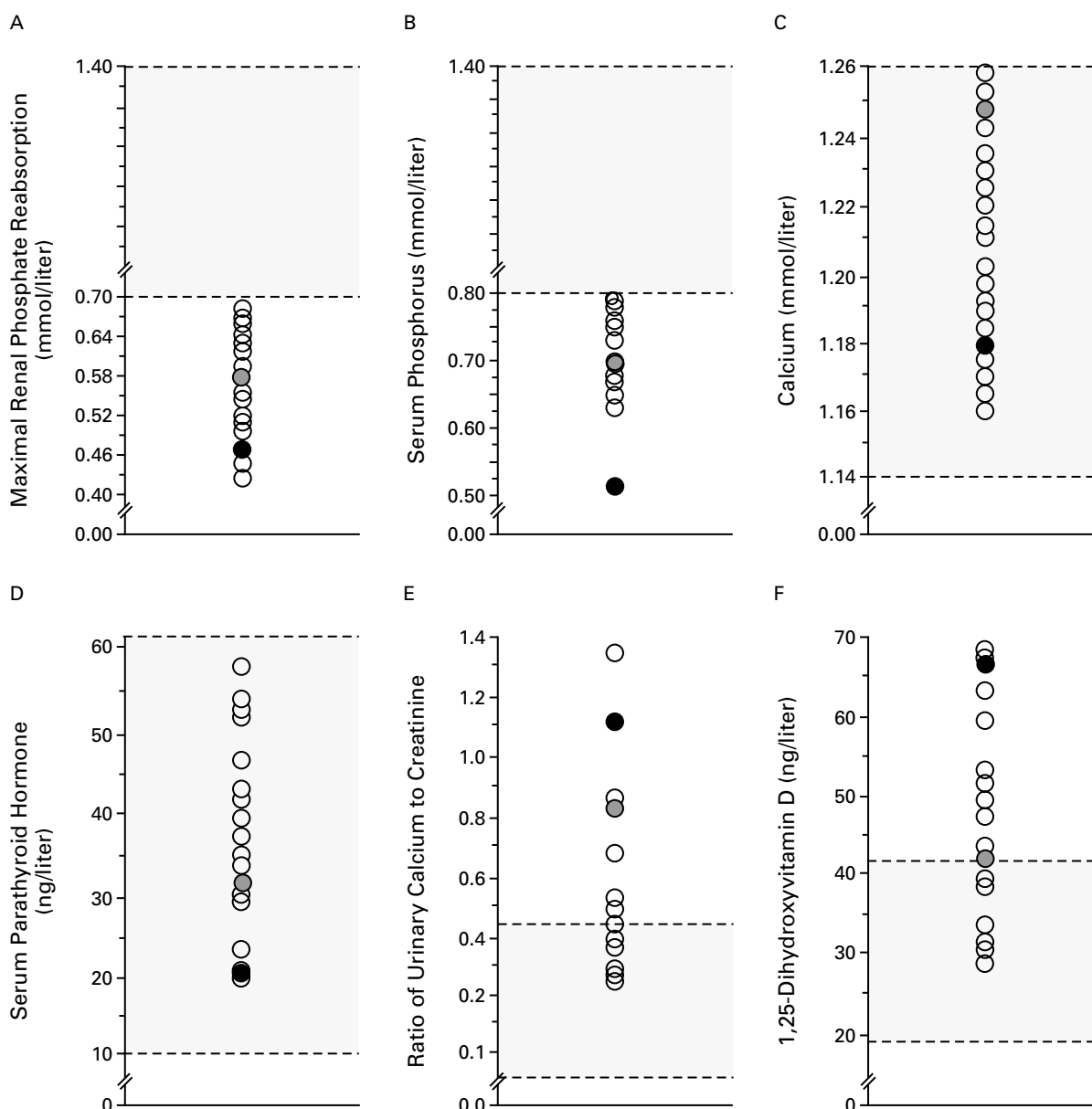


Figure 5. Laboratory Values in the 20 Patients with Hypophosphatemia and Reduced Maximal Renal Phosphate Reabsorption, According to Whether Mutations in NPT2a Were Identified.

Open circles represent patients with wild-type NPT2a, solid circles the patient with the A48F mutation, and shaded circles the patient with the V147M mutation. In some cases, two or more patients had the same value. The shaded area in each panel shows the range of normal values. Data are mean values for measurements in at least three different samples. To convert the values for serum phosphorus to milligrams per deciliter, divide by 0.3229.

V147M mutation and the correlation between functional and biologic data support the hypothesis that the *NPT2a* mutations were responsible for the hypophosphatemia and loss of renal phosphate. The patients were heterozygous for the mutations, suggesting a dominant effect of the mutant allele. This is consistent with the observation of a dominant negative effect of the mutant proteins on the function of the wild-type transporter, leading to substantial renal phosphate losses in heterozygous patients. The underlying mechanism may involve either a competition between wild-type and mutant transporters for the interaction with a rate-limiting intracellular protein or a direct interaction between wild-type and mutant transporters, although there are conflicting data with regard to the existence of dimers of *NPT2a*.^{32,33}

The reason why renal phosphate loss may lead to either the formation of calcium stones or bone demineralization is unknown, but it might be due to sex, environmental factors, or other genetic differences. Only 2 of our 20 patients with idiopathic renal phosphate loss had mutations in the *NPT2a* gene. Although it is possible that we did not detect all *NPT2a* mutations in our patients (e.g., mutations in introns or regulatory regions), other genes may be involved in the renal phosphate loss in these patients. In patients who have hereditary hypophosphatemic rickets with hypercalciuria, an autosomal recessive disorder, no mutation in the coding region of the *NPT2a* gene has been found.^{25,26} Similarly, mutations in the *NPT2a* gene have been ruled out in two additional forms of familial hypophosphatemia: X-linked hypophosphatemic rickets³⁴ and autosomal dominant hereditary rickets.³⁵ These disorders are associated with mutations in the phosphate-regulating gene with homologies to endopeptidases on the X chromosome (*PHEX*) and the gene encoding fibroblast growth factor 23 (*FGF-23*), respectively.^{34,35}

Our study provides genetic evidence that heterozygous *NPT2a* mutations are involved in hypophosphatemia resulting from idiopathic renal phosphate loss and indicates that *NPT2a* plays a major part in phosphate homeostasis. The identification of functional variants of the *NPT2a* gene in patients with hypophosphatemia associated with urolithiasis or bone demineralization also provides genetic evidence that a defect in renal phosphate reabsorption may contribute to the pathogenesis of these two common disorders.

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