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The *Gy* mutation: Another cause of X-linked hypophosphatemia in mouse

(phosphate transport/kidney/vitamin D-resistant rickets/inner ear defect)

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ABSTRACT **An X-linked dominant mutation (gyro, gene symbol *Gy*) in the laboratory mouse causes hypophosphatemia, rickets/osteomalacia, circling behavior, inner ear abnormalities, and sterility in males and a milder phenotype in females. *Gy* maps closely (crossover value 0.4–0.8%) to another X-linked gene (*Hyp*) that also causes hypophosphatemia in the mouse. *Gy* and *Hyp* genes have similar quantitative expression in serum phosphorus values, renal excretion of phosphate, and impairment of Na^+/phosphate cotransport by renal brush-border membrane vesicles. These findings indicate that independent translation products of two X-linked genes serve phosphate transport in mouse kidney and thereby control phosphate content of extracellular fluid. The *Gy* translation product, unlike the *Hyp* product, is also expressed in the inner ear. These findings have implications for our understanding of the human counterpart known as "X-linked hypophosphatemia."**

Renal reabsorption of phosphate anion is a major determinant of its extracellular homeostasis in mammals, and mutations that have a primary effect on renal phosphate transport identify polypeptides controlling the process (1). The *Hyp* mutation in the laboratory mouse impairs renal transport of phosphate at the brush-border membrane and causes hypophosphatemia (2, 3); the *Hyp* gene is at a distal locus on the X chromosome (2). The conventional view holds that corresponding genes on the X chromosome in man and mouse encode polypeptides with homologous functions (4). Accordingly, the murine phenotype associated with the *Hyp* gene is a useful model to study the human counterpart known as "X-linked hypophosphatemia" (5). We now report evidence for a second locus on the X chromosome controlling renal transport and homeostasis of phosphate in the laboratory mouse. Since the second gene (designated *Gy*) occurs at a locus different from *Hyp*, we deduce that at least two different polypeptides, both encoded by DNA on the X chromosome, are, in some way, involved in the process serving renal reabsorption of phosphate. It follows that human X-linked hypophosphatemia may be a more heterogeneous phenotype than suspected hitherto.

MATERIALS AND METHODS

Mice. Animals were bred in the Medical Research Council Radiobiology Unit at Harwell. The *Hyp* gene was transferred from the C57BL/6J background (in The Jackson Laboratory strain) (2) to the *Gy* strain in the Harwell colony. By appropriate crosses, the *Gy* mutation was shown to be dominantly inherited and X-linked. All studies reported here were performed on Harwell strain male hemizygotes (*Gy*/Y and *Hyp*/Y), *Gy* females (*Gy*/+), and control littermates.

Diet. Mice in England were adapted to a diet containing 1.26% (wt/wt) calcium and 0.65% available phosphorus. Studies in the Canadian laboratory were carried out within 1 week after transfer of mice by air from the Harwell Unit and following a minimum 36-hr equilibration period on a diet of Wayne Lab Blox (Allied Miller, Chicago) containing 1.2% calcium, 0.99% phosphorus, and 4.4 international units of vitamin D_3 per g.

Body Weight and Tail Length. Weight was measured on a tared pan balance with digital readout; tail length was measured in a restraining apparatus that exposed the tail.

Analytical and Procurement. Blood samples were collected in the morning, after normal overnight feeding, by retro-orbital sinus puncture, but when necessary from neck blood after decapitation. Serum was analyzed for inorganic phosphorus by a micromethod (Pierce); serum calcium, by fluorometric analysis (Corning calcium analyzer model 940); and alkaline phosphatase, at 37°C with *p*-nitrophenyl phosphate as substrate. Serum sodium, potassium, and bicarbonate were measured by conventional methods. Serum immunoreactive parathyroid hormone was measured with a rodent-specific mid-molecule radioimmunoassay (Immuno Nuclear, Stillwater, MN). We collected urine overnight from individual mice fed and housed in metabolic cages to measure phosphorus, calcium, creatinine, glucose, and amino acids by conventional methods (2, 3). Adenosine 3',5'-cyclic monophosphate (cAMP) was measured by competitive binding assay with a kit (Amersham). Phosphate excretion was normalized to the corresponding urine creatinine and serum phosphorus values to calculate the "phosphate excretion index" (2).

Histology. Undecalcified sections of bone (8–10 μm thick) from rib, pelvis, femur, and vertebrae were prepared and stained by conventional methods (6). Wedges of renal cortex and medulla were examined by light and electron microscopy after conventional fixation. Inner ear structures were prepared for light microscopy by methods to be described elsewhere (G. Truslove and M. S. Deol, personal communication).

Renal Brush-Border Membrane Vesicles. Vesicles were prepared and used to measure solute uptake as described (3, 7).

Statistical Analysis. Differences were analyzed by two-tailed Aspin–Welch or Student *t* tests or by paired *t* test.

RESULTS

Origin of Gyro Strain. The original mutant animal was a circling female found among the offspring of a female that had received 194 cGy of x-rays when a fetus of 17 days gestation (8). When mated with a normal male, a proportion of her sons were severely affected animals; among the daughters there

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were 3 mildly affected out of 28. This suggested that the phenotype was due to an X-linked gene, and this was confirmed by linkage tests with X-linked markers. The gene was named gyro (symbol *Gy*).

Genetic Location of *Gy* on X Chromosome. Linkage tests between *Gy* and the X chromosome markers tabby (*Ta*) and blotchy (Mo^{blo}) gave crossover values of 20.9% with *Ta* and 24.6% with Mo^{blo} (9). This placed the map position of *Gy* very close to that of *Hyp*. To test whether the two genes were allelic, a search was made for crossovers between *Gy* and *Hyp* among the offspring of *Gy*+/+*Hyp* females mated to tabby (*Ta*/Y) males. We measured serum phosphorus to classify offspring as *Gy*, *Hyp*, or normal. Among 239 male offspring, 238 had low serum phosphorus; 66 were classified as *Gy*/Y, on the basis of strong circling behavior, and 172 as *Hyp*/Y. A single male, with normal behavior and a normal serum phosphorus value (1.86 mmol/liter; range of values in the mutants, 0.65–1.52 mmol/liter) was a presumed non-*Gy*, non-*Hyp* crossover. He was crossed with a tabby X0 female; there were two types of daughters: $X^{Ta}X$, which looked visibly tabby, and X0, which looked non-tabby and had their father's X only. All of five such non-tabby X0 female offspring had normal serum phosphorus values (range, 1.71–2.3 mmol/liter), thus confirming that the father's X chromosome did not carry either the *Gy* or the *Hyp* gene and that he was a true crossover. Accordingly, *Gy* and *Hyp* are not allelic but are closely linked loci. We were unable to score for potential crossovers of the complementary type carrying both *Gy* and *Hyp*. Thus, since only half the possible male crossovers were being scored by the method used, the crossover value was 2 × 1/239, or 0.8%.

Among the 266 female offspring, it was not possible to distinguish reliably, by major phenotype, those carrying *Gy* from those carrying *Hyp*. The range of serum phosphorus in these heterozygotes was quite wide (0.5–1.55 mmol/liter), but no animals had values clearly outside the range of the remainder. With hindsight, the value 1.55 mmol/liter fell within the normal range, and this female may have been a crossover, carrying neither *Gy* nor *Hyp*. She was not tested genetically. The two heterozygotes with the next highest values (1.42 and 1.43 mmol/liter) showed other signs of abnormality (mild circling behavior in one and short femur length in the other). Thus, among the 266 female offspring, the number of crossovers was either 1 or 0. For male and female offspring combined, the number of observed crossovers was 1 or 2 in 505. As only half the possible crossovers were detected the crossover value was 2 × (1 or 2)/505, or 0.4–0.8%. The *Gy* and *Hyp* genes are located in the region F2-ter on the X chromosome, about 6 map units proximal to the most distal mapped gene, cream (symbol *Crm*) (10, 11).

General Phenotype of Affected Gyro Animals. *Males.* Three litters of gyro and normal males were weighed at various ages from 1–14 days. The gyro males were already smaller at 1 day, and the slopes of the regressions of weight against age for gyro and normal males (not shown) differed at a high level of significance ($t_{15} = 9.72$; $P = 3 \times 10^{-8}$). Thus, gyro males are smaller than their normal sibs at all times from birth onward. By age 3 months, gyro males have about half the body weight of normal sibs. Gyro males weigh less than *Hyp* males at similar ages but have equivalent shortening of tail length (Fig. 1).

Gyro males have strong circling behavior, accompanied by head shaking, and extreme hyperactivity when awake. They lack postural reflexes; an absent Preyer reflex indicates deafness. The head shape is abnormal, with bulging under the eyes and abnormal eyelids predisposing to blepharitis. Males have reduced viability from birth, and sudden death occurs in adults. Seven males were tested for fertility by mating with 1 or 2 normal females for 1–4 months; vaginal plugs occurred,

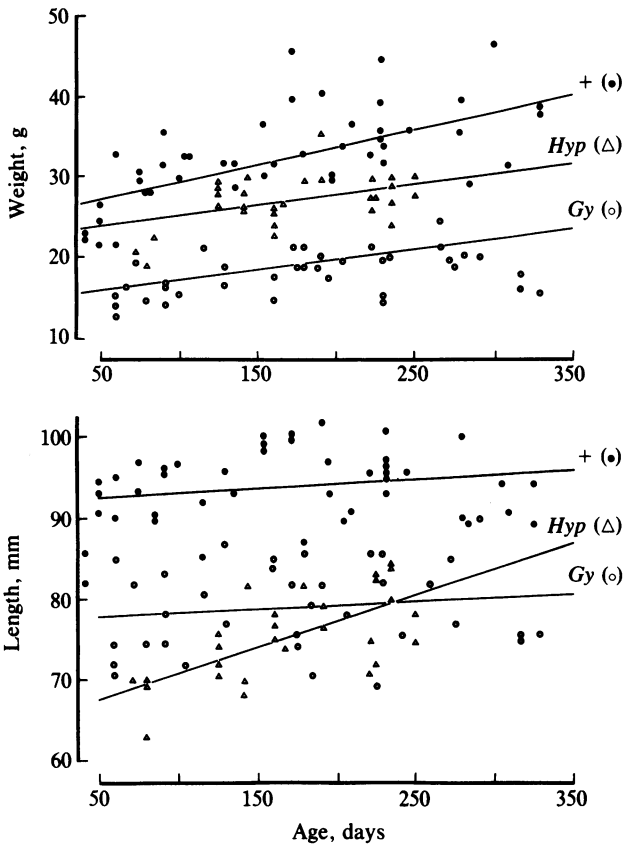

FIG. 1. Age-related weights (*Upper*) and the corresponding tail lengths (*Lower*) of male mice, classified as *Gy*, *Hyp*, or normal (+) by the appropriate tests. Regressions of values vs. age were calculated by regression analysis.

but all males were sterile. Beechey (12) has shown that testis weight and sperm number are reduced.

Females. Some, but not all, heterozygous *Gy*/+ females show mild circling behavior. Serum phosphorus values are low and there is significant reduction of femur length and body weight (Table 1). These findings indicate that the *Gy* mutation is expressed dominantly.

Comparison of *Gy*/Y and *Hyp*/Y Phenotypes. *Inner ear lesions.* *Gy*/Y mice have three abnormalities in the organ of Corti (G. Truslove and M. S. Deol, personal communication); the acoustic ganglion is deficient in cell bodies, hair cells are degenerate, and the tectorial membrane is detached. Vestibular sense organs are also abnormal; sacculus and

Table 1. Expression of *Gy* allele in female mice

	Mean value ± SD (no. of mice)		
	Gy/+	+/+	*P* value*
Serum phosphorus,† mmol/liter	0.92 ± 0.03 (27)	1.6 ± 0.03 (15)	10^{-10}
Femur length, mm	14.5 ± 0.1 (23)	16.0 ± 0.1 (14)	10^{-9}
Body weight, g	24.1 ± 0.6 (27)	27.9 ± 0.6 (15)	0.00016

Age range: *Gy*/+ mice, 89–123 days; +/+ littermates, 90–131 days. There was no significant evidence of a regression with age, in the range studied, in any sample.

*Two-tailed *t* test.

† Serum phosphorus values in females cannot be compared with values in males (Table 2) because diet, age, and other factors were different in the two studies.

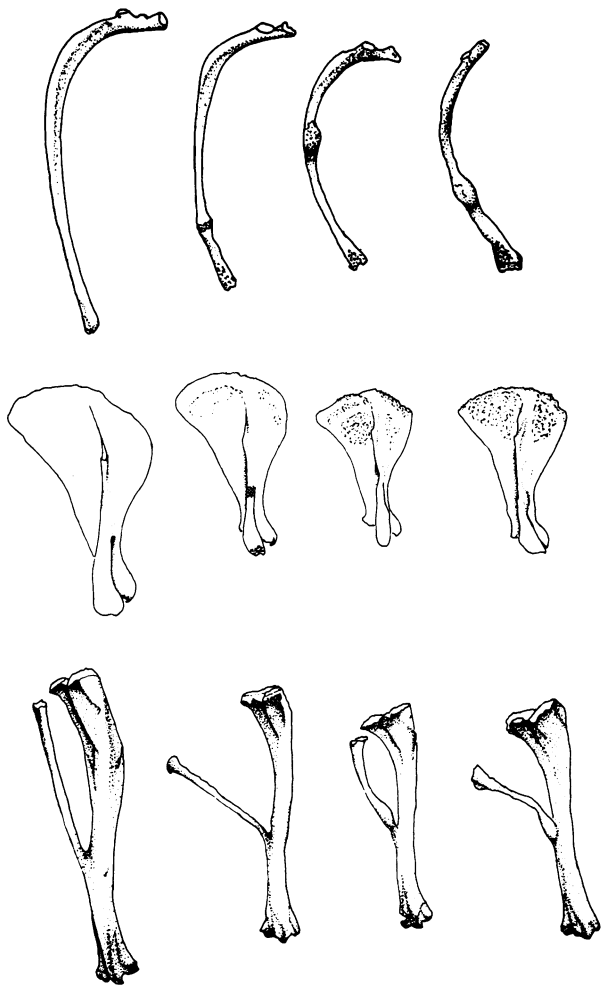

FIG. 2. Camera lucida appearance of papain-treated bones from normal +/Y (left-hand specimen) and *Gy*/Y male littermates (remaining specimens). Rib, scapula, and fibula plus tibia are shown in top, middle, and bottom rows, respectively. The rachitic deformations resemble those found in hypophosphatemic *Hyp*/Y males at the corresponding age.

utriculus are atrophic, and the semicircular canals are stenotic. *Hyp*/Y mice do not have these abnormalities.

Skeleton and bone histology. Rachitic deformations were seen in bones of weaned *Gy*/Y mice (Fig. 2). The changes are similar to those seen in *Hyp*/Y mice. They include uneven thickness of ribs and fibula and splayed rib ends. The long bones are shortened, thickened, and abnormally soft. Teeth are also abnormal. Microscopy findings in *Gy* and *Hyp* bone were similar. Osteoid seams are widened in gyro mice, without evidence of increased resorptive activity along trabecular surfaces or of marrow fibrosis. Although we did not perform tetracycline double-labeling studies to measure the rate of osteoid production, the findings, in combination with elevated serum alkaline phosphatase values (see below), are compatible with defective mineralization (osteomalacia) and exclude hyperparathyroid bone disease in gyro mice.

Serum phosphorus, calcium, parathyroid hormone, and alkaline phosphatase. Age influences these values. Age distributions were similar in the groups in which the measurements were made [*Gy*/Y, 181 ± 12 days (mean ± SD, n = 37); *Hyp*/Y, 171 ± 55 days (n = 31), +/Y, 166 ± 65 days (n = 45)]. *Gy*/Y and *Hyp*/Y mice had equivalent hypophosphatemia and both mutants were also significantly hypocalcemic (Table 2). Serum immunoreactive parathyroid hormone values were similar in gyro and control animals [202 ± 209 pmol/liter (mean ± SD, n = 13) and 277 ± 275 pmol/liter (n = 14), respectively]. Serum alkaline phosphatase values were elevated in the mutants [*Gy*/Y, 64–153 units; *Hyp*/Y, 97–296; +/Y, 41–59 (range of four measurements in each group)].

Urinary excretion of phosphate, calcium, and cAMP. Renal loss of phosphate was significantly elevated in *Gy*/Y and *Hyp*/Y mice relative to controls (Table 2). Urinary calcium related to creatinine excretion was similar in *Gy* and *Hyp* males but low relative to normal males. Urinary cAMP was elevated in both mutants.

Renal function and histology. Gy, Hyp, and control males had similar values for serum potassium and bicarbonate, urine-concentrating ability, and urine content of glucose and amino acids. No abnormalities were detected by light or electron microscopy in glomerular or tubular structures of kidney from *Gy* and *Hyp* males.

Renal brush-border membrane transport. We compared the uptake of phosphate, D-glucose, and L-alanine by renal brush-border membrane vesicles prepared from *Gy*/Y, *Hyp*/Y, and +/Y mice. Paired uptakes of phosphate and glucose, and of alanine and glucose, were measured simultaneously by the double-label method (7) in vesicles prepared on the same day. The inward-oriented, Na^+-gradient-dependent uptake was deficient for phosphate and normal for glucose in vesicles prepared from *Gy* mice (Fig. 3). The initial rates in the presence of Na^+ were normalized to the corre-

Table 2. Serum and urine values in *Gy*, *Hyp*, and control male mice

	Mean value ± SD (no. of mice)			*P* value		
	Gy/Y (A)	*Hyp*/Y (B)	+/Y (C)	A vs. B	A vs. C	B vs. C
		Serum values				
Calcium, mmol/liter	3.44 ± 0.16 (14)	3.40 ± 0.08 (15)	3.64 ± 0.12 (15)	NS	0.0002	0.0001
Phosphorus, mmol/liter	1.45 ± 0.42 (34)	1.45 ± 0.16 (31)	2.23 ± 0.45 (44)	NS	0.0004	0.0001
		Urine values				
FEI_{P_i}*	74.7 ± 33.4 (23)	67.0 ± 20.4 (14)	47.5 ± 18.6 (26)	NS	0.0004	0.002
cAMP, nmol/mg of creatinine	41.8 ± 26.5 (14)	32.5 ± 9.9 (14)	25.8 ± 8.2 (16)	NS	0.0147	0.0256
Calcium/creatinine	0.28 ± 0.32 (11)	0.27 ± 0.17 (6)	0.53 ± 0.29 (7)	NS	0.07	0.01

NS, not significant.

*The fractional excretion index for phosphate (FEI_{P_i}) was used instead of the fractional excretion value because measurement of serum creatinine in mice by conventional methods is unreliable. FEI_{P_i} = [urinary phosphorus (mg/liter)/urinary creatinine (mg/liter)]/plasma phosphorus (mg/ml).

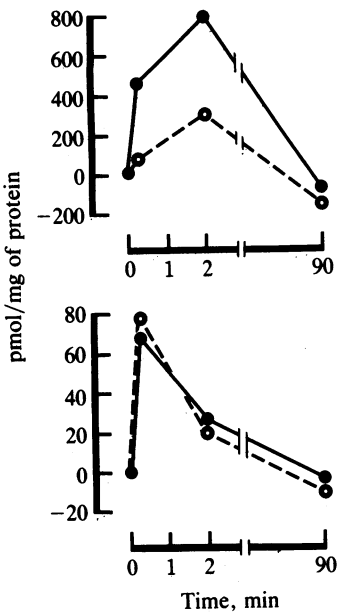

FIG. 3. Time course for Na^+-gradient-dependent uptake of phosphate (100 μM, *Upper*) and D-glucose (10 μM, *Lower*) by renal brush-border membrane vesicles prepared from normal (+/Y, ●—●) and mutant (*Gy*/Y, ○---○) male littermates at age ≈150 days. A deficiency of phosphate transport is observed in the gyro membranes.

sponding equilibrium values at 90 min; this value in the mutant was then related to the corresponding value in control vesicles. In this way, we accommodated analytic variation between experiments. Our findings (Table 3) demonstrate a similar deficit of phosphate transport in *Gy* and *Hyp* vesicles. *Hyp* mice (Harwell strain) had enhanced uptake of D-glucose. L-Alanine uptake was normal in the mutant mice.

DISCUSSION

Ohno (4) proposed that different mammalian species will exhibit homologous functions for the polypeptide products of corresponding genes on the X chromosome. Knowledge gained from study of the *Hyp* mutation in mouse has been used to interpret the renal transport dysfunction in the human counterpart known as X-linked hypophosphatemia (1, 5). It follows that there may be a human counterpart of the murine *Gy* mutation yet to be recognized. There is no certainty that the corresponding human locus will show close linkage with *Hyp* in man, since the human and mouse X chromosomes differ in the arrangement of genes (13). The locus for the human X-linked hypophosphatemia gene (*Hyp* counterpart) has been mapped to the short arm of the X chromosome (14).

Gy and *Hyp* alleles do not confer identical general phenotypes in the mouse. *Gy* animals have the earlier onset of impaired weight gain (cf. Fig. 1 and ref. 15) and there are abnormalities of the inner ear. Accordingly, we anticipate a difference in the putative human counterparts of *Gy* and *Hyp* phenotypes. The search could focus initially on inner ear function. Sensorineural deafness has been reported in humans with X-linked hypophosphatemia (16), but it is also found in autosomal recessive hypophosphatemic rickets (17). Therefore, the finding by itself is not necessarily a marker for the *Gy* gene. Deafness in hypophosphatemic patients may reflect disturbed biochemical composition of the inner ear fluid, due to abnormal ion transport, or occlusion of the internal auditory meatus, due to bone overgrowth. The latter can be ruled out by tomography.

The *Gy* mutation of mouse provides insight into the genetic control of renal mechanisms dedicated to phosphate homeostasis. First, since *Hyp* and *Gy* are not alleles on the X chromosome of mouse, we deduce that there are two translation products of X-linked genes involved in a renal component of phosphate homeostasis and that they are both expressed in the renal brush-border membrane. Second, the mutations in *Gy* and *Hyp* genes studied here have similar quantitative effects on transmembrane transport of phosphate. Whether the respective translation products are operative at different steps in the transport process or in their putative controlling mechanisms or are separate components of a heteropolymer at a single step remains to be discovered. Third, the *Gy* mutation has independent expression in the inner ear of mouse; the *Hyp* gene is not similarly expressed. Since the effects of both genes on phosphate homeostasis are similar, perturbation of phosphate homeostasis itself is apparently not the cause of the inner ear lesion in the *Gy* mouse. The nature of the *Gy* translation product that is common to inner ear and renal brush-border membrane is unknown. Fourth, *Gy* and *Hyp* mutations are both expressed in some heterozygotes and can be recognized by the resultant hypophosphatemia; the *Gy* allele is evidently also expressed in the inner ear in heterozygotes, since some have circling behavior.

The phosphate-transport defect, apparent *in vivo* in the *Gy* mouse, is also expressed *in vitro* in renal brush-border

Table 3. Uptake of phosphate, D-glucose, and L-alanine by renal brush-border membrane vesicles of *Gy*, *Hyp*, and + (control) male mice

	Mean value ± SD (no. of experiments*)			*P* value		
	Gy/Y (A)	*Hyp*/Y (B)	+/Y (C)	A vs. B	A vs. C	B vs. C
Phosphate (0.1 mM)						
% equilibrium†	106 ± 41 (4)	125 ± 46 (3)	259 ± 134 (4)	0.2940	0.0289‡	0.0605‡
% +/Y value	44 ± 14	47 ± 10		0.3890		
D-Glucose (0.01 mM)						
% equilibrium	598 ± 160 (7)	880 ± 209 (5)	575 ± 146 (7)	0.0122	0.3696‡	0.0409‡
% +/Y value	107 ± 27	141 ± 35		0.0408		
L-Alanine (0.1 mM)						
% equilibrium	563 ± 72 (3)	600, 520 (2)	637 ± 97 (3)	0.4801	0.1767	0.1998
% +/Y value	91 ± 14	91, 98		0.4243		

*Each with a new preparation of vesicles. Each experiment had 4–6 replicate analyses.
†Total uptake in Na^+-containing medium, with inward-oriented Na^+ gradient at zero time, measured at 15 sec and normalized to uptake at 90 min (equilibrium).
‡*P* value obtained by paired *t* test; otherwise, *P* value represents two-sample *t* test.

membrane vesicles. Other Na^+-gradient-coupled transport processes (for example, transport of D-glucose and of L-alanine) are not affected by the *Gy* mutation. Accordingly, the oculocerebrorenal syndrome in man (18), in which there is impairment of renal transport for phosphate and several other solutes, is not the human counterpart of the *Gy* mutation. Moreover, a characteristic morphologic lesion in the nephron, seen in human patients with this X-linked syndrome, is absent in the male gyro mouse.

The gyro mouse reveals that the renal transport system for phosphate controlled by the X chromosome is more complex than we had anticipated. Since the *Gy* and *Hyp* genes occur at closely linked loci on the mouse X chromosome and are dedicated to equivalent physiological roles, their evolution, structure, and expression merit attention.

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