

## Screening for increased plasma urea levels in a large-scale ENU mouse mutagenesis project reveals kidney disease models

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**Aigner B, Rathkolb B, Herbach N, Kemter E, Schessl C, Klaften M, Klempt M, Hrabé de Angelis M, Wanke R, Wolf E.** Screening for increased plasma urea levels in a large-scale ENU mouse mutagenesis project reveals kidney disease models. *Am J Physiol Renal Physiol* 292: F1560–F1567, 2007. First published January 30, 2007; doi:10.1152/ajprenal.00213.2006.—Kidney diseases lead to the failure of urinary excretion of metabolism products. In the Munich ethylnitrosourea (ENU) mouse mutagenesis project, which is done on a C3H inbred genetic background, blood samples of more than 15,000 G1 offspring and 500 G3 pedigrees were screened for alterations in clinical-chemical parameters. We identified 44 animals consistently exhibiting increased plasma urea concentrations. Transmission analysis of the altered phenotype of 23 mice to subsequent generations led to the establishment of five mutant lines. Both sexes were affected in these lines. Urinary urea levels were decreased in the mutants. In addition, most mutants showed increased plasma and decreased urinary creatinine levels. Pathological investigation of kidneys from the five mutant lines revealed a broad spectrum of alterations, ranging from no macroscopic and light microscopic kidney alterations to decreased kidney weight-to-body weight ratio, dilation of the renal pelvis, and severe glomerular lesions. Thus screening for elevated plasma urea levels in a large-scale ENU mouse mutagenesis project resulted in the successful establishment of mouse strains which are valuable tools for molecular studies of mechanisms involved in urea excretion or which represent interesting models for kidney diseases.

ethylnitrosourea; nephropathy; renal failure

ANIMAL MODELS ARE REQUIRED for studying the pathobiology of diseases as well as for the development and evaluation of therapeutic strategies. Specific models for known genetic diseases can be generated by genetic engineering techniques which result in defined alterations of the mouse genome. However, the resulting phenotypes of the mutant mice cannot be predicted and do not always mirror the respective human disease. A complementary strategy is to generate new alleles by random mutagenesis and to screen for clinically relevant phenotypes. The underlying mutations are then identified by forward genetics strategies and may lead to the identification of genes/alleles which may have counterparts relevant for human diseases (2, 24, 28).

Ethylnitrosourea (ENU) has been used in various mouse mutagenesis programs to produce random mutations. Specific pathological states have been identified by appropriate routine procedures allowing the screening of large numbers of mice

for a broad spectrum of parameters (9, 19). In the Munich ENU mouse mutagenesis project (<http://www.gsf.de/ieg/groups/genome/enu.html>), a screening profile of clinical chemistry blood parameters was established for the analysis of offspring of chemically mutagenized mice to detect phenotypic variants with defects of various organ systems or changes in metabolic pathways. Breeding of the affected mice and screening of the offspring confirmed the transmission of the altered phenotype to subsequent generations, thereby revealing a mutation as cause for the aberrant phenotype (21, 22).

Urea is the waste product of protein catabolism. In mammals, it is excreted in the urine thereby representing the most abundant urinary solute. Compared with blood, urea is highly concentrated in the urine of humans and rodents and plays an important role in the urine concentration mechanism. The ability to concentrate urine depends on the accumulation of urea in the renal medulla by specific transporter proteins (26). Urea excretion per unit body weight in mice is several times higher than in humans which relies on an effective excretory system (33). Increased blood urea levels may be caused by prerenal, renal, and postrenal alterations. Regarding renal diseases, blood urea concentrations increase if kidney function is severely reduced (14).

Nephropathies include various multifactorial disorders caused by genetic and/or environmental factors. Several monogenic disorders are known to result in progressive renal insufficiency. Among these are glomerular basement membrane defects as well as tubular and interstitial alterations. Once chronic renal insufficiency is established, it tends to progress to end-stage kidney failure, which is characterized by progressive glomerulosclerosis irrespective of the initiating nephropathy (25). Few appropriate animal models are available to date for the examination of genes involved in chronic kidney diseases (1, 4, 13, 30, 32). Quantitative trait loci (QTL) in rodents were found to be concordant with kidney disease loci in humans, suggesting conserved disease genes in these species (12).

To date published ENU project results did not include reports of generating mutant lines by measuring increased plasma urea levels. Here, we present the successful establishment and comparative phenotypic characterization of mutant lines exhibiting increased plasma urea levels which were derived by the clinical chemical screen within the Munich ENU mouse mutagenesis project.

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## MATERIALS AND METHODS

**Mutagenesis and breeding of mice.** The Munich ENU mouse mutagenesis project was carried out on the inbred C3HeB/FeJ (C3H) genetic background as described (16). Briefly, 10-wk-old male mice were injected intraperitoneally with ENU. The screen for dominant mutations was performed on G1 animals which were derived by mating of mutagenized G0 males to wild-type C3H females. Inheritance of abnormal phenotypes as detected by clinical-chemical studies of plasma samples was tested in G2 offspring from the mating of the affected G1 mouse and wild-type C3H mice. The screen for recessive mutations was carried out on G3 mice produced in a two-step breeding scheme from G1 mice, which were excluded to harbor dominant mutations in the phenotypic mutation analysis of the project. Inheritance of phenotypes in the inbred C3H genetic background was tested on G4 intercross or G4 × G3 backcross offspring. Mouse husbandry was done under a continuously controlled specific pathogen-free (SPF) hygiene standard according to the FELASA protocols (<http://www.felasa.org>) (18). The standard rodent diet 1314 (Altromin, Lage, Germany; [http://www.altromin.de/downloads/1000\\_ratten\\_maeuse.pdf](http://www.altromin.de/downloads/1000_ratten_maeuse.pdf)) and water were provided ad libitum. All animal experiments were carried out with permission from the responsible animal welfare authority (Regierung von Oberbayern).

**Clinical chemistry analysis.** Blood samples from 3-mo-old G1 and G3 mice fasted overnight were obtained by puncture of the retro-orbital sinus under ether anesthesia. Plasma from Li-heparin-treated blood was analyzed using an Olympus AU400 autoanalyzer (Olympus, Hamburg, Germany) and the adapted reagents (Olympus). Calibration and quality control were performed according to the manufacturer's protocols. Physiological parameter values were determined in male and female inbred C3H controls. Plasma urea concentrations were examined using the reagent OSR6134 (Olympus) with the linear measurement range of 5–300 mg/dl.

Moreover, the clinical chemistry screen contained additional plasma parameters including substrates, electrolytes, and enzymes. Furthermore, hematologic parameters including erythrocyte indexes and white blood cell counts were measured in EDTA-treated blood using an ABC blood analyzer (Scil, Viernheim, Germany) (11).

Urine parameters were measured in 24-h urine samples collected in metabolic cages (Tecniplast, Buguggiate, Italy; UREHD1: *n* = 3 male and 1 female mutants, UREHR1: *n* = 3 males and 1 female, UREHR3: *n* = 2 males and 2 females) and spot urine samples were collected in the morning (UREHD1: *n* = 2 male and 2 female mutants, UREHR1: *n* = 1 male and 2 females, UREHR2: *n* = 3 males and 4 females, UREHR3: *n* = 6 males and 4 females). Examination of albuminuria in phenotypic mutants was carried out by the qualitative analysis of urinary proteins using spot urine samples and SDS-PAGE (23).

**Morphological kidney analysis of the mutant lines.** Animals were killed by ether inhalation. Kidneys of animals of the lines UREHR1-R4 were fixed with 4% paraformaldehyde in PBS (pH 7.2) via orthograde vascular perfusion as described previously (7). Following perfusion fixation, the kidneys were removed, weighed to the nearest milligram, postfixed by immersion in 4% paraformaldehyde for 24 h, and cut perpendicular to the longitudinal axis into 1- to 2-mm-thick slices. The kidney samples were processed in a Shandon Citadel 1000 (Thermo Electron, Waltham, MA) and subsequently embedded in plastic as described (6). Plastic sections (1.5 μm) were stained with hematoxylin and eosin (H&E) as well as periodic-acid Schiff (PAS). In addition, kidney slices as well as representative samples of lung, heart, liver, spleen, salivary gland, stomach, and intestine were routinely processed and embedded in paraffin according to standard procedures. Paraffin sections were stained with H&E, as well as Giemsa, Masson-Trichrom, PAS, and Congo red.

Kidneys derived from the line UREHD1 were fixed by immersion in 10% buffered formalin for 24 h, cut perpendicular to the longitu-

dinal axis into 2-mm-thick slices, routinely processed, and embedded in paraffin.

Immunohistochemical analysis was carried out on paraffin sections mounted on sialinized slides (Superfrost Plus; Menzel Gläser, Braunschweig, Germany), using the indirect immunoperoxidase technique. Sections were deparaffinized, blocked with 1% H<sub>2</sub>O<sub>2</sub> for 15 min, and pretreated with Proteinase K for 10 min. Immunohistochemistry was performed with a polyclonal rabbit anti-IgA, IgM, and C1q antibody (DAKO, Hamburg, Germany), respectively. Immunoreactivity was visualized using 3,3'-diaminobenzidine tetrahydrochloride dihydrate.

For electronmicroscopic analysis, kidneys of 3-mo-old UREHR1 mice were fixed with 3% glutaraldehyde in PBS (pH 7.2) via orthograde vascular perfusion, and 1-mm<sup>3</sup> samples of the renal cortex were postfixed by immersion in the same fixative for 1 day and processed as described previously (7).

**Linkage analysis of the mutation.** In the lines UREHR3 and UREHR4, homozygous phenotypic mutant C3H males were mated to C57BL/6 (for line UREHR4) or BALB/c (for line UREHR3) female mice. The resulting G1 hybrid mice were bred. G2 offspring were phenotypically classified in mice exhibiting normal or increased plasma urea levels, respectively. After death, tissue samples were collected for subsequent DNA isolation.

Tail clip samples were incubated overnight with a lysis buffer consisting of 10 mM Tris·HCl (pH 8.0), 1% (wt/vol) SDS, 50 mM EDTA, and 300 μg/ml Proteinase K (Sigma, Taufkirchen, Germany). Automated DNA extraction from the lysates was performed using the AGOWA Mag Maxi DNA Isolation Kit (AGOWA, Berlin, Germany). For linkage analysis, a genome-wide mapping panel consisting of 149 single nucleotide polymorphism (SNP) markers was applied. Genotyping of this panel was performed using MassExtend, a MALDI-TOF high-throughput genotyping system supplied by Sequenom (San Diego, CA).

**Statistical analysis.** The statistical analysis of the data was carried out using the software program Microsoft Excel 2000. Values are presented as means ± SD unless stated otherwise. Statistical significance (defined as *P* < 0.05) was evaluated using Student's *t*-test.

## RESULTS

**ENU-induced phenotypic variants showing increased plasma urea.** The search for mutant mice showing increased plasma urea levels in the Munich ENU mouse mutagenesis project was carried out on the inbred C3H genetic background 3 mo postpartum after overnight fasting of the animals. Mean total urea levels in plasma from male and female C3H controls corresponded to 48 and 41 mg/dl, respectively (11). In females, the 95% range of the values contained higher values than the data range including two standard deviations above and below the mean. Therefore, the values within the 95% range were defined to be physiological thereby eliminating outlier data (14). Thus pathological plasma urea levels were defined in our test for both male and female mice showing values above the cut-off level of 70 mg/dl in two measurements within a 3-wk interval (Table 1).

Table 1. Physiological range of plasma urea (mg/dl) in 3-mo-old C3H wild-type mice

Sex	<i>n</i>	Range	Mean	SD	Mean ± 2 SD	Median	95% Range
Male	192	29–79	48	11	26–70	47	31–71
Female	235	20–76	41	11	19–63	39	23–71

Cut-off level for males and females: 70 mg/dl.

The search for phenotypic variants observed increased plasma urea levels in the two measurements in 17 of more than 15,000 G1 offspring of ENU-treated mice screened for dominant mutations. For revealing recessive mutations, G3 offspring from more than 500 different pedigrees of ENU-treated G0 mice were examined. Twenty-seven G3 offspring exhibited increased plasma urea levels in two measurements. Referring to the number of G1 mice and G3 pedigrees examined, a much higher number of G1 phenotypic variants was expected in proportion to the number of G3 phenotypic variants detected. In total, 30 of 44 observed phenotypic variants (68%) were male mice (Table 2). This may be partly due to the use of the same cut-off level for both males and females despite the lower physiological mean plasma urea level in female mice.

*Transmission of the increased plasma urea phenotype to subsequent generations.* The inheritance of the increased plasma urea levels of 23 mice originally exhibiting this phenotype was analyzed on G2 offspring derived from the mating of the G1 phenotypic variants to wild-type mice in the screen for dominant mutations and on G4 intercross or G4 × G3 backcross offspring after breeding the G3 phenotypic variants to wild-type mice in the screen for recessive mutations. In the case no offspring were obtained, the respective phenotypic variants were mated to another wild-type animal. Eleven of the mated phenotypic variants (48%) produced no offspring due to severe illness and/or sterility in repeated breeding approaches (Table 2).

Twelve of the 23 mated mice showing increased plasma urea levels (52%) produced offspring. A mutation as cause for the increased plasma urea levels was assumed in the phenotypic variants when plasma urea values above the cut-off level (70 mg/dl) were detected in the offspring in two measurements within a 3-wk interval. Five of the mice which produced offspring (42%) transmitted the increased plasma urea levels to subsequent generations (Table 2). Establishment of another mutant line from a female variant derived from the recessive screen failed due to the low penetrance of the mutation. Therefore, this variant was classified as negative (Table 2). In our project, breeding of almost five phenotypic variants was necessary to establish one mutant line exhibiting an increased plasma urea level.

The mutant line derived from the screen for dominant mutations and the four mutant lines derived from the screen for recessive mutations were designated UREHD1 and UREHR1-R4, respectively. Mutant offspring showing pathological plasma urea levels were produced by mating heterozygous mutants to wild-type animals (line UREHD1) and by breeding heterozygous mutant mice (lines UREHR1-R4). The phenotypic mu-

tants of the five lines showed mean plasma urea levels between 83 and 106 mg/dl, respectively (Table 3).

The phenotypic penetrance of the increased plasma urea levels in the five lines was analyzed by defining 100% phenotypic penetrance in the case of the appearance of 50 and 25% offspring exhibiting the phenotypic deviation after mating phenotypic mutants to wild-type mice and breeding heterozygous mutant mice in the lines with dominant and recessive mutations, respectively. A suitable phenotypic penetrance of the pathological urea levels above 50% was observed in all five lines which facilitates the effective subsequent phenotypic and molecular genetic analyses (Table 3). Similar results were found for both sexes within all lines. Reanalysis of the animals after a few weeks increased the phenotypic penetrance of pathological urea levels. The 5 lines were bred for 7 (lines UREHR2 and UREHR4) and more than 10 (lines UREHD1, UREHR1, and UREHR3) generations starting from the ENU mutagenized G0 founder animal without losing the abnormal phenotype. In all five lines, the numbers of animals exhibiting plasma urea levels above the cut-off level were significantly ( $\chi^2$ -test,  $P < 0.001$ ) higher than the expected 2.5% of the C3H mouse population (Table 3).

*Additional clinical chemical alterations in the mutant lines.* For the analysis of additional plasma parameters, mean values and standard deviations of the means of phenotypic mutants within the litters were compared with those of sex-matched littermate controls. Plasma creatinine levels were increased in the phenotypic mutants of all five lines (Fig. 1). The Jaffé method used is described to overestimate the creatinine level in mice. In the comparative analysis of phenotypic mutants and controls, further techniques might improve the detected differences of the plasma and urinary creatinine levels (5, 15). The plasma electrolytes sodium, potassium, and chloride showed line- and/or sex-specific differences. A significant alteration in both sexes ( $P < 0.05$ ) was only obtained for the decrease of chloride in the phenotypic mutants of line UREHR3 ( $P < 0.001$ ). Reduced plasma glucose levels were observed in the phenotypic mutants of all lines examined (lines UREHD1, UREHR1, UREHR2, and UREHR3). In addition, the mutant mice of line UREHR1 showed strong hypercholesterolemia ( $P < 0.001$ ) which was linked to the plasma urea retention phenotype of this line.

The clinical examination showed obvious additional deviations in the phenotypic mutants of the lines UREHD1, UREHR1, and UREHR3. In line UREHD1, the mutants exhibited increased food intake but decreased body weight gain and decreased body fat. The mutant mice of line UREHR1 had a decreased lifetime and commonly died at 5 mo of age

Table 2. Screen and analysis of the inheritance for increased plasma urea in ENU mutant mice

Screen	n	n Pedigrees	Variants*: n (m/f)	Analysis of Inheritance†: n (m/f)			
				Variants mated	No offspring	Negative	Mutants‡
Dominant (G1)	>15,000	nd	17 (13/4)	8 (7/1)	2 (1/1)	5 (5/0)	1 (1/0)
Recessive (G3)	nd	>500	27 (17/10)	15 (10/5)	9 (5/4)	2 (1/1)	4 (4/0)
Total			44 (30/14)	23 (17/6)	11 (6/5)	7 (6/1)	5 (5/0)

\*G1/G3 offspring of ENU-treated mice showing urea levels of 2 measurements within a 3-wk interval above the cut-off level of 70 mg/dl. m/f, Males/females. †A mutation as cause for the appearance of the abnormal phenotype was diagnosed in phenotypic variants with offspring showing plasma urea levels of 2 measurements within a 3-wk interval above the cut-off level of 70 mg/dl. ‡Variants inheriting increased plasma urea to the subsequent generations. nd, Not determined.

Table 3. Phenotypic penetrance of the increased plasma urea levels in the mutant lines

Line*	mg/dl†	Offspring Tested‡: n	Increased Plasma Urea§, mg/dl			
			% Penetrance	n	Range	Mean
UREHD1	114	228	73	83	70–117	84
UREHR1	97	213	58	31	72–169	91
UREHR2	108	69	70	12	71–126	93
UREHR3	117	123	91	28	70–146	106
UREHR4	139	30	60	9	70–114	88
		41	73	30	71–105	83

\*UREH, line showing increased plasma urea levels; D1, dominant mutation; R1–R4, recessive mutation. †The mutant lines are listed according to the mean plasma urea level (mg/dl) of both measurements of the founder G1/G3 mutant of the line. ‡Offspring after mating heterozygous mutants to wild-type mice (dominant mutation D1) and offspring of heterozygous mutant mice (recessive mutations R1, R2, and R3). In line R4, offspring were produced after mating phenotypic homozygous mutants to heterozygous mutant mice (n = 30) and after mating homozygous mutants (n = 41). §Increased plasma urea in the offspring was defined by the appearance of mean levels of 2 measurements within a 3-wk interval above the cut-off level of 70 mg/dl. 100% phenotypic penetrance is defined by 50 and 25% offspring of dominant and recessive mutants, respectively, showing increased plasma urea values. The absolute numbers of observed phenotypic mutants are given (n).

probably due to renal failure. The penetrance of the plasma urea retention phenotype increased in older animals, whereas complete penetrance of hypercholesterolemia was already present in 12-wk-old mice (16). In the phenotypic mutants of line UREHR3, polydipsia and polyuria were observed.

Examination of the 24-h urine collected in metabolism cages was carried out in the lines UREHD1, UREHR1, and UREHR3. Additional spot urine samples were analyzed in these lines and in line UREHR2. The phenotypic mutants showing increased plasma urea levels were compared with sex-matched littermate controls.

All phenotypic mutants showed increased urine volumes (Fig. 2A) and water consumption in the metabolism cage. As a tendency, urinary creatinine (Fig. 2B) and urea/creatinine (Fig. 2C) were mostly decreased in the 24-h urine in the phenotypic mutants of both sexes. Analysis of spot urine samples of additional animals confirmed these findings. The glomerular filtration rate of creatinine showed the same changes in both sexes only for line UREHR1, where decreased values were

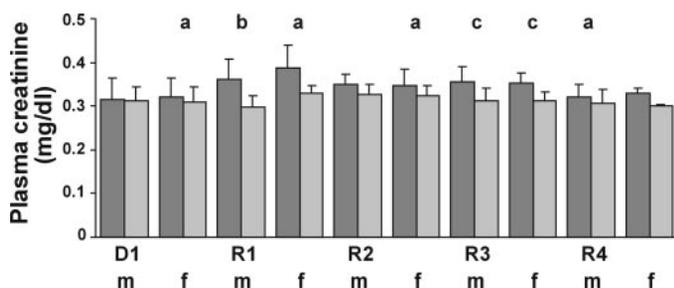


Fig. 1. Plasma creatinine levels of the phenotypic mutants (filled bars) and their wild-type controls (open bars) in the mutant lines UREHD1 and UREHR1–R4. Mean levels (mg/dl) of 12-wk-old animals are shown together with the standard deviations. Plasma creatinine analysis included male and female mutants (UREHD1: n = 25 males and 19 females, UREHR1: n = 9 males and 5 females, UREHR2: n = 5 males and 5 females, UREHR3: n = 11 males and 12 females, UREHR4: n = 4 males and 3 females). m, Males; f, females. *t*-Test of the values of the phenotypic mutants and their wild-type littermates: <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01, <sup>c</sup>*P* < 0.001.

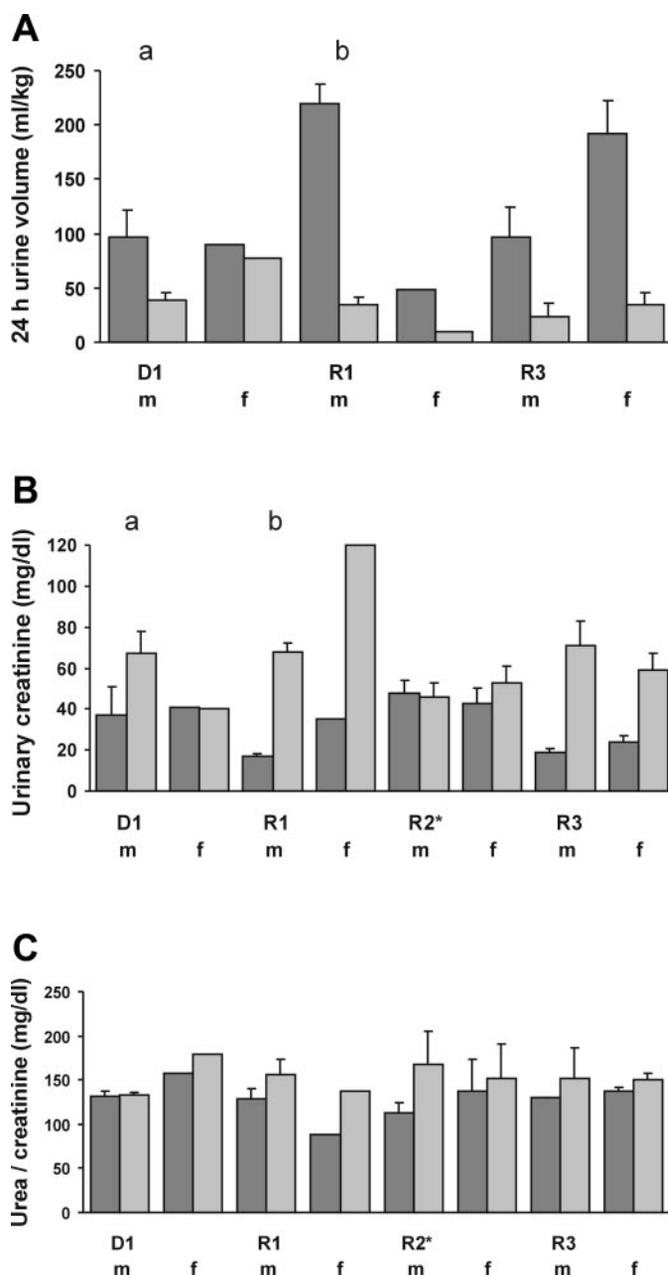


Fig. 2. Urine analysis including volume (A), creatinine (B), and urea/creatinine (C) from 24-h metabolism cage experiments of the phenotypic mutants (filled bars) and their wild-type controls (open bars) in the mutant lines UREHD1 (n = 3 males and 1 female mutants), UREHR1 (n = 3 males and 1 females), and UREHR3 (n = 2 males and 2 females). In addition, creatinine and urea levels of line UREHR2 (n = 3 males and 4 females) are shown from spot urine samples (indicated by \*). Mean levels (mg/dl) are shown together with the standard deviations. *t*-Test of the values of the phenotypic mutants and their wild-type littermates: <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01.

found for the phenotypic mutants. Thus renal clearance has to be analyzed in additional animals. The urinary sodium, potassium, and chloride levels were also analyzed in relation to the creatinine level. As a tendency, urinary sodium/creatinine and chloride/creatinine ratios of the phenotypic mutants were decreased in the 24-h urine and spot urine samples of all lines, whereas urinary potassium/creatinine ratio was increased in the 24-h urine and spot urine samples of the lines UREHR1 and UREHR3.

The phenotypic mutants of the lines UREHD1 and UREHR1 were examined for albuminuria using SDS-PAGE. Severe albuminuria was found in the phenotypic mutants of line UREHR1, whereas this was not detected in line UREHD1 (23).

**Nephropathological findings in the mutant lines.** Morphological analysis was carried out on kidneys from phenotypic mutants exhibiting increased plasma urea levels as well as sex-matched littermates with normal plasma urea levels. Kidneys from the phenotypic mutant mice of the lines UREHD1 ( $n = 4$ ; 8 mo postpartum) and UREHR4 ( $n = 2$ ; 3 mo postpartum) were both macroscopically and light microscopically indistinguishable from those of littermate controls (data not shown).

Compared with sex-matched littermate controls showing no pathological kidney changes (Fig. 3A), 3-mo-old phenotypic mutant mice of line UREHR1 ( $n = 7$ ) consistently showed renal lesions including both glomerular and tubulointerstitial changes (Fig. 3, B and C). Light microscopic examination revealed diffuse glomerular lesions with considerable variation among glomeruli. Prominent findings of less damaged glomeruli included glomerular hypertrophy and segmental to global mesangiolysis with widened and reticulated mesangial areas. The capillary walls were thickened, sometimes to many times of their original width, due to the accumulation of a pale staining material beneath the endothelium, sometimes leading to obliteration of capillary lumina. Thickened capillary walls frequently showed double-contour formation of the glomerular basement membrane. Further glomerular lesions included extravasation into the mesangial compartment, glomerular obsolescence, hyalinosis, as well as sclerosis with segmental to



Fig. 4. Macroscopic appearance of the kidneys of mice of the line UREHR2. The kidneys of the phenotypic mutants are smaller than those of sex-matched littermate controls.

circumferential synechiae between the glomerular tuft and Bowman's capsule. Occasionally, capillaries contained microthrombi. No amyloid deposits were detectable using Congo red stain. Tubulointerstitial lesions included tubular atrophy, hyaline droplets in proximal tubular epithelia, and proteinaceous casts in distal tubules. Furthermore, larger intrarenal arterial vessels showed cellular intimal thickening and media hypertrophy.

Immunohistochemical analysis revealed a positive staining of IgA, IgM, and C1q in glomeruli of phenotypic mutant UREHR1 mice (data not shown).

Predominant electronmicroscopic findings of glomeruli of phenotypic mutant UREHR1 mice (Fig. 3D) were capillary

Fig. 3. Pathomorphological findings in kidneys of mice of the line UREHR1. Overview of the renal cortex of a 3-mo-old healthy littermate control (A) and a phenotypic mutant (B) mouse. Glomerular hypertrophy, thickening of capillary walls, glomerulosclerotic changes with circumferential adhesion between the glomerular tuft and Bowman's capsule, and glomerular obsolescence as well as tubular atrophy and numerous proteinaceous casts are evident in the mutant mouse kidney (B). A, B: H&E, magnification  $\times 10$  objective. C: glomerular profile of a phenotypic mutant mouse, showing mesangiolytic changes and thickening of capillary walls due to accumulation of pale staining material beneath the endothelium (H&E, magnification  $\times 40$  objective). D: transmission electron microscopy of a phenotypic mutant mouse. Glomerular capillary walls are widened by accumulation of lucent material. Mesangial cell interposition into widened subendothelial space and endothelial cells swelling is shown. Final magnification  $\times 3,800$ .

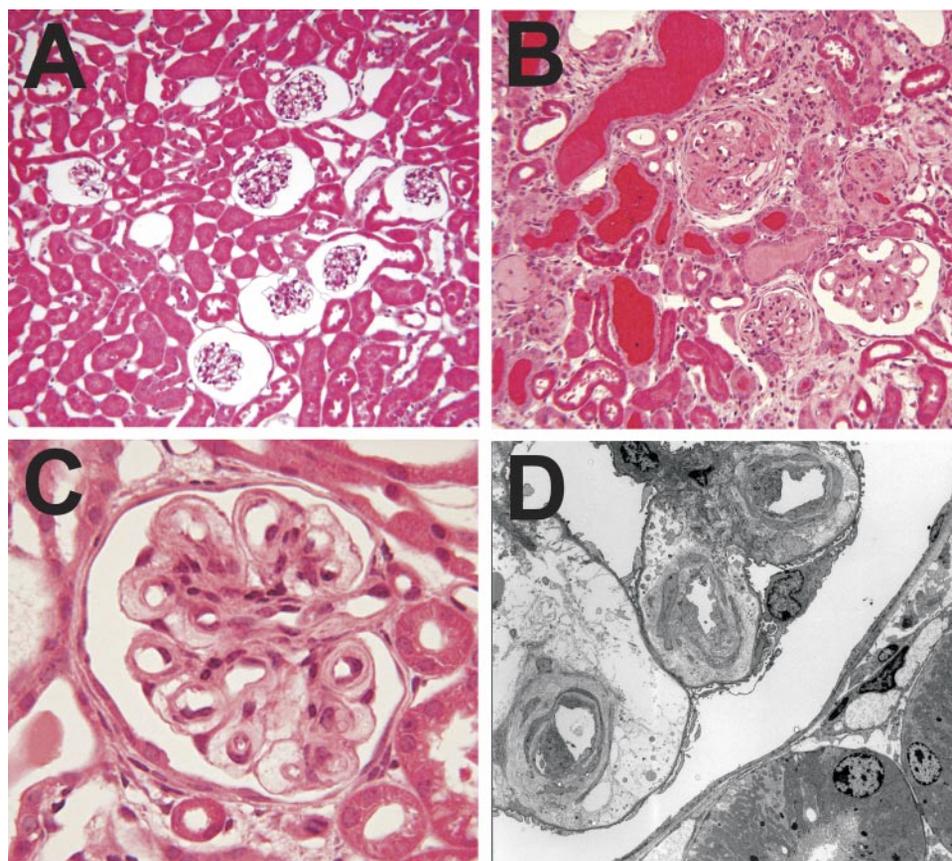


Table 4. *Body weight, kidney weight, and relative kidney weight of 4-mo-old female mice of the line UREHR2*

Phenotype*	n	Body Weight, g	Kidney Weight, mg	Relative Kidney Weight
Mutant	4	21.7±2.2	251±32	1.15±0.07
Wild-type	4	28.5±6.2†	429±36†	1.54±0.20†

Values are means ± SD. \*Offspring of heterozygous mutant mice were classified according to their plasma urea levels. † $P < 0.05$ .

widening by subendothelial accumulation of lucent, finely granular material, sometimes containing fibrin or cell fragments. Mesangial cell interposition was frequently observed into widened subendothelial space. Furthermore, endothelial cells appeared swollen, and a variety of podocyte changes was detected including pseudocyst formation, protrusions of the epithelial surface, and foot process effacement.

Furthermore, in some phenotypic mutant mice of line UREHR1, the ascending aorta appeared thickened and showed inflammatory infiltration of the vascular wall. Lightmicroscopic examination of lungs, liver, spleen, salivary gland, stomach, and intestine revealed no alterations in phenotypic mutant mice.

Four-month-old phenotypic mutants of line UREHR2 ( $n = 4$ ) showed a decreased kidney weight (Fig. 4) and kidney-to-body weight ratio (Table 4) compared with littermate controls. Histologically, no renal alterations were evident.

In 4-mo-old ( $n = 5$ ) and 10-mo-old ( $n = 2$ ) phenotypic mutant male mice of line UREHR3, uni- or bilateral dilation of the renal pelvis was found, which was not seen in an additional 10-mo-old phenotypic mutant female. Histologically, atrophy of the renal papilla and focal calcification of tubular epithelial cells were prominent findings in the phenotypic mutant mice of this line. There were no signs of obstruction of the urinary tract (Fig. 5).

*Linkage analysis of the causative mutation.* According to the availability of resources in the ongoing ENU project, linkage analysis of the chromosomal site of the mutation was carried out in the lines UREHR3 and UREHR4. Use of BALB/c instead of C57BL/6 showing high physiological plasma urea levels as second inbred strain in the breeding scheme facilitated the phenotypic classification of the offspring to the

groups harboring normal or increased plasma urea levels. The genome-wide linkage analysis of 85 and 136 informative single nucleotide polymorphism (SNP) marker assays were analyzed in 47 and 45 phenotypic mutant mice for the lines UREHR3 and UREHR4, respectively. In line UREHR3, the examination revealed a strong linkage [ $\chi^2=125.5$ ;  $-\log_{10}(P) = 27.25$ ] of the mutation to a defined single chromosomal site on chromosome 2 represented by the marker rs4223511 at 132 Mb (mouse genome build 36.1). In line UREHR4, a strong linkage [ $\chi^2=43.8$ ;  $-\log_{10}(P) = 9.51$ ] of the mutation to a defined single chromosomal site on chromosome 7 represented by the marker rs3654133 at 115 Mb was detected.

## DISCUSSION

In this paper, we describe the successful establishment of five mouse lines harboring dominant and recessive mutations by the high-throughput screening of a large number of randomly mutagenized mice for increased plasma urea levels.

Elevated plasma urea levels were mostly confirmed in the reanalysis of the affected animals. The breeding approaches for the establishment of mutant lines detected more than half of the fertile phenotypic variants which did not transmit the increased plasma urea levels to the offspring. A high ratio in the failure of the transmission of the defect to the offspring was also found for other phenotypic deviations in our clinical chemistry screen (16). One reason may be the occurrence of not genetically fixed increased plasma urea levels due to the inherent impossibility of carrying out complete standardization of husbandry and experiment.

The established lines showed an incomplete penetrance of the mutant phenotype of various degrees. Appearance of the pathological plasma urea levels due to the interaction of multiple unlinked mutations leads to lower numbers of affected offspring. However, all five lines were bred for multiple generations starting from the ENU-mutagenized G0 founder animal without losing the abnormal phenotype. In addition, reanalysis of the lines after a few weeks increased the phenotypic penetrance of the pathological urea levels, thereby indicating that the penetrance was generally underestimated in the analysis after 12 wk of age.

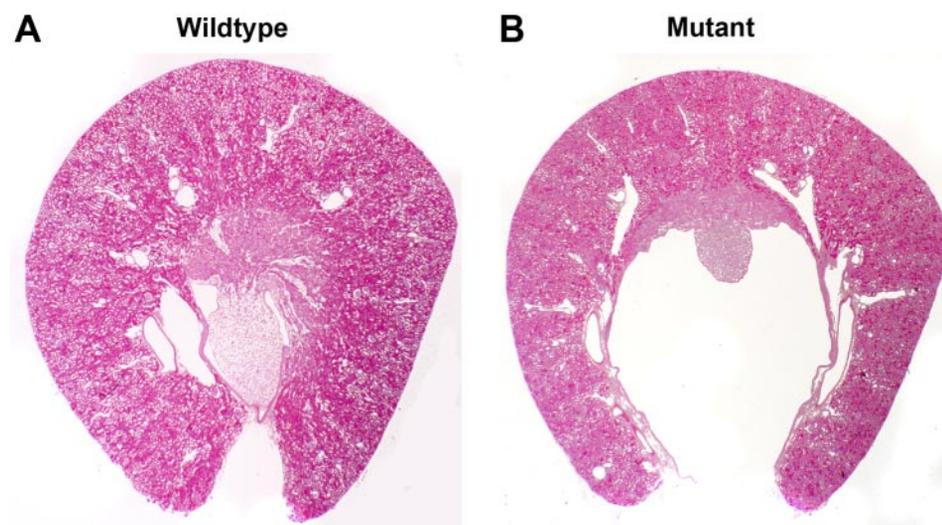


Fig. 5. Histology of the kidneys of mice of the line UREHR3. Overview of the kidney of a control (A) and phenotypic mutant (B) mouse. The mutants show dilation of the renal pelvis and atrophy of the papilla. Plastic section, H&E, final magnification  $\times 20$ .

Deviations in the number of offspring per litter and/or in the sex ratio of the offspring indicating early loss of mutants as alternative reason for the decreased frequency of mice showing an increased plasma urea level were not examined. The phenotypic analysis of the mutation may be improved by analyzing the animals more frequently and/or by challenge experiments (31). Strain-specific susceptibility to renal damage (1) and/or strain-specific variations in the plasma urea concentration (<http://www.jax.org/phenome>; <http://www.eumorphia.org>) (20) have been found. Therefore, generation and subsequent analysis of hybrids and/or congenic strains may give rise to variations in the appearance of increased plasma urea levels.

In addition to the clinical chemistry screen in the Munich ENU mouse mutagenesis project, a systematic clinical chemistry analysis has been described to date in another ENU mouse mutagenesis program (19). The appearance of lines exhibiting increased plasma urea values has not yet been reported on a different genetic background (BALB/c × C3H) (8). Other ENU-based screens for renal dysfunction (3, 12) used primary albuminuria examination with urinary dipstick and ELISA techniques and subsequent calculation of the ratio of urinary albumin to urinary creatinine (<http://mouse-genome.bcm.tmc.edu>). Alternatively, renal dysfunction was analyzed by searching for abnormalities in blood electrolytes and creatinine combined with urinalysis (<http://www.cmhd.ca>) (3).

We recently carried out the large-scale analysis of ENU-induced mouse mutants for albuminuria in the Munich ENU project by using qualitative SDS-PAGE. Examination of more than 2,000 G1 animals for dominant mutations and nearly 50 G3 pedigrees for recessive mutations resulted in two lines showing a low phenotypic penetrance of albuminuria. This might be caused by the appearance and segregation of multiple causative mutations within the lines. However, the analysis demonstrated the principle feasibility to identify nephropathy phenotypes in ENU-mutagenized mice by screening for albuminuria (23).

The morphological analysis of the kidneys revealed different results for the established lines. The kidney pathology of line UREHR1 obviously explains the observed plasma urea retention. The morphological features of UREHR1 mutants resemble the findings that have been suggested to be classified as thrombotic microangiopathy (17). The reduction of the kidney weight and kidney-to-body weight ratio in line UREHR2 may contribute to the altered phenotype but per se does not appear to be sufficient to cause the plasma urea retention. Therefore, further investigations concerning ultrastructural and pathophysiological changes of the kidneys are required to clarify the etiology of the clinical chemistry plasma alteration in line UREHR2. The structural changes of the kidneys of line UREHR3 may be primarily responsible for the increased plasma urea levels and for polydipsia and polyuria, or they may be a secondary change due to polydipsia and polyuria. In the lines UREHD1 and UREHR4, no structural kidney changes were apparent; therefore, pathophysiological alterations have to be taken into account as a cause for increased plasma urea levels. Further phenotyping of the renal defects will include kidney function analysis (31 and refs. therein) as well as expression profiling of isolated tissues and/or cultured cells from the mutant lines (27, 29).

The number and the chromosomal position of the causative mutations in ENU projects are examined by linkage analysis.

The results in the lines UREHR3 and UREHR4 revealing a single chromosomal site as cause for the altered phenotype showed the feasibility of the project with plasma urea as parameter. Further linkage analysis on the defined chromosomal site and candidate gene analysis is currently performed to identify the causative mutation (10, 28).

Due to the triggering of random mutations by ENU, discovery of genes not yet known to be involved in the plasma urea excretion may occur in our mouse mutants. The mutants may also carry hypomorphic, hypermorphic, or null alleles of known genes which may be useful in the further understanding of the role of these genes in kidney diseases. After having carried out the chromosomal linkage analysis, the subsequent identification of the causative mutations including the analysis of their functional relevance by reverse genetics methods as well as the examination of the pathological consequences of the mutations may establish suitable models for the subsequent research on human nephropathies.

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#### REFERENCES

1. Anders H, Schlondorff D. Murine models of renal disease: possibilities and problems in studies using mutant mice. *Exp Nephrol* 8: 181–193, 2000.
2. Brown SD, Balling R. Systematic approaches to mouse mutagenesis. *Curr Opin Genet Dev* 11: 268–273, 2001.
3. Clark AT, Goldowitz D, Takahashi JS, Vitaterna MH, Siepka SM, Peters LL, Frankel WN, Carlson GA, Rossant J, Nadeau JH, Justice MJ. Implementing large-scale ENU mutagenesis screens in North America. *Genetica* 122: 51–64, 2004.
4. De Winther MP, Hofker MH. New mouse models for lipoprotein metabolism and atherosclerosis. *Curr Opin Lipidol* 13: 191–197, 2002.
5. Dunn SR, Qi Z, Bottinger EP, Breyer MD, Sharma K. Utility of endogenous creatinine clearance as a measure of renal function in mice. *Kidney Int* 65: 1959–1967, 2004.
6. Hermanns W, Liebig K, Schulz LC. Postembedding immunohistochemical demonstration of antigen in experimental polyarthritis using plastic embedded whole joints. *Histochemistry* 73: 439–446, 1981.
7. Hoeflich A, Weber MM, Fisch T, Nedbal S, Fottner C, Elminger MW, Wanke R, Wolf E. Insulin-like growth factor binding protein 2 (IGFBP-2) separates hypertrophic and hyperplastic effects of growth hormone (GH)/IGF-I excess on adrenocortical cells in vivo. *FASEB J* 16: 1721–1731, 2002.
8. Hough TA, Nolan PM, Tspouri V, Toye AA, Gray IC, Goldsworthy M, Moir L, Cox RD, Clements S, Glenister PH, Wood J, Selley RL, Strivens MA, Vizor L, McCormack SL, Peters J, Fisher EM, Spurr N, Rastan S, Martin JE, Brown SD, Hunter AJ. Novel phenotypes identified by plasma biochemical screening in the mouse. *Mamm Genome* 13: 595–602, 2002.
9. Hrabé de Angelis M, Flaswinkel H, Fuchs H, Rathkolb B, Soewarto D, Marschall S, Heffner S, Pargent W, Wuensch K, Jung M, Reis A, Richter T, Alessandrini F, Jakob T, Fuchs E, Kolb H, Kremmer E, Schaeble K, Rollinski B, Roscher A, Peters C, Meitinger T, Strom T, Steckler T, Holsboer F, Klopstock T, Gekeler F, Schindewolf C, Jung T, Avraham K, Behrendt H, Ring J, Zimmer A, Schughart K, Pfeffer K, Wolf E, Balling R. Genome-wide, large-scale production of mutant mice by ENU mutagenesis. *Nat Genet* 25: 444–447, 2000.
10. Keays DA, Clark TG, Flint J. Estimating the number of coding mutations in genotypic- and phenotypic-driven N-ethyl-N-nitrosourea (ENU) screens. *Mamm Genome* 17: 230–238, 2006.
11. Klempt M, Rathkolb B, Fuchs E, Hrabé de Angelis M, Wolf E, Aigner B. Genotype-specific environmental impact on the variance of blood values in inbred and F<sub>1</sub> hybrid mice. *Mamm Genome* 17: 93–102, 2006.

12. **Korstanje R, DiPetrillo K.** Unraveling the genetics of chronic kidney disease using animal models. *Am J Physiol Renal Physiol* 287: F347–F352, 2004.
13. **Kriz W, LeHir M.** Pathways to nephron loss starting from glomerular diseases—insights from animal models. *Kidney Int* 67: 404–419, 2005.
14. **Loeb WF, Quimby FW.** *The Clinical Chemistry of Laboratory Animals*. Philadelphia: Taylor & Francis, 1999.
15. **Meyer MH, Meyer RA Jr, Gray RW, Irwin RL.** Picric acid methods greatly overestimate serum creatinine in mice: more accurate results with high-performance liquid chromatography. *Anal Biochem* 144: 285–290, 1985.
16. **Mohr M, Klempt M, Rathkolb B, Hrabe de Angelis M, Wolf E, Aigner B.** Hypercholesterolemia in ENU-induced mouse mutants. *J Lipid Res* 45: 2132–2137, 2004.
17. **Morita T, Yamamoto T, Churg J.** Mesangiolytic: an update. *Am J Kidney Dis* 31: 559–573, 1998.
18. **Nicklas W, Baneux P, Boot R, Decelle T, Deeny AA, Fumanelli M, Illgen-Wilcke B.** Recommendations for the health monitoring of rodent and rabbit colonies in breeding and experimental units. *Lab Anim* 36: 20–42, 2002.
19. **Nolan PM, Peters J, Strivens M, Rogers D, Hagan J, Spurr N, Gray IC, Vizer L, Brooker D, Whitehill E, Washbourne R, Hough T, Greenaway S, Hewitt M, Liu X, McCormack S, Pickford K, Selley R, Wells C, Tymowska-Lalanne Z, Roby P, Glenister P, Thornton C, Thaug C, Stevenson JA, Arkell R, Mburu P, Hardisty R, Kiernan A, Erven A, Steel KP, Voegeling S, Guenet JL, Nickols C, Sadri R, Nasse M, Isaacs A, Davies K, Browne M, Fisher EM, Martin J, Rastan S, Brown SD, Hunter J.** A systematic, genome-wide, phenotype-driven mutagenesis programme for gene function studies in the mouse. *Nat Genet* 25: 440–443, 2000.
20. **Paigen K, Eppig JT.** A mouse phenome project. *Mamm Genome* 11: 715–717, 2000.
21. **Rathkolb B, Decker T, Fuchs E, Soewarto D, Fella C, Heffner S, Pargent W, Wanke R, Balling R, Hrabe de Angelis M, Kolb HJ, Wolf E.** The clinical-chemical screen in the Munich ENU Mouse Mutagenesis Project: screening for clinically relevant phenotypes. *Mamm Genome* 11: 543–546, 2000.
22. **Rathkolb B, Fuchs E, Kolb HJ, Renner-Muller I, Krebs O, Balling R, Hrabe de Angelis M, Wolf E.** Large-scale *N*-ethyl-*N*-nitrosourea mutagenesis of mice—from phenotypes to genes. *Exp Physiol* 85: 635–644, 2000.
23. **Rathkolb B, Tran TV, Klempt M, Hrabe de Angelis M, Wanke R, Wolf E, Aigner B.** Large-scale albuminuria screen for nephropathy models in chemically induced mouse mutants. *Nephron Exp Nephrol* 100: e143–e149, 2005.
24. **Russ A, Stumm G, Augustin M, Sedlmeier R, Wattler S, Nehls M.** Random mutagenesis in the mouse as a tool in drug discovery. *Drug Discov Today* 7: 1175–1183, 2002.
25. **Saborio P, Scheinman J.** Genetic renal disease. *Curr Opin Pediatr* 10: 174–183, 1998.
26. **Sands JM.** Renal urea transporters. *Curr Opin Nephrol Hypertens* 13: 525–532, 2004.
27. **Seltmann M, Horsch M, Drobyshv A, Chen Y, Hrabe de Angelis M, Beckers J.** Assessment of a systematic expression profiling approach in ENU-induced mouse mutant lines. *Mamm Genome* 16: 1–10, 2005.
28. **Silver LM.** *Mouse Genetics: Concepts and Applications*. New York: Oxford Univ. Press, 1995.
29. **Soutourina O, Cheval L, Doucet A.** Global analysis of gene expression in mammalian kidney. *Pflügers Arch* 450: 13–25, 2005.
30. **Terzi F, Burtin M, Friedlander G.** Using transgenic mice to analyze the mechanisms of progression of chronic renal failure. *J Am Soc Nephrol* 11, Suppl 16: S144–S148, 2000.
31. **Vallon V.** In vivo studies of the genetically modified mouse kidney. *Nephron Physiol* 94: 1–5, 2003.
32. **Wolf E, Wanke R.** Growth hormone overproduction in transgenic mice: phenotypic alterations and deduced animal models. In: *Welfare of Transgenic Animals*, edited by van Zutphen LFM and van der Meer M. Heidelberg: Springer-Verlag, 1996, p. 26–47.
33. **Yang B, Bankir L.** Urea and urine concentrating ability: new insights from studies in mice. *Am J Physiol Renal Physiol* 288: F881–F896, 2005.

