The bladder as a bioreactor: Urothelium production and secretion of growth hormone into urine

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Uroplakin genes are expressed in a bladder-specific and differentiation-dependent fashion. Using a 3.6-kb promoter of mouse uroplakin II gene, we have generated transgenic mice that express human growth hormone (hGH) in their bladder epithelium, resulting in its secretion into the urine at 100–500 ng/ml. The levels of urine hGH concentration remain constant for longer than 8 months. hGH is present as aggregates mostly in the uroplakin-delivering cytoplasmic vesicles that are targeted to fuse with the apical surface. Using the bladder as a bioreactor offers unique advantages, including the utility of all animals throughout their lives. Using urine, which contains little protein and lipid, as a starting material facilitates recombinant protein purification.

Keywords: genetic engineering, transgenic mice, agricultural biotechnology

Ten years ago, Gordon et al.[1] reported that transgenic animal technology could be used for the production of pharmaceutical proteins in milk. This technology has now advanced from transgenic mice to transgenic livestock, and milk-derived pharmaceuticals are now being evaluated in human clinical trials[2–4]. The major benefits of biopharming over cell culture–based bioreactors are the lower cost of production, and the relative ease of scale up. The production of transgenic farm animals, however, requires a considerable investment in time. With milk-based bioreactor animals this time commitment includes the interval from the birth of the transgenic animal to its first lactation, which is typically 12, 14, and 26 months for pigs, sheep or goats, and cattle, respectively. Lactation occurs only in females and is noncontinuous, typically lasting 2, 6, and 10 months, respectively, and with 2-month to 6-month intervals between subsequent lactation periods. Finally, milk is a complex substance usually containing 3% to 6% total protein and 3% to 5% lipid, and thus requires the development of extensive purification schemes to obtain the transgene-derived protein[5].

Another easily collectable fluid from transgenic livestock is urine. Consideration of urine for biopharming would likely require product synthesis within the bladder urothelium and its direct secretion into the urine. The bladder urothelium produces, and expresses on its apical surface, a group of membrane proteins known as the uroplakins[6–10], which are thought to function as a permeability barrier and as a physical stabilizer of the urothelial apical surface[11,12]. Bladder specificity of the uroplakins was observed by immunohistochemistry and reverse transcription polymerase chain reaction (RT-PCR)[7,8,10,13]. Recently, the 3.6-kb, 5′ flanking region of the mouse uroplakin II (UPII) gene was used to express a bacterial reporter gene (lacZ) in transgenic mice[13]. Reporter gene expression was found in the suprabasal cell layers of the urothelium and to a much lesser extent in the hypothalamus, but not in other tissues examined.

A major advantage of bladder production of pharmaceuticals is the ability to harvest the product soon after birth and throughout the life of the animal without regard to its sex or reproductive status. Also, livestock urine is a proven, currently utilized source of pharmaceuticals. It is estimated that urine is being collected from 75,000 pregnant horses annually as a source of estrogenic compounds for postmenopausal hormone replacement therapy[14].

We assessed the possibility of using a urothelium-specific promoter to direct expression of a foreign protein into the urine of transgenic animals as a first step in assessing this approach for biopharming. We specifically chose to express human growth hormone (hGH) because the consequences of ectopic or "leaky" expression are easily detected.

Results

Production of transgenic mice. The UPII-hGH transgene was microinjected into fertilized eggs and resulted in the production of nine transgenic mice. The five female founder animals failed to produce offspring, a phenotype often observed in transgenic rodents expressing biologically active GH. We failed to detect hGH in urine of one founder male or in his offspring. The three remaining male founders which had detectable hGH in their urine, were used to establish lines for further study. The transgene was inherited by approximately 50% of offspring, and the level of expression in founders and their progeny was similar, suggesting that the founders were not mosaic. Southern blot analysis suggested that the transgene was inserted at only one integration site as multicopy arrays in each of these three lines. A predominant 5.7-kb band was detected in Bam HI digested DNA, which presumably resulted from head-to-tail concatemers of the transgene (not shown). Phosphoimager quantification of the 5.7-kb band indicated that lines GHP1, GHP2, and GHP3 had approximately 5, 50, and 100 copies of the transgene, respectively.

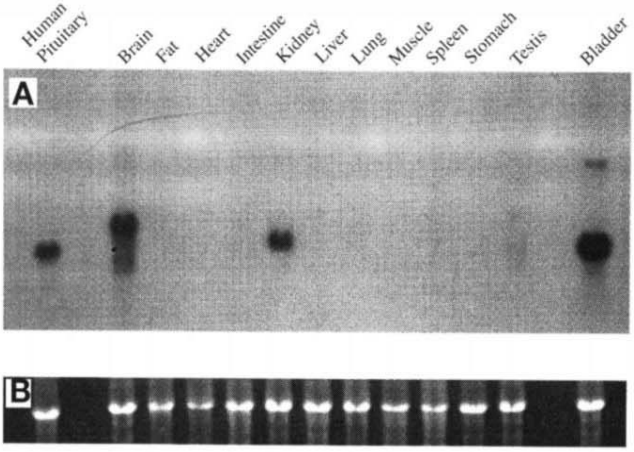

Figure 1. Northern blot analysis of RNA extracted from various tissues of male mice from line GHP2. The blot was probed with ^{32}P-labeled hGH cDNA. (A) The human pituitary lane contained 1 ng of human pituitary poly A$^+$ RNA. Other lanes contained 15 µg of total RNA from various tissues, except for the bladder lane, which contained 300 ng of total RNA. (B) Ethidium bromide staining of the 18S ribosomal RNA subunit.

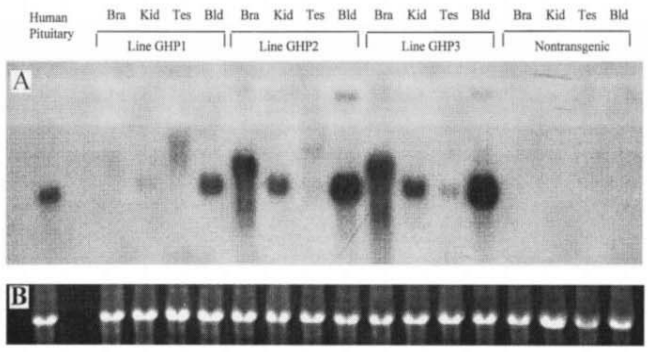

Figure 2. Northern blot analysis of total RNA extracted from the brain (Bra), kidney (Kid), testes (Tes), and bladder (Bld) from three lines of transgenic mice containing the UPII-hGH transgene, and from a nontransgenic mouse. The blot was probed with ^{32}P-labeled hGH cDNA. (A) The human pituitary lane contained 1 ng of human pituitary poly A$^+$ RNA, the bladder lanes contained 300 ng of bladder total RNA and other lanes contained 15 µg total RNA. (B) Ethidium bromide staining of the 18S ribosomal RNA subunit.

Urothelial expression of the hGH transgene. Northern blot analysis of hGH expression in various tissues of line GHP2 indicated substantial expression in the bladder with about 100-fold less expression in the brain, kidney, and testes (Fig. 1; note that the bladder RNA had been diluted 50-fold with nontransgenic mouse liver RNA). Similar distribution patterns were observed in the other two lines (Fig. 2). The major band observed in bladder and kidney RNA (Fig. 2) was of similar size to authentic hGH mRNA obtained from human pituitary gland. The transcript size of the brain was consistently larger, while that of testes was variable among lines. Quantification of the major bands corrected for the dilution of bladder RNA, showed that bladder expression was approximately two orders of magnitude greater than in brain or kidney. As kidneys were obtained by gross dissection, hGH expression in these samples could have been due to contamination by urothelium in the renal pelvis or ureter.

Immunofluorescent staining of bladder sections using an antiserum to hGH resulted in the intense labeling of all the suprabasal urothelial cell layers—which normally express the uroplakin II gene (Fig. 3). Electron microscopy of transgenic urothelium revealed the presence of many electron-dense granules in the uroplakin-delivering cytoplasmic vesicles (Fig. 4). These granules were decorated by the anti-hGH antibody, and therefore represent aggregated hGH (Fig. 4). Some of the hGH granules seemed to localize outside the uroplakin vesicles, but appeared to be enclosed by a limiting membrane. No such hGH granules were seen in the urothelium of control mice. These results indicate that the UPII-hGH transgene was indeed expressed quite specifically in the differentiated mouse urothelial cells.

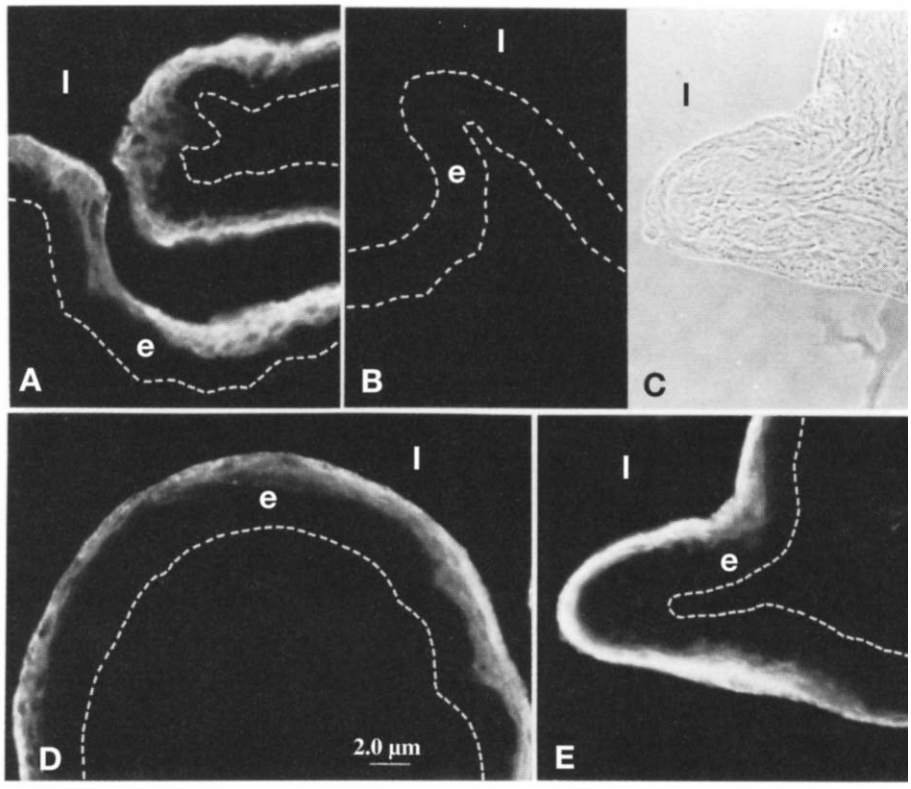

Figure 3. Immunofluoresent staining of cryostat sections of bladders from transgenic mice. Sections were stained with (A) rabbit antiserum to total bovine uroplakins, (B) pre-immune serum, (D and E) rabbit antiserum to hGH. (C) Phase contrast image of the section shown in (E). e and l denote bladder epithelium and lumen, respectively. All sections are at the same magnification.

Secretion of hGH into the urine. Apical secretion of hGH into urine was determined by measuring urinary hGH concentrations by radioimmunoassay (RIA). While no hGH was detectable in the urine of control animals, urine of UPII-hGH transgenic mice contained hGH at concentrations of up to 500 ng/ml. Moreover, the

urinary concentration of hGH of each animal was relatively stable from the first sampling at approximately 6 weeks of age to the last sampling at approximately 8 months (Fig. 5). The relative expression levels of the founder animals were preserved in their offspring; thus, animals from line GHP1 generally had the lowest hGH concentrations, while those from line GPH3 generally had the highest (Table 1). A sexual dimorphism was observed among founders, with males containing approximately twice as much hGH in their urine as female founders (120 ng/ml vs. 59 ng/ml, n=5, p=0.029). However, no such sex difference was observed in the first generation offspring (Table 1). Our attempts to visualize the urinary hGH by Western blot analysis were hampered by the fact that hGH comigrates during SDS-PAGE with the so-called "major urinary protein," which is present in extremely high concentrations in mouse urine.

The total urinary protein concentration in a group of F1 mice representing both sexes and all three lines averaged 1.5±0.2 mg/ml and ranged from 0.4 to 3.0 mg/ml (Table 1). The urinary hGH in these 18 animals expressed as a percentage of total protein ranged from 0.002% to 0.049%, and averaged 0.018% of total urinary protein. For comparative purposes, we assayed the protein content of cattle and swine urine. Average total urinary protein in five cow samples and four sow samples was 172±26 µg/ml and 51±22 µg/ml, respectively.

Plasma samples were obtained from six F1 animals representing the three transgenic lines. Circulating concentrations of hGH were approximately 100-fold less than urine concentrations, and ranged from 2 to 7 ng/ml (Mean ±S.E., 4.7±0.8 ng/ml).

Discussion

We have demonstrated that the 3.6-kb 5′-flanking region of the mouse UPII gene can be used to direct the production of a foreign protein in the urothelium of transgenic mice and—perhaps unexpectedly given that the urothelium is normally considered a nonsecretory tissue—its secretion into the urine. Our data provide the first indication that the bladder of transgenic animals can be transformed into a bioreactor.

We found that hGH was accumulated in urine up to 0.5 µg/ml, which represented 0.02% of the total urinary protein. Although this percentage of specific protein may seem low as a starting material for recombinant protein

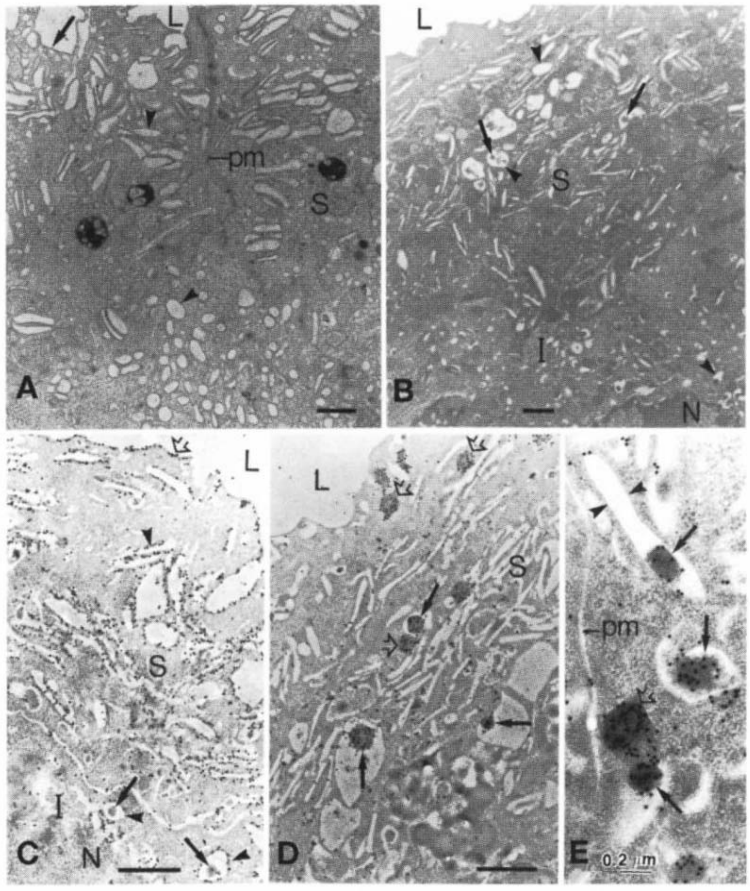

Figure 4. The fine structure and immunogold labeling of the bladder epithelium of normal and UPII-hGH transgenic mice. (A) A section of normal urothelium demonstrating two adjoining superficial umbrella cells containing numerous cytoplasmic vesicles (arrowheads) involved in the transport of uroplakin-containing asymmetric unit membranes (AUM) to the apical surface (arrow). (B) Ultrastucture of the urothelium of a UPII-hGH transgenic mouse showing many electron-dense granules (arrows) packaged in AUM-lined vesicles (arrowheads). (C) Immunogold labeling of uroplakins in the urothelium of a UPII-hGH transgenic mouse demonstrating numerous uroplakin-containing cytoplasmic vesicles (arrowheads) and the apical urothelial plaques (open arrow). Arrows indicate the lack of staining of the hGH granules enclosed in AUM vesicles. (D and E) Show the immunogold-labeling of human growth hormone in the urothelium of a transgenic mouse. Arrows indicate the labeling of the electron-dense granules that are packaged in the AUM-lined cytoplasmic vesicle (arrowheads), as well as some packaged in another type of vesicles not lined by AUM (open arrows). I, S, PM, and N mark intermediate cell, superficial cell, plasma membrane and nucleus, respectively. Scale bars in (A–D) are 1.0 µm.

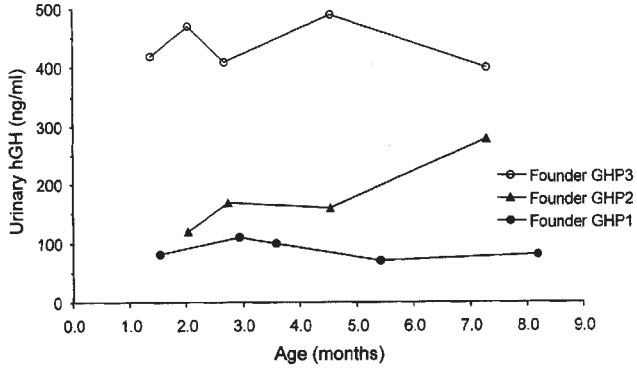

Figure 5. Time course of hGH concentration in urine from UPII-hGH transgenic mice.

Table 1. Human growth hormone (hGH), total protein, and hGH as a percentage of total protein in urine of F1 transgenic mice.

Line	Sex	n*	Urinary hGH† (ng/ml) mean	S.E.	Urinary protein‡ (mg/ml) mean	S.E.	hGH as % of protein‡ mean
GHP1	m	7	130	20	0.57	0.07	0.022
	f	5	70	10	1.68	0.66	0.007
GHP2	m	5	240	40	1.81	0.65	0.019
	f	2	350		0.66		0.034
GHP3	m	3	330	30	2.13	0.57	0.020
	f	7	130	10	1.63	0.16	0.014

*Number of individual F1 mice sampled. Mice were sampled once or twice; if twice, then an average value for that mouse was calculated. †Least square mean hGH concentration differed among lines (GHP1, 100 ng/ml; GHP2, 288 ng/ml; GHP3, 219 ng/ml; p<0.01), but did not differ among sexes (females, 200 ng/ml; males, 253 ng/ml; p=0.286). ‡Urinary protein was determined in three male and three female mice from each line, except that urine was collected from only two female mice of line GHP2.

purification, the mouse excretes 10- to 50-fold more protein in its urine than do other species[15]. This is due to the presence of a group of closely related proteins termed the major urinary proteins (MUPs). The MUP concentration in urine can approach 5 mg/ml. MUPs are produced by the liver[16] as the products of a multigene family consisting of approximately 35 genes and pseudogenes[17].

Mouse urine contains about 10-fold more total protein than urine from cows and sows. Thus, it is likely that transgenic protein in urine of farm animals could represent 0.1% to 1.0% of total urinary protein. In comparison, mammary-specific transgenes designed for the production of pharmaceuticals in milk often express in the mg/ml range, which represents approximately 1% to 10% of total milk protein[4]. The lower specific protein percentage in urine could be offset by advantages in downstream processing. At least one step, the fat separation, would not be required for urine processing. The relative effort involved in purifying a transgenic protein from milk or urine will likely be protein dependent. Although unknown, it is possible that post-translational modification of proteins may differ between mammary and bladder epithelium, making one the production site of choice. Adequate post-translational modification of product will be of increasing concern as the bioreactor industry targets more complex proteins[4].

Precedents for purifying biologicals from urine are numerous, with estrogenic compounds from pregnant mare urine (PMU) and gonadotropins from postmenopausal women's urine being currently used in human medicine. The PMU industry is well established in western Canada and the north-central United States where it is estimated that urine is currently being collected from 75,000 mares. Approximately 500 L of urine are collected annually from each pregnant mare. If the urine from these horses were to contain the desired transgenic protein at a concentration of 0.5 mg/L, then 19 kg of product would be contained in the starting material. Even with a 50% purification loss, a relatively small herd of 200 transgenic cattle, with each animal producing 20 L/day of urine containing 0.5 mg/L of product, could produce the approximately 300 grams of human blood-clotting factor VIII needed to supply the entire world market on an annual basis. Recently, we estimated that it would take approximately 7 years from the time of embryo microinjection to generate a production herd of transgenic cows producing a pharmaceutical in milk[4]. In contrast, to generate a production herd of cattle producing a transgene in urine would require approximately 3 years.

The very limited distribution of tissues expressing the UPII-hGH transgene indicates that the 3.6-kb, 5′-flanking region of the UPII gene contains most of the sequence elements required for bladder-specific expression. Kidney, brain, and testes expression was approximately two orders of magnitude less than in bladder. The kidney expression was likely the result of urothelial contamination of the kidney sample, while brain expression has also been observed in UPII-LacZ transgenic mice[13]. Ectopic expression could potentially be abolished by incorporating transgene insulator sequences such as matrix attachment regions into the transgene[20], as has been previously demonstrated with a prostate specific transgene[21]. It is also possible that the ectopic expression would be reduced with different structural genes.

Although we demonstrated in this study the potential of mammalian bladder as a bioreactor using a 3.6-kb 5′-regulatory sequence, the promoters of other bladder-specific genes may also be useful. These include the genes encoding the three other uroplakins (i.e., the 27-kDa UPIa, the 28-kDa UPIb[9], and the 47-kDa UPIII[8]). These three uroplakins, together with UPII[6], form the 16-nm protein particles arranged in two-dimensional crystalline arrays[22] that constitute urothelial plaques covering approximately 70% to 80% of the apical surface of the urothelium. Thus, the uroplakins represent the major differentiation products of mammalian urothelium. Moreover, uroplakin genes have been shown to be highly conserved in nine mammalian species including mouse, rabbit, human, cattle, and sheep.

The transgenic animal bioreactor industry has focused primarily on directing expression of their products to the milk, though at least one organization has explored the possibility of isolating products from blood[24]. Our study provides a third alternative (i.e., using the bladder as a bioreactor) that has the same advantage of mammary gland bioreactors: straightforward, noninvasive collection of the product. In addition, this alternative offers the main advantage of blood based systems: the ability to harvest products shortly after birth from both sexes. Furthermore, the most significant benefit of a bladder bioreactor may be the cost-effectiveness of purification compared with purification schemes required for milk or blood-borne products.

Experimental protocol

Construction of the UPII-hGH fusion gene. The 3.6-kb 5′ flanking region of the murine UPII gene was isolated from pUPII-LacZ[13] by digestion of the plasmid with Kpn I, removal of the 3′ overhang with T4 DNA polymerase, and then digestion with BamHI. The fragment was ligated to the 5′ end of the hGH structural gene between a blunted Sal I site and the BamHI site of the promoterless p0GH plasmid (Nichols Institute, San Juan Capistrano, CA). The resulting pUPII-hGH plasmid was digested with Hind III and EcoR I to isolate the 5.7-kb UPII-hGH fusion gene for microinjection.

Generation of transgenic mice. Mouse embryo collection (strain C57BL/6 x SJL), microinjection, and transfer to pseudopregnant recipients were performed by standard techniques. Offspring were screened for fusion gene incorporation by PCR using DNA from tail tips. The primers spanned the junction region between the UPII promoter region and the hGH gene (upstream primer; 5′-GGTCCAGAAAGAGCCATACTCC-3′, downstream primer; 5′GTCCACAGGACCCTGAGTG-3′). Following a 72° hot start, PCR conditions included 30 cycles of 95° for 1 min, 57.5° for 30 sec, and 72° for 30 sec.

Southern and Northern blot analysis. Transgene copy number was estimated by Southern blot analysis using 10 μg of tail DNA that had been digested with BamHI. The gel included a standard lane, which contained 10 μg of BamHI-digested, nontransgenic mouse DNA spiked with 10 pg of Hind III/EcoRI-digested pUPII-hGH. Transgene expression was analyzed by Northern blot using 15-μg samples of total RNA extracted from various tissues. A standard lane, included on each blot, contained 1 ng of human pituitary poly $(A)^+$ RNA (Clontech, Palo Alto, CA) diluted in 15 μg of nontransgenic mouse liver total RNA. Northern and Southern blots were probed with a ^{32}P-labeled hGH cDNA insert and then analyzed with a phosphoimager (Storm, Molecular Dynamics, Sunnyvale, CA).

hGH RIA. The concentration of hGH in plasma and urine samples was determined by RIA. Fresh urine samples were clarified by centrifugation (12,000 G, 10 min), diluted 1:100 in 3% BSA-PBS, and stored (-20°). Antisera to hGH (NIDDK-anti-hGH-2), purified hGH (NIDDK-hGH-RP-1) were kindly provided by the Hormone Distribution Program of the NIDDK (Bethesda, MD). Radiolabeled (^{125}I)-hGH was obtained from NEN/Dupont. All samples were run in a single assay (intra-assay C.V. = 6.0%). Recovery of hGH, added to male or female nontransgenic mouse urine, incubated at 37° for 1 h prior to clarification and assay, was greater than 95%. Recovery of hGH added to nontransgenic mouse plasma was 61%.

Immunohistochemistry. Mouse bladders were fixed in ice-cold Zamboni's solution, embedded in OCT medium (Miles Scientific, Naperville, IL), cryostat-sectioned at 7 μm, and stained by the indirect immunofluorescence technique[25]. hGH was detected using rabbit antisera to hGH (NIDDK-anti-hGH-IC-3), and mouse UPII was detected with a rabbit antiserum against total bovine uroplakins[13].

Transmission electron microscopy and immunoelectron microscopy. Mouse bladders were fixed in 2.5% paraformaldehyde in PBS, postfixed with 2% osmium tetroxide buffered with 0.1 M sodium cacodylate buffer (pH 7.4), and embedded in Epon 812 (Polysciences Inc., Warrington, PA)[26]. For mouse bladders were fixed for 4 h at 4° in a freshly prepared fixative containing 4% paraformaldehyde, 0.1% glutaraldehyde, 4% sucrose, and 0.1 M sodium cacodylate buffer (pH 7.4), followed by four 15 min washes at 4° in 0.1 M sodium cacodylate containing 4% sucrose. The embedding, sectioning, and staining were performed as described[10,25].

Total urinary protein determination. The concentration of total protein in mouse, pig, and cow urine was determined by a modification of the Lowry method[27]. Fresh urine samples were clarified by centrifugation (5 min, 12,000 G) and frozen. Thawed samples were diluted in 0.2% Triton X-100; then, total protein was precipitated with chilled TCA (final concentration, 7%) and solubilized in 1N NaOH. Bovine serum albumin was used as a standard.

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