## RESEARCH COMMUNICATION

# Uromodulin promoter directs high-level expression of biologically active human $\alpha_1$-antitrypsin into mouse urine

Halina M. ZBIKOWSKA, Nadia SOUKHAREVA, Reza BEHNAM, Henryk LUBON, David HAMMOND and Serguei SOUKHAREV[1]

Plasma Derivatives Department, Holland Laboratory, American Red Cross, 15601 Crabbs Branch Way, Rockville, MD 20855, U.S.A.

We have recently shown that the regulatory sequence of the uromodulin gene, containing the 3.7 kb promoter, exon 1 and a part of exon 2, provided for kidney-specific expression of the reporter *lacZ* gene in transgenic mice [Zbikowska, Soukhareva, Behnam, Chang, Drews, Lubon, Hammond and Soukharev (2002) Transgenic Res., in the press]. In the present study, we generated transgenic mice harbouring the regulatory sequence of the uromodulin gene to direct the expression of human $\alpha_1$-antitrypsin ($\alpha_1$AT) into urine. Of the 13 founder mice that tested positive by PCR, seven showed the presence of the human protein in their urine. The concentration of the recombinant human (rh) $\alpha_1$AT in the urine, estimated by using ELISA, ranged from 0.5 to 14 $\mu$g/ml in the $F_0$-generation mice, and reached up to 65 $\mu$g/ml in the $F_1$ generation. The transgenically produced rh $\alpha_1$AT was found to be N-glycosylated and biologically active. The N-terminal sequence analysis confirmed the identity of the human protein and revealed that the recombinant $\alpha_1$AT was correctly processed with the signal peptide cleaved off. Our results demonstrate for the first time that the uromodulin regulatory sequence provides a very attractive option for the potential large-scale production of functional therapeutic proteins in livestock.

Key words: transgenic mice, uromodulin promoter, targeting recombinant proteins into urine.

## INTRODUCTION

Using technologies developed during the last decade, the feasibility of producing human therapeutic proteins such as $\alpha_1$-antitrypsin ($\alpha_1$AT) [1,2], plasminogen activator [3] or protein C [4] in the milk of transgenic livestock have offered an attractive alternative to production in mammalian cell-culture systems. More recently, attention has focused on urine-based expression systems as a much more cost-effective technology. Successful application of this technology, however, requires the definition of several crucial regulatory elements that direct production of the protein of interest into the urinary tract. To date, two proteins, human growth hormone (GH) and granulocyte–macrophage colony-stimulating factor (GM-CSF), have been targeted into animal urine by expression into the bladder epithelium of transgenic mice [5,6]. In both studies the 3.6 kb, 5′ flanking region of the mouse uroplakin II (UPII) gene was used as a promoter. Expression of human GH into murine bladder epithelium resulted in its secretion into murine urine at 100–500 ng/ml. This low level of expression was considered inadequate to meet commercial needs. Consequently, a new regulatory element was necessary to increase the yield of recombinant protein. We used for the first time the uromodulin promoter regulatory system and our goal was to evaluate the efficiency of this new promoter for the potential application of the kidney as a protein bioreactor. The uromodulin gene seemed to be a good candidate for this purpose, since uromodulin (known as the Tamm–Horsfall protein) is recognized as the most abundant protein in human urine [7,8]. The expression of uromodulin is specific to the cells of the ascending limbs of Henle's loop in the kidney, and uromodulin mRNA is found as a major transcript of the medullar part of the kidney [9].

Our previous preliminary studies showed that a 5.6 kb fragment of the uromodulin gene, containing the 3.7 kb promoter, could provide for kidney-specific expression of the reporter *lacZ* gene in transgenic mice [10]. Here, we used the same regulatory sequences of the uromodulin gene to direct expression of human $\alpha_1$-antitrypsin into the urine of transgenic mice. We extended the preliminary studies on this model system to demonstrate the production of a therapeutically relevant protein. Moreover, the initial characterization of this protein shows it to be biologically active. $\alpha_1$AT is one of the major protease inhibitors in human plasma and is used for treatment of congenital deficiency of $\alpha_1$AT. The primary importance of $\alpha_1$AT as a therapeutic protein used for treatment of many genetically inherited diseases, including pulmonary emphysema and cystic fibrosis, has been summarized in [11].

## EXPERIMENTAL

### Construction of the expression plasmids

P1 clone #630 containing the uromodulin gene was obtained from the human genomic library at Incytes Genomic (St Louis, MO, U.S.A.). The 6.72 kb *Eco*RI fragment from the P1 clone, comprising the uromodulin promoter, exons 1 and 2, was ligated to the pBlueScript II KS+ at the *Eco*RI site. Sites *Sca*I and *Cla*I were introduced directly in front of the ATG codon and were used for cloning the encoding fragment of the $\alpha_1$AT genes. The 9.5 kb *Eco*RI fragment of the #24665 clone containing the complete $\alpha_1$AT gene, which was isolated by Incytes Genomic,

Abbreviations used: rh $\alpha_1$AT, recombinant human $\alpha_1$-antitrypsin; GH, growth hormone; GM-CSF, granulocyte–macrophage colony-stimulating factor; UPII, uroplakin II; PNGase F, peptide N-glycosidase F; PPE, porcine pancreatic elastase; ARC, American Red Cross.

[1] To whom correspondence should be addressed (e-mail Soukharevs@usa.redcross.com).

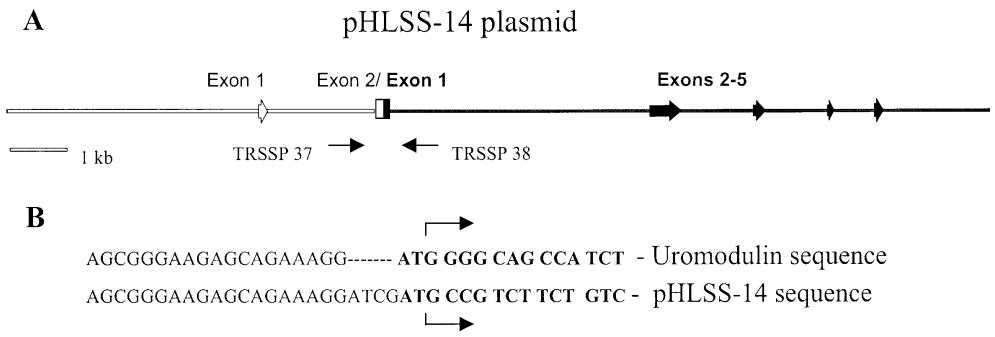


**Figure 1 (A) Schematic diagram of the expressing vector and (B) the sequence around the ATG codon for the uromodulin and the pHLSS14 plasmid**

(**A**) The uromodulin part is indicated as an open bar and the blocks (exons); the $\alpha_1$AT part is indicated as a dark bar and the blocks. (**B**) Start of translation is indicated by arrow.

was subcloned into the pBlueScript II KS vector. The *Cla*I site was introduced directly in front of the ATG codon. The map of the final plasmid, pHLSS 14, and the sequence around the ATG codon, are presented in Figures 1(A) and 1(B).

### Production of transgenic mice

CD-1 mice obtained from Charles River Laboratories (Wilmington, MA, U.S.A.) were used as the donors and the recipients of the eggs. A linear fragment of DNA containing the transgenic construct was separated from the pBluescript DNA in a 0.8 %-agarose gel. The DNA band was purified from the gel using the Gel Extraction Kit (Qiagen). Purified DNA was microinjected into the mouse eggs by a standard procedure [12]. All mice were raised and kept under specific-pathogen-free conditions. The genomic DNA was extracted from the offspring by an ear biopsy and the founders were identified by PCR with the specific primers. TRSSP 37 (AAA GCA CTC CTT CCA GCT GTG G) and TRSSP 38 (GAG GTT GAA ATT CAG GCC CTC C) primers were used for detection of transgenic animals.

### Urine and blood collection, Western-blot analysis and measurement of recombinant human (rh) $\alpha_1$AT by ELISA

Urine samples were collected daily from an individual mouse according to the standard procedure [13]. Fresh urine samples were clarified by centrifugation (14000 ***g***, 10 min), desalted on Micro Bio-Spin chromatography columns (Bio-Rad) and stored (−80°C) until analysed. Concentrations of human $\alpha_1$AT in urine were measured by Western blot and by ELISA. For Western blotting, desalted urine samples were freeze-dried and redissolved in a sample buffer. The proteins were separated by SDS/PAGE on 8–16 % (w/v) gels under reducing conditions [14] and transferred on to a nitrocellulose membrane in Tris/Glycine buffer/20 % methanol [15]. The blots were blocked with 10 % non-fat dry milk in TBS-T [20 mM Tris/137 mM NaCl (pH 7.5)/0.1 % Tween 20]. Human $\alpha_1$AT was detected using a peroxidase-conjugated goat IgG fraction to human $\alpha_1$AT (ICN Pharmaceuticals) and the LumiGLO Chemiluminescent Substrate Kit (Kirkegaard & Perry Laboratories, Gaithersburg, MD, U.S.A.). For the ELISA assays, mouse anti-human $\alpha_1$AT antibodies (US Biologicals) were used as the capture antibody at a concentration of 2.5 $\mu$g/ml. Goat anti-human $\alpha_1$AT horseradish peroxidase-conjugated antibodies (US Biologicals) were diluted 1:5000 and applied as the detection antibody. Commercially available $\alpha_1$AT from human plasma (Calbiochem) was used as a standard.

Samples of blood were obtained from the retro-orbital venous plexus by using Pasteur pipettes wetted with EDTA. Plasma was obtained by centrifugation of fresh blood anticoagulated with 50 mM EDTA (1:9, v/v). The ELISA, as described previously, was used to estimate the concentration of human $\alpha_1$AT in mouse plasma.

### Deglycosylation of rh $\alpha_1$AT

Samples of desalted urine (an equivalent of approx. 150 ng of $\alpha_1$AT in a total volume of 25 $\mu$l) were deglycosylated with peptide N-glycosidase F (PNGase F; New England BioLabs) according to the manufacturer's protocol. Briefly, 25 units of PNGase F was added to each pre-denaturated urine sample and the mixture was incubated for 1.5 h at 37 °C. Alternatively, control samples of urine were prepared and treated in exactly the same way, except that no PGNase F was added. All samples were separated by SDS/PAGE and then analysed by Western blotting as described above.

### N-terminal amino acid sequence determination

A desalted sample of urine from the transgenic mouse, which was then deglycosylated with PNGase F was subjected to SDS/8 %-PAGE and electroblotted on to a ProBlott membrane (Applied Biosystems) according to the manufacturer's protocol. Proteins were visualized by Coomassie Brilliant Blue staining, and a band corresponding to the human $\alpha_1$AT was cut out. The N-terminal sequence of the separated protein was performed using a model 1494 Procise protein sequencer (Applied Biosystems).

### rh $\alpha_1$AT activity assays

Two bioassays were applied to test the biological activity of the rh $\alpha_1$AT. These included the Western-blot analysis of the complexes formed between the proteinase inhibitor and a porcine pancreatic elastase (PPE), as well as the elastase-inhibitory activity of the rh $\alpha_1$AT. In both assays urine from transgenic mice was compared with control mouse urine spiked with plasma-derived human $\alpha_1$AT obtained from the American Red Cross (ARC). To analyse the PPE–$\alpha_1$AT complexes, transgenic-mouse urine (an equivalent of 100 ng of the proteinase inhibitor) or control-mouse urine spiked with the 100 ng of the plasma-derived $\alpha_1$AT was mixed with PPE (Sigma) diluted with Tris/HCl buffer, pH 8.0, at different molar ratios. The mixture was incubated for 10 min at room temperature, then the reaction was stopped by adding the sample buffer with 2 % $\beta$-mercapto-

ethanol. After boiling for 5 min, samples were separated by SDS/PAGE, and then analysed by Western blot as described above.

The elastase-inhibitory activity of the rh $\alpha_1$AT was measured using EnzCheck Elastase Assay Kit (Molecular Probes) following the manufacturer's protocol. A known amount of the PPE (0.1 unit/ml) was titrated with the increasing volumes of transgenic-, control- or $\alpha_1$AT-spiked-mouse urine. Aliquots of the DQ elastin substrate at the final concentration of 25 $\mu$g/ml were added and the samples were incubated for 3 h at room temperature. Fluorescence was measured using the Spectra Max GeminiXS fluorescence microplate reader for excitation at 485 nm, and emission detection at 530 nm. Background fluorescence, determined for a no-enzyme control reaction, has been subtracted from each value. The fluorescence of the no-proteinase-inhibitor control was taken as 100 %. The concentration of the $\alpha_1$AT in urine of transgenic mice was calculated from ELISA estimates.

## RESULTS AND DISCUSSION

### Generation of transgenic mice

The primary structure of the uromodulin promoter has been recently elucidated by subcloning and sequencing the human genomic fragment containing the promoter itself and the two first exons [10]. Since there are more than 1 million nephrons in each kidney, targeting of a protein into the cells lining the thick limb of Henle's loop seems to be more productive than targeting proteins to be expressed in the bladder epithelium. In an attempt to improve the yield of the urinary expression system, we have generated transgenic mice carrying the $\alpha_1$AT construct under the regulatory sequence of the uromodulin promoter. A general map of the transgene construct is presented in Figure 1(A). Of 75 mice obtained after the microinjection of pHLSS14 DNA, 13 founders tested positive by PCR with TRSSP 37–TRSSP 38 oligonucleotides.

### Production of human $\alpha_1$AT in urine

Of all 13 founders, seven animals showed the presence of the human protein in their urine. Estimates of the total rh $\alpha_1$AT production, made by comparison of the band intensities in transgenic-mouse urine samples with the intensities of $\alpha_1$AT-spiked-control-mouse urine, ranged from approx. 0.5 $\mu$g/ml to 20 $\mu$g/ml among the founders (Figure 2A). The measurements of the expression level of rh $\alpha_1$AT by ELISA (Table 1) confirmed the values estimated by Western blot. No cross-reactivity with the mouse $\alpha_1$AT was observed, since $\alpha_1$AT was not detected in the serum samples of the non-transgenic control mice (Figure 2A). After breeding with intact CD-1 mates, six founders were found to have transmitted the transgene to the F1 generation. No pups were obtained from founder #3767. Generally, the F1 generation of transgenic mice has produced similar or higher levels of human $\alpha_1$AT in urine when compared with the founder mouse (Figure 2B and Table 1). Of offspring coming from the best founder (#3481), three mice (#1817, #1819 and #1820) had expression levels up to 35 $\mu$g/ml of rh $\alpha_1$AT in their urine, and one mouse (#1809) had up to 65 $\mu$g/ml in his urine. The production of rh $\alpha_1$AT in the urine of founders and $F_1$ generation of transgenic mice was found to be relatively constant during 4 months, with variations of 100–200 % during different days of urine collection (results not shown). Of all founders and tested offspring, only two founder mice were noted to have rh $\alpha_1$AT in their plasma. In the plasma of mouse #3333, 0.29 $\mu$g/ml of the human protein was found. The concentration of rh $\alpha_1$AT in plasma of founder #3767 was approx. 10-fold lower (0.035 $\mu$g/ml).

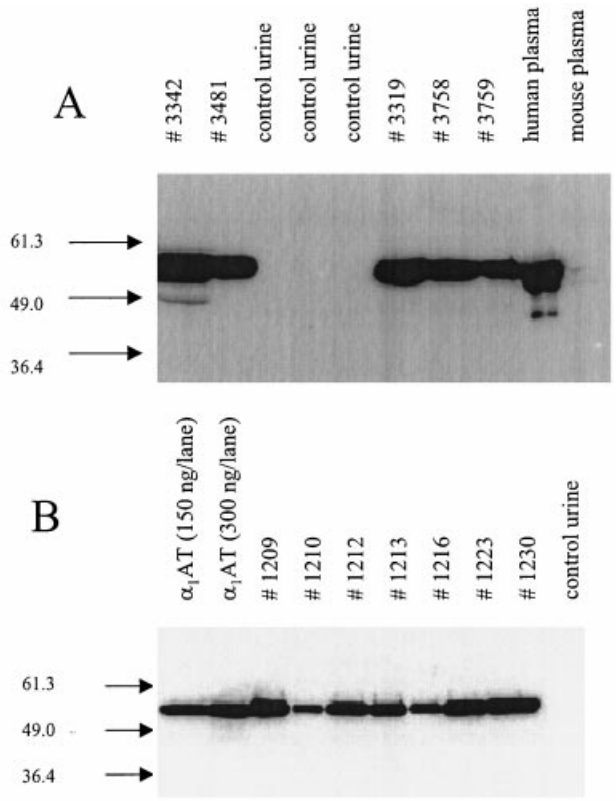


**Figure 2 (A) Expression of the rh $\alpha_1$AT into urine of transgenic mice and (B) Western-blot analysis of the urine samples from the transgenic F1 offspring**

(**A**) Protein from 50 $\mu$l (except from sample #3481, 15 $\mu$l) of the urine sample from transgenic founders was analysed by Western blot. The molecular size markers are shown as kDa. A 10 $\mu$l portion of human or mouse plasma was loaded on to the gel. (**B**) Protein from 25 $\mu$l of urine was loaded on to the gel.

A 5.7 kb fragment of the uromodulin promoter was demonstrated to be sufficient for the effective production of up to 65 $\mu$g/ml of the rh $\alpha_1$AT protein in the urine of transgenic mice. The expression level of the rh $\alpha_1$AT in the urine of the mice was two orders of magnitude higher than that observed in transgenic mice harbouring the UPII promoter fused to a human-GH gene (up to 500 ng/ml) [6] or to a human GM-CSF gene (up to 180 ng/ml) [7]. On the other hand, 10-fold (up to 100-fold) higher concentration of the rh $\alpha_1$AT (0.5 mg/ml; up to 7 mg/ml in one transgenic line) has been reported in the milk of mice transgenic for the ovine $\beta$-lactoglobulin gene/human $\alpha_1$AT sequences [19].

### The N-terminal amino acid sequence of rh $\alpha_1$AT

To confirm the identity of the rh $\alpha_1$AT, N-terminal amino acid sequencing was performed. For this purpose, the rh $\alpha_1$AT was separated from the urine sample of founder #3481 and sequenced. The N-terminal amino acid sequence of the urine-derived rh $\alpha_1$AT has been identified as EDPQGDAAQKT (Figure 3A). This corresponds to the completely processed human $\alpha_1$AT, indicating the correct cleaving of the signal peptide. For comparison, there is a low homology with the N-terminal sequence of the processed murine $\alpha_1$AT (EDVQETDTSQK).

**Table 1 The expression level of rh $\alpha_1$AT in urine of the transgenic founders and $F_1$ generation determined by ELISA**

PCR-positive founders and $F_1$ generation mice are indicated by the **bold** and regular font respectively. Data on the $F_1$-generation mice originating from their respective founders are located within each relevant block (M, male; F, female).

| Mouse identification | [rh $\alpha_1$AT] ($\mu$g/ml) |
|---|---|
| **3758 F** | **1.42 ± 0.89** |
| 3065 F | 4.82 ± 0.18 |
| **3759 M** | **0.41 ± 0.03** |
| 3077 M | 1.13 ± 0.21 |
| **3767 M** | 2.63 ± 0.74 |
| – | – |
| **3319 F** | **2.57 ± 1.06** |
| 1230 M | 10.08 ± 2.88 |
| 1232 M | 7.09 ± 1.46 |
| 1233 F | 23.88 ± 1.36 |
| **3333 M** | **6.73 ± 2.10** |
| 1209 F | 12.33 ± 2.62 |
| 1210 F | 6.19 ± 2.01 |
| 1212 M | 10.05 ± 1.81 |
| 1213 F | 11.94 ± 3.22 |
| **3342 M** | **4.92 ± 2.67** |
| 1216 M | 8.52 ± 0.44 |
| 1223 M | 10.36 ± 1.04 |
| **3481 M** | **13.88 ± 3.32** |
| 1809 M | 64.05 ± 14.51 |
| 1817 M | 34.52 ± 7.36 |
| 1819 F | 31.89 ± 6.85 |
| 1820 F | 38.50 ± 12.42 |

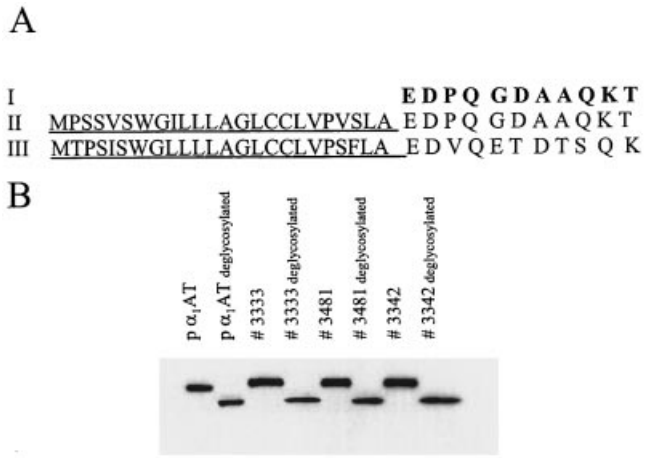


**Figure 3 (A) N-terminal sequencing of the urine-derived $\alpha_1$AT and (B) Western-blot analysis of the glycosylated and deglycosylated $\alpha_1$AT**

(**A**) I, the estimated sequence of the urine-derived $\alpha_1$AT; II, theoretical sequence of the $\alpha_1$AT from human plasma; III, theoretical sequence of the mouse $\alpha_1$AT. The signal peptides for the human and mouse $\alpha_1$AT sequence are underlined. (**B**) Abbreviation: p $\alpha_1$AT, plasma-derived $\alpha_1$AT.

## Glycosylation of rh $\alpha_1$AT

The electrophoretic pattern of the rh $\alpha_1$AT produced by transgenic mice appeared to be very similar to the $\alpha_1$AT present in human plasma (Figures 2A and 2B). One major band at a molecular mass of 56.5 ± 2.6 kDa, versus 53.7 ± 2.7 kDa for the plasma-derived $\alpha_1$AT, was detected. An additional slightly more visible band at the lower molecular mass probably represented a degraded form of the protein (Figure 2A, founder #3342). Since the N-terminal sequence analysis confirmed that the urine-derived rh $\alpha_1$AT was processed in a correct manner and a signal peptide of the foreign protein was properly cleaved off, the detected discrepancy in the molecular mass between the glycosylated recombinant and the plasma-derived $\alpha_1$AT might have come from some differences in the glycosylation potency of the mouse kidney and human liver cells.

After de-glycosylation with PNGase F, no differences in electrophoretic patterns were noted between the plasma-derived and the transgenic-mouse $\alpha_1$AT produced by all three tested founders (Figure 3B). The apparent molecular mass of 43.8 ± 1.5 kDa has been estimated for the deglycosylated samples of different origin. Thus our data indicate that rh $\alpha_1$AT was glycosylated. Three N-linked carbohydrate side chains in human $\alpha_1$AT at positions 46, 83 and 247 of the polypeptide were identified [16]. The minor discrepancy in the electrophoretic mobility between the plasma-derived and transgenic-mouse glycosylated $\alpha_1$AT is most likely due to differences in the total oligosaccharide content. Shorter incubation with PNGase F yielded three bands in the case of both plasma-derived and transgenic material (results not shown), suggesting that all samples possessed three carbohydrate chains.

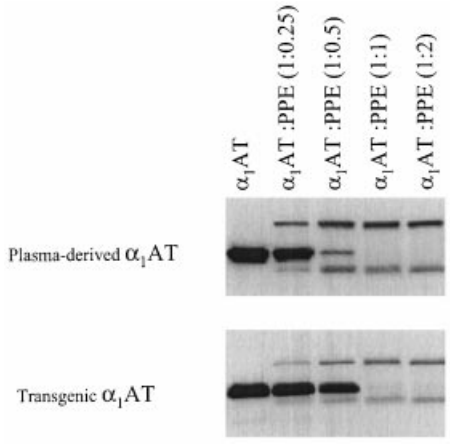


**Figure 4 Complex formation between the $\alpha_1$AT and PPE**

The sample preparation and the Western-blot procedure were as detailed in the Experimental section.

## Activity of the urine-derived rh $\alpha_1$AT

It is well known that plasma-derived $\alpha_1$AT inhibits elastase, its target proteinase, with the formation of a stable equimolar complex [17]. As shown in Figure 4, the rh $\alpha_1$AT formed a complex with elastase in a pattern similar to the plasma-derived $\alpha_1$AT. In both cases, at an $\alpha_1$AT/PPE molecular ratio of 1:1, all of the proteinase inhibitor was converted into an SDS-stable complex of a higher molecular mass and to a proteolytically modified form of the inhibitor of a lower molecular mass. This reaction constitutes a typical interaction of $\alpha_1$AT with elastase. The majority of the proteinase inhibitor is converted into a complex; however, a part of it serves as a substrate for the

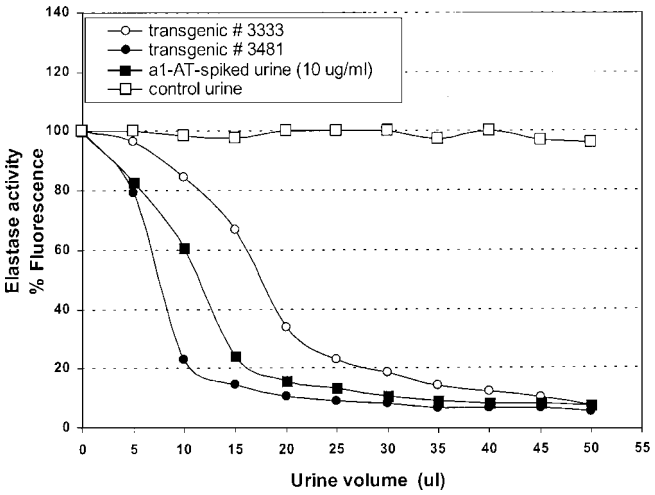


**Figure 5 Elastase inhibition assay using the EnzCheck Elastase Assay Kit (Molecular Probes)**

The elastase-inhibitory capacity was measured as described in the Experimental section.

elastase to form a product in which inhibitory capacity is abolished [17,18]. Elastase loaded on to the gel alone did not react with the antibody used in the assay (results not shown).

In an additional assay, PPE activity was measured after incubation with a fluorescent substrate in the presence of either transgenic- or control-mouse urine spiked with plasma-derived $\alpha_1$AT. The recombinant $\alpha_1$AT produced in mouse urine was shown to be very similar to a plasma-derived proteinase inhibitor in its ability to inhibit elastase (Figure 5). The mouse urine proteins and urine itself did not inhibit elastase, whereas the urine from transgenic-mice and $\alpha_1$AT-spiked urine demonstrated significant inhibition of elastase. The $IC_{50}$ calculated for the $\alpha_1$AT-spiked urine, transgenic mouse #3481 and transgenic mouse #3333 urine were 0.61, 0.60 and 0.55 $\mu$g/ml respectively. The results obtained indicate that the minor differences in glycosylation and/or other potential post-translational modifications did not alter the activity of the rh $\alpha_1$AT.

Previous studies have shown the possibility of accumulating active therapeutic proteins in urine using an uroplakin promoter [6,7]. Several possible advantages of the urinary system have been pointed out in previous reports [5,20]. First, the product could be harvested shortly after birth, throughout the life of the animal, and from both sexes. Secondly, since the concentration of protein and fat in urine is marginal, it will be possible to simplify the isolation and purification process. With these benefits, even a 100-fold lower yield production, when compared with milk-based systems, makes a urinary technology applicable, even advantageous, on an industrial scale. Our results demonstrate that the quantitative effectiveness of a urine-based bioreactor is comparable with milk-based systems when the regulatory sequence of the uromodulin promoter is applied. Therefore the use of the uromodulin regulatory sequence makes a urine-based bioreactor a very attractive option for the potential large-scale production of functional therapeutic proteins in livestock.

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## REFERENCES

1 Wright, G., Carver, A., Cottom, D., Reeves, D., Scott, A., Simons, P., Wilmut, I., Garner, I. and Colman, A. (1991) High level expression of active human alpha-1-antitrypsin in the milk of transgenic sheep. Bio/Technology **9**, 830–834
2 Carver, A. S., Dalrymple, M. A., Wright, G., Cottom, D. S., Reeves, D. B., Gibson, Y. H., Keenan, J. L., Barrass, J. D., Scott, A. R., Colman, A. et al. (1993) Transgenic livestock as bioreactors: stable expression of human alpha-1-antitrypsin by a flock of sheep. Bio/Technology **11**, 1263–1270
3 Ebert, K. M., Selgrath, J. P., DiTullio, P., Denman, J., Smith, T. E., Memon, M. A., Schindler, J. E., Monastersky, G. M., Vitale, J. A. and Gordon, K. (1991) Transgenic production of a variant of human tissue-type plasminogen activator in goat milk: generation of transgenic goats and analysis of expression. Bio/Technology **9**, 835–838
4 Velander, W. H., Johnson, J. L., Page, R. L., Russell, C. G., Subramanian, A., Wilkins, T. D., Gwazdauskas, F. C., Pittius, C. and Drohan, W. N. (1992) High-level expression of a heterologous protein in the milk of transgenic swine using the cDNA encoding human protein C. Proc. Natl. Acad. Sci. U.S.A. **89**, 12003–12007
5 Kerr, D. E., Liang, F., Bondioli, K. R., Zhao, H., Kreibich, G., Wall, R. J. and Sun, T.-T. (1998) The bladder as a bioreactor: urothelium production and secretion of growth hormone into urine. Nat. Biotechnol. **16**, 75–79
6 Ryoo, Z. Y., Kim, M. O., Kim, K. E., Bahk, Y. Y., Lee, J. W., Park, S. H., Kim, J. H., Byun, S. J., Hwang, H. Y., Youn, J. and Kim, T. Y. (2001) Expression of recombinant human granulocyte macrophage-colony stimulating factor (hGM-CSF) in mouse urine. Transgenic Res. **10**, 193–200
7 Pennica, D., Kohr, W. J., Kuang, W. J., Glaister, D., Aggarwall, B. B., Chen, E. Y. and Goeddel, D. V. (1987) Identification of human uromodulin as the Tamm–Horsfall urinary glycoprotein. Science **236**, 83–87
8 Kumar, S. and Muchmore, A. (1990) Tamm–Horsfall protein – uromodulin (1950–1990). Kidney Int. **37**, 1395–1401
9 Virlon, B., Cheval, L., Buhler, J. M., Billon, E., Doucet, A. and Elalouf, J. M. (1999) Serial microanalysis of renal transcriptomes. Proc. Natl. Acad. Sci. U.S.A. **96**, 15286–15291
10 Zbikowska, H. M., Soukhareva, N., Behnam, R., Chang, R., Drews, R., Lubon, H., Hammond, D. and Soukharev, S. (2002) Using uromodulin promoter to target production of recombinant proteins into urine of transgenic animals. Transgenic Res., in the press
11 Doring, G. (1999) Serine proteinase inhibitor therapy in $\alpha_1$-antitrypsin inhibitor deficiency and cystic fibrosis. Pediatr. Pulmonol. **28**, 363–375
12 Hogan, B., Constantini, F. and Lacy, E. (1986) Microinjection of DNA into pronuclei. In Manupulating the Mouse Embryo, A Laboratory Manual (Hogan, B., Beddington, R., Costantini, F. and Lacy, E., eds.), pp. 157–182, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
13 Kurien, B. T. and Scofield, R. H. (1999) Mouse urine collection using clear plastic wrap. Lab. Anim. **33**, 83–86
14 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**, 680–685
15 Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354
16 Travis, J. and Salvesen, G. S. (1983) Human plasma proteinase inhibitors. Annu. Rev. Biochem. **52**, 655–709
17 Gettins, P., Patston, P. A. and Schapira, M. (1992) Structure and mechanism of action of serpins. Hematol./Oncol. Clin. North Am. **6**, 1393–1408
18 Mast, A. E., Enghild, J. J., Pizzo, S. V. and Salvesen, G. (1991) Analysis of the plasma elimination kinetics and conformational stabilities of native, proteinase-complexed, and reactive site cleaved serpins: comparison of alpha-1-proteinase inhibitor, alpha-1-antichymotrypsin, antithrombin III, alpha-2-antiplasmin, angiotensinogen, and ovalbumin. Biochemistry **30**, 1723–1730
19 Archibald, A. L., McClenaghan, M., Hornsey, V., Simons, J. P. and Clark, A. J. (1990) High-level expression of biologically active human alpha-1-antitrypsin in the milk of transgenic mice. Proc. Natl. Acad. Sci. U.S.A. **87**, 5178–5182
20 Meade, H. and Ziomek, C. (1998) Urine as a substitute for milk? Nat. Biotechnol. **16**, 21–22