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High-level expression of biologically active human $a_1$-antitrypsin in the milk of transgenic mice

(embryos/ elastase/ antiprotease/ recombinant DNA/ therapeutic proteins)

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ABSTRACT Reduced circulating levels of $a_1$-antitrypsin ($a_1$AT) are associated with certain $a_1$AT genotypes and increased susceptibility to emphysema. Unfortunately, the amounts of $a_1$AT that would be required for replacement therapy are beyond the capacity of plasma fractionation and mammalian cell culture systems. Thus, we have examined the potential of transgenic animals as an alternative means of producing human $a_1$AT. A hybrid gene constructed by using sequences from the ovine milk protein gene $\beta$-lactoglobulin fused to an $a_1$AT “minigene” was used to generate transgenic mice. Of 13 independent transgenic mice and mouse lines, 5 expressed the hybrid gene in the mammary gland, 5 in the salivary glands, and 2 in both these tissues. Human $a_1$AT was secreted into the milk of each of the 7 mice and mouse lines that expressed the hybrid gene in the mammary gland. Four of these mammary-expressing transgenic mice and mouse lines produced concentrations of at least 0.5 mg of $a_1$AT per ml in their milk; one line (AAATB 35) produced 7 mg of this protein per ml. $a_1$AT from transgenic mouse milk was similar in size to human plasma-derived $a_1$AT and showed a similar capacity to inhibit trypsin. Expression at equivalent levels in transgenic sheep or cattle would yield sufficient $a_1$AT for therapeutic purposes.

Genetic deficiencies of $a_1$-antitrypsin ($a_1$AT) in humans are common and result in an increased susceptibility to emphysema (1). Human $a_1$AT is a 394-amino acid glycoprotein that acts as a suicide inhibitor of a wide range of serine proteases. In humans, the $a_1$AT gene is expressed in a variety of tissues, including macrophages, kidney, small intestine, pancreas, and liver; the latter is the primary site of expression (1, 2). In normal humans, more than 2 g of $a_1$AT is synthesized daily, resulting in a serum concentration of $\approx 2$ mg/ml.

The primary function of $a_1$AT is to inhibit neutrophil elastase and thus prevent this protease from causing excessive tissue damage (1). The S and Z $a_1$AT alleles are relatively common ($=0.03$ and 0.02, respectively) and encode proteins that have reduced stability (S) or are poorly secreted (Z), although they exhibit normal antiprotease activity. Individuals with the SZ and ZZ genotypes have significantly reduced concentrations of $a_1$AT ($<0.8$ mg/ml) and are at risk of developing the degenerative lung disease emphysema, particularly if they smoke.

Since $a_1$AT normally circulates at 2 mg/ml and has a half-life of 6 days, considerable quantities ($=4$ g per week per patient) would be required for replacement therapy for afflicted individuals (3), which amounts to 4000–8000 kg annually to treat the ZZ homozygote population of the United States (4). Such large amounts of protein will be available only if recombinant DNA technology is used for production. However, although $a_1$AT does not require its carbohydrate side chains for activity, the in vivo half-life of nonglycosylated $a_1$AT (expressed in yeast) is 50-fold lower than that of plasma-derived $a_1$AT (4). Therefore it would seem prudent to produce $a_1$AT in a mammalian expression system capable of making the appropriate posttranslational modifications. Unfortunately, large-scale culture of mammalian cells is expensive and technically demanding and thus far has failed to match the yields necessary for high dosage therapeutics, as exemplified by $a_1$AT.

As an alternative to genetically engineered cell lines, Palmiter et al. (5) proposed that valuable proteins could be harvested from transgenic animals. We have argued that the mammary gland is the organ of choice for the expression of recombinant proteins (6, 7) because large amounts of protein can be synthesized by the mammary gland, secreted into milk, and collected easily without detriment to the animal. We have decided to use sheep for this purpose and have recently demonstrated the production of human factor IX and $a_1$AT in the milk of transgenic sheep (8, 9). In these sheep, and also in transgenic mice carrying the same hybrid genes, the levels of expression of the transgenes were low. The comparisons of the performance of these hybrid genes (FIXA and AATA) in transgenic sheep and mice are the subject of separate studies (M.M., A.L.A., S. Harris, J.P.S., B. White- law, I. Wilmut, and A.J.C., unpublished results; M.M., H. Bassos, C. Prowse, J.P.S., B. Whitalow, I. Wilmut, and A.J.C., unpublished results).

As money and time preclude the use of large animals to test and refine DNA constructs for efficient expression, we have elected to carry out these experiments in transgenic mice. We previously showed that the gene encoding a sheep milk protein, $\beta$-lactoglobulin (BLG), was expressed efficiently and abundantly in the mammary gland of transgenic mice (10). Here we show that sequences derived from this gene can be used to direct expression of human $a_1$AT sequences in the mammary gland, yielding high levels of human $a_1$AT in milk.

METHODS

Hybrid Gene Construction and Production of Transgenic Mice. A hybrid gene (referred to as AATB, see Fig. 1) was elaborated in which the $Pvu$ II site within the 5′ untranslated sequences of the ovine BLG clone SSI (11, 12) was fused to the Taq I site in the 5′ untranslated sequences of $a_1$AT. The first $a_1$AT intron was excluded by using DNA sequences from a cDNA clone, p81ppg, which encodes the M1 variant of $a_1$AT (13), as the source of the first 80 base pairs of $a_1$AT sequences, extending up to the BamHI site in the second exon. The remainder of the $a_1$AT “minigene” comprises a

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6.5-kilobase (kb) BamHI fragment from the human α₁AT genomic clone pATp7 [also encoding the M₁ variant of α₁AT (14)]. The construct was elaborated in the vector pPOLY-III-1 (15), enabling excision of the 10.6-kb insert by using Not I sites in the polylinker sequences. Gel-purified insert DNA was microinjected into pronuclear mouse eggs [collected from (C57BL/6 × CBA)F₁ mice after mating with F₁ stud males] in order to generate transgenic mice (10, 16). Lines were propagated by mating with F₁ mice.

**DNA and RNA Analysis.** DNA (for Southern blot analysis) prepared from tail biopsies was digested with restriction enzyme(s), subjected to agarose gel electrophoresis, blotted to Hybond N (Amersham) nylon membranes, and probed with 32P-labeled AATB DNA sequences. RNA was prepared from lactating mice 11 days after parturition by standard methods (17, 18). Aliquots (10 μg) of total RNA were fractionated on denaturing Mops/formaldehyde (1–1.5%) agarose gels, transferred to Hybond N membranes (Amersham), and probed with a 32P-labeled 243-base-pair TaqI–Pst I fragment derived from the 3' end of p821pG (13), which allows mouse and human α₁AT mRNAs to be distinguished. RNA probes were labeled by using random primers (19), and hybridizations were carried out as described by Church and Gilbert (20).

**Analysis of Milk.** Milk was collected from lactating females 11 days after parturition as described by Simons et al. (10). Mouse milk was diluted 1:5 in distilled water, and fat was removed after centrifugation. To prepare whey, 1.0 M HCl was added to give a final pH of 4.5, to precipitate the caseins, which were then removed by centrifugation.

Diluted milk or whey samples were solubilized by boiling in loading buffer prior to discontinuous SDS/polyacrylamide (8% or 10%) gel electrophoresis (21) and immunoblotting analysis (22). Human α₁AT was identified on immunoblot filters by using goat anti-α₁AT serum [Protein Reference Unit (PRU), Royal Hallamshire Hospital, Sheffield S10 2JF] and anti-sheep/goat IgG serum conjugated to horseradish peroxidase [Scottish Antibody Production Unit (SAPU), Law Hospital, Carluke, Lanarkshire, ML8 5ES]. Amounts of human α₁AT in mouse milk were measured by radial immunodiffusion (RID) and radioimmunoassay (RIA). RID estimates were obtained by using LC-Partigen RID plates (Behring Diagnostics) according to the manufacturer’s instructions. RIAś were performed according to standard procedures (23) with goat anti-human α₁AT antiserum (PRU) and donkey anti-goat IgG (SAPU). Human α₁AT, purified from plasma by using a modification of the method described by Laurell et al. (24), was iodinated by using chloramine T and used as the tracer, and pooled human plasma was employed for calibration. The detection limits of these methods were 40 μg/ml (RID) and 5 μg/ml (RIA), respectively, when applied to defatted murine milk samples, and results were validated by using known amounts of human plasma/serum added to control mouse milk.

**Trypsin Inhibitory Activity.** Dilutions of defatted milk or plasma (40 μl) were incubated at room temperature with

![Fig. 1.](image1)

**Fig. 1.** The AATB construct comprises ≈4.0 kb of the 5' end of the ovine BLG clone SSI (11, 12) fused to a minigene encoding human α₁AT. Thick line, 5' BLG sequences; open box, BLG exon 1 sequences; hatched boxes, α₁AT exons; thin lines, α₁AT introns and 3' flanking regions. The position of the BLG TATA box and also the α₁AT initiation codon, stop codon, and polyadenylation site are shown.

**RESULTS**

**Generation of Transgenic Mice.** To direct expression of α₁AT to the mammary gland of transgenic mice, a hybrid gene (AATB) was elaborated, comprising, ≈4.0 kb of the 5' end of the ovine BLG gene fused to a minigene encoding human α₁AT (Fig. 1). The hybrid gene (AATB) was microinjected into pronuclei of fertilized mouse eggs (n = 993). Analysis of RNA prepared from tail biopsies showed that 21 of the 122 generation zero (G₀) animals carried the AATB construct.

**Expression of the AATB Transgene.** Expression of the transgene was assessed by analyzing RNA and milk from lactating females that were generally either G₀ animals or the transgenic G₁ offspring of G₀ males. Three patterns of human α₁AT RNA expression were observed after Northern blot analysis (Fig. 2). In some animals and lines, expression was limited to the mammary gland, whereas in others it was confined to the salivary gland. There were two lines where transcripts were seen in both the mammary and the salivary glands (Table 1). As judged by comparison with human liver RNA and HepG2 RNA, both mammary and salivary transcripts were, as expected, the same size as human liver α₁AT mRNA. One line in particular, AATB 35, showed extremely high levels of expression of α₁AT mRNA in the mammary gland, comparable to the level observed in human liver.

**Production of Human α₁AT in Milk.** Milk was analyzed by SDS/PAGE and immunoblotting for the presence of human α₁AT protein (Fig. 3a). Human α₁AT was present in milk from all the transgenic animals that had detectable levels of human α₁AT mRNA in the mammary gland but was not detected in those that did not express the transgene or expressed it only in the salivary gland. The antisem to human α₁AT cross-reacted with an endogenous mouse protein present in milk, probably murine α₁AT. The most
Table 1. Summary of the pattern of expression of AATB RNA in transgenic mice

<table>
<thead>
<tr>
<th>Animal/line</th>
<th>Sex*</th>
<th>Copy no.</th>
<th>Mammary</th>
<th>Salivary</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>M</td>
<td>3</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>17</td>
<td>M</td>
<td>10</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>26</td>
<td>M</td>
<td>20</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>28</td>
<td>M</td>
<td>3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>35</td>
<td>M</td>
<td>1</td>
<td>+</td>
<td>+</td>
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<tr>
<td>44</td>
<td>F</td>
<td>15</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>45</td>
<td>F</td>
<td>2</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>65</td>
<td>F</td>
<td>3</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>69</td>
<td>F</td>
<td>2</td>
<td>+</td>
<td>-</td>
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<tr>
<td>78</td>
<td>M</td>
<td>10</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>79</td>
<td>M</td>
<td>15</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>105</td>
<td>F</td>
<td>20</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>107</td>
<td>F</td>
<td>15</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Expression was analyzed by Northern blot analysis of tissues from lactating G0 females or, where the founder was male, from G1 females that had inherited the transgene. No human α1AT RNA was detected in liver, spleen, kidney, or heart. Copy numbers were estimated by Southern blotting relative to copy number controls.

*Sex of the G0 animals.
†α1AT transcripts were detected only in poly(A)* RNA in one of two animals analyzed.

prominent human α1AT bands in transgenic mouse milk had electrophoretic mobilities similar, but not identical, to the major bands observed in samples of purified human α1AT or pooled human plasma.

The concentrations of human α1AT in transgenic mouse milk were measured by RID and RIA (Table 2). The results obtained with the two methods of measurement were similar. Concentrations ranged from 6 μg/ml (mouse 15.20) to more than 7 mg/ml (mouse 35.3). Of the seven animals and lines that expressed the transgene in the mammary gland, four yielded concentrations of α1AT of at least 0.5 mg/ml.

**Milk from Transgenic Mice Has Enhanced Trypsin-Inhibitory Activity.** Milk samples from line AATB 35 were shown to have high levels of trypsin-inhibitory activity when compared with milk from nontransgenic mice (Fig. 4a). When milk from line AATB 35 mice was compared with human plasma, it was evident that equivalent amounts of plasma and milk α1AT had similar biological activities (Fig. 4b). Milk from line AATB 17 mice was also shown to have greater levels of antitrypsin activity than milk from control mice. The trypsin-inhibitory capacities of milk from lines 17 and 35 were in accord with expectations based on the α1AT contents of these milks as measured by immunological methods.

**DISCUSSION**

For the reasons outlined above, we sought to harness the high protein synthetic capacity of the mammary gland of transgenic animals as a source of recombinant α1AT. To this end, we elaborated a hybrid gene (AATB) by fusing the promoter and 5' flanking sequences from the abundantly expressed ovine milk protein BLG to a human α1AT minigene. The construction of such hybrid genes and their excision from vectors is eased if the component sequences are kept as short as possible. However, introns have been found to be important for the expression of transgenes (ref. 27; B. Whitelaw, M.M., A.L.A., S. Harris, J.P.S., and A.J.C., unpublished results). Nevertheless, the deletion of some intron(s) may still allow high-level expression while facilitating transgene construction. The omission of the first α1AT (5.3-kb) intron made the elaboration of the construct simpler and excluded a 429-base-pair open reading frame, an Alu repeat, and a pseudo transcription initiation sequence (28).

![Fig. 3. Electrophoretic analysis of milk proteins. (a) Immunoblot. Wheys, equivalent to 1.5 μl of milk, from transgenic mice (numbered lanes) and control mice (CM), 0.25 μg of purified human α1AT (AT) (Sigma), and 0.05 μl of pooled human sera (H) were immunoblotted and probed for human α1AT. (b) SDS/PAGE gel. Defatted milk and whey samples from control mice (CM) and two transgenic G1 females from line 35 (numbered lanes) were electrophoresed alongside dilutions of purified human α1AT (Sigma; lane 1, 5 μg; lane 2, 2.5 μg; lane 3, 1 μg) and molecular weight markers (M) (GIBCO, BRL) and stained with Coomassie blue.](image-url)

The finding of mammary gland expression of the AATB construct in seven transgenic individuals and lines confirmed the efficacy of the construct design. However, salivary expression using the BG3 promoter was not anticipated. We

Table 2. Measurements of human α1AT present in transgenic mouse milk as determined by immunoblotting (Blot), RID, and RIA

<table>
<thead>
<tr>
<th>Animal/line</th>
<th>Generation</th>
<th>Blot</th>
<th>RID, μg/ml (n)</th>
<th>RIA, μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>15.10</td>
<td>G1</td>
<td>+</td>
<td>—(1)</td>
<td>6</td>
</tr>
<tr>
<td>15.20</td>
<td>G1</td>
<td>—</td>
<td>—(1)</td>
<td>—</td>
</tr>
<tr>
<td>17.23</td>
<td>G1</td>
<td>+</td>
<td>463 (5)</td>
<td>ND</td>
</tr>
<tr>
<td>17.24</td>
<td>G1</td>
<td>+</td>
<td>556 (6)</td>
<td>520</td>
</tr>
<tr>
<td>17.5.1</td>
<td>G1</td>
<td>+</td>
<td>990 (2)</td>
<td>1055</td>
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<tr>
<td>17.5.4</td>
<td>G2</td>
<td>+</td>
<td>407 (2)</td>
<td>390</td>
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<td>17.5.9</td>
<td>G2</td>
<td>+</td>
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<td>490</td>
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<td>730 (2)</td>
<td>680</td>
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<td>35.3</td>
<td>G1</td>
<td>+</td>
<td>7738 (2)</td>
<td>9000</td>
</tr>
<tr>
<td>35.11</td>
<td>G1</td>
<td>+</td>
<td>6215 (2)</td>
<td>5700</td>
</tr>
<tr>
<td>44</td>
<td>G0</td>
<td>+</td>
<td>879 (2)</td>
<td>920</td>
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<td>45</td>
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<td>59</td>
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<tr>
<td>65</td>
<td>G0</td>
<td>+</td>
<td>83 (2)</td>
<td>46</td>
</tr>
<tr>
<td>69</td>
<td>G0</td>
<td>+</td>
<td>695 (2)</td>
<td>445</td>
</tr>
</tbody>
</table>

n, Number of assays performed; ND, not determined.
eliminated mammary gland contamination of salivary gland RNA as the cause of this result by reproping with mammary-specific probes (data not shown). We have not detected significant salivary expression of a variety of other transgenes comprising the BLG gene or its hybrid derivatives (ref. 10; A.J.C., A.L.A., S. Harris, M.M., J.P.S., and B. Whitehead, unpublished observations). All these other transgenes share 3' BLG sequences that are absent from the AATB construct. Interestingly, transgenic mice carrying the human α1-AT gene show expression in the salivary gland (29). Several other groups have also reported salivary gland expression of hybrid genes in which the promoter and 5' flanking sequences were also derived from mammary-specific genes (30–33). Thus, it is possible that the downstream BLG sequences contain a salivary gland-specific negative regulatory element or that positive regulatory elements within the α1-AT sequences direct expression to this tissue.

Although the electrophoretic mobilities, in SDS/PAGE, of α1-AT from transgenic mouse milk or human plasma are similar, the electrophoretic pattern of α1-AT proteins observed in transgenic mouse milk appears more complex. This may reflect differences in the posttranslational modifications of the proteins produced in human liver and mouse mammary gland. Alternatively, human α1-AT produced in mouse milk may be more susceptible to degradation during secretion or storage.

Within lines of transgenic mice, some variation in expression was observed. In line 15, low-level α1-AT expression was detected in only one of the two animals analyzed, and in line 17, in which both G1 and G2 animals were analyzed, an ≈2-fold variation in α1-AT concentration was observed. This may simply reflect a variation in the total protein content of individual milk samples (10) or variation in the level of transgene expression within a line due to nonuniform genetic backgrounds. We have also noted considerable variation in the level of transgene expression within a line of mice carrying the BLG gene (M.M., unpublished observations).

The level of human α1-AT in the milk of line 35 mice is very high, as expected from the level of α1-AT mRNA observed in the mammary gland. The α1-AT is clearly evident on Coomassie blue-stained gels of total milk proteins (Fig. 3b). Densitometry of stained gels showed that human α1-AT comprises ≈10% of total milk proteins and more than 30% of the whey proteins. These proportions compare favorably with those obtained for expression of α1-AT in bacteria (15% of total cell protein) and yeast (3% of total soluble proteins) and particularly so when compared with eukaryotic cell culture expression (<1 μg per 10⁶ cells per 24 hr) (34–37).

The human α1-AT present in the milk of transgenic mice from lines 17 and 35 was shown to be biologically active in a trypsin-inhibition assay. When transgenic mouse milk (mouse 35.11) and pooled human plasma were compared, it was evident that equivalent amounts of plasma and recombinant α1-AT had similar capacities to inhibit trypsin (Fig. 4b), indicating that α1-AT synthesized in the mammary gland and secreted into milk is as biologically active as that derived from plasma.

In December 1987, the U.S. Food and Drug Administration licensed the use of α1-AT in replacement therapy (1). The methods by which α1-AT might be delivered to the critical lung locations include intravenous infusions, aerosol sprays, and gene therapy (38, 39). It seems likely that gene therapy will be very expensive and not readily available to the many individuals suffering from α1-AT deficiency. For replacement therapy by means of aerosols or intravenous infusions to be generally available, large quantities of biologically active and correctly glycosylated α1-AT will be required.

In this paper we have described the production of transgenic mice expressing high levels of biologically active human α1-AT in their milk. The levels of expression in line 35 are of particular interest and, to our knowledge, represent one of the highest levels of expression of a recombinant protein in any mammalian expression system, including transgenic mice and sheep (9, 40, 41). High-milk-yielding breeds of sheep, such as the East Friesland, can produce up to 400 liters per lactation. Therefore transgenic sheep that express α1-AT at the levels observed in line 35 could produce up to 3 kg of α1-AT at each lactation, a level of production that might be capable of supplying the large quantities required for replacement therapy.

We thank Jen Anderson, John Bowman, Wendy Shepherd, and Roberta Wallace for their skilled technical assistance; Steven Moore for facilitating the RIA work; Anthea Springbett for statistical advice; and Prof. R. Cortese and Dr. G. Kelsey for clones p8α1ppg and pATp7, respectively. This work was supported, in part, by Pharmaceutical Proteins, Ltd.