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High-level expression of biologically active human $\alpha_1$-antitrypsin in the milk of transgenic mice  

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

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ABSTRACT  Reduced circulating levels of $\alpha_1$-antitrypsin ($\alpha_1$AT) are associated with certain $\alpha_1$AT genotypes and increased susceptibility to emphysema. Unfortunately, the amounts of $\alpha_1$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 $\alpha_1$AT. A hybrid gene constructed by using sequences from the ovine milk protein gene $\beta$-lactoglobulin fused to an $\alpha_1$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 $\alpha_1$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 $\alpha_1$AT per ml in their milk; one line (AATB 35) produced 7 mg of this protein per ml. $\alpha_1$AT from transgenic mouse milk was similar in size to human plasma-derived $\alpha_1$AT and showed a similar capacity to inhibit trypsin. Expression at equivalent levels in transgenic sheep or cattle would yield sufficient $\alpha_1$AT for therapeutic purposes.

Genetic deficiencies of $\alpha_1$-antitrypsin ($\alpha_1$AT) in humans are common and result in an increased susceptibility to emphysema (1). Human $\alpha_1$AT is a 394-amino acid glycoprotein that acts as a suicide inhibitor of a wide range of serine proteases. In humans, the $\alpha_1$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 $\alpha_1$AT is synthesized daily, resulting in a serum concentration of ~2 mg/ml.

The primary function of $\alpha_1$AT is to inhibit neutrophil elastase and thus prevent this protease from causing excessive tissue damage (1). The S and Z $\alpha_1$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 $\alpha_1$AT (<0.8 mg/ml) and are at risk of developing the degenerative lung disease emphysema, particularly if they smoke.

Since $\alpha_1$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 ZZZ 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 $\alpha_1$AT does not require its carbohydrate side chains for activity, the in vivo half-life of nonglycosylated $\alpha_1$AT (expressed in yeast) is 50-fold lower than that of plasma-derived $\alpha_1$AT (4). Therefore it would seem prudent to produce $\alpha_1$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 $\alpha_1$AT.

As an alternative to genetically engineered cell lines, Palmeter 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 $\alpha_1$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. Bessos, C. Prowse, J.P.S., B. Whitesel, 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 $\alpha_1$AT sequences in the mammary gland, yielding high levels of human $\alpha_1$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 $Psv$ II site within the 5′ untranslated sequences of the ovine BLG clone SS1 (11, 12) was fused to the $tau$ A site in the 5′ untranslated sequences of $\alpha_1$AT. The first $\alpha_1$AT intron was excluded by using DNA sequences from a cDNA clone, $pB\alpha$1ppg, which encodes the M1 variant of $\alpha_1$AT (13), as the source of the first 80 base pairs of $\alpha_1$AT sequences, extending up to the $Bam$HI site in the second exon. The remainder of the $\alpha_1$AT "minigene" comprises a

Abbreviations: $\alpha_1$AT, $\alpha_1$-antitrypsin; BLG, $\beta$-lactoglobulin; G, generation; n, radial immunodiffusion.

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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 pPOLYIII-I (15), enabling excision of the 10.6-kb insert by using Nor 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 (Amerham) nylon membranes, and probed with 32P-labeled α1AT 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 (Amerham), and probed with a 32P-labeled 243-base-pair TagI–PstI fragment derived from the 3′ end of p8AT1ps (13), which allows mouse and human α1AT mRNAs to be distinguished. DNA 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 radioimmunooassay (RIA). RID estimates were obtained by using LC-Partigen RID plates (Behring Diagnostics) according to the manufacturer’s instructions. RIA’s 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 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

![Northern blot analysis of total RNA from transgenic mice (numbers AATB 35.3, AATB 26.1, and AATB 17.20) and a control C57BL/6 mouse (CM). The tissues analyzed were mammary (M), liver (L), spleen (Sp), kidney (K), and salivary (Sa). Control lanes: HL, human liver RNA; HG, HepG2 RNA (26). The ~1400-nucleotide α1AT transcripts are indicated by an arrow. Ten micrograms of total RNA was loaded except for HL, 35.3 M, and HG, which contain 1 μg of sample RNA with 9 μg of control mouse mammary RNA.](attachment:image)
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>+(^{\dagger})</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>
</tr>
<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>
</tr>
<tr>
<td>78</td>
<td>M</td>
<td>10</td>
<td>-</td>
<td>+</td>
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<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.

\(^{\dagger}\)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 G0 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 BLG 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>
</tr>
<tr>
<td>17.5.4</td>
<td>G2</td>
<td>+</td>
<td>407 (2)</td>
<td>390</td>
</tr>
<tr>
<td>17.5.9</td>
<td>G2</td>
<td>+</td>
<td>606 (3)</td>
<td>490</td>
</tr>
<tr>
<td>17.5.16</td>
<td>G2</td>
<td>+</td>
<td>730 (2)</td>
<td>680</td>
</tr>
<tr>
<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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<tr>
<td>45</td>
<td>G0</td>
<td>+</td>
<td>84 (2)</td>
<td>59</td>
</tr>
<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 reprobing 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. White, unpublished observations). All these other transgenes share 3' BLG sequences that are absent from the AATB construct. Interestingly, transgenic mice carrying the human α1AT 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 α1AT sequences direct expression to this tissue.

Although the electrophoretic mobilities, in SDS/PAGE, of α1AT from transgenic mouse milk or human plasma are similar, the electrophoretic pattern of α1AT 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 α1AT 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 α1AT 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 α1AT 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 α1AT in the milk of line 35 mice is very high, as expected from the level of α1AT mRNA observed in the mammary gland. The α1AT is clearly evident on Coomassie blue-stained gels of total milk proteins (Fig. 3b). Densitometry of stained gels showed that human α1AT comprises ∼10% of total milk proteins and more than 30% of the whey proteins. These proportions compare favorably with those obtained for expression of α1AT 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 α1AT 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 α1AT had similar capacities to inhibit trypsin (Fig. 4b), indicating that α1AT 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 α1AT in replacement therapy (1). The methods by which α1AT 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 α1AT deficiency. For replacement therapy by means of aerosols or intravenous infusions to be generally available, large quantities of biologically active and correctly glycosylated α1AT will be required.

In this paper we have described the production of transgenic mice expressing high levels of biologically active human α1AT 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 α1AT at the levels observed in line 35 could produce up to 3 kg of α1AT 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 p8αlpp and pATp7, respectively. This work was supported, in part, by Pharmaceutical Proteins, Ltd.