Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 5178–5182, July 1990
Genetics

High-level expression of biologically active human α_1-antitrypsin in the milk of transgenic mice

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

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Communicated by Mary F. Lyon, March 26, 1990 (received for review February 16, 1990)

ABSTRACT **Reduced circulating levels of α_1-antitrypsin (α_1AT) are associated with certain α_1AT genotypes and increased susceptibility to emphysema. Unfortunately, the amounts of α_1AT 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 α_1AT. A hybrid gene constructed by using sequences from the ovine milk protein gene β-lactoglobulin fused to an α_1AT "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 α_1AT 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 α_1AT per ml in their milk; one line (AATB 35) produced 7 mg of this protein per ml. α_1AT from transgenic mouse milk was similar in size to human plasma-derived α_1AT and showed a similar capacity to inhibit trypsin. Expression at equivalent levels in transgenic sheep or cattle would yield sufficient α_1AT for therapeutic purposes.**

Genetic deficiencies of α_1-antitrypsin (α_1AT) in humans are common and result in an increased susceptibility to emphysema (1). Human α_1AT is a 394-amino acid glycoprotein that acts as a suicide inhibitor of a wide range of serine proteases. In humans, the α_1AT 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 α_1AT is synthesized daily, resulting in a serum concentration of $\approx$2 mg/ml.

The primary function of α_1AT is to inhibit neutrophil elastase and thus prevent this protease from causing excessive tissue damage (1). The S and Z α_1AT alleles are relatively common ($\approx$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 α_1AT (<0.8 mg/ml) and are at risk of developing the degenerative lung disease emphysema, particularly if they smoke.

Since α_1AT normally circulates at 2 mg/ml and has a half-life of 6 days, considerable quantities ($\approx$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 α_1AT does not require its carbohydrate side chains for activity, the *in vivo* half-life of nonglycosylated α_1AT (expressed in yeast) is 50-fold lower than that of plasma-derived α_1AT (4). Therefore it would seem prudent to produce α_1AT 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 α_1AT.

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 α_1AT 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. Whitelaw, I. Wilmut, and A.J.C., unpublished results; M.M., H. Bessos, C. Prowse, J.P.S., B. Whitelaw, 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, β-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 α_1AT sequences in the mammary gland, yielding high levels of human α_1AT 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 SS1 (11, 12) was fused to the *Taq* I site in the 5′ untranslated sequences of α_1AT. The first α_1AT intron was excluded by using DNA sequences from a cDNA clone, p8α1ppg, which encodes the M_1 variant of α_1AT (13), as the source of the first 80 base pairs of α_1AT sequences, extending up to the *Bam*HI site in the second exon. The remainder of the α_1AT "minigene" comprises a

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Abbreviations: α_1AT, α_1-antitrypsin; BLG, β-lactoglobulin; G_n, generation n; RID, radial immunodiffusion.

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6.5-kilobase (kb) *Bam*HI fragment from the human α_1AT genomic clone pATp7 [also encoding the M_1 variant of α_1AT (14)]. The construct was elaborated in the vector pPOLYIII-I (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)F_1 mice after mating with F_1 stud males] in order to generate transgenic mice (10, 16). Lines were propogated by mating with F_1 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 ^{32}P-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 ^{32}P-labeled 243-base-pair *Taq* I–*Pst* I fragment derived from the 3′ end of p8α1ppg (13), which allows mouse and human α_1AT 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 α_1AT was identified on immunoblot filters by using goat anti-α_1AT 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 α_1AT 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. RIAs were performed according to standard procedures (23) with goat anti-human α_1AT antiserum (PRU) and donkey anti-goat IgG (SAPU). Human α_1AT, 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 equal volumes of trypsin (bovine pancreatic type III, Sigma) at 200 units/ml in 0.1 M Tris/0.15 M NaCl/3 mM sodium azide, pH 8.0 for 5 min, before addition of 40 μl of chromogenic substrate S-2222 (KabiVitrum) (25). After 2.5 min, acetic acid was added to a final concentration of 8.5% to stop the reaction, and absorbances at 405 nm were read immediately.

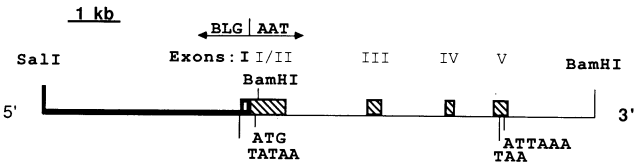

FIG. 1. The AATB construct comprises ≈4.0 kb of the 5′ end of the ovine BLG clone SS1 (11, 12) fused to a minigene encoding human α_1AT. Thick line, 5′ BLG sequences; open box, BLG exon 1 sequences; hatched boxes, α_1AT exons; thin lines, α_1AT introns and 3′ flanking regions. The position of the BLG TATA box and also the α_1AT initiation codon, stop codon, and polyadenylylation site are shown.

RESULTS

Generation of Transgenic Mice. To direct expression of α_1AT 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 α_1AT (Fig. 1). The hybrid gene (AATB) was microinjected into pronuclei of fertilized mouse eggs (n = 993). Analysis of DNA prepared from tail biopsies showed that 21 of the 122 generation zero (G_0) 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 G_0 animals or the transgenic G_1 offspring of G_0 males. Three patterns of human α_1AT 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 α_1AT mRNA. One line in particular, AATB 35, showed extremely high levels of expression of α_1AT mRNA in the mammary gland, comparable to the level observed in human liver.

Production of Human α_1AT in Milk. Milk was analyzed by SDS/PAGE and immunoblotting for the presence of human α_1AT protein (Fig. 3*a*). Human α_1AT was present in milk from all the transgenic animals that had detectable levels of human α_1AT 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 antiserum to human α_1AT cross-reacted with an endogenous mouse protein present in milk, probably murine α_1AT. The most

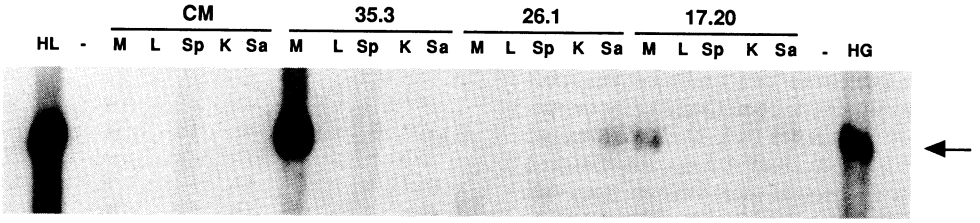

FIG. 2. Northern blot analysis of total RNA from transgenic mice (numbers AATB 35.3, AATB 26.1, and AATB 17.20) and a control C57BL/6 mouse (CM). The tissues analyzed were mammary (M), liver (L), spleen (Sp), kidney (K), and salivary (Sa). Control lanes: HL, human liver RNA; HG, HepG2 RNA (26). The ≈1400-nucleotide α_1AT 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.

Table 1. Summary of the pattern of expression of AATB RNA in transgenic mice

Animal/line	Sex*	Copy no.	Mammary	Salivary
15	M	3	+†	−
17	M	10	+	+
26	M	20	−	+
28	M	3	−	−
35	M	1	+	+
44	F	15	+	−
45	F	2	+	−
65	F	3	+	−
69	F	2	+	−
78	M	10	−	+
79	M	15	−	+
105	F	20	−	+
107	F	15	−	+

Expression was analyzed by Northern blot analysis of tissues from lactating G_0 females or, where the founder was male, from G_1 females that had inherited the transgene. No human α_1AT RNA was detected in liver, spleen, kidney, or heart. Copy numbers were estimated by Southern blotting relative to copy number controls.
*Sex of the G_0 animals.
†α_1AT transcripts were detected only in poly(A)$^+$ RNA in one of two animals analyzed.

prominent human α_1AT bands in transgenic mouse milk had electrophoretic mobilities similar, but not identical, to the major bands observed in samples of purified human α_1AT or pooled human plasma.

The concentrations of human α_1AT 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 α_1AT 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. 4*a*). When milk from line AATB 35 mice was compared with human plasma, it was evident that equivalent amounts of plasma and milk α_1AT had similar biological activities (Fig. 4*b*). 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 α_1AT 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 α_1AT. 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 α_1AT 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 α_1AT (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).

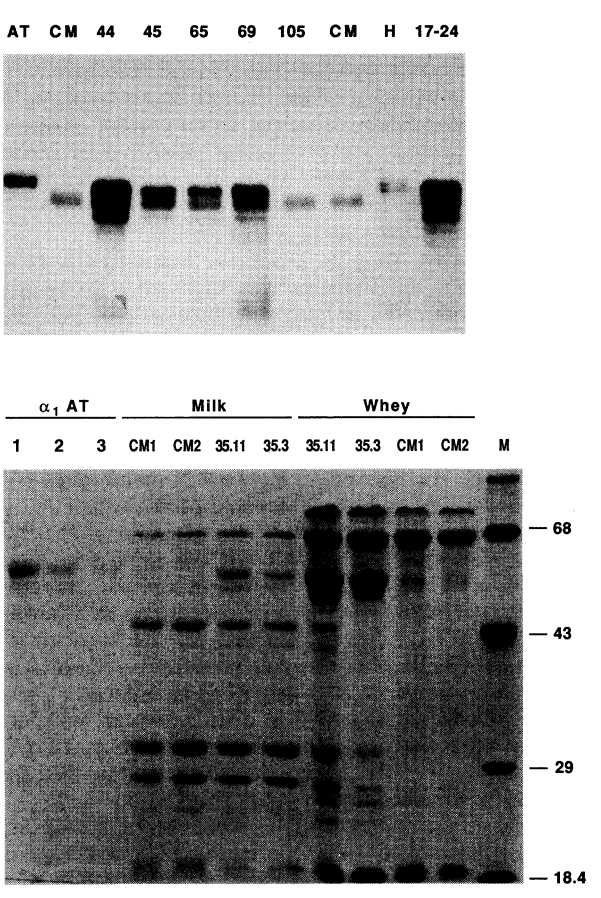

FIG. 3. Electrophoretic analysis of milk proteins. (*a*) Immunoblot. Wheys, equivalent to 1.5 μl of milk, from transgenic mice (numbered lanes) and control mice (CM), 0.25 μg of purified human α_1AT (AT) (Sigma), and 0.05 μl of pooled human sera (H) were immunoblotted and probed for human α_1AT. (*b*) SDS/PAGE gel. Defatted milk and whey samples from control mice (CM) and two transgenic G_1 females from line 35 (numbered lanes) were electrophoresed alongside dilutions of purified human α_1AT (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.

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 α_1AT present in transgenic mouse milk as determined by immunoblotting (Blot), RID, and RIA

Animal/line	Generation	Blot	RID, μg/ml (*n*)	RIA, μg/ml
15.10	G_1	+	— (1)	6
15.20	G_1	−	— (1)	—
17.23	G_1	+	463 (5)	ND
17.24	G_1	+	556 (6)	520
17.5.1	G_2	+	990 (2)	1055
17.5.4	G_2	+	407 (2)	390
17.5.9	G_2	+	606 (3)	490
17.5.16	G_2	+	730 (2)	680
35.3	G_1	+	7738 (2)	9000
35.11	G_1	+	6215 (2)	5700
44	G_0	+	879 (2)	920
45	G_0	+	84 (2)	59
65	G_0	+	83 (2)	46
69	G_0	+	695 (2)	445

n, Number of assays performed; ND, not determined.

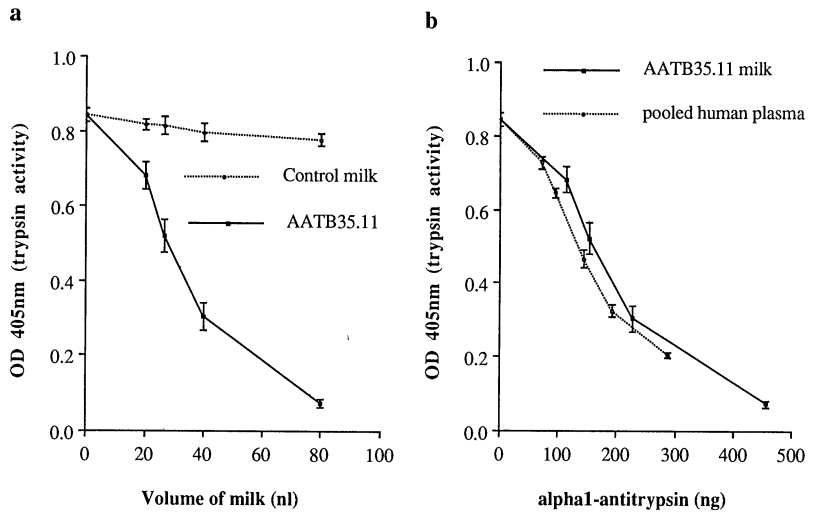

FIG. 4. Trypsin-inhibitory activity of transgenic mouse milk. (*a*) A comparison of the trypsin-inhibitory capacities of milk from transgenic mouse AATB 35.11 and pooled milk from nontransgenic mice. (*b*) A comparison of the trypsin-inhibitory capacities of the human α_1AT in milk from mouse AATB 35.11 and in pooled human plasma; the human α_1AT contents of the diluted milk and plasma samples were calculated from RID estimates of the concentrations of the corresponding milk and plasma.

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. Whitelaw, unpublished observations). All these other transgenes share 3′ BLG sequences that are absent from the AATB construct. Interestingly, transgenic mice carrying the human α_1AT 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 α_1AT sequences direct expression to this tissue.

Although the electrophoretic mobilities, in SDS/PAGE, of α_1AT from transgenic mouse milk or human plasma are similar, the electrophoretic pattern of α_1AT 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 α_1AT 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 α_1AT expression was detected in only one of the two animals analyzed, and in line 17, in which both G_1 and G_2 animals were analyzed, an $\approx$2-fold variation in α_1AT 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 α_1AT in the milk of line 35 mice is very high, as expected from the level of α_1AT mRNA observed in the mammary gland. The α_1AT is clearly evident on Coomassie blue-stained gels of total milk proteins (Fig. 3*b*). Densitometry of stained gels showed that human α_1AT comprises $\approx$10% of total milk proteins and more than 30% of the whey proteins. These proportions compare favorably with those obtained for expression of α_1AT 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 10^6 cells per 24 hr) (34–37).

The human α_1AT 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 α_1AT had similar capacities to inhibit trypsin (Fig. 4*b*), indicating that α_1AT 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 α_1AT in replacement therapy (1). The methods by which α_1AT 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 α_1AT deficiency. For replacement therapy by means of aerosols or intravenous infusions to be generally available, large quantities of biologically active and correctly glycosylated α_1AT will be required.

In this paper we have described the production of transgenic mice expressing high levels of biologically active human α_1AT 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 α_1AT at the levels observed in line 35 could produce up to 3 kg of α_1AT 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.

1. Crystal, R. G. (1989) *Trends Genet.* **5**, 411–417.
2. Carlson, J. A., Rogers, B. B., Sifers, R. N., Hawkins, H. K.,

Finegold, M. J. & Woo, S. L. C. (1988) *J. Clin. Invest.* **82,** 26–36.
3. Gadek, J. E. & Crystal, R. G. (1983) *Am. Rev. Respir. Dis.* **127,** S45–S46.
4. Casolaro, M. A., Fells, G., Wewers, M., Pierce, J. E., Ogushi, F., Hubbard, R., Sellers, S., Forstrom, J., Lyons, D., Kawasaki, G. & Crystal, R. G. (1987) *J. Appl. Physiol.* **63,** 2015–2023.
5. Palmiter, R. D., Brinster, R. L., Hammer, R. E., Trumbauer, M. E., Rosenfeld, M. G., Birnberg, N. C. & Evans, R. M. (1982) *Nature (London)* **300,** 611–615.
6. Lathe, R., Clark, A. J., Archibald, A. L., Bishop, J. O., Simons, P. & Wilmut, P. (1986) in *Exploiting New Technologies in Animal Breeding*, eds. Smith, C., King, J. W. B. & McKay, J. C. (Oxford Univ. Press, Oxford), pp. 91–102.
7. Clark, A. J., Simons, P., Wilmut, I. & Lathe, R. (1987) *Trends Biotechnol.* **5,** 20–24.
8. Simons, J. P., Wilmut, I., Clark, A. J., Archibald, A. L., Bishop, J. O. & Lathe, R. (1988) *Bio/Technology* **6,** 179–183.
9. Clark, A. J., Bessos, H., Bishop, J. O., Brown, P., Harris, S., Lathe, R., McClenaghan, M., Prowse, C., Simons, J. P., Whitelaw, C. B. A. & Wilmut, I. (1989) *Bio/Technology* **7,** 487–492.
10. Simons, J. P., McClenaghan, M. & Clark, A. J. (1987) *Nature (London)* **328,** 530–532.
11. Ali, S. & Clark, A. J. (1988) *J. Mol. Biol.* **199,** 415–426.
12. Harris, S., Ali, S., Anderson, S., Archibald, A. L. & Clark, A. J. (1988) *Nucleic Acids Res.* **16,** 10379–10380.
13. Ciliberto, G., Dente, L. & Cortese, R. (1985) *Cell* **41,** 531–540.
14. Kelsey, G. D., Povey, S., Bygrave, A. E. & Lovell-Badge, R. H. (1987) *Genes Dev.* **1,** 161–171.
15. Lathe, R., Vilotte, J.-L. & Clark, A. J. (1987) *Gene* **57,** 193–201.
16. Hogan, B., Costantini, F. & Lacy, E. (1986) *Manipulating the Mouse Embryo: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
17. Chirgwin, J. M., Pryzbyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979) *Biochemistry* **18,** 5294–5299.
18. Chomezynski, P. & Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
19. Feinberg, A. P. & Vogelstein, B. (1983) *Anal. Biochem.* **132,** 6–13.
20. Church, G. M. & Gilbert, W. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 1991–1995.
21. Laemmli, U. K. (1970) *Nature (London)* **277,** 680–685.
22. Khyse-Anderson, J. (1984) *J. Biochem. Biophys. Methods* **10,** 203–209.
23. Hornsey, V., Micklem, L. R., McCann, M. C., James, K., Dawes, J., McClelland, D. B. L. & Prowse, C. V. (1985) *Thromb. Haemostasis* **54,** 510–514.
24. Laurell, C.-B., Pierce, J., Persson, U. & Thulin, E. (1975) *Eur. J. Biochem.* **57,** 107–113.
25. Friberger, P. (1982) *Scand. J. Clin. Lab. Invest.* **42,** Suppl. 162.
26. Knowles, B. B., Howe, C. C. & Aden, D. P. (1980) *Science* **209,** 497–499.
27. Brinster, R. L., Allen, J. M., Behringer, R. R., Gelinas, R. E. & Palmiter, R. D. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 836–840.
28. Long, G. L., Chandra, T., Woo, S. L. C., Davie, E. W. & Kurachi, K. (1984) *Biochemistry* **23,** 4828–4837.
29. Koopman, P., Povey, S. & Lovell-Badge, R. H. (1989) *Genes Dev.* **3,** 16–25.
30. Andres, A.-C., Schonenberger, C.-A., Groner, B., Hennighausen, L., LeMeur, M. & Gerlinger, P. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 1299–1303.
31. Muller, W. J., Sinn, E., Pattengale, P. K., Wallace, R. & Leder, P. (1988) *Cell* **54,** 105–115.
32. Pittius, C. W., Hennighausen, L., Lee, E., Westphal, H., Nicols, E., Vitale, J. & Gordon, K. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 5874–5878.
33. Lee, K.-F., Atiee, S. H. & Rosen, J. M. (1989) *Mol. Cell. Biol.* **9,** 560–565.
34. Courtney, M., Buchwalder, A., Tessier, L.-H., Jaye, M., Benavente, A., Balland, A., Kohli, V., Lathe, R., Tolstoshev, P. & Lecocq, J.-P. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 669–673.
35. van der Straten, A., Falque, J.-C., Loriau, R., Bollen, A. & Cabezon, T. (1986) *DNA* **5,** 129–136.
36. Garver, R. I., Chytil, A., Karlsson, S., Fells, G. A., Brantly, M. L., Courtney, M., Kantoff, P. W., Nienhuis, A. W., Anderson, W. F. & Crystal, R. G. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 1050–1054.
37. Pavirani, A., Skern, T., Le Meur, M., Lutz, Y., Lathe, R., Crystal, R. G., Fuchs, J.-P., Gerlinger, P. & Courtney, M. (1989) *Bio/Technology* **7,** 1049–1054.
38. Hubbard, R. C., Casolaro, M. A., Mitchell, M., Sellers, S. E., Arabia, F., Matthay, M. A. & Crystal, R. G. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 680–684.
39. Garver, R. I., Chytil, A., Courtney, M. & Crystal, R. G. (1987) *Science* **237,** 762–764.
40. Gordon, K., Lee, E., Vitale, J. A., Smith, A. E., Westphal, H. & Hennighausen, L. (1987) *Bio/Technology* **5,** 1183–1187.
41. Yu, S.-H., Deen, K. C., Lee, E., Hennighausen, L., Sweet, R. W., Rosenberg, M. & Westphal, H. (1989) *Mol. Biol. Med.* **6,** 255–261.