and 0.6 m*M* $MgCl_2$], *p*H ~6.0, to develop for the conditioned medium. Acidifying conditioned medium to *p*H 6.0 after conditioning has no effect. We also found no effect or a slightly deleterious effect from using medium conditioned by a threefold greater density of cells than described (*11*), changing the conditioned medium 1, 2, 4, or 6 hours after starvation, starving initially in MES-PDF and then changing to conditioned medium 1, 2, 4, or 6 hours after starvation, adding histidine [S. G. Bradley, M. Sussman, H. L. Ennis, *J. Protozool.* **3**, 33 (1956)] or bovine serum albumin to the conditioned medium, or adding 0.1 to 3 m*M* NH_4Cl at the time of cAMP addition. Higher levels of NH_4Cl appeared to inhibit prespore and prestalk gene expression.
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Clonal Gene Therapy: Transplanted Mouse Fibroblast Clones Express Human α1-Antitrypsin Gene in Vivo

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A retroviral vector was used to insert human α1-antitrypsin (α1AT) complementary DNA into the genome of mouse fibroblasts to create a clonal population of mouse fibroblasts secreting human α1AT. After demonstrating that this clone of fibroblasts produced α1AT after more than 100 population doublings in the absence of selection pressure, the clone was transplanted into the peritoneal cavities of nude mice. When the animals were evaluated 4 weeks later, human α1AT was detected in both sera and the epithelial surface of the lungs. The transplanted clone of fibroblasts could be recovered from the peritoneal cavities of those mice and demonstrated to still be producing human α1AT. Thus, even after removal of selective pressure, a single clone of retroviral vector–infected cells that expressed an exogenous gene in vitro, continued to do so in vivo, and when recovered, continued to produce the product of the exogenous gene.

THE CHARACTERIZATION OF A growing number of heritable disorders at the gene and protein level has led to the conceptualization of therapies that are directly aimed at correcting the molecular defects. One example is the hereditary form of emphysema associated with α1-antitrypsin (α1AT) deficiency. In this disorder, inheritance of "deficiency" or "null" α1AT alleles from both parents results in low α1AT serum levels (*1, 2*). When this occurs, α1AT, the major inhibitor of neutrophil elastase in the human body, is not present in sufficient amounts to protect the lower respiratory tract from the destructive potential of neutrophil elastase (*3–5*). As a result, there is unimpeded destruction of the lung parenchyma, resulting in emphysema by the third to fourth decades (*1*). Therapies for preventing or slowing the development of emphysema in this disorder are directed toward increasing the serum and lung levels of α1AT (*6, 7*).

The phenotypic abnormality (the α1AT deficiency state) could theoretically be corrected by the addition of a normal human α1AT gene to the genome of cells of deficient individuals, such that the target cells would express the α1AT gene and secrete α1AT. Current concepts of gene therapy have focused on the use of retroviral vectors, which utilize two long terminal repeat (LTR) regions for integration of nonviral genes between them, to permanently integrate the α1AT gene into the genome of the target mammalian cells (*8*). In this regard, most interest has focused on bone marrow precursors as the target cell (*9*). Genetic disorders would be corrected by the addition of a normal gene to autologous bone marrow by in vitro infection, followed by transplantation of the infected cells into the host (*10*). By means of the N2 vector, a retroviral construct derived from an incomplete Moloney murine leukemia virus (*11*), human α1AT complementary DNA (cDNA) has been successfully integrated into the genome of mouse fibroblasts (*12*). These fibroblasts secrete a glycosylated human α1AT molecule that functions as an inhibitor of human neutrophil elastase and has a half-life in serum similar to α1AT purified from normal human plasma (*12*). We hypothesized that such cells could potentially be transplanted into the peritoneum of a recipient with the result that the integrated α1AT gene in the transplanted cells would direct the synthesis of α1AT. The α1AT would be secreted into the peritoneal cavity, diffuse into the blood, and be carried to organs throughout the body, including the lung. If possible, such an approach would have several advantages over the strategy of using autologous bone marrow cells as the target for in vitro introduction of a foreign gene. First, the process of retrovirus infection results in integration of the foreign gene in random sites in the target cell genome (*8*), and thus, the population of infected bone marrow cells to be transplanted would contain a mixture of several clonal populations, in each of which native genes might have been activated or inactivated in a deleterious fashion (*13–15*). In contrast, a monoclonal population of cells containing the integrated foreign gene could be characterized extensively in vitro prior to transplantation. Second, the level of gene

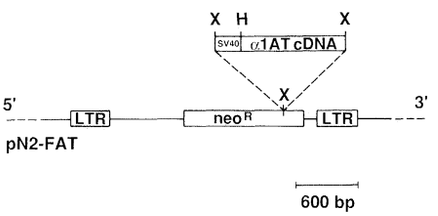

Fig. 1. Retroviral vector used to integrate the human α1AT cDNA into mouse fibroblasts. Vector pN2-FAT contained the SV40 early promoter in tandem with the forward orientation of the α1AT cDNA. The human α1AT cDNA, derived from the human α1AT-containing plasmid pTG 603 (*19*), was modified by standard techniques to create a 5′ Hind III ("H") site and 3′ Xho I ("X") site. Sequence analysis confirmed the fidelity of the α1AT protein coding region. The SV40 early promoter, with a 5′ Xho I site and 3′ Hind III site, was derived from pLSDL (provided by A. D. Miller, Fred Hutchinson Cancer Center) (*20*). The α1AT cDNA and SV40 early promoter fragments were cut from their respective plasmids and simultaneously ligated into the Xho I site of the retroviral vector N2 (provided by E. Gilboa, Memorial Sloan-Kettering Cancer Center) containing a 5′ and 3′ long terminal repeat (LTR) (*11, 12*). The orientation of the construct was established by restriction mapping. This vector also contains the gene encoding neomycin resistance, permitting infected cells to be identified by their ability to survive in medium containing the neomycin analog, G418 (*21*).

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expression in target cells such as fibroblasts has generally been better than with primate bone marrow stem cells (*16*).

To evaluate the possibility of using a monoclonal population of fibroblasts in this manner, we transplanted a monoclonal population of murine fibroblasts secreting human α1AT into the peritoneal cavities of nude mice. The monoclonal population of murine fibroblasts, NIH/3T3-FAT4, was derived from a single NIH/3T3 fibroblast infected with a retroviral vector that contained a full-length human α1AT cDNA and a neomycin resistance gene (Fig. 1). The NIH/3T3-FAT4 clone was identified under the selective pressure of G418, the neomycin analog.

Evaluation of the NIH/3T3-FAT4 clone demonstrated that it contained a single copy of the integrated N2-FAT provirus, produced human α1AT messenger RNA (mRNA) transcripts, and secreted human α1AT. These characteristics were unchanged after the clone had been amplified in nonselective conditions, transplanted by injection into the peritoneal cavities of mice, and then recovered 4 weeks later. The uninfected NIH/3T3 cells did not contain any DNA detectable with the α1AT cDNA probe (Fig. 2, lane 1), but both the untransplanted clone and the post-transplant G418-resistant cells had an identical, single integration site of the N2-FAT provirus (Fig. 2, lanes 2 and 3). Similarly, the uninfected NIH/3T3 cells did not express human α1AT transcripts (Fig. 2, lane 4), but the untransplanted NIH/3T3-FAT4 clone and the post-transplant G418-resistant cells produced three transcripts with human α1AT sequences (Fig. 2, lanes 5 and 6). The transcripts (largest to smallest) probably represent the genomic message, a transcript generated by a spliced genomic message, and transcription of the spliced message driven by the SV40 promoter (*12*). Finally, while the uninfected NIH/3T3 cells did not secrete any human α1AT (Fig. 2, lane 7), both the post-transplant–resistant cells and the original clone secreted human α1AT (Fig. 2, lanes 8 and 9). As previously described, the 52-kD human α1AT secreted by similar clonal populations of mouse fibroblasts containing the N2-FAT construct in the genome was not only identical in size to normal human α1AT, but was glycosylated, inhibited human neutrophil elastase, and had a half-life in vivo similar to α1AT purified from human plasma (*12*). Thus, the NIH/3T3-FAT4 clone had a single copy of integrated N2-FAT which produced message that was translated into human α1AT. Furthermore, after transplantation, a clonal population of G418-resistant cells was recovered that was identical to the untransplanted clone without evidence of proviral rearrangement or reinfection.

The NIH/3T3-FAT4 cells were also able to express the α1AT gene and secrete α1AT in vivo. In this regard, evaluation of the serum and lungs of nontransplanted mice, or in mice 4 weeks after transplantation of normal NIH/3T3 cells demonstrated no human α1AT (Fig. 3). In striking contrast, in the 11 mice that had received transplants of the monoclonal population of NIH/3T3-FAT4 cells 4 weeks previously, human α1AT was easily detected in the serum as well as on the epithelial surface of the lung. The amount of human α1AT in the epithelial fluid of the lung of these animals was 0.96 ± 0.04 μg/ml (mean ± standard deviation), 29% of the human α1AT levels in the serum of the same mice (3.3 ± 1.1 μg/ml), this would suggest that the human α1AT produced by the transplanted NIH/3T3-FAT4 cells diffused from the peritoneum to blood and across the lung parenchyma to the lung epithelial surface. Thus, the in vitro behavior was predictive of the in vivo functioning of the clone produced by retroviral infection, suggesting that this approach may be useful as a means of assessing the stability of other clones infected with other retroviral constructs.

These observations also suggest that gene therapy of such disorders of circulating protein as α1AT deficiency does not have to be targeted directly toward the cells that naturally produce the protein. In the model used, the human α1AT gene was inserted into mouse fibroblasts, cells that do not normally express the α1AT gene but are more conve-

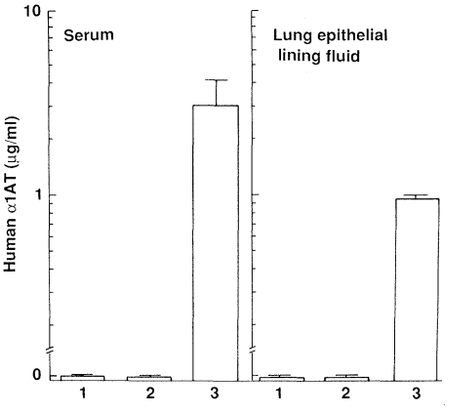

Fig. 3. Human α1AT was detected in serum and lungs of mice transplanted with NIH/3T3-FAT4 cells. Four weeks after transplantation (carried out as described in the legend to Fig. 2), the mice were sacrificed, the tracheas cannulated, and total lung lavage performed with three 300-μl aliquots of phosphate-buffered saline, *p*H 7.4. The amount of epithelial lining fluid in the recovered lavage fluid was determined by the urea method (*25*). Serum was isolated from blood obtained by cardiac puncture. Human α1AT levels in lung epithelial lining fluid and serum were determined with a human α1AT specific enzyme immunoassay (*26*). Shown are the amounts of human α1AT present in the serum and lung epithelial lining fluids of mice that did not receive any transplanted cells (lanes 1, $n = 3$), mice transplanted with NIH/3T3 (lanes 2), and mice transplanted with NIH/3T3-FAT4 cells (lanes 3).

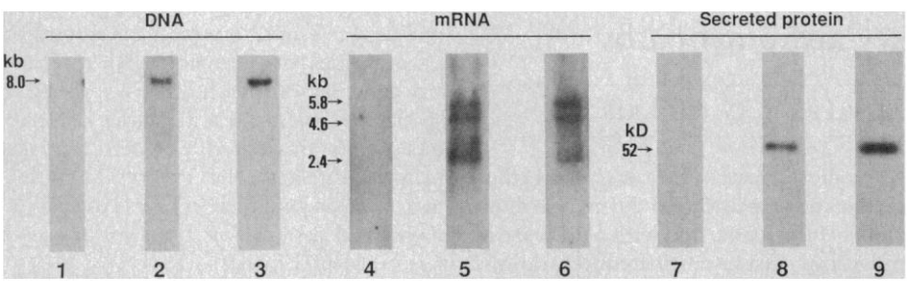

Fig. 2. Characterization of cells before and 4 weeks after transplantation into athymic nude mice. The NIH/3T3-FAT4 clone was produced by infection of NIH/3T3 fibroblasts with the N2-FAT virus; the virus was secreted from a clone of ψ2 cells (*22*) that had been transfected with pN2-FAT as previously described (*12*). This clone did not produce helper virus detectable by an XC assay (*23*). The clone was amplified in nonselective conditions (Iscove's minimal essential medium, 10% calf serum) on microcarriers (Cytodex I, Pharmacia), trypsinized (0.25%), separated from the microcarriers by unit gravity filtration through a 100-μm mesh, washed, and counted. Each mouse (16 to 23 g) received transplants of 3×10^6 cells per gram of body weight of the cell suspension (NIH/3T3, $n = 6$; NIH/3T3-FAT4, $n = 11$) by intraperitoneal injection. The mice were killed after 4 weeks by lethal ether anesthesia, then underwent peritoneal lavage with 1 ml of selective medium (nonselective medium plus G418 at 0.5 g/liter) via a single peritoneal incision. The recovered cells were then serially passaged in selective media to eliminate all cells not resistant to G418 (2 to 3 weeks). Southern blot analysis was performed with genomic DNA digested with the restriction endonuclease Hind III and hybridized with a ^{32}P-labeled human α1AT cDNA probe as described (*12*). Poly(A) selection was used to isolate mRNA for Northern analysis with a ^{32}P-labeled human α1AT cDNA probe (*24*). Secretion of human α1AT by the cells was evaluated by immunoprecipitation of supernates from [^{35}S]methionine-labeled cells with antibody to human α1AT antibody (*24*). DNA analysis: lane 1, normal NIH/3T3 cells; lane 2, pre-transplant NIH/3T3-FAT4 cells; and lane 3, post-transplant recovered G418-resistant cells. Messenger RNA analysis: lane 4, normal NIH/3T3 cells; lane 5, pre-transplant NIH/3T3-FAT4 cells; and lane 6, post-transplant recovered G418-resistant cells. Secreted protein analysis: lane 7, normal NIH/3T3 cells; lane 8, pre-transplant NIH/3T3-FAT4 cells; and lane 9, post-transplant recovered G418-resistant cells.

nient to culture than the cells normally responsible for producing α1AT, such as hepatocytes and mononuclear phagocytes (*2, 3, 17*). Nevertheless, the fibroblasts produced human α1AT that diffused into the blood and, more importantly, reached the lower respiratory tract of the lung. Although large amounts would have to be produced by such cells in order to effectively treat α1AT deficiency, modifications such as the transplantation of clones containing multiple copies of the integrated gene could make this a feasible approach (*18*).

In addition to the long-term application of this approach to the therapy of hereditary disorders, transplantation of retroviral vector-produced clones secreting other proteins active in the extracellular milieu might provide a new approach to study the in vivo effects of such hormones and growth factors.

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Myocardial Failure in Cats Associated with Low Plasma Taurine: A Reversible Cardiomyopathy

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Thousands of pet cats die each year with dilated cardiomyopathy, the cause of which is unknown. Although taurine is present in millimolar concentrations in the myocardium of all mammals, taurine depletion has not previously been associated with a decrease in myocardial function in any species. In this study, low plasma taurine concentrations associated with echocardiographic evidence of myocardial failure were observed in 21 cats fed commercial cat foods and in 2 of 11 cats fed a purified diet containing marginally low concentrations of taurine for 4 years. Oral supplementation of taurine resulted in increased plasma taurine concentrations and was associated with normalization of left ventricular function in both groups of cats. Since myocardial concentrations of taurine are directly related to plasma concentrations and low plasma concentrations were found to be associated with myocardial failure in cats, a direct link between decreased taurine concentration in the myocardium and decreased myocardial mechanical function is proposed.

DILATED CARDIOMYOPATHY (DCM), a degenerative disease of the myocardium, results in decreased myocardial contractility (myocardial failure). DCM has been reported in humans, dogs, cats, and other species (*1*). The etiology of primary DCM in most cases, regardless of the species, is unknown and the prognosis is poor (*1*). The incidence of DCM in domestic cats is unknown, but a retrospective study at a large urban veterinary referral center revealed that 193 (3.0%) of 6385 consecutive necropsy reports on pet cats that died between January 1962 and December 1977 indicated a gross pathologic diagnosis of DCM (*2*).

Taurine (2-aminoethanesulfonic acid) is an essential nutrient for cats and possibly primates, including humans (*3*). The most prominent clinical sign associated with taurine deficiency in humans and animals is photoreceptor cell degeneration (*3*). Taurine is not a constituent of mammalian proteins and its major metabolic role has been attributed to bile salt conjugation (*4*). Myocardium and retina have the highest concentration of free taurine in the body, ranging between 100 and 400 times that found in plasma. This concentration gradient is maintained by an energy-dependent transport process that is saturable and is mediated, at least in part, by adrenergic mechanisms (*5*). Taurine may play a role in the inotropic, metabolic, and osmotic regulation of the myocardium (*6*). In addition, congestive heart failure in humans, dogs, and rabbits is associated with increased myocardial taurine concentrations (*7*). Although several studies imply that taurine affects the inotropic properties of the heart in vivo and in vitro in several species, including humans (*8*), the basic physiologic function of taurine in the heart is unknown.

Taurine depletion is difficult to induce in most species. In cats, however, the biosynthesis of taurine is minimal and conjugation of bile acids with taurine is obligatory. Feeding taurine-deficient diets to cats results in low concentrations of taurine in plasma and tissues, including the retina and myocardium (*3, 9*). Taurine depletion for more than 6 months may produce feline central retinal degeneration (FCRD) (*3, 9*). In taurine-depleted rats, no mechanical cardiac abnormalities have been noted, and mechanical function of the heart has not been specifically investigated in taurine-depleted cats (*9*). In this report we present results that implicate low concentrations of taurine in the plasma [and therefore by deduction in myocardial tissues (*5, 9*)] as a major causal factor of DCM in cats.

Twenty-three cases of DCM were diagnosed at the University of California Veterinary Medical Teaching Hospital between 1 December 1986 and 1 April 1987. DCM was diagnosed by echocardiography in 21 client-owned cats (group 1) and in 2 of 11 female cats maintained in a specific pathogen-free (SPF) colony fed a purified diet containing marginal concentrations of taurine (250 or 500 mg per kilogram of dry diet) for 4 years (group 2). M-mode echocardiograms, indirect funduscopic examinations, plasma taurine concentrations, and dietary histories were obtained for all cats.

Four cats died from congestive heart failure within hours of arriving at the clinic.

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