In vivo retroviral gene transfer into human bronchial epithelia of xenografts.

J F Engelhardt, … , J R Yankaskas, J M Wilson

J Clin Invest. 1992;90(6):2598-2607. https://doi.org/10.1172/JCI116155.

Research Article

Cystic fibrosis (CF) is the most common lethal inherited disease in the Caucasian population with an incidence of approximately 1 in 2,500 live births. Pulmonary complications of CF, which are the most morbid aspects of the disease, are caused by primary abnormalities in epithelial cells that lead to impaired mucociliary clearance. One potential therapeutic strategy is to reconstitute expression of the CF gene in airway epithelia by somatic gene transfer. To this end, we have developed an animal model of the human airway using bronchial xenografts and have tested the efficiency of in vivo retroviral gene transfer. Using the LacZ reporter gene, we find the efficiency of in vivo retroviral gene transfer to be dramatically dependent on the regenerative and mitotic state of the epithelium. Within an undifferentiated regenerating epithelium in which 40% of nuclei labeled with BrdU, 5-10% retroviral gene transfer was obtained. In contrast, no gene transfer was noted in a fully differentiated epithelium in which 1% of nuclei labeled with BrdU. These findings suggest that retroviral mediated gene transfer to the airway in vivo may be feasible if the proper regenerative state can be induced.

In Vivo Retroviral Gene Transfer into Human Bronchial Epithelia of Xenografts

John F. Engelhardt,* James R. Yankaskas,‡ and James M. Wilson*
**Departments of Internal Medicine and Biological Chemistry, University of Michigan, Ann Arbor, Michigan 48109-0650; and ‡Division of Pulmonary Diseases, The University of North Carolina, Chapel Hill, North Carolina 27599-7020*

Abstract

Cystic fibrosis (CF) is the most common lethal inherited disease in the Caucasian population with an incidence of ~ 1 in 2,500 live births. Pulmonary complications of CF, which are the most morbid aspects of the disease, are caused by primary abnormalities in epithelial cells that lead to impaired mucociliary clearance. One potential therapeutic strategy is to reconstitute expression of the CF gene in airway epithelia by somatic gene transfer. To this end, we have developed an animal model of the human airway using bronchial xenografts and have tested the efficiency of in vivo retroviral gene transfer. Using the LacZ reporter gene, we find the efficiency of in vivo retroviral gene transfer to be dramatically dependent on the regenerative and mitotic state of the epithelium. Within an undifferentiated regenerating epithelium in which 40% of nuclei labeled with BrdU, 5–10% retroviral gene transfer was obtained. In contrast, no gene transfer was noted in a fully differentiated epithelium in which 1% of nuclei labeled with BrdU. These findings suggest that retroviral mediated gene transfer to the airway in vivo may be feasible if the proper regenerative state can be induced. (*J. Clin. Invest.* 1992. 90:2598–2607.) Key words: gene therapy • cystic fibrosis • recombinant retroviruses • airway epithelial cells • gene transfer

Introduction

Cystic fibrosis (CF)[1] is an inherited disease that affects several organ systems. The pulmonary manifestations of CF, which are believed to be caused by defects in mucociliary clearance, are the most morbid manifestations of the disease (1). Isolation of the gene responsible for CF suggested possible therapeutic strategies based on lung-directed somatic gene transfer (2, 3, 4). Functional correction of the defect in cultured CF cells by viral transduction of a gene encoding a normal version of the cystic fibrosis transmembrane conductance regulator (CFTR) provided support for the feasibility of this approach (5, 6).

Rational development of somatic gene therapies for CF requires a better understanding of the distribution of CF gene expression in the human lung. Experience with the clinical manifestations and pathologic consequences of CF strongly suggests that the cells of the airway are directly involved in pathogenesis (1). Several studies have supported this hypothesis, including our recent characterization of normal human bronchus, which localized CFTR RNA and protein to both the surface epithelium and submucosal glands (6a). The surface epithelium is affected by CF (7, 8) and has features that make it an attractive but challenging target for direct gene transfer. It may be possible to achieve efficient access of gene transfer substrates to the surface cells of the airway through relatively noninvasive means such as aerosol inhalation or bronchial lavage. However, the surface epithelium is a complex structure that normally has a low level of cell cycling (9).

Several approaches for delivering genes to the airway have been proposed including retroviruses (6, 10, 11), adenoviruses (12), adeno-associated viruses (13, 14), and liposomes (15). While each has its potential advantages, no single approach has emerged as a method of choice for CF. The largest experience in other models of gene therapy has been with retroviruses. Many animal models (16) and all of the clinical applications (17) of gene therapy to date are based on transplantation of autologous cells modified ex vivo with retroviruses. More recently, retroviruses have been used to directly introduce genes into blood vessels and liver (18, 19). An important consideration in the application of retroviruses to in vivo airway gene transfer relates to the extensive experience with these reagents in other models and their relative safety. Disadvantages include relatively low viral titers and the presumed requirement for active replication of the recipient cell to achieve gene transduction.

Critical to the development of effective gene therapies of CF is the availability of animal models to evaluate the feasibility, efficacy, and safety of the various approaches. Epithelial cells derived from the airway of a variety of animals have been shown to develop into fully differentiated epithelia after they are seeded onto denuded trachea and implanted into nu/nu mice (20, 21). Our initial attempt at using the xenograft system for studying gene transfer was based on the reconstitution of denuded trachea with rat tracheal cells exposed to retroviruses while in primary culture (10). Grafts seeded with the genetically modified cells developed a fully differentiated epithelium with patches of transgene expressing cells possibly representing clonal outgrowth of retroviral transduced progenitor cells. We

Address correspondence to James M. Wilson, M.D., Ph.D., The University of Michigan Medical Center, 4554 MSRB II, Box 0650, 1150 West Medical Center Drive, Ann Arbor, MI 48109-0650.

Received for publication 3 June 1992 and in revised form 15 July 1992.

1. *Abbreviations used in this paper:* BrdU, 5-bromo-2′-deoxyuridine-5′-monophosphate; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; DS, donkey serum.

J. Clin. Invest.

0021-9738/92/12/2598/10 $2.00
Volume 90, December 1992, 2598–2607

report in this study a xenograft animal model based on the generation of a fully differentiated human epithelium in athymic mice. This model was used to assess the potential of retroviruses for delivering recombinant genes into the human airway.

Methods

Epithelial cell isolation and culture. Human bronchial tissue (three different bronchial samples were analyzed) was obtained from healthy donor lungs at the time of lung transplantation. Primary cultures were prepared according to a previous protocol with modifications (22). The tissue was soaked and rinsed in modified Eagle's medium containing 50 U/ml penicillin, 50 μg/ml streptomycin, 40 μg/ml tobramycin, 50 μg/ml ceftazidime, 2.5 μg/ml amphotericin B, 10 μg/ml DNAse, and 0.5 mg/ml dithiothreitol for 4–12 h at 4°C. The tissue was then transferred to the same medium supplemented with 0.1% protease-14 and incubated for an additional 30–34 h at 4°C. FCS was then added to a final concentration of 10%, and the cells were removed by agitation and blunt scraping. Cells were pelleted and washed two times in Ham's F12 containing 10% FCS. Cells were resuspended in Ham's F12 hormonally defined medium containing 1 μM hydrocortisone, 10 μg/ml insulin, 30 nM thyroxine, 5 μg/ml transferrin, 25 ng/ml epidermal growth factor, 3.75 μg/ml endothelial cell growth supplement, 10 ng/ml cholera toxin, 50 U/ml penicillin, 50 μg/ml streptomycin, 40 μg/ml tobramycin, 50 μg/ml ceftazidime, and 2.5 μg/ml amphotericin B, and plated at a density of 2×10^6 cells per 100-mm plate (uncoated plastic). The medium on cultures was replaced every 24 h. Cultures were harvested on day 4 by treatment of 0.1% trypsin followed by the addition of 10% FCS/Ham's F12. Cells were pelleted and resuspended in hormonally defined medium for seeding into denuded rat tracheas.

Establishment of xenografts. Donor rat tracheas were harvested from CO_2 asphyxiated male Fisher 344 rats (200–250 g) and denuded by three rounds of freeze thawing followed by rinsing of the lumen with modified Eagle's medium. Tracheas were ligated to tubing at both ends after seeding of 2×10^4 or 2×10^6 primary bronchial epithelial cells in 30 μl of hormonally defined medium. These "open-ended" grafts were then inplanted subcutaneously in the flanks of female nu/nu BALB/c mice anesthetized by intraperitoneal injections of 100 mg/kg ketamine and 20 mg/kg xylazine. Implants were sutured such that the ends of the tubing exited through the back of the neck (Fig. 1 *A*). Grafts were flushed weekly with Ham's F12 to remove excess mucus.

Analysis of grafts by electron microscope. Xenografts were excised rinsed briefly in PBS followed by overnight fixation in 2.5% glutaraldehyde, 1.5% paraformaldehyde, 0.02% $CaCl_2$ in 0.1 M sodium cacodylate (pH 7.4) at 4°C. After fixation, the tissue was washed repeatedly in 0.1 M sodium cacodylate, postfixed in 1% osmium tetroxide (1 h at 0°C), dehydrated in alcohols, and embedded in epoxy resins. Sections were stained with uranyl acetate and lead citrate before being viewed and photographed in an electron microscope (model 11a; Hitachi Sci. Instrs., Mountain View, CA).

BrdU labeling and detection of mitotically active cells. Mitotically active cells were labeled at the time of BAG retroviral infection by the addition of 50 μg/ml 5-bromo-2′-deoxyuridine-5′-monophosphate (BrdU) to the retroviral medium. Grafts seeded with 2×10^4 and 1×10^6 cells were labeled with BrdU at 1 and 4 wk after transplantation, respectively. Infections were performed in the presence of BrdU for 24 hrs after which the tracheas were flushed with 2 ml F12 medium followed by expulsion of all fluid from the lumen of the graft with air. Grafts were harvested 3 d after infection and fresh frozen in optimal cutting temperature embeding compound. Sections (4-μm) were fixed in methanol for 10 min at −20°C followed by air drying. Sections were then treated with 2 *N* HCl for 1 h at 37°C and neutralized in 0.1 M NaBorate pH 8.5 for 10 min. After rinsing in PBS for 10 min, sections were blocked in 20% donkey serum (DS)/PBS for 30 min. Mouse anti–BrdU-FITC conjuagated monoclonal antibody (Boehringer Mannheim Corp., Indianapolis, IN) was incubated with the sections (50 μg/ml) in PBS containing 1.5% DS for 90 min at room temperature. Sections were washed in 1.5% DS/PBS and coverslipped in mounting medium (Citifluor Inc., London, England). Colocalization of lacZ positive cells with BrdU label was not possible because of HCl destruction of anti-lacZ reactive epitopes.

The percent of BrdU labeling was calculated by counting 30,000 cells (as evident by nuclei under Nomarski optics) from 12 different sections of two fully differentiated grafts and 3,000 cells from 12 sections of two regenerating grafts. The averages and standard deviations of this analysis are reported in the text.

Localization of cell specific cytokeratins and CFTR. Fresh frozen xenograft tissue was sectioned at 6 μm onto poly(L-lysine)-coated slides and fixed in acetone for 10 min at −20°C followed by air drying. Sections were blocked in 20% DS in PBS for 30 min followed by the addition of the following primary antibodies for 90 min. CFTR was localized by indirect double immunofluorescence using an affinity purified rabbit polyclonal antibody to a COOH-terminal peptide (residues 1468–1480) of CFTR (23, 24, 25). This previously characterized antibody is designated α-1468 and shows reactivity in normal bronchial epithelium in a subset of keratin-14 positive cells (6 a). Cytokeratin-14 (RCK107) and cytokeratin-18 (RGE53) monoclonal hybridoma supernatants were kindly provided by Dr. Frans Ramekers University of Limburg, Maastricht, The Netherlands and have been previously characterized (26, 27). Hybridoma supernatants were used undiluted and α-1468 was used at a concentration of 5 μg/ml. After primary antibody incubation the sections were washed three times and incubated with 5 μg/ml of FITC–anti-mouse or FITC–anti-rabbit for 30 min. Sections were washed and coverslipped in mounting medium.

Peptide competition experiments were performed with α-1468 by preincubating 0.5 mg/ml peptide (residues 1468–1480 of CFTR protein) with 0.25 mg/ml of α-1468 in PBS containing 1.5% DS overnight at 4°C before immunofluorescent staining. Control uncompeted antibody was incubated under the same conditions but without peptide.

Production of virus and retroviral infection. Previously described amphotropic packaging cell lines harboring the BAG lacZ containing provirus (28) and low density lipo-protein receptor containing provirus (29) were grown in DME containing 10% FCS and 1 μg/ml penicillin/streptomycin. In the BAG construct lacZ is expressed from the viral long terminal repeat. Virus was harvested from a confluent monolayer of cells by the addition of fresh medium for 18 h. After harvesting, stocks were supplemented with 3 μg/ml polybrene and passed through 0.2-μm filters. Viral stocks were used fresh for direct infusion into xenografts. Xenografts were exposed to 1 ml of retrovirus by slow infusion for 1 h in all cases except for retroviral infection in the presence of BrdU in which the lumen of the grafts were filled with virus and incubated for 24 h. After infection, grafts were rinsed with 2 ml of Ham's F12 followed by expulsion of all fluid from the graft with air.

Analysis of grafts for expression of lacZ. Xenografts were cytochemically analyzed for lacZ transgene expression using a previously described protocol (10). At various times after retroviral infections xenografts were excised from euthanized mice and directly fixed in 0.5% glutaraldehyde/PBS for 10 min followed by rinsing in 1 mM $MgCl_2$/PBS for two 15-min washes. Xenograft rings were then stained in Xgal solution (10) for 4 h. Tissue was then rinsed in PBS and postfixed in 4% paraformaldehyde/PBS overnight before embedding in glycomethacrylate by standard protocol.

Results and Discussion

Epithelial cells derived from main stem bronchi of donor organs were plated in culture and subsequently harvested and seeded onto denuded rat trachea that were implanted into nu/nu mice. Several problems were encountered in our initial experiments. When ligated at both ends, the implanted tracheas accumulated increasing quantities of mucus that eventually led

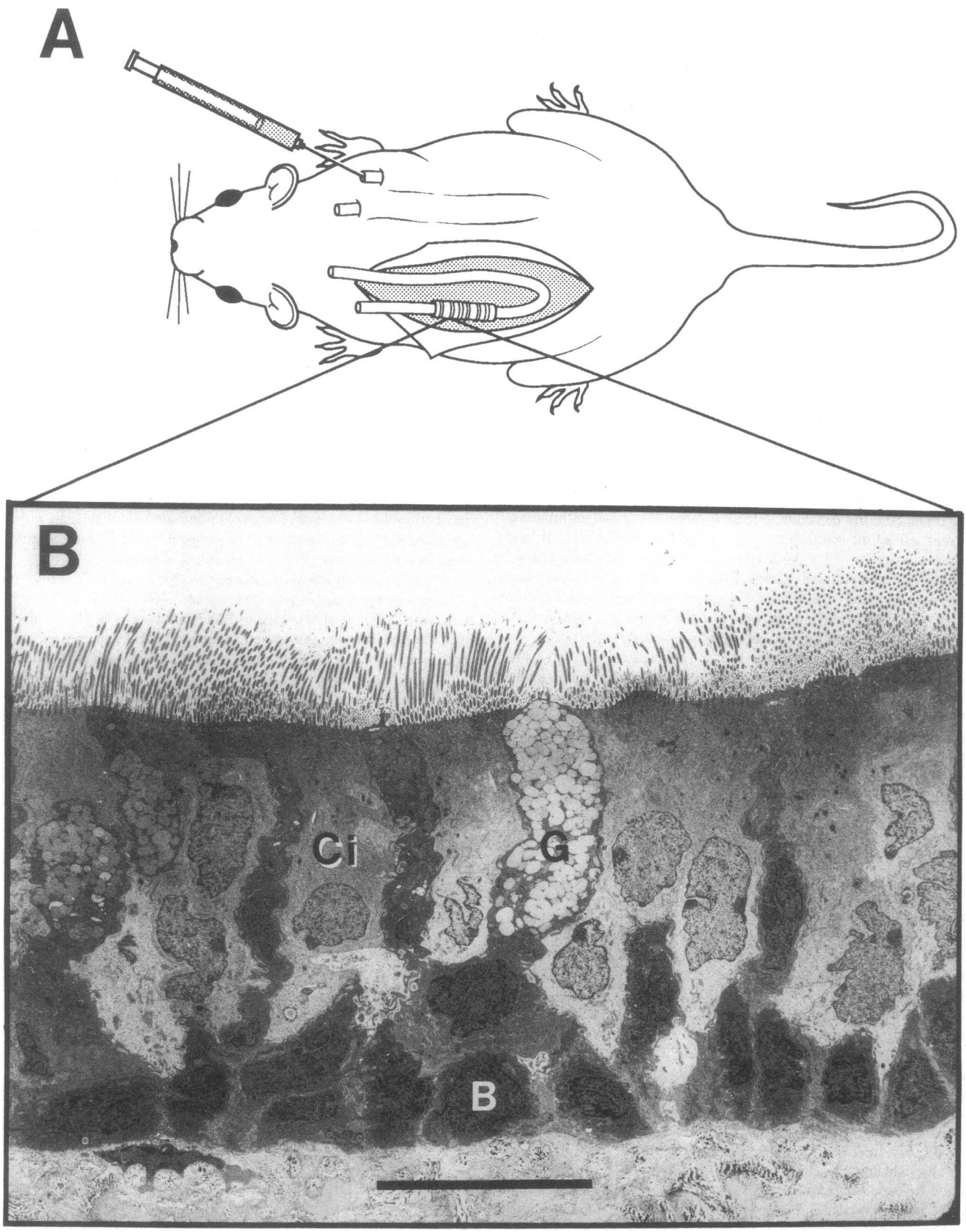


Figure 1. Open-ended xenografts. (*A*) Xenografts were ligated to flexible plastic tubing and implanted subcutaneously. Implants were sutured such that the ends of the tubing exited through the back of the neck. (*B*) Transmission electron micrograph of a fully differentiated bronchial xenograft epithelium. Xenograft was seeded with 2×10^6 cells and allowed to develop for 3 wk. *Ci*, ciliated cell; *G*, goblet cell; and *B*, basal cell (bar = 20 μm).

to distortion of the graft. After 3–6 wk incubation, the reconstituted epithelium contained major areas of a squamous or cuboidal epithelium, as well as pseudostratified morphology. To prevent the intraluminal accumulation of mucus and provide easy access to the xenograft, we modified the system in the following manner. The ends of the implanted tracheas were kept open to the surface via plastic tubing that was tied to the proximal and distal ports of the graft, tunneled subcutaneously, and exited percutaneously. Fig. 1 *A* illustrates this modification. Xenografts established using this technique were irrigated regularly with isotonic buffer to prevent accumulation of mucus. The resulting fully mature graft produced a pseudostratified epithelium along the entire surface of the the trachea that was indistinguishable from that of a normal bronchus. An electron micrograph of a typical xenograft is presented in Fig. 1 *B*. All cell types were represented including a layer of basal cells, ciliated cells, secretory goblet cells, and nonciliated columnar cells. A more complete morphological description of the human xenograft system will be provided elsewhere.

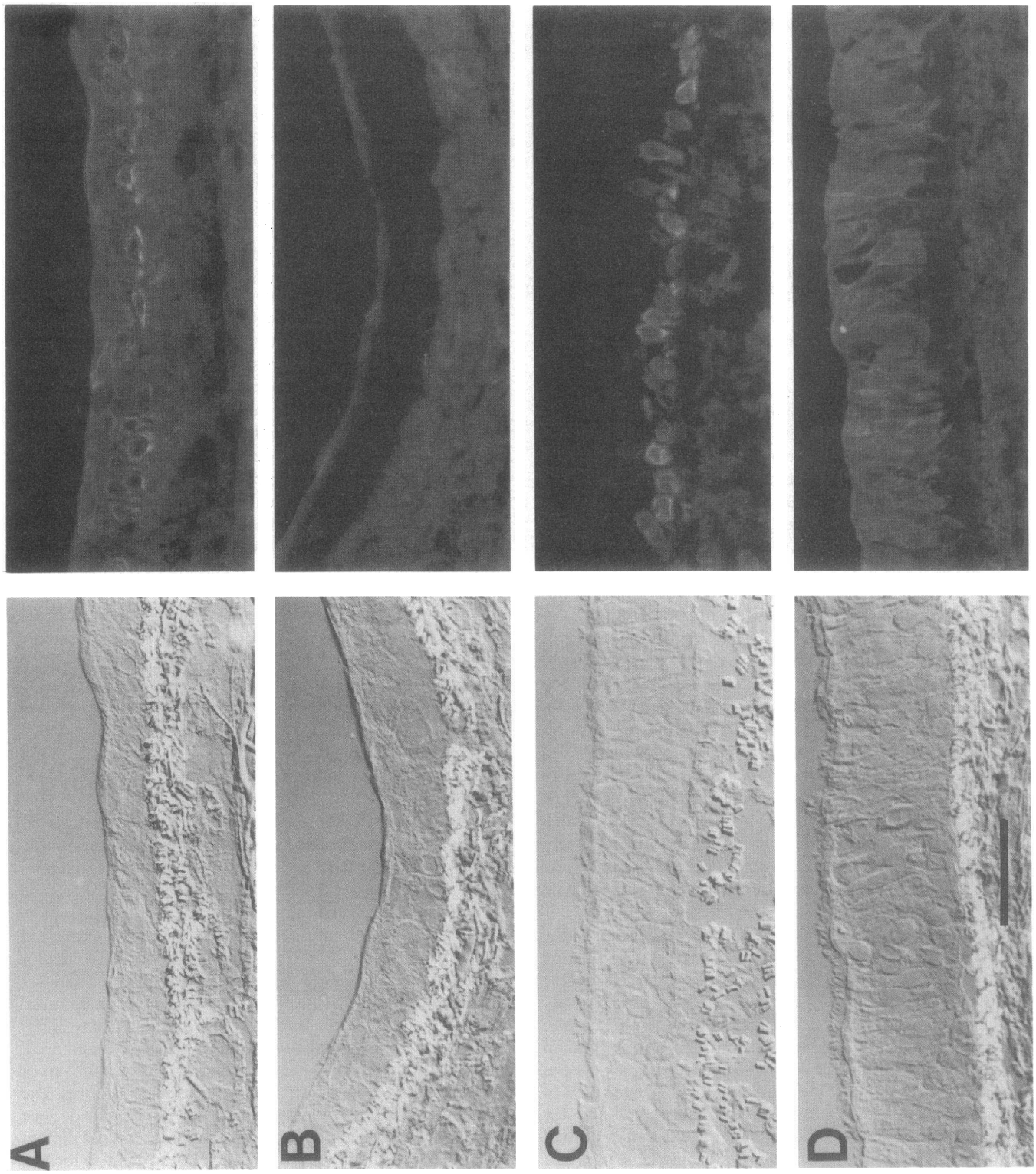
The modified xenografts were used to study the biology of retroviral-mediated gene transfer. Two different biological substrates for gene transfer were established: grafts were seeded with a low density of cells (2×10^4 cells) and maintained for one week in the nu/nu mice to produce a regenerating epithelium, or were seeded with a high density of cells (2×10^6 cells) and maintained for 3 wk in the nu/nu mice to provide a fully differentiated, relatively quiescent epithelium.

Analysis of morphology and proliferative activity of regenerating and differentiated grafts. Light microscopic analysis of grafts seeded at low density revealed relatively undifferentiated morphology. Cells were either squamous or cuboidal in shape and were frequently stratified into multiple layers. Approximately > 95% of the cells reacted with antibodies for the basal cell specific marker keratin-14 (Fig. 2 *A*), while infrequent squamous cells lining the lumen of this cuboidal epithelium (< 2% of total cells) reacted with a cytokeratin-18 antibody (Fig. 2 *B*). None of the cells appeared to polarize, contain cilia, or react to alcian blue or periodic acid-Schiff (data not shown). When grafts seeded at low density were incubated with BrdU to assess the overall state of cell proliferation at the time of retroviral infection, 40±4% of the cells were mitotically active (Fig. 3 *A*). These studies indicate that after 1 wk, grafts seeded with 2×10^4 cells contain an undifferentiated epithelium that is rapidly regenerating (subsequently referred to as regenerating grafts). If left in the nu/nu mice for 6 wk, these grafts became quiescent and fully differentiated (Fig. 5 *B*).

Grafts seeded at high density (2×10^6 cells) develop a fully differentiated epithelium within 3 wk. The pattern of staining seen in fully differentiated grafts with the basal cell specific keratin-14 antibody (Fig. 2 *C*) and the keratin-18 antibody (Fig. 2 *D*), which is specific for differentiated columnar cells, was equivalent to the pattern of staining in normal human bronchus (data not shown) and that reported in other species such as rat (26, 27). In addition to characterizing the pattern of cytokeratin staining, we felt it was important to compare the localization of CFTR in the fully differentiated graft to normal human bronchus, since this xenograft system will be used as a model for studying CF gene therapies. Fig. 2 *E* shows the pattern of staining within a fully differentiated graft to a polyclonal antibody made to the COOH terminus of CFTR. This pattern is virtually identical to that seen in normal human bronchial samples (6 a), and is competed by preincubation of the antibody with peptide (Fig. 2 *F*). Low level staining is detected on the apical surface of columnar epithelial cells, while a higher level of staining is seen in basal and intermediate cells. Incubation of these grafts with BrdU resulted in labeling of 1.1±0.2% of the cells (Fig. 3 *B*), a number similar to that found in the bronchi of several types of animals including rat and mouse (9). We conclude that after 3 wk, grafts seeded at high density produce an epithelium that closely resembles that of a normal, unperturbed human main stem bronchus (subsequently referred to as differentiated grafts).

Analysis of retroviral gene expression in regenerating and differentiated grafts. Regenerating grafts were exposed to lacZ retrovirus and subsequently analyzed histochemically for retroviral gene expression. Analysis of grafts 3 d after infection revealed lacZ expression in 5–10% of the reconstituted epithelium (Figs. 4 *A* and 5 *A*). Expression of lacZ was demonstrated in cells located throughout the depth of the apparently undifferentiated stratified epithelium (Fig. 5 *A*). When these grafts were allowed to develop into a fully differentiated epithelium (42 d after exposure to virus) the pattern of Xgal staining changed from frequent positive single cells to a limited number of large aggregates of transgene expressing cells with occasional positive single cells. The total surface area of the differentiated graft that was repopulated with lacZ expressing cells varied from 1–5% between three different tissue samples analyzed. This pattern of staining is compatible which clonal expansion of retroviral transduced progenitor cells. Within these aggregates, all cell types were found to express the transgene including basal cells, goblet cells, ciliated cells, and nonciliated columnar cells. The intensity of Xgal staining in undifferentiated grafts appeared to diminish when compared with staining of the same grafts allowed to differentiate for 42 d after infection. This difference may be caused by variation in long terminal repeat transcription effected by the differentiated state of the epithelium. Experiments in which retroviruses were exposed to fully differentiated epithelium produced dramatically different results. Fully differentiated grafts exposed to lacZ retroviruses failed to demonstrate any evidence for retroviral gene transfer or expression based on the Xgal histochemical stain (Figs. 4 *C* and 5 *C*).

These studies demonstrate the feasibility of directly delivering recombinant genes to a human airway epithelium with retroviruses. The efficiency of gene transfer into an undifferentiated regenerating epithelium was only 5–10-fold less than that achieved when the same virus was exposed to a rapidly dividing culture of murine fibroblasts for 12 h (data not shown). Importantly, the transgene continued to express for the life of the xenograft (5 wk after exposure to virus) and was distributed in all cell types (Figs. 4 *B* and 5 *B*). In contrast, retroviral mediated gene transfer into a quiescent, fully differentiated graft was remarkably inefficient (Figs. 4 *C* and 5 *C*). This may be explained in part by the extremely low numbers of dividing cells in these graft at the time of exposure to virus. The requirement of cell division for retroviral-mediated gene transfer has been demonstrated in vitro using cultured diploid fibroblasts (30). Other factors may have contributed to the low efficiency of gene transfer in the differentiated grafts including diminished expression of the receptor for the amphotropic virus or localization of the receptor to inaccessible areas of the cell such as intracellular compartments or the basolateral sur-


A
B
C
D

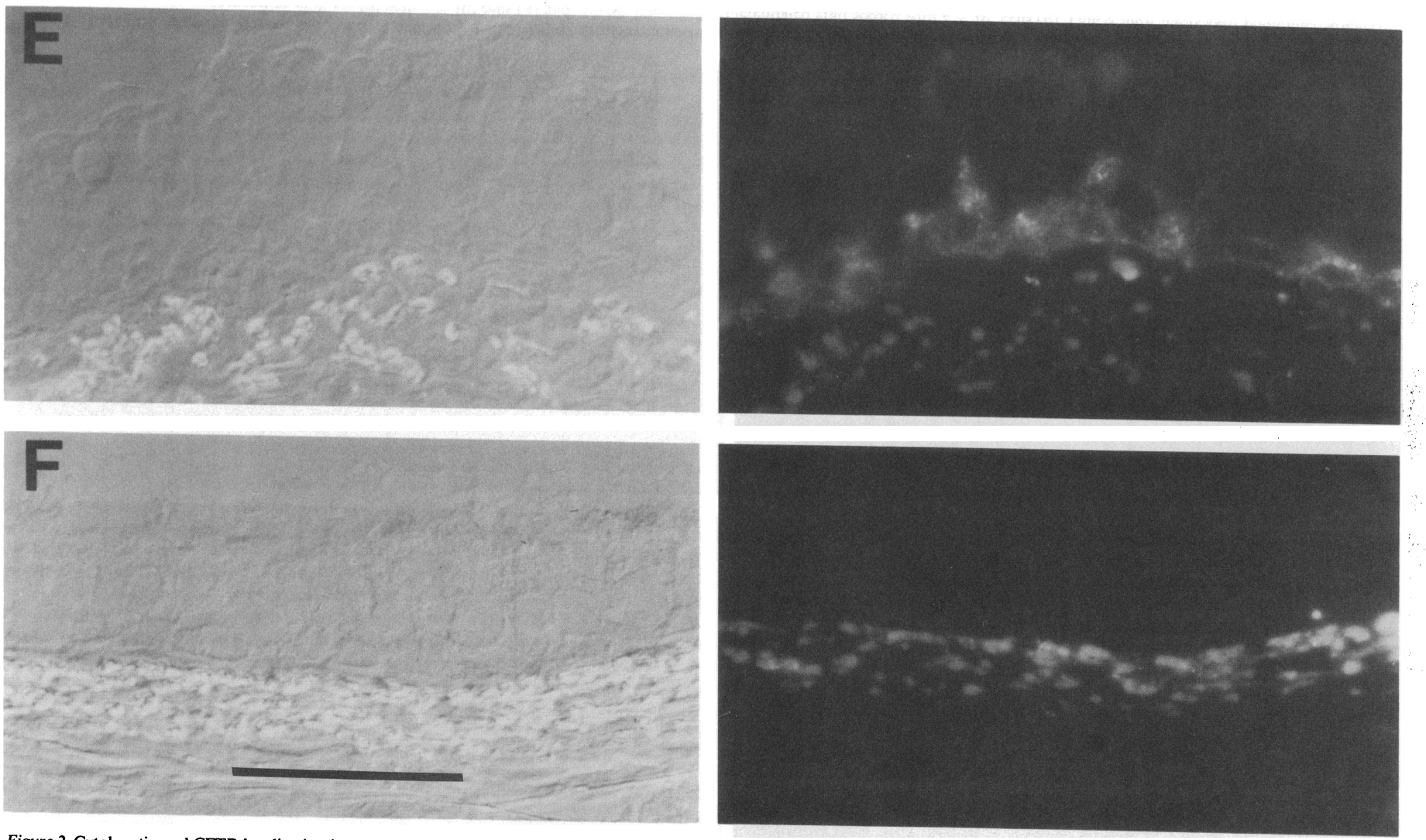


Figure 2. Cytokeratin and CFTR localization in xenografts. Photomicrographs show indirect immunofluorescent detection of cytokeratin-14 (*A* and *C*) and cytokeratin-18 (*B* and *D*) staining in regenerating (*A* and *B*) and fully differentiated (*C* and *D*) xenograft epithelia. Localization of CFTR within a fully differentiated xenograft epithelium in the absence of peptide antigen (*E*) and after preincubation of the α-1468 antibody with peptide antigen (*F*). Each set of pictures consists of a Nomarski (*left*) and immunofluorescent (*right*) photomicrograph. Microphot-FXA Nikon microscope (bar = 60 μm).

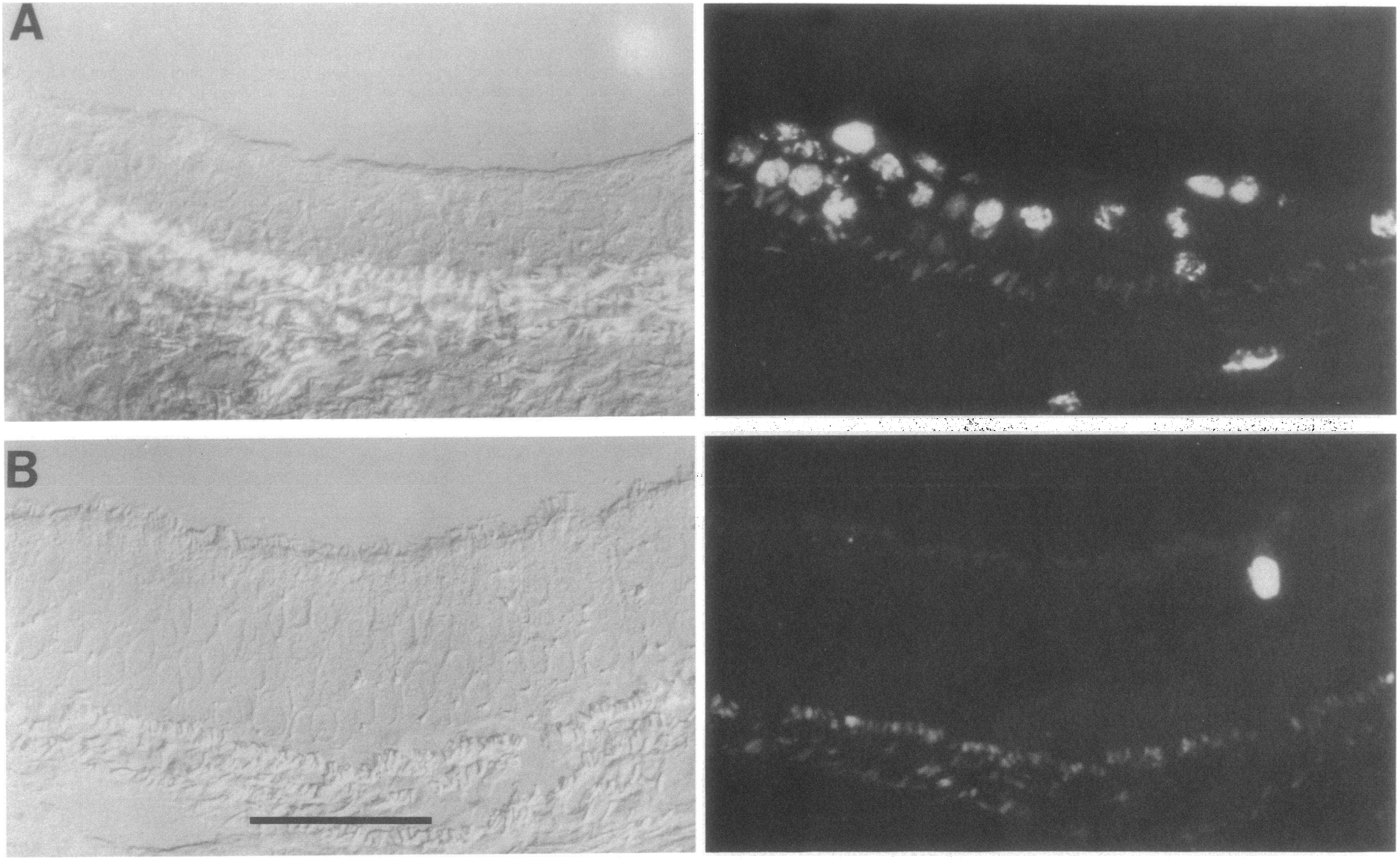


Figure 3. BrdU incorporation into mitotically active cells of regenerating and fully differentiated xenograft epithelia. Mitotically active cells were labeled with BrdU during a 24-h retroviral infection with BAG at 1 wk into a regenerating graft seeded with 2×10^4 cells (*A*) and at 4 wk into a fully differentiated graft seeded with 2×10^6 cells (*B*). Panels show fluorescent photomicrographs of anti-BrdU-FITC fluorescence (*right*), and Nomarski photomicrographs of the same field (*left*). Microphot-FXA Nikon microscope (bar = 60 μm).

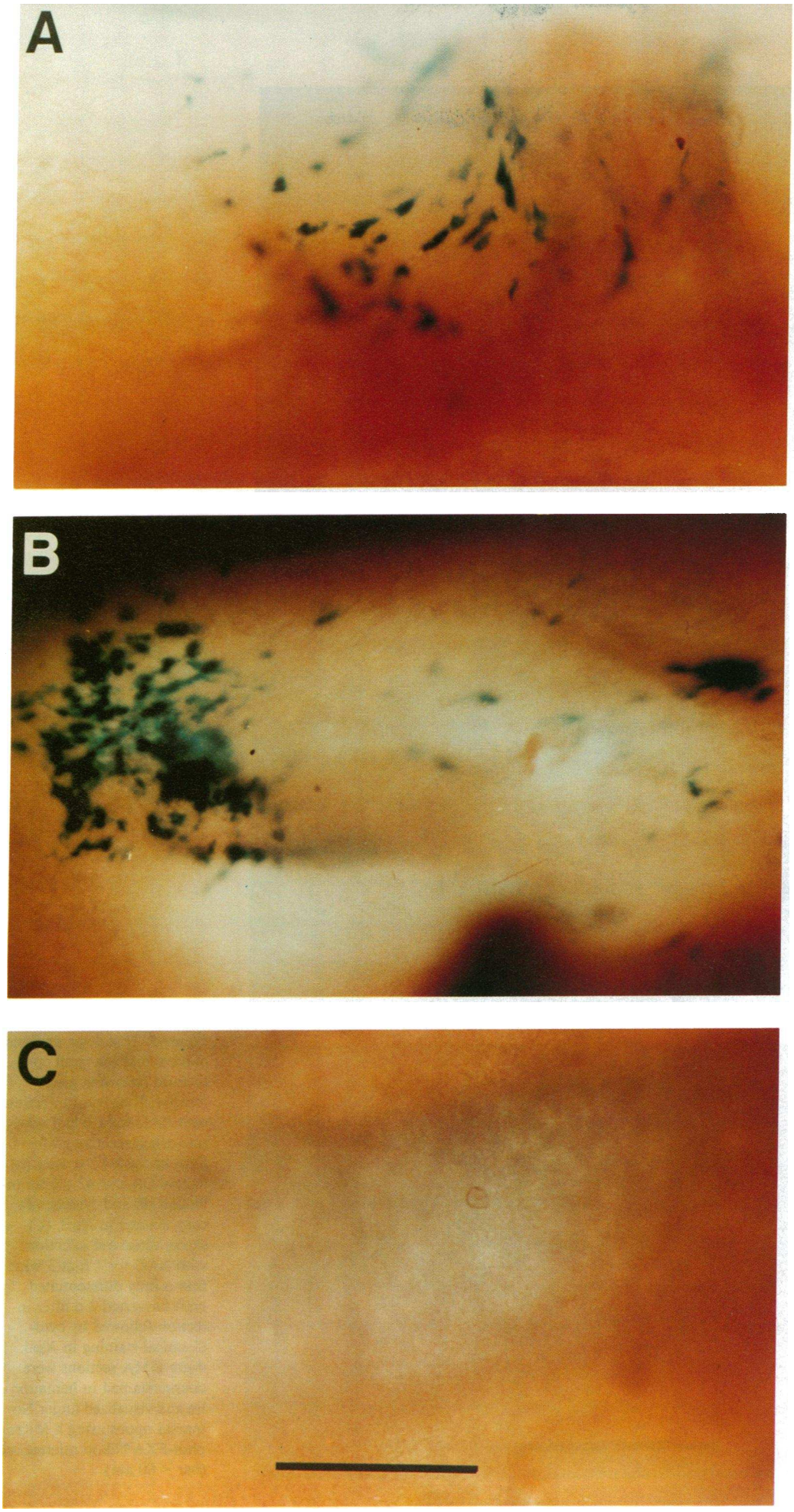


Figure 4. Retroviral infection into regenerating and fully differentiated epithelium. Micrographs show the luminal surface of grafts seeded with 2×10^4 cells, infected with BAG retrovirus for 1 h, and harvested 3 d (*A*) and 42 d (*B*) after infection followed by cytochemical staining in Xgal. (*C*) shows the luminal surface of a graft seeded with 2×10^6 cells, infected with BAG retrovirus for 1 h, and harvested 3 d after infection followed by cytochemical staining in Xgal. No Xgal staining was seen in BAG infected fully differentiated grafts harvested 21 d after infection or in any grafts (regenerating or fully differentiated) infected with LDLR retrovirus (data not shown). Microphot-FXA Nikon microscope (bar = 300 μm).

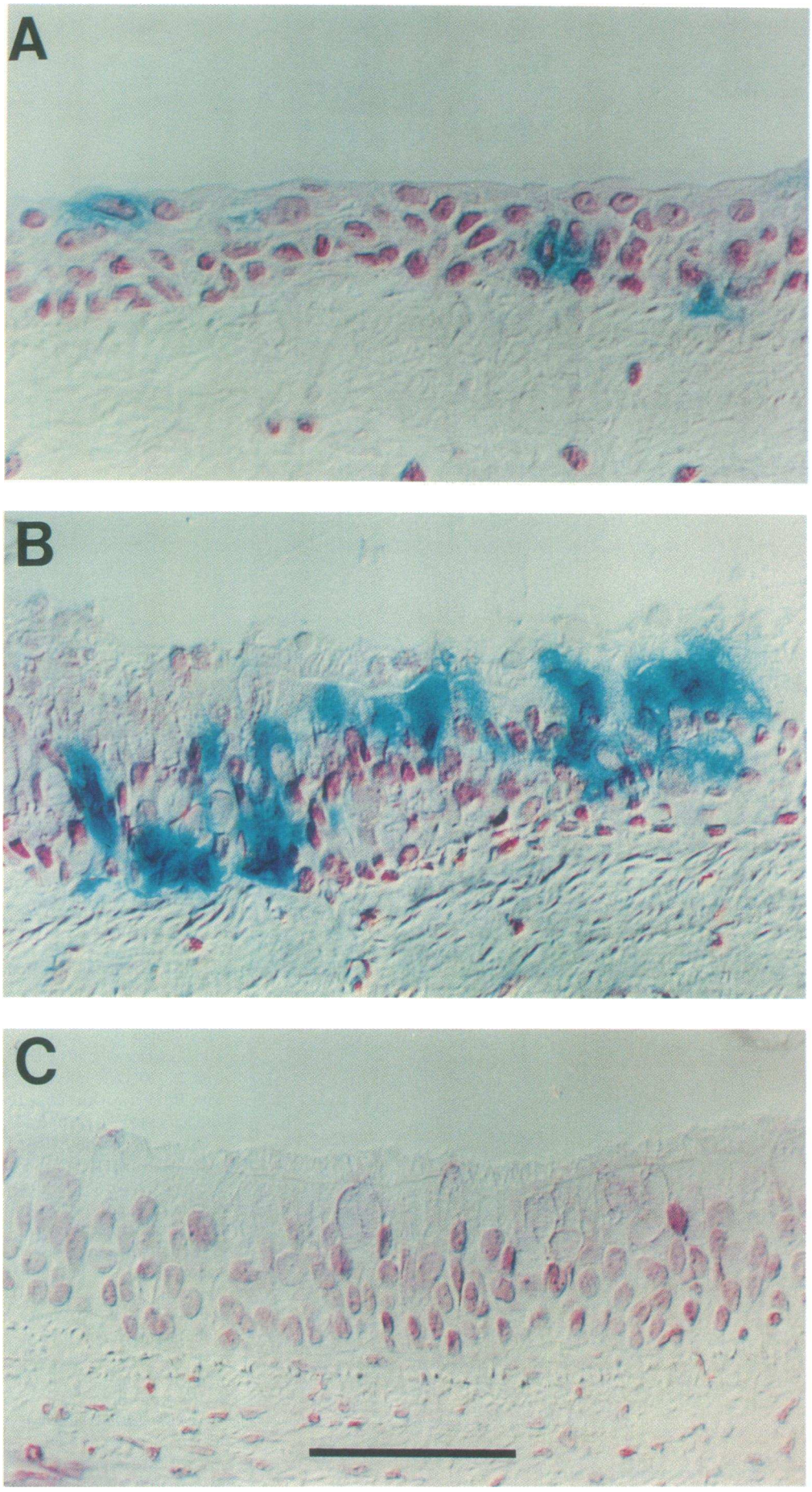


Figure 5. Histologic analysis of bronchial xenograft epithelium following gene transfer. Retroviral infection with BAG for 1 h at 1 wk into a regenerating epithelium harvested 3 d after infection (*A*) and 42 d after infection (*B*) and cytochemically stained in Xgal. (*C*) shows retroviral infection with BAG for 1 h at 3 wks into a fully differentiated graft harvested 3 d after infection followed by cytochemical staining in Xgal. 4-μm GMA sections were counterstained in hematoxylin and visualized under Nomarski optics using a Microphot-FXA Nikon microscope (bar = 60 μm).

face. It is impossible to study these potential mechanisms because the amphotropic virus receptor has not been identified.

The important challenge is to translate these observations in the xenografts to practical approaches of gene transfer into the airway in vivo. These data strongly suggest that it will be necessary to significantly perturb the biology of the airway in order to achieve significant levels of gene transfer. Various models of airway injury have been described which lead to compensatory increases in the proliferation of various cell types of the upper airway including basal cells and various secretory cells (9). These usually involve transient exposure to noxious stimuli such as toxic chemicals, mechanical irritation, or viral pathogens. The clinical feasibility of these interventions remains to be demonstrated. Alternatively, it may be possible to deliver genes via retroviruses to the undifferentiated epithelium of fetuses in utero, circumventing the need for airway injury in the adult. The relevance of the regenerating graft to models of airway injury and the feasibility of accomplishing gene transfer in an endogenous airway must await additional studies in animals. In addition, it is not clear if the efficiency of gene transfer accomplished in the undifferentiated graft (i.e., 5–10%) would be sufficient to achieve a therapeutic effect. Boucher et al. (31) have achieved significant functional correction in a polarized monolayer of CF cells when only 10% of the cells have been corrected by gene transfer.

In summary we describe an animal model for evaluating the feasibility and potential efficacy of somatic gene therapies for CF. Our results support the use of recombinent retroviruses in these therapies if the recipient airway epithelium is relatively undifferentiated and rapidly regenerating.

Acknowledgments

We thank Lorita Dudus for technical support and Ed Allen (Cell Morphology Core) for his contribution with electron microscopy. The support and advice of Francis Collins were greatly appreciated.

This work was supported by the Cystic Fibrosis Foundation and the National Institutes of Health (HL-34322).

References

1. Boat, T. F., M. J. Welsh, and A. L. Beaudet. 1989. Cystic fibrosis. *In* The Metabolic Basis of Inherited Disease. 6th ed. C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle, editors. McGraw-Hill, New York. 2649–2680.

2. Rommens, J. M., M. C. Iannuzzi, B-S. Kerem, M. L. Drumm, G. Melmer, M. Dean, R. Rozmahel, J. L. Cole, D. Kennedy, N. Hidaka, et al. 1989. Identification of the cystic fibrosis gene: chromosome walking and jumping. *Science (Wash. DC).* 245:1059–1065.

3. Riordan, J. R., J. M. Rommens, B-S. Kerem, N. Alon, R. Rozmahel, Z. Grzelczak, J. Zielenski, S. Lok, N. Plavsic, J-L. Chou, et al. 1989. Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science (Wash. DC).* 245:1066–1072.

4. Kerem, B-S., J. M. Rommens, J. A. Buchanan, D. Markiewicz, T. K. Cox, A. Chakravarti, M. Buchwald, and L-C. Tsui. 1989. Identification of the cystic fibrosis gene: genetic analysis. *Science (Wash. DC)* 245:1073–1080.

5. Rich, D. P., M. P. Anderson, R. J. Gregory, S. H. Cheng, S. Paul, D. M. Jefferson, J. D. McCann, K. W. Klinger, A. E. Smith, and M. J. Welsh. 1990. Expression of cystic fibrosis transmembrane conductance regulator corrects defective chloride channel regulation in cystic fibrosis airway epithelial cells. *Nature (Lond.).* 347:358–363.

6. Drumm, M. T., H. A. Pope, W. H. Cliff, J. M. Rommens, S. A. Marvin, L-C. Tsui, F. S. Collins, R. A. Frizzell, and J. M. Wilson. 1990. Correction of the cystic fibrosis defect in vitro by retrovirus-mediated gene transfer. *Cell.* 62:1227–1233.

6a. Engelhardt, J. F., J. R. Yankaskas, S. A. Ernst, Y. Yang, C. R. Marino, R. C. Boucher, J. A. Cohn, and J. M. Wilson. 1992. Submucosal glands are the predominant site of CFTR expression in the human bronchus, *Nature Genetics.* 2:240–248.

7. Knowles, M., J. Gatzy, and R. Boucher. 1983. Relative ion permeability of normal and cystic fibrosis nasal epithelium. *J. Clin. Invest.* 71:1410–1417.

8. Boucher, R. C., C. U. Cotton, J. T. Gatzy, M. R. Knowles, and J. R. Yankaskas. 1988. Evidence for reduced Cl^- and increased Na^+ permeability in cystic fibrosis human primary cell cultures. *J. Physiol.* 405:77–103.

9. Ayers, M. M., and P. K. Jeffery. 1988. Proliferation and differentiation in mammalian airway epithelium. *Eur. Respir. J.* 1:58–80.

10. Engelhardt, J. F., E. D. Allen, and J. M. Wilson. 1991. Reconstitution of tracheal grafts with a genetically modified epithelium. *Proc. Natl. Acad. Sci. USA.* 88:11192–11196.

11. Stanley, C., M. B. Rosenberg, and T. Friedmann. 1991. Gene transfer into airway epithelial cells using retroviral vectors. *Somatic Cell Mol. Genet.* 17:185–190.

12. Rosenfeld, M. A., K. Yoshimura, B. C. Trapnell, K. Yoneyama, E. R. Rosenthal, W. Dalemans, M. Fukayama, J. Bargon, L. E. Stier, L. Stratford-Perricaudet, et al. 1992. In vivo transfer of the human cystic fibrosis transmembrane conductance regulator gene to the airway epithelium. *Cell.* 68:143–155.

13. Samulski, R. J., L. S. Chang, and T. Shenk. 1989. Helper-free stocks of recombinant adeno-associated viruses: normal integration does not require viral gene expression. *J. Virol.* 63:3822–3828.

14. Flotte, T., P. Zeitlin, S. Afione, R. Solow, M. Drumm, W. Guggino, and B. Carter. 1991. Complementation of the CF defect in bronchial epithelial cells using an AAV vector: CFTR expression and restoration of forskolin-stimulated chloride efflux. *Pediatr. Pulmonol.* 89(Suppl. 6):247. (Abstr.)

15. Brigham, K. L., B. Meyrick, B. Christman, M. Magnuson, G. King, and L. C. Berry. 1989. In vivo transfection of murine lungs with a functioning prokaryotic gene using a liposome vehicle. *Am. J. Med. Sci.* 298:278–281.

16. Friedmann, T. 1989. Progress toward human gene therapy. *Science (Wash. DC).* 244:1275–1281.

17. Anderson, W. F. 1992. Human gene therapy. *Science (Wash. DC).* 256:808–813.

18. Nabel, E. G., G. Plautz, and G. J. Nabel. 1990. Site-specific gene expression in vivo by direct gene transfer into the arterial wall. *Science (Wash. DC).* 249:1285–1288.

19. Ferry, N., O. Duplessis, D. Houssin, O. Danos, and J-M. Heard. 1991. Retroviral-mediated gene transfer into hepatocytes *in vivo. Proc. Natl. Acad. Sci. USA.* 88:8377–8381.

20. Inayama, Y., G. E. Hook, A. R. Brody, A. M. Jetten, T. Gray, J. Mahler, and P. Nettesheim. 1988. The differentiation potential of tracheal basal cells. *Lab. Invest.* 58:706–717.

21. Inayama, Y., G. E. Hook, A. R. Brody, A. M. Jetten, T. Gray, J. Mahler, and P. Nettesheim. 1989. In vitro and in vivo growth and differentiation of clones of tracheal basal cells. *Am. J. Pathol.* 134:539–549.

22. Yankaskas, J. R., M. R. Knowles, J. T. Gatzy, and R. C. Boucher. 1985. Persistence of abnormal chloride ion permeability in cystic fibrosis nasal epithelial cells in heterologous culture. *Lancet.* i:954–956.

23. Cohn, J. A., O. Melhus, L. J. Page, K. L. Dittrich, and S. R. Vigna. 1991. CFTR: development of high-affinity antibodies and localization in sweat gland. *Biochem. Biophys. Res. Commun.* 181:36–43.

24. Marino, C. R., L. M. Matovcik, F. S. Gorelick, and J. A. Cohn. 1991. Localization of the cystic fibrosis transmembrane conductance regulator in pancreas. *J. Clin. Invest.* 88:712–716.

25. Cohn, J. A., A. C. Nairn, C. R. Marino, O. Melhus, and J. Kole. 1992. Characterization of the cystic fibrosis transmembrane conductance regulator in a colonocyte cell line. *Proc. Natl. Acad. Sci. USA.* 89:2340–2344.

26. Randell, S. H., C. E. Comment, C. S. Ramaekers, and P. Nettesheim. 1991. Properties of rat tracheal epithelial cells separated based on expression of cell surface α-galactosyl end groups. *Am. J. Respir. Cell Mol. Biol.* 4:544–554.

27. Rutten, A. A. J. J. L., J. P. Bruyntjes, and F. C. S. Ramaekers. 1988. Effect of cigarette smoke condensate and vitamin A depletion on keratin expression patterns in cultured hamster tracheal epithelium. *Virchows Archiv. B. Cell. Pathol.* 56:111–117.

28. Price, J., D. Turner, and C. Cepko. 1987. Lineage analysis in the vertebrate nervous system by retrovirus-mediated gene transfer. *Proc. Natl. Acad. Sci. USA.* 84:156–160.

29. Chowdhury, J. R., M. Grossman, S. Gupta, N. R. Chowdhury, J. R. Baker, Jr., and J. M. Wilson 1991. Long-term improvement of hypercholesterolemia after ex vivo gene therapy in LDLR-deficient rabbits. *Science (Wash. DC).* 254:1802–1805.

30. Miller, D. G., M. A. Adam, and A. D. Miller. 1990. Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. *Mol. Cell Biol.* 10:4239–4242.

31. Johnson, L. G., J. C. Olsen, B. Surkadi, K. L. Moore, R. Swanstrom, and R. C. Boucher. 1992. Efficiency of gene transfer for restoration of normal airway epithelial function in cystic fibrosis. *Nature Genetics.* 2:21–25.