Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 11192–11196, December 1991
Cell Biology

Reconstitution of tracheal grafts with a genetically modified epithelium

(cystic fibrosis/gene therapy/recombinant retroviruses)

JOHN F. ENGELHARDT, EDWARD D. ALLEN, AND JAMES M. WILSON*

Departments of Internal Medicine and Biological Chemistry, Howard Hughes Medical Institute, The University of Michigan Medical Center, Ann Arbor, MI 48109

Communicated by Stuart H. Orkin, September 12, 1991 (received for review July 3, 1991)

ABSTRACT **A rational approach to the development of gene therapies for cystic fibrosis requires a better understanding of the cellular targets for gene transfer in the airway epithelium. We have used recombinant retroviruses to study the dynamics and lineage relationships of a regenerating rat tracheal epithelium. Primary cultures of tracheal epithelial cells were exposed to *lacZ*-transducing retroviruses and subsequently seeded into denuded trachea that were implanted into BALB/c (*nu/nu*) mice. The grafts developed a fully differentiated mucociliary epithelium containing large clones of *lacZ*-expressing cells with virtually all cell types represented within each clone. These data are most consistent with gene transfer into a putative progenitor cell that is capable of extensive self renewal and pleuripotent development. Vector-specific variation in transgene expression was noted in the various cell types.**

Cystic fibrosis (CF) is a lethal inherited disease caused by abnormalities in epithelial cell function (1, 2). Patients usually die of respiratory failure due to recurrent and destructive pneumonia. The primary cellular abnormality in the lung is believed to reside in the epithelium of the airways. The gene responsible for CF was recently isolated, providing insight into the molecular basis of the disease (3–5). Many of the mutations responsible for CF have been identified (6); however, the precise function of the gene product—i.e., the CF transmembrane regulator (CFTR)—remains controversial. We and others have shown that viral transduction of a normal CFTR cDNA into cultured CF epithelial cells can correct the basic cellular abnormality in cAMP-regulated Cl transport (7, 8). The isolation of the CF gene and the *in vitro* correction of Cl transport suggest a possible therapeutic approach based on CFTR gene transfer into airway epithelial cells *in vivo*.

A rational strategy for gene therapy of CF requires an understanding of the targets for gene transfer, the cells that comprise the airway epithelium. The proximal airway contains a complex epithelium with (*i*) nondividing terminally differentiated ciliated cells, (*ii*) nonciliated columnar cells with secretory (serous and goblet cells) or without secretory granules (intermediate and brush cells), and (*iii*) squamous appearing basal cells that contain little cytoplasmic specialization and a high nuclear/cytoplasmic ratio (9, 10). In contrast, the distal airway consists only of ciliated cells and a single type of secretory cell called the Clara cell.

The cellular dynamics and lineage relationships within the proximal airway epithelium are not entirely understood but it is generally believed that basal and secretory cell populations have the ability to divide. Two experimental approaches, pulse thymidine labeling (11–13) and cell purification followed by graft reconstitution (14–16), have been used to demonstrate the existence of progenitor cells in the airway. Despite intensive research in this area, the precise progenitor(s) and lineage relationships remain unclear. In contrast, the distal airway progenitor has been identified as the Clara cell (9).

We have developed an *in vivo* model to study recombinant gene expression in a fully differentiated epithelium. Primary cultures of epithelial cells are established from explanted rat trachea and subsequently exposed to recombinant retroviruses. The genetically modified cells are seeded into a denuded tracheal graft and implanted into a BALB/c (*nu/nu*) mouse, where they develop into a fully differentiated epithelium. Using this approach, we have studied the cellular dynamics of graft repopulation with respect to progenitor cell proliferative capacity and differentiating potential. In addition, we have identified vector-dependent transgene expression in the various cell types of the tracheal epithelium.

MATERIAL AND METHODS

Recombinant Retroviruses. Three *lacZ* retroviral vectors were used to produce amphotropic virus: BAG, in which the retroviral long terminal repeat is responsible for *lacZ* transcription (17), CMV/ADA-*lacZ* (Shu-Jen Chen and J.M.W., unpublished data), which contains the cytomegalovirus (CMV) enhancer and a portion of the adenosine deaminase (ADA) promoter, and CMV/BA-*lacZ* (Shu-Jen Chen and J.M.W., unpublished data), which contains the CMV enhancer and the chicken β-actin (BA) promoter. The rabbit low density lipoprotein receptor (rLDLR) retroviral vector CMV/BA-rLDLR (Mariann Grossman and J.M.W., unpublished data) was used as a *lacZ* negative control. Amphotropic virus was produced in the Ψ crip packaging line as described (18). The relative titers of the producer cell lines were estimated by infecting NIH 3T3 cells with limiting dilutions of viral supernatants. Colony-forming units (cfu) were visualized by 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) staining for *lacZ*-expressing virus or fluorescent LDL uptake for LDLR-expressing virus. Freshly prepared viral stocks had the following titers: BAG, 5×10^5 cfu/ml; CMV/BA-*lacZ*, 4×10^4 cfu/ml; CMV/ADA-*lacZ*, 3×10^5 cfu/ml; and CMV/BA-rLDLR, 2×10^6 cfu/ml. The viral stocks used in this study were free of replication-competent virus based on the previously described *lacZ* mobilization assay (18).

Cell Culture and Tracheal Grafts. Primary cultures of tracheal epithelial cells from male Fisher 344 rats (175–200 g) were prepared as described (19) with the following modifications. Freshly harvested cells were cultivated on collagen-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: CF, cystic fibrosis; CFTR, CF transmembrane regulator; CMV, cytomegalovirus; BA, β-actin; LDLR, low density lipoprotein receptor; rLDLR, rabbit LDLR; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; AEC, 3-amino-9-ethylcarbazole; RTE, rat tracheal epithelial; EM, electron microscopy; GMA, glycol methacrylate; cfu, colony-forming unit(s); ADA, adenosine deaminase.

*To whom reprint requests should be addressed.

coated (Vitrogen) plates in Hams F12 medium containing 5% fetal calf serum and 1% penicillin/streptomycin (conditioned 16 hr on a confluent monolayer of NIH 3T3 cells) and supplemented with fresh vitamin A (0.1 μg/ml), hydrocortisone (1 μg/ml), insulin (1 μg/ml), and fungizone (3.1 μg/ml). The cells were infected with supernatants collected in the same medium at 24, 48, 72, and 96 hr following the initial plating. Cells were harvested in 0.05% trypsin containing 0.53 mM EDTA on day 5 and seeded into denuded tracheas from syngeneic rats (16) at a density of 5×10^5 cells per trachea. These tracheas were transplanted subcutaneously into the flanks of female (19–22 g) *nu/nu* BALB/c mice.

Cytochemical Analysis for β-Galactosidase Activity. Tracheal grafts were excised and sectioned into 2-mm rings, rinsed briefly in phosphate-buffered saline (PBS, pH 7.4), fixed for 10 min in 0.5% glutaraldehyde in PBS, rinsed twice for 15 min in PBS containing 1 mM $MgCl_2$, and incubated in 1 mg of X-Gal per ml, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, and 1 mM $MgCl_2$ in PBS (pH 7.4) for 5 hr. Samples were postfixed in 4% paraformaldehyde/0.5% glutaraldehyde in PBS overnight at 4°C; this was followed by embedding in paraffin or glycol methacrylate (GMA) by standard procedures. All light microscopy was performed on a Microphot-FXA Nikon microscope.

Immunocytochemical Analysis with Anti-β-Galactosidase Antibody. Tracheal grafts were divided into 2-mm rings and fresh-frozen in OCT embedding compound. Serial frozen sections (6 μm) were prepared on poly(L-lysine)-coated slides and postfixed either in 0.5% glutaraldehyde/PBS for 10 min at 4°C or in acetone for 10 min at −20°C. Glutaraldehyde-postfixed sections were stained with X-Gal as described above, whereas β-galactosidase was detected in acetone-postfixed sections by indirect immunoperoxidase staining using the Vectastain ABC method (Vector Laboratories) using a primary rabbit polyclonal antibody to β-galactosidase (5 Prime → 3 Prime, Inc.). The sections were developed in 3-amino-9-ethylcarbazole (AEC) for 15 min.

Electron Microscopy (EM). Following X-Gal staining as described above, tracheal rings were fixed in 2.5% glutaraldehyde/1.5% paraformaldehyde/0.02% $CaCl_2$ in 0.1 M sodium cacodylate (pH 7.4) at 4°C overnight. Following fixation, the rings were washed repeatedly in 0.1 M sodium cacodylate, postfixed in 1% osmium tetroxide (1 hr 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 a Hitachi 11a electron microscope.

RESULTS

Retroviral Infection of Primary Rat Tracheal Epithelial (RTE) Cells. Optimal transduction efficiency, as measured by X-Gal staining, was 10–15% using the BAG retrovirus. Lower levels of transduction, 1–2% and 7–10%, were achieved with CMV/BA-*lacZ* and CMV/ADA-*lacZ*, respectively. Infection of RTE cells with CMV/BA-rLDLR, as measured by uptake of fluorescent labeled ligand, gave 20–25% transduction. Cells were harvested in trypsin to give a single cell suspension as detected by phase-contrast microscopy. A large number of cells (5×10^5) were seeded into denuded tracheas to promote rapid repopulation of the graft and to minimize expansion of individual clones.

Cytochemical Analysis for β-Galactosidase Activity. X-Gal-stained tracheas harvested at 9 days after grafting exhibited clonal patches of blue cells varying in size from 10 to 15,000 cells (data not shown). Given a constant repopulation density, the number of blue clonal patches appeared to be directly proportional to the titer of retroviral stock used to infect the primary cultures. Seeding with 5×10^4 cells per trachea led to fewer, but larger, transgene clone sizes (data not shown). BAG and CMV/ADA-*lacZ* produced 80–160 blue clones accounting for ≈5% of the luminal surface area, whereas CMV/BA-*lacZ* grafts contained 8–20 blue clones. No significant increase in clonal size occurred between 21- and 42-day grafts. Fig. 1*A* shows a section (≈1/16 of the entire graft) of a BAG-infected graft explant at day 42. A high magnification of a BAG-infected clone of cells can be seen in Fig. 1*B*. Similar clones were seen in CMV/ADA-*lacZ*- and CMV/BA-*lacZ*-infected grafts. No clones of this type were seen in CMV/BA-rLDLR- or mock-infected grafts (data not shown). Endogenous "β-galactosidase-like" activity began to appear in 21-day grafts and was most evident in 42-day grafts (Fig. 1*C*). The extent of this punctate endogenous staining was highly variable but visible in all grafts and persisted despite modifications of the X-Gal cytochemical analysis.

The cellular distribution of X-Gal staining was analyzed by paraffin (5 μm) and GMA (4 μm) sections. Fig. 2 shows the progression of epithelial reconstitution of BAG-infected grafts. Grafts harvested 9 days after implantation consisted of a single layer of squamous cells lining the lumen of the entire graft (Fig. 2*A*). The grafts began to differentiate by day 21, as evidenced by infrequent ciliated cells and a low cuboidal epithelium one or two cells thick (Fig. 2*B*). By day 42 the epithelium predominantly consisted of a pseudostratified layer of basal, ciliated, and nonciliated columnar cells (Fig. 2C). Analysis of *lacZ*-transduced grafts 42 days after implantation revealed contiguous patches of blue-staining cells. Most clones produced from each vector contained basal, ciliated, and nonciliated cells. However, a few clones contained a restricted subset of cells such as basal and ciliated cells or basal and nonciliated columnar cells.

There was marked vector-specific variation in the relative expression of *lacZ* within the different cell types of each clone. BAG (Fig. 2*C*) and CMV/ADA-*lacZ* (Fig. 3*A*) produced intense activity in basal and nonciliated columnar cells with less activity in ciliated cells. This variation in the level of transgene expression was consistent in all of the clones

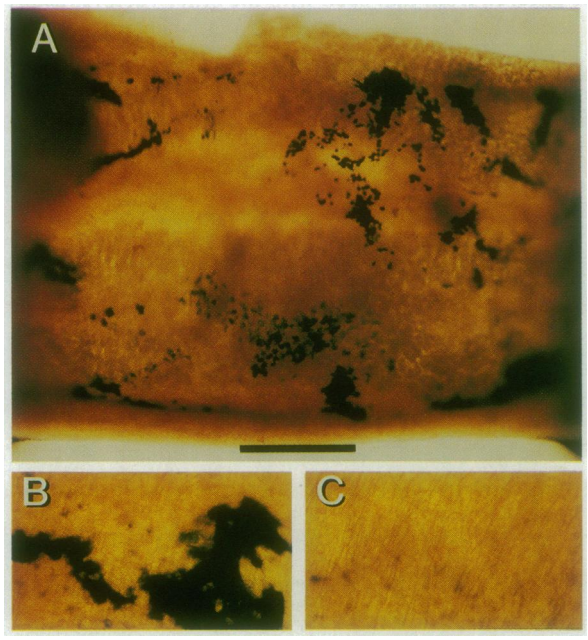

FIG. 1. Clones of retroviral-infected epithelial cells. (*A*) Low-power photomicrograph of a 42-day graft lumen seeded with BAG-infected primary tracheal epithelial cells. (Nikon PlanApo 4× objective; bar = 400 μm.) (*B*) Higher magnification of a *lacZ*-positive clone shown in *A*. (Nikon PlanApo 10× objective; bar = 80 μm.) (*C*) Higher magnification of a *lacZ*-negative portion of the graft shown in *A*. (Nikon PlanApo 10× objective; bar = 80 μm.)

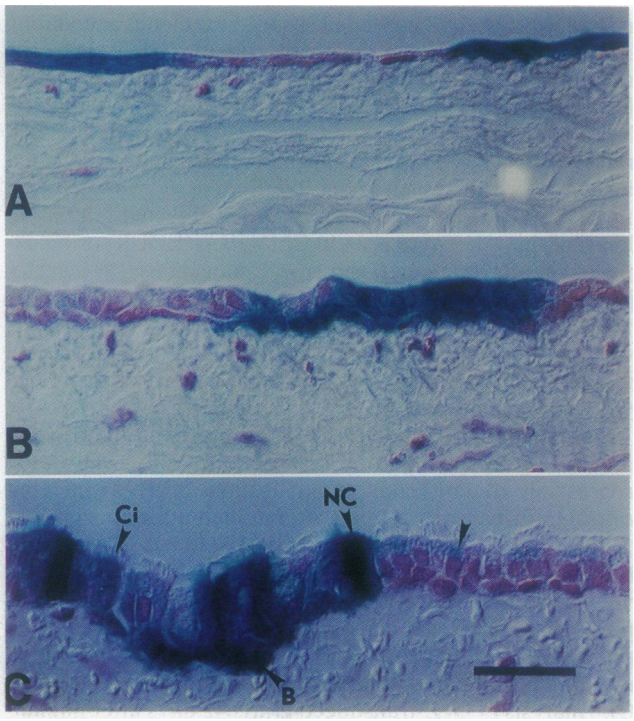

FIG. 2. Progression of graft repopulation and epithelial differentiation. BAG-infected grafts were stained in X-Gal buffer, embedded in paraffin, sectioned at 5 μm, and counterstained in hematoxylin. Grafts were harvested at 9 days (*A*), 21 days (*B*), and 42 days (*C*). The unlabeled arrowhead marks endogenous X-Gal staining seen also with the CMV/BA-rLDLR vector (data not shown). Ci, ciliated cell; NC, nonciliated columnar cell; B, basal cell. (Nikon PlanApo 60× objective, Nomarski optics; scale bar = 25 μm.)

analyzed from two independent retroviral infections of primary cultures. The pattern observed with CMV/BA-*lacZ* virus differed from that seen with the other vectors. Expression of β-galactosidase in ciliated cells was equal to or greater than that observed in nonciliated columnar and basal cells (Fig. 3*B*). The higher level of variability in expression seen with the CMV/BA-*lacZ* vector in ciliated cells, when compared to nonciliated cells, may be due to classification of preciliated cells as nonciliated columnar. In addition, the CMV/BA enhancer/promoter combination may be more susceptible to position effects of transgene integration. Endogenous X-Gal staining at 42 days was present predominantly in ciliated cells and localized in a perinuclear manner (see arrowhead, Fig. 2*C*).

Immunocytochemical Colocalization of X-Gal-Positive Clones. To further address the presence of endogenous β-galactosidase-like activity, as evidenced by perinuclear staining in ciliated cells, we used a polyclonal antibody to bacterial β-galactosidase to detect transgene expression. Serial sections from BAG-infected grafts were analyzed for the presence of β-galactosidase activity by X-Gal staining or immunoperoxidase staining using anti-bacterial β-galactosidase. Fig. 4*A* shows serial sections with red immunoperoxidase staining colocalized within the same region of the tracheal ring as the blue X-Gal stain (Fig. 4*B*). This confirms that the cytoplasmic X-Gal staining in *lacZ*-infected grafts is due to *lacZ*-encoded β-galactosidase and not endogenous β-galactosidase-like activity. No positive cells were detected by immunoperoxidase in CMV/BA-rLDLR-infected sections (data not shown).

Cell-Specific Markers. The functional status of individual cell types within the reconstituted epithelium was analyzed using monoclonal antibodies to cell-specific markers. Grafts explanted at day 42 were characterized with respect to expression of the basal cell-specific marker cytokeratin 14 and the cytokeratin 18 antigen, which is expressed in most cells of the rat tracheal epithelium except basal cells (14). The immunofluorescent staining pattern of monoclonal anti-cytokeratin 14 and anti-cytokeratin 18 in 42-day grafts was indistinguishable to that found in tracheas from normal Fisher rats (data not shown).

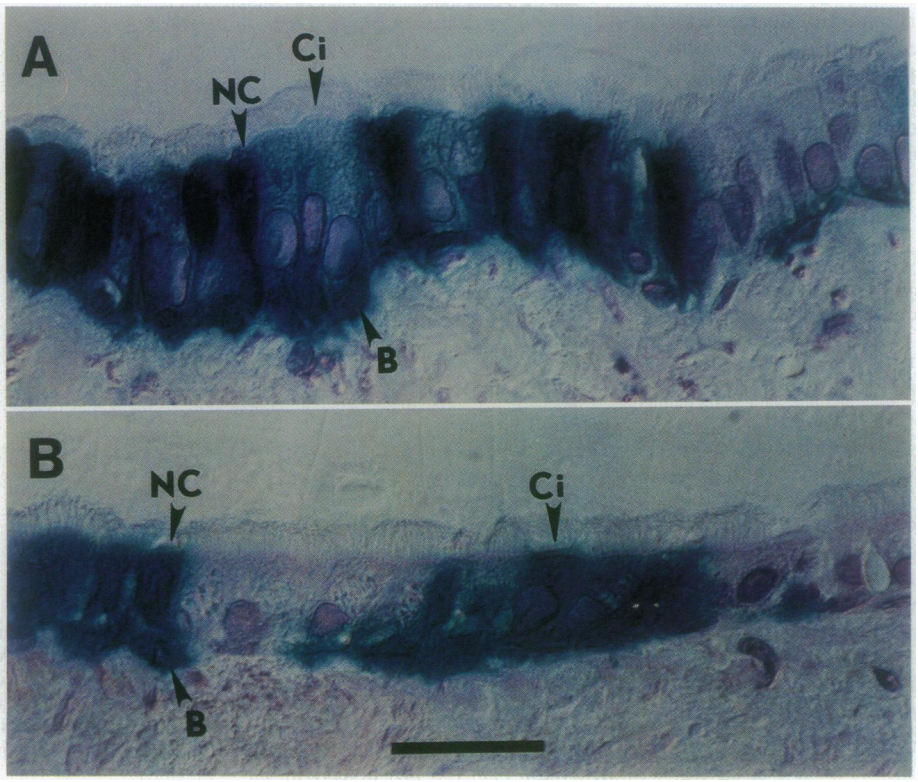

FIG. 3. Vector-dependent expression in 42-day differentiated epithelium. BAG-infected grafts were stained cytochemically in X-Gal buffer, embedded in GMA, sectioned at 4 μm, and counterstained in hematoxylin. Epithelium was reconstituted with CMV/ADA-*lacZ*-infected (*A*) and CMV/BA-*lacZ*-infected (*B*) primary tracheal epithelial cells. Ci, ciliated cell, NC, nonciliated columnar cell; B, basal cell. (Nikon PlanApo 60× objective; bar = 25 μm.)

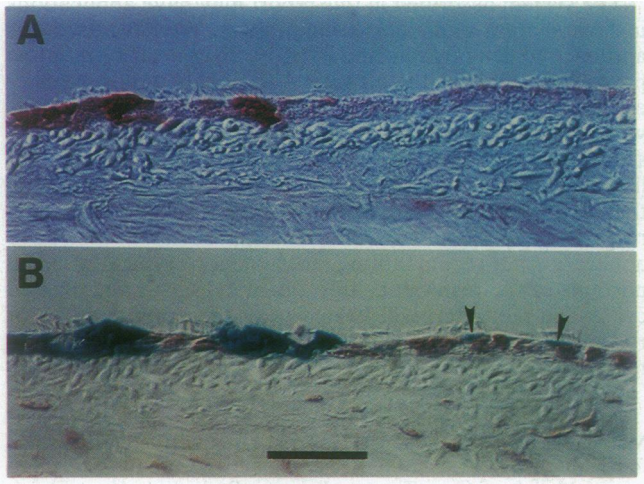

FIG. 4. Immunocytochemical colocalization of X-Gal-positive clones. Serial sections from 42-day BAG-infected grafts were stained cytochemically by indirect immunoperoxidase with polyclonal anti-β-galactosidase to give a red precipitate in positive cells (no counterstain) (*A*) or with X-Gal to give a blue precipitate in positive cells followed by counterstaining in hematoxylin (*B*). Arrowheads mark endogenous X-Gal staining in ciliated cells. (Nikon PlanApo 60× objective, Nomarski optics; bar = 25 μm.)

EM Analysis. To facilitate identification of nonciliated columnar cell types not distinguishable at the light microscopy level, we examined 42-day grafts by EM. Fig. 5 is an electron micrograph illustrating three types of nonciliated columnar cells: serous cells containing dense secretory granules and two types of intermediate cells with light and dense cytoplasm without secretory granules. Occasional brush cells were seen (data not shown); however, goblet cells (as identified by lucent secretory granules) were absent. The mucociliary epithelium consisted of predominantly ciliated cells, serous cells, and basal cells. All cell types in the graft were ultrastructurally normal with the exception of large lipid-like inclusions present in the cytoplasm of some ciliated cells.

lacZ expression was visualized at the EM level by the presence of electron-dense precipitates in X-Gal-stained grafts. Fig. 6 compares the cytoplasmic X-Gal precipitate present in a serous cell and intermediate cell to cells lacking the precipitate. The transgene-specific ultrastructural pattern of X-Gal precipitate was demonstrated in ciliated cells, basal cells, serous cells, and intermediate cells. Endogenous β-galactosidase-like activity produced a sparse X-Gal precipitate localized apically around the nucleus in ciliated cells of CMV/BA-rLDLR- and BAG-infected grafts (data not shown). Standard staining times with uranyl acetate and lead citrate partially obscured the X-Gal precipitate (Fig. 6). However, a short (<10 min) exposure to uranyl acetate alone provided enough detail to determine cell type without obscuring the X-Gal precipitate (data not shown).

DISCUSSION

Recent progress in the molecular biology of CF has led to renewed optimism for a potential treatment based on somatic gene transfer. The realization of this goal will require the development of safe and efficient *in vivo* gene delivery systems. The rational development of these techniques will be greatly facilitated by a better understanding of the cell biology of CF. It is generally believed that the airway epithelium is the appropriate target for gene transfer; however, the biology of this tissue is not well understood. We have developed a system in which retroviral-transduced epithelial cells within the setting of a fully differentiated epithelium can be used to answer important biologic questions related to the development of gene therapies for CF.

The reporter gene *lacZ* was used in graft repopulation studies to characterize putative progenitor cells in terms of their proliferative and differentiating capacity. The fully differentiated graft was repopulated with large clusters of *lacZ*-expressing cells. Cell types represented within each cluster were characterized by morphology (light and EM) as well as with cell-specific markers. Ciliated and basal cells appeared to be distributed within each clone at frequencies that were indistinguishable from the normal rat trachea. The presence of three types of nonciliated columnar cells within

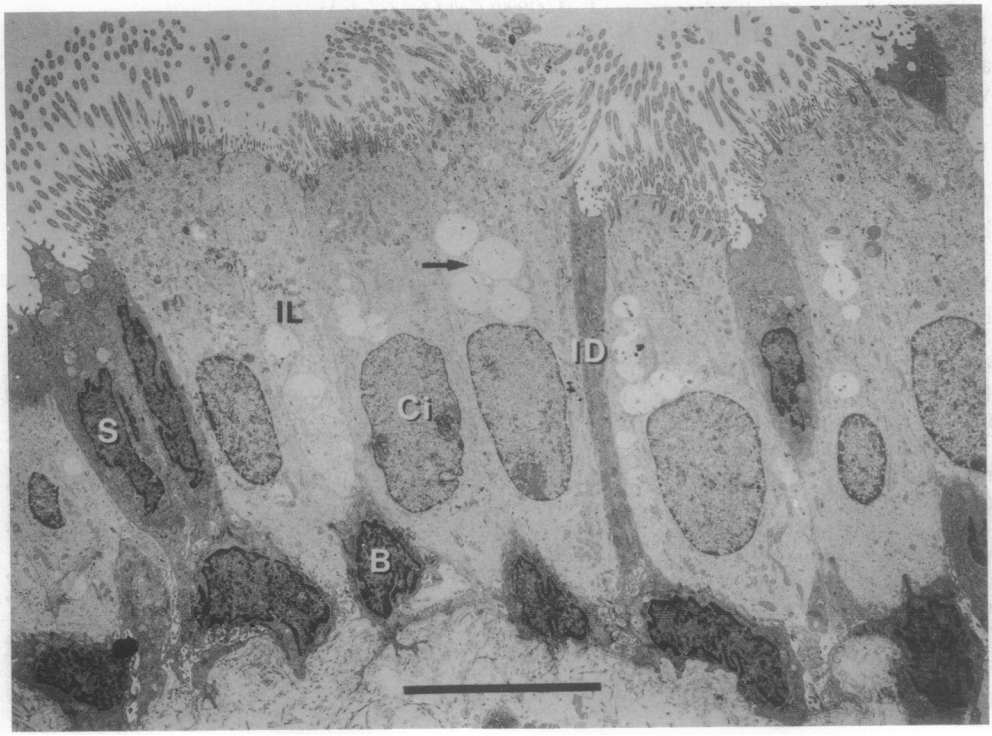

FIG. 5. Electron microscopic identification of nonciliated columnar cell types. Micrograph of a *lacZ*-negative region of the epithelium from a 42-day BAG-infected graft stained with X-Gal. Sections were stained in uranyl acetate and lead citrate. Note the presence of three nonciliated columnar cells types: S, serous cell; ID, dense intermediate cell; IL, lucent intermediate cell. The predominant cell types are ciliated cells (Ci) and basal cells (B). The arrow marks the lipid-like inclusions present only in ciliated cells and not seen in normal rat trachea. (Bar = 5 μm.)

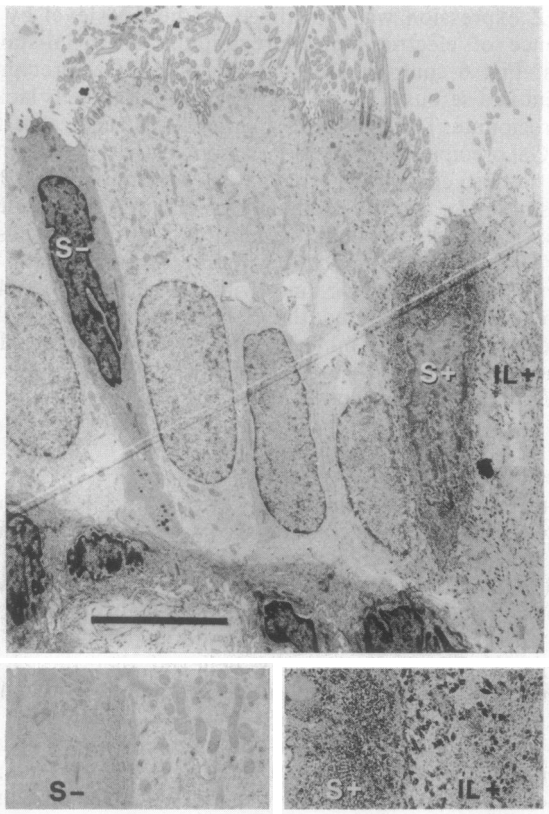

FIG. 6. Electron micrograph of a *lacZ*-positive region of the epithelium. The section was stained in uranyl acetate and lead citrate. Note the presence of intense staining within a serous cell (S+) as compared to a negatively staining serous cell (S−). LI+, lightly stained lucent intermediate cell. Serial 1-μm sections examined by light microscopy exhibited blue X-Gal stain in the cells marked positive (+) (data not shown). (*Insets*) Higher magnification of X-Gal-positive and -negative regions. (Bar = 5 μm.)

the *lacZ*-positive clones, including serous cells and intermediate cells (dense and lucent), was confirmed by EM. Goblet cells, which are normally <1% of the rat tracheal epithelium (10), were not seen in this study; this may be the result of insufficient sampling or restricted developmental potential of the transduced cell type(s). Our data provide direct evidence for the clonal expansion of a retroviral-transduced cell that is capable of extensive self renewal and pleuripotent development. The exact identity of this putative progenitor cell is unclear; however, it may be an attractive target for gene transfer in the treatment of CF. An alternative explanation for these results is the pseudoclonal appearance of *lacZ*-transduced cells due to seeding of aggregates. However, since repopulation was performed with a single cell suspension, such a hypothesis would require preferential clustering of *lacZ*-expressing cells.

Light microscopic analysis of X-Gal-stained 42-day grafts revealed consistently higher levels of expression in nonciliated columnar cells with the long terminal repeat- and CMV/ADA-based vectors. In contrast, the CMV/BA-*lacZ* vector expressed β-galactosidase at equal to higher levels in ciliated cells as compared to nonciliated columnar cells. Vector-dependent variation in transgene expression is most likely a result of the transcriptional elements contained within the retroviral vectors. However, it is also possible that translational efficiencies may vary for the different proviral-derived *lacZ* transcripts. The relatively low activity of the long terminal repeat in ciliated cells remains unexplained.

The system described in this report may have applications in several areas of medicine and biology. We have used this approach to demonstrate and initially characterize a putative progenitor cell(s) in the airway. Additional studies are necessary to define the precise lineage relationships of cells within the airway epithelium. Previous reports have demonstrated the feasibility of reconstituting differentiated epithelium from other species including humans (20, 21), indicating that it may be feasible to study gene transfer in human respiratory epithelium *ex vivo*. This approach may also have applications in reconstituting airways more typical of distal portions of the respiratory tree—namely, Clara and ciliated cells. The next step will be to study the consequences of recombinant CFTR gene expression in the context of a reconstituted CF epithelium.

We thank Sonia Janich and Lorita Dudus for technical support, Mariann Grossman for the CMV/BA-rLDLR virus, Shu-Jen Chen for the *lacZ* viruses, and the Cell Morphology Core of the Experimental Models of Gene Therapy Program Project (A. Kent Christensen and Kay Brabec) for advice and technical support. Steve Ernst provided important consultation regarding the interpretation of electron micrographs. The support and advice of Francis Collins were greatly appreciated. Work was supported by the Cystic Fibrosis Foundation, the National Institutes of Health, and the Howard Hughes Medical Institute.

1. Boat, T. F., Welsh, M. J. & Beaudet, A. L. (1989) *The Metabolic Basis of Inherited Disease II* (McGraw-Hill, New York), 6th Ed., pp. 2649–2680.
2. Taussig, L. M. (1984) in *Cystic Fibrosis*, ed. Taussig, L. M. (Thieme–Stratton, New York), pp. 1–9.
3. Rommens, J. M., Iannuzzi, M. C., Kerem, B.-S., Drumm, M. L., Melmer, G., Dean, M., Rozmahel, R., Cole, J. L., Kennedy, D., Hidaka, M., Zsiga, M., Buchwald, M., Riordan, J. R., Tsui, L.-C. & Collins, F. S. (1989) *Science* **245**, 1059–1065.
4. Riordan, J. R., Rommens, J. M., Kerem, B. S., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J , Lok, S., Plavsic, N., Chou, J.-L., Drumm, M. L., Iannuzzi, M. C., Collins, F. S. & Tsui, L.-C. (1989) *Science* **245**, 1066–1073.
5. Kerem, B.-S., Rommens, J. M., Buchanan, J. A., Markiewicz, D., Cox, T. K., Chakravarti, A., Buchwald, M. & Tsui, L.-C. (1989) *Science* **245**, 1073–1080.
6. McColley, S. A., Rosenstein, B. J. & Cutting, G. R. (1991) *Am. J. Dis. Child.* **145**, 94–97.
7. Drumm, M. L., Pope, H. A., Cliff, W. H., Rommens, J. M., Marvin, S. A., Tsui, L.-C., Collins, F. S., Frizzell, R. A. & Wilson, J. M. (1990) *Cell* **62**, 1227–1233.
8. Anderson, M. P., Rich, D. P., Gregory, R. J., Smith, A. E. & Welsh, M. J. (1991) *Science* **251**, 679–682.
9. Kauffman, S. L. (1980) *Int. Rev. Exp. Pathol.* **22**, 131–191.
10. Jeffery, P. K. & Reid, L. (1975) *J. Anat.* **120**, 295–320.
11. Beuer, R., Zajicek, G., Christensen, T. G., Lucey, E. C. & Snider, G. L. (1990) *Am. J. Respir. Cell. Mol. Biol.* **2**, 51–58.
12. Donnelly, G. M., Haack, D. G. & Heird, C. S. (1982) *Cell Tissue Kinet.* **15**, 119–130.
13. Boren, H. G. & Paradise, L. J. (1978) in *Pathogenes and Therapy of Lung Cancer*, ed. Harris, C. C. (Dekker, New York), pp. 369–418.
14. Randell, S. H., Comment, C. E., Ramaekers, C. S. & Nettesheim, P. (1991) *Am. J. Respir. Cell. Mol. Biol.* **4**, 544–554.
15. Johnson, N. F. & Hubbs, A. F. (1990) *Am. J. Respir. Cell. Mol. Biol.* **3**, pp. 579–585.
16. Inayama, Y., Hook, G. E. R., Brody, A. R., Cameron, G. S., Jetten, A. M., Gilmore, L. B., Gray, T. & Nettesheim, P. (1988) *Lab. Invest.* **58**, 706–717.
17. Price, J., Turner, D. L. & Cepko, C. L. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 156–160.
18. Danos, O. & Mulligan, R. C. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 6460–6464.
19. Terzaghi, M., Nettesheim, P. & Williams, M. L. (1978) *Cancer Res.* **38**, 4546–4553.
20. Obara, T., Conti, C. J., Baba, M., Resau, J. H., Trifillis, A. L., Trump, B. F. & Klein-Szanto, A. J. P. (1986) *Am. J. Pathol.* **122**, 386–391.
21. Klein-Szanto, A. J. P., Terzaghi, M., Mirkin, L. D., Martin, D. & Shiba, M. (1982) *Am. J. Pathol.* **108**, 231–239.