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Expression of $\alpha_v\beta_5$ Integrin Is Necessary for Efficient Adenovirus-Mediated Gene Transfer in the Human Airway

MITCHELL J. GOLDMAN AND JAMES M. WILSON*

Institute for Human Gene Therapy, and Departments of Medicine and Molecular and Cellular Engineering, University of Pennsylvania Medical Center, and Wistar Institute, Philadelphia, Pennsylvania 19104

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Recombinant adenoviruses are being evaluated for gene therapy of cystic fibrosis lung disease with the goal of reconstituting the expression of the cystic fibrosis transmembrane conductance regulator in pulmonary epithelia by direct administration of the virus into the airway. The therapeutic potential of recombinant adenoviruses is limited in part by the relative inefficiency by which gene transfer occurs. This study uses a human bronchial xenograft model to study adenovirus infection in the human airway in an attempt to define the molecular events that limit gene transfer. Our studies of the human airway confirm previous observations of cell lines that have indicated a two-step process for adenovirus entry, which begins with the binding of the virus to the cell through the fiber protein and continues with internalization via interactions among cellular integrins and an RGD motif (Arg-Gly-Asp) in the penton base. Furthermore, the level of maturity of the epithelia in xenografts has a major impact on gene transfer. Undifferentiated epithelia express high levels of $\alpha_v\beta_5$ integrins and are easily infected with recombinant adenoviruses; gene transfer is completely inhibited with excess fiber and partially inhibited with RGD peptide and $\alpha_v\beta_5$ integrin antibody. Pseudostratified epithelia do not express $\alpha_v\beta_5$ integrin in differentiated columnar cells and are relatively resistant to adenovirus-mediated gene transfer; what little gene transfer occurs is inhibited by fiber but not by RGD peptide or $\alpha_v\beta_5$ integrin antibody. These studies suggest that the expression of integrins in human airway epithelia limits the efficiency of gene transfer with recombinant adenoviruses. However, low-level gene transfer can occur in fully mature epithelia through $\alpha_v\beta_5$ integrin-independent pathways.

Recombinant forms of human adenoviruses are being evaluated for gene therapy of cystic fibrosis (CF). These nonenveloped viruses contain well-characterized 36-kb double-stranded DNA genomes that can be rendered replication defective for applications of gene therapy by deletion of the genes encoding E1a and E1b (3, 15). Biological features of human adenoviruses important in their use as vectors in CF gene therapy are that they have a natural tropism to lung tissue, are capable of infecting nondividing cells, and can be highly purified and grown in large quantities.

E1-deleted recombinant adenoviruses have been evaluated extensively for animal models and to a lesser extent for humans (3, 6, 12, 22). Immunologic responses to the vector and genetically corrected cells have emerged as problems that could affect safety and limit efficacy (3, 20, 21). Studies are under way to define the basic immunology of CF gene therapy and to design strategies to overcome these limitations. Despite the deletion of E1 sequences, recombinant adenoviruses express viral proteins that activate destructive cellular immune responses which lead to the elimination of the corrected cells (21). The development of improved recombinant adenoviruses with disabling mutations in other essential genes should help overcome this problem. In vivo administration of recombinant adenoviruses is also associated with the activation of T helper and B cells against the input viral proteins and with the eventual production of neutralizing antibodies that inhibit gene transfer (20). Strategies are being developed to prevent this activation by the coadministration of immune modulators with the virus.

* Corresponding author. Mailing address: Institute for Human Gene Therapy, 204 Wistar Institute, 3601 Spruce St., Philadelphia, PA 19104-4268. Phone: (215) 898-1979. Fax: (215) 898-6588.

The development of recombinant adenoviruses as therapeutic agents for gene therapy of CF will require strategies that allow the administration of truly effective doses of virus that has little toxicity. The biology of the recombinant virus is a primary determinant of this therapeutic index, with efficiency of gene transfer determining therapeutic efficacy and with host responses (i.e., nonspecific and antigen-specific responses) influencing safety. Studies of both animals and humans suggest that some airway epithelia such as those present in the nasal cavity are relatively resistant to adenovirus-mediated gene transfer (10, 12). Modifications that enhance gene transfer should improve the therapeutic index.

Recent progress has been made elucidating the steps involved in adenovirus entry, which appears to occur via two separate receptors, one mediating attachment and the other mediating internalization (5, 14, 17, 19). Adenoviruses attach to cells by way of the fiber capsid protein, as demonstrated by the ability of soluble fiber or antifiber antibodies to block a productive infection (5, 14). While the primary cell receptor that binds adenovirus fiber protein is still unknown, α_v integrins have recently been identified as the secondary receptor responsible for internalization (19). Adenovirus binding to α_v integrins is mediated by five RGD (Arg-Gly-Asp) sequences in the penton base, a tripeptide motif also present in a number of cellular adhesion molecules (11). A recent study by Wickham et al. showed that cells expressing α_v integrins were more infectable than their α_v-negative counterparts (19). In addition, adenovirus infection was inhibited by the penton base, soluble RGD peptides, and function-blocking monoclonal antibodies (MAbs) against $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins. These data revealed that the interaction of the penton base with this subset of α_v-containing integrins promotes virus infection.

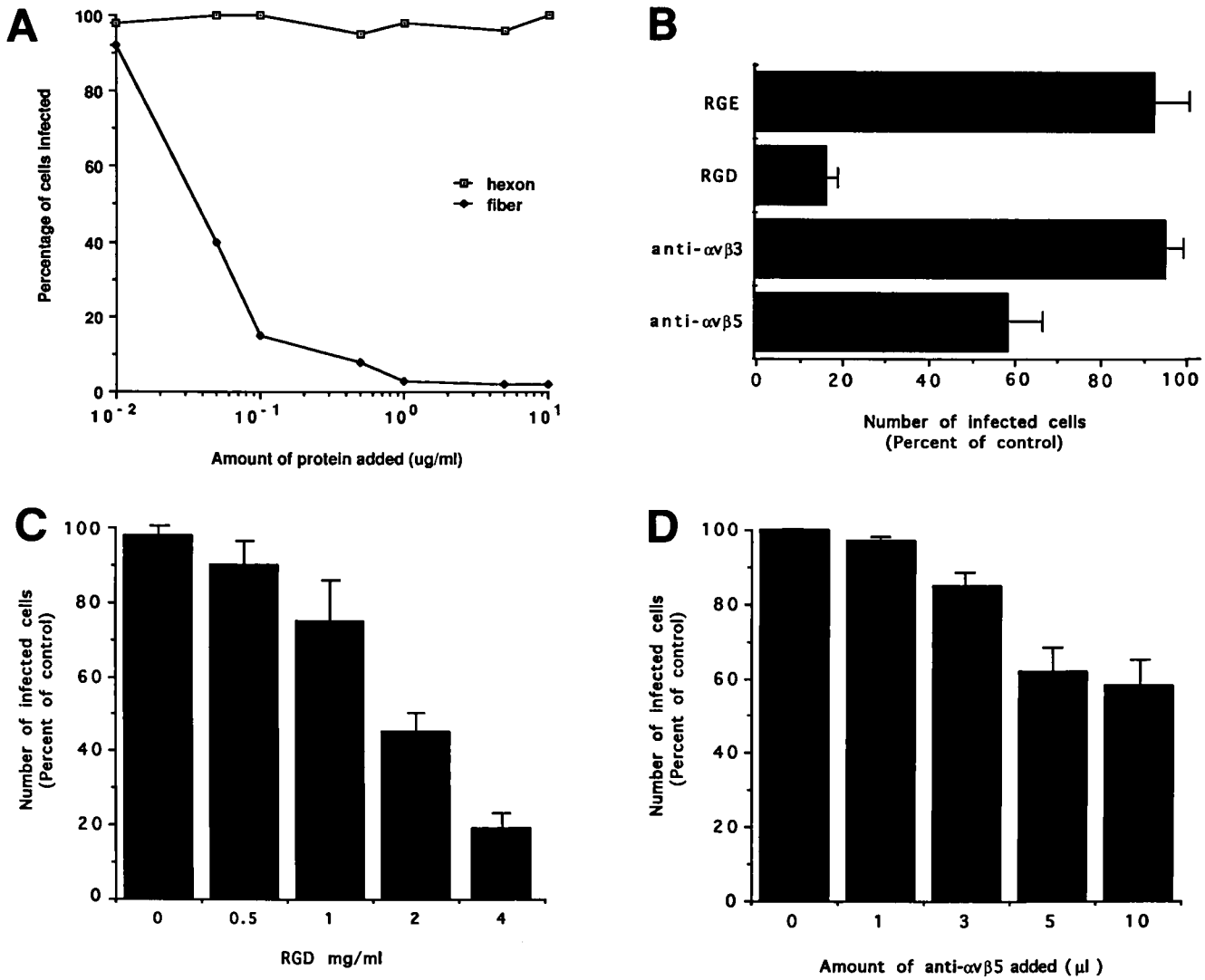


FIG. 1. Biology of adenovirus infection of cultured human bronchial epithelial cells. (A) Inhibition with fiber. (B) Inhibition of $\alpha_v\beta_5$ integrin function. (C) Dose response of inhibition with RGD peptide. (D) Dose response of inhibition with $\alpha_v\beta_5$ integrin antibody. In panel A, human bronchial epithelium cells were exposed to the presence of increasing concentrations of either hexon or fiber for 1 h, washed, and then infected with *lacZ* adenovirus. The cells were then fixed and stained 24 h later with X-Gal. In panels B to D, human bronchial epithelial cells were exposed to an appropriate amount of synthetic peptide or function-blocking MAb to $\alpha_v\beta_5$ or $\alpha_v\beta_3$, before infection with *lacZ* adenovirus. Infection was again quantitated 24 h later by X-Gal staining. Maximal inhibiting quantities of soluble peptide or MAb were used in generating the data shown in panel B. Data are expressed relative to control studies in which human bronchial epithelial cells were infected with virus, but not exposed to any other agent. Results shown in each graph are the averages and standard errors of triplicate determinations.

This study uses a human bronchial xenograft system to study the biology of recombinant adenovirus infection in the human airway. We observed that the efficiency of adenovirus infection was very high in developing grafts that consist of a relatively simple epithelium, whereas the pseudostratified epithelium of fully developed mature grafts was poorly infectable. To probe the importance of α_v integrins in adenovirus-mediated gene transfer to bronchial grafts, we colocalized the transgene product with the distribution of these cellular integrins. We also examined whether adenoviral infections of primary bronchial cells and xenografts were inhibited by soluble fiber, synthetic RGD-containing peptides, or an α_v integrin function-blocking MAb. Our results suggest that both fiber attachment and an available distribution of α_v integrins are necessary for efficient adenoviral gene transfer to a human bronchial epithelium.

MATERIALS AND METHODS

Recombinant adenovirus. The structure and production of the *lacZ* E1-deleted virus, with the cytomegalovirus promoter in a sub360 viral genome, has previously been described (7).

Human bronchial xenografts. Human bronchial tissues were obtained from explanted lungs at the time of lung transplantation, and primary cell cultures were prepared as described previously (7, 8). After several days, bronchial epithelial cells were seeded into denuded rat tracheas and implanted subcutaneously into the flanks of athymic nude mice in which they develop into a mature, fully differentiated, functional bronchial epithelium in 3 to 4 weeks. These tracheas had been ligated to flexible tubing, allowing access to the graft's lumen.

Antibodies and peptides. The MAb LM142 (Chemicon) was used to detect α_v-containing integrins. The MAbs P1F6 (Chemicon) and LM609 (Chemicon) were used as blocking antibodies against functional epitopes on $\alpha_v\beta_5$ and $\alpha_v\beta_3$, respectively. The MAb B5-IVF2, directed against the β_5 integrin, was the kind gift of Martin Hemler. The MAb E7P6, directed against the β_6 subunit, was the kind gift of Dean Sheppard. A rabbit polyclonal antibody was used to detect *Escherichia coli* β-galactosidase expression (5′,3′, Inc.). The synthetic peptides

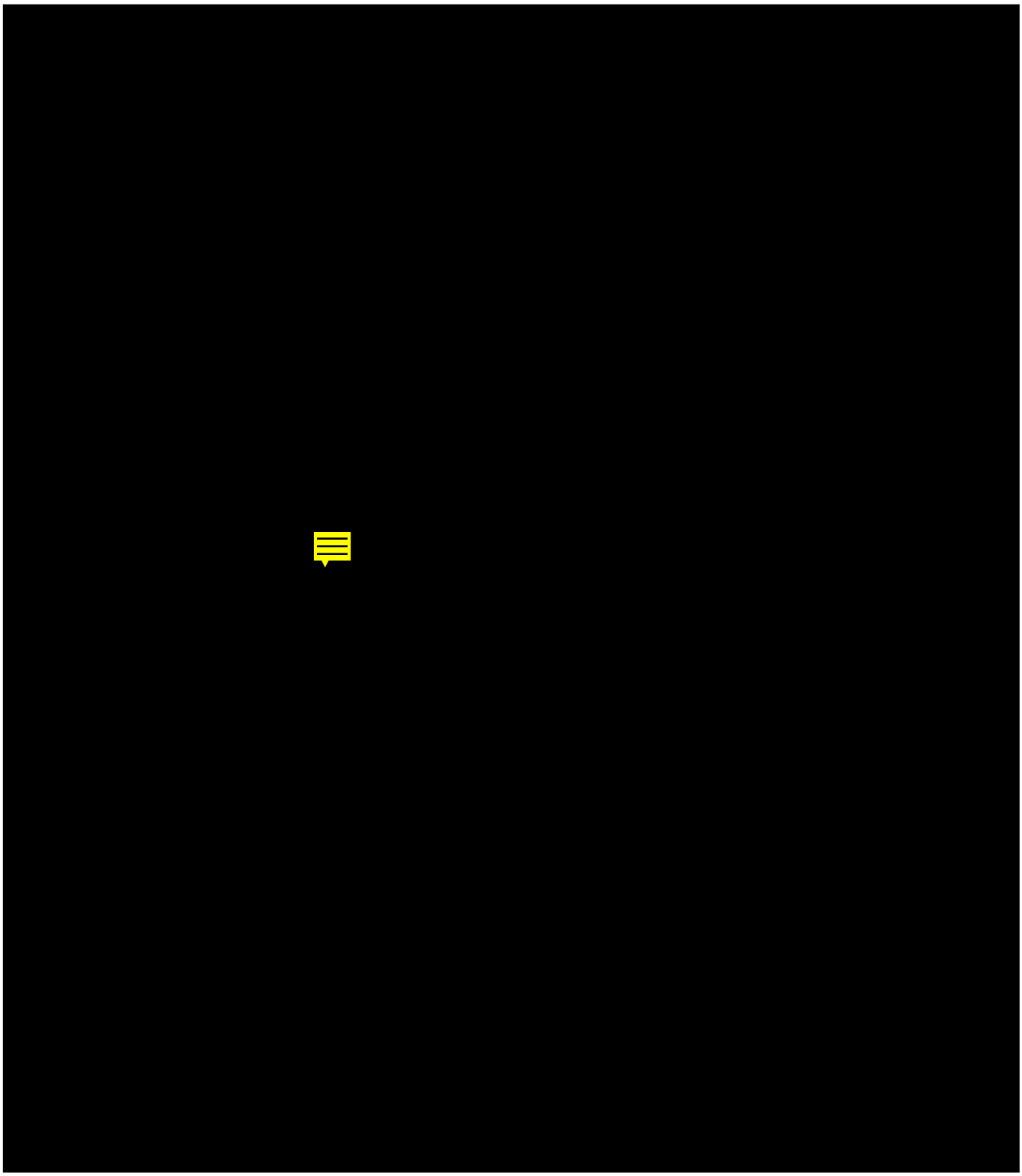


FIG. 2. Adenovirus-mediated gene transfer as a function of xenograft differentiation. (A and B) Immature graft. (C and D) Graft of intermediate state of differentiation. (E and F) Fully mature graft. (A, C, and E) High-magnification (×80) Nomarski photomicrograph. (B, D, and F) Low-magnification (×10) X-Gal-stained fresh-frozen section. Closed arrows, basement membrane. Open arrow, ciliated epithelium.

GRGDSP and GRGESP were obtained from Gibco-BRL. Prior to their use, these hexapeptides were dissolved in media and adjusted to a pH of 7.4 with 1 N NaOH.

Infectivity assays. The infection of human bronchial epithelial cells was performed as previously described (1, 19) with the following modifications. Cells were released with 0.5 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, pH 8.0] and incubated with an appropriate amount of synthetic peptide or soluble MAb at 4°C for 1 h, followed by the addition of *lacZ* adenovirus (50 PFU per cell) at 4°C. After 1 h, the cells were placed at 37°C, treated with trypsin 30 min later to remove noninternalized virus, washed, and plated onto poly-D-lysine-coated chamber slides. Twenty-four hours later the

cells were fixed with 0.5% glutaraldehyde in phosphate-buffered saline (PBS; pH 7.4) and stained in 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) solution. Infectivity was calculated as the percentage of *lacZ*-positive cells in the experimental sample divided by the number of *lacZ*-positive cells in the control sample. To determine the effect of adenovirus capsid proteins on adenoviral infection, human bronchial epithelial cells were incubated with increasing concentrations of partially purified fiber or hexon fractions in the presence of *lacZ* virus (50 PFU per cell). *lacZ* expression was quantitated 24 h later by X-Gal histochemistry. In each experiment, mock-treated bronchial cells infected with the same dose of recombinant adenovirus were used as controls. To determine the effects of soluble MAbs, synthetic peptides, or adenovirus capsid proteins on infection in bronchial xenografts, the appropriate agent was instilled into the lumen of a graft, and then the xenografts were instilled with recombinant virus 30 min later. The virus was then expelled with air 1 h later. Grafts were administered virus at a multiplicity of infection of 100. Determinations regarding the estimated number of cells in xenografts were previously reported (8). Two days after adenoviral infection, the xenografts were explanted, fresh frozen in an embedding compound, and cryosectioned for X-Gal histochemistry or immunocytochemical analysis.

Histochemical and immunocytochemical analysis of xenografts. Cytochemical localization of *lacZ* expression was performed as follows. Frozen sections (6 μm) were mounted onto slides, fixed with 0.5% glutaraldehyde in PBS (pH 7.4), and stained in X-Gal solution. Immunocytochemical localization of β-galactosidase with α_v and β_5 integrins was performed as follows. Sections of fresh-frozen tissue (6 μm) were postfixed in ice-cold methanol for 10 min, air dried, and blocked in PBS (pH 7.4) containing 20% donkey serum for 20 min. Sections were then incubated in a mixture of a rabbit anti-β-galactosidase and an α_v or β_5 integrin mouse MAb for 1 h and then incubated for 30 min in 5 μg of donkey anti-rabbit rhodamine-conjugated and donkey anti-mouse fluorescein isothiocyanate-conjugated (Jackson Laboratories) secondary antibodies per ml. After three 5-min washes in PBS (pH 7.4), sections were mounted in Citifluor antifadent and visualized on a Microphet-FXA Nikon fluorescent microscope.

RESULTS

Pathway of internalization for recombinant adenoviruses in primary human bronchial epithelial cells. Steps involved in efficient adenovirus-mediated gene transfer in the human airway were studied initially in primary cultures of human bronchial epithelial cells. Previous studies have indicated that the majority of cells in these cultures express a profile of cytokeratins that is most consistent with basal cells, i.e., cytokeratin 14^+ and cytokeratin 18^- (23). Additionally, immunocytochemical studies and flow cytometry demonstrated high levels of both α_v and β_5 integrins in 75 to 90% of primary cells (data not shown).

The importance of receptor-specific binding to adenovirus was studied in competition studies (Fig. 1). In the absence of interference, >80% of bronchial epithelial cells were infected with *lacZ* recombinant adenoviruses introduced at a multiplicity of infection of 50. Incubating the cells with increasing concentrations of partially purified fiber completely eliminated gene transfer, whereas no effect was seen with equal quantities of the hexon fraction (Fig. 1A). The importance of α_v cellular integrins in adenovirus-mediated gene transfer of human airway epithelial cells was then studied. E1-deleted recombinant adenovirus expressing *lacZ* was bound to a suspension of human bronchial epithelial cells; following a 30-min incubation, the noninternalized virus was destroyed with trypsin and the cells were plated and histochemically stained 24 h later with X-Gal to detect infection. Recombinant viral infection was partially inhibited in the presence of RGD peptide (Fig. 1B and C) or an antibody blocking $\alpha_v\beta_5$ integrin (Fig. 1B and D); no inhibition of binding was observed with RGE (Arg-Gly-Glu) peptide or with antibody blocking $\alpha_v\beta_3$ integrin, confirming the specificity of the assay (Fig. 1B). The $\alpha_v\beta_3$ integrin-blocking antibody served as an appropriate control, as this integrin is not present in human bronchial epithelial cells (4, 13). The extent of inhibition with RGD peptide or $\alpha_v\beta_5$ integrin antibody was proportional to dose, reaching a maximum of 82 and 48% inhibition, respectively (Fig. 1C and D).

TABLE 1. Quantitation of *lacZ*-positive cells in bronchial xenografts in an intermediate or fully mature state of differentiation

Agent instilled into bronchial grafts	% *lacZ*-positive cells[a] in:	
	Intermediate-level grafts	Mature grafts
Control	15 ± 3	5 ± 2
RGE	18 ± 5	7 ± 2
RGD	3 ± 2	7 ± 1
$\alpha_v\beta_5$ MAb	5 ± 2	5 ± 3
Hexon	15 ± 3	5 ± 1
Fiber	<1	<1

[a] The percentage of positive cells was obtained by counting *lacZ*-expressing cells and dividing this number by the number of total cells present in the sections. Twenty representative sections from each infected graft were analyzed, and the values from three separate experiments are shown. Control grafts were infected with recombinant adenovirus but were not exposed to any other agent. The data are means ± 1 standard deviation.

Adenovirus-mediated gene transfer in the human airway is dependent on the differentiated state of the epithelium and correlates with expression of integrins. Principles of adenovirus-mediated gene transfer delineated in primary cultures were further evaluated in the more relevant in vivo setting of the human bronchial xenograft. This system facilitates a quantitative and dynamic evaluation of recombinant virus gene transfer.

The initial study assessed efficiency of gene transfer as a function of the differentiated state of the epithelium. Xenografts were seeded at subconfluent density and explanted at subsequent times, approximating different stages of differentiation. Figure 2 presents representative photomicrographs of three states of differentiation, including the immature state, a monolayer of squamous-like cells of the basal cell phenotype (Fig. 2A); the intermediate stage, a simple cuboidal epithelium of nonciliated cells resting on a layer of squamous cells of basal cell characteristics (Fig. 2C); and the mature stage, a fully differentiated pseudostratified epithelium (Fig. 2E). There was a dramatic difference in the efficiency of gene expression among these three states of epithelial differentiation when identical doses of virus were instilled into the lumen of xenografts (Table 1; Fig. 2). Immature xenografts were efficiently infected with the *lacZ* virus, achieving gene transfer in 90% of the cells, with the gene transfer diminishing as the xenograft differentiates to 15% of infected cells in intermediate grafts and 5% of infected cells in fully differentiated grafts. The efficiency of gene transfer correlated with the expression of α_v and β_5 integrins (Fig. 3). Virtually every cell in an immature xenograft expressed both integrin subunits. All basal cells and a significant number of cuboidal cells in the intermediate graft expressed both integrin subunits; gene transfer in these grafts was almost always restricted to the cuboidal cells that expressed the integrin subunits. The expression of α_v integrin in the fully differentiated xenografts was exclusively restricted to the basal cells; the rare columnar cells transduced with recombinant virus did not express this integrin.

Gene transfer in human airway epithelia is limited by expression of α_v integrin. Competition studies were performed in human bronchial xenografts to further assess the role of fiber binding and α_v integrin-mediated internalization in adenovirus-mediated gene transfer. Soluble adenovirus capsid proteins, synthetic peptides, or function-blocking MAb was instilled into the lumen of xenografts 30 min before the introduction of virus. Two days later the xenografts were explanted and evaluated for *lacZ* expression. Representative photomicrographs of the xenografts are presented in Fig. 4 and 5, and a summary of the morphometric analysis of these grafts is

FIG. 3. Immunocytochemical localization of α_v and β_5 integrins in bronchial xenografts infected with recombinant adenovirus. Immature grafts (top row), grafts of the intermediate state of differentiation (middle row), and fully mature grafts (bottom row) were infected with *lacZ* adenovirus, harvested 2 days postinfection, and analyzed by immunofluorescence with a rabbit β-galactosidase (βgal) polyclonal antibody detected with rhodamine (in red) or α_v or β_5 integrin mouse MAb detected with fluorescein isothiocyanate (in green). The data shown represent localization of these proteins in either the same or serial sections.

FIG. 4. Inhibition of recombinant adenoviral infection by soluble fiber. Bronchial xenografts representing either an intermediate state of differentiation (A and B) or a fully mature state (C and D) were exposed to soluble hexon (A and C) or fiber (B and D) before infection with *lacZ* virus. Two days later the xenografts were explanted and fresh-frozen sections were analyzed for *lacZ* expression. The pictures shown are representative of multiple sections obtained from each graft.

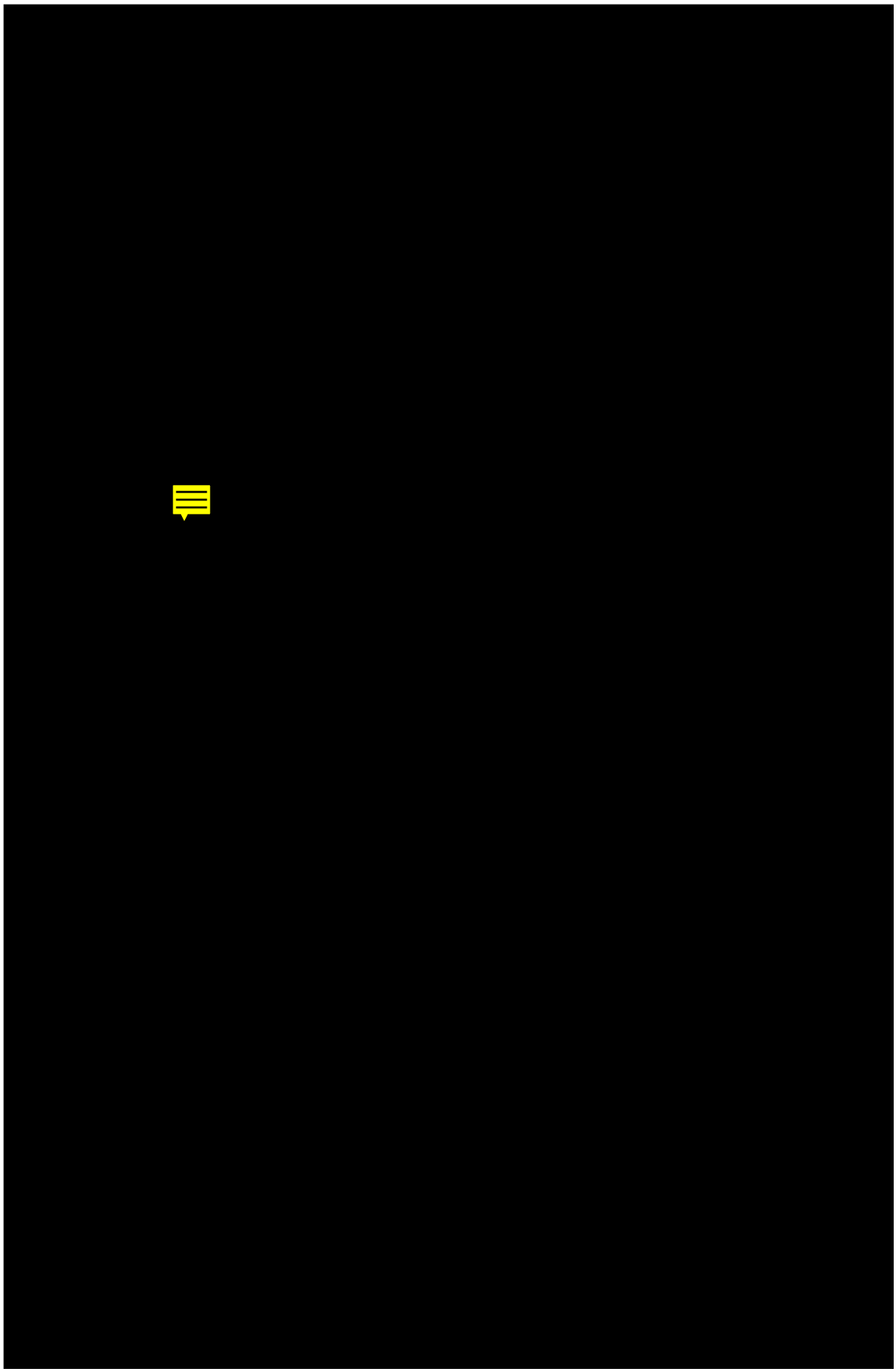
presented in Table 1. Excess fiber completely inhibited gene transfer into xenografts regardless of their state of differentiation (Fig. 4B and D); the introduction of partially purified hexon at similar doses had no effect (Fig. 4A and C). The importance of $\alpha_v\beta_5$ integrin availability for efficient gene transfer was most clearly documented in relatively undifferentiated grafts; gene transfer was inhibited approximately 4-fold and 2.5-fold with RGD peptide (Fig. 5C) and antibody to $\alpha_v\beta_5$ integrin (Fig. 5D), respectively, whereas RGE peptide had no detectable effect (Fig. 5B). Low-level gene transfer detected in fully differentiated grafts was not affected by the preinstillation of RGE peptide (Fig. 5F), RGD peptide (Fig. 5G), or $\alpha_v\beta_5$ integrin antibody (Fig. 5H).

DISCUSSION

Critical to the success of recombinant adenovirus for gene therapy of CF is the development of strategies that enable therapeutically meaningful genetic reconstitution to occur in the absence of toxicity. Studies to date suggest this so-called therapeutic index is highly dependent on the biology of the target epithelia. Preclinical and preliminary clinical studies of CF transmembrane conductance regulator gene transfer to the nasal cavity of genetically deficient recipients suggest that the therapeutic index may be narrow, in part because of the relative resistance of the nasal epithelia to gene transfer (10, 12). Similar studies of adenovirus-mediated gene transfer in lungs of CF mice and humans are more encouraging, with gene transfer being achieved with lower doses of virus (3, 21). The human bronchial xenograft model was used in this study to evaluate the biology of recombinant adenovirus-mediated gene transfer in the human proximal conducting airway.

Recent experiments in human cell lines have begun to delineate the steps involved in the internalization of human ad-

FIG. 5. Effects of soluble RGD peptides and an anti-$\alpha_v\beta_5$ MAb on recombinant adenovirus infection of bronchial xenografts. Intermediate-level (A to D) or mature (E to H) xenografts were instilled with either synthetic hexapeptides or a function-blocking MAb against $\alpha_v\beta_5$ integrin 30 min before infection with *lacZ* adenovirus. Two days later the xenografts were explanted and fresh-frozen sections were analyzed for *lacZ* expression. Control grafts were infected with *lacZ* virus but were not exposed to other agents. (A and E) Control grafts; (B and F) RGE-exposed grafts; (C and G) RGD-exposed grafts; (D and H) anti-$\alpha_v\beta_5$ MAb-exposed grafts. The pictures shown are representative of multiple sections obtained from each graft.



enoviruses (2, 9, 17, 19). These studies indicate that two molecular interactions at the plasma membrane of the target cell are necessary for efficient infection. The primary event is high-affinity binding of an unknown cellular receptor to the fiber capsid protein of the virus (5, 14). This is followed by lower-affinity binding of a cellular $\alpha_v\beta_5$ integrin to the RGD motif of the penton base, which facilitates internalization (19). This model of viral entry was considered when investigating adenovirus-mediated gene transfer into the airway of human bronchial xenografts.

The most striking finding was the dramatic effect that the differentiated state of the epithelia had on efficiency of gene transfer. Undifferentiated cells exposed to the lumen of the graft were substantially more infectable than either fully differentiated ciliated or secretory cells or the lower-lying basal cells not exposed to the luminal surface. Immunocytochemical studies suggested a strong correlation in luminal epithelial cells between expression of α_v integrin and infection with recombinant adenovirus. The relative importance of molecular interactions at the cell surface to efficient gene transfer in the airway was directly assessed through competitive binding studies which demonstrated that (i) the binding of a cellular receptor to fiber is necessary for viral entry in all epithelial cells, (ii) the availability of α_v integrins in adluminal epithelial cells determines the efficiency of adenovirus-mediated gene transfer, and (iii) the infrequent gene transfer that occurs in fully differentiated epithelia is dependent on binding to fiber but is independent of α_v integrin binding to the penton base.

These studies have important implications in the development of gene therapies for CF. The effect of epithelial cell differentiation on gene transfer may confound the interpretation of in vivo gene transfer studies. Imperfections in the integrity of the intrapulmonary CF epithelia due, in part, to chronic inflammation and injury may lead to a patchy distribution of gene transfer which will be difficult to quantify from biopsies or bronchial brushings. More important, however, is the observation that the fully differentiated pseudostratified epithelium of the proximal pulmonary conducting airway is relatively resistant to adenovirus infection because of the absence of available $\alpha_v\beta_5$ integrin. The importance of this finding to the simpler epithelia of the distal conducting airway is unclear.

If efficiency of gene transfer limits the success of CF gene therapy, strategies will have to be designed to enhance virus uptake in intrapulmonary epithelia. One approach is to upregulate the expression of the necessary integrin prior to the administration of virus. This has been accomplished in human peripheral blood lymphocytes by activating $\alpha_v\beta_5$ expression with granulocyte-macrophage colony-stimulating factor (16). Another approach is to exploit pathways of viral entry that are not dependent on $\alpha_v\beta_5$. Several serotypes of human adenoviruses, such as adenovirus type 40, are missing RGD motifs in the penton base, suggesting α_v integrin-independent entry or interaction of the virus with other cell surface molecules. Pulmonary epithelial cells express a wide variety of related but distinct $\alpha\beta$ integrins that could be targeted as coreceptors for adenovirus internalization (4, 13, 18).

In summary, studies of the biology of adenovirus entry in the human bronchial xenograft model have delineated cell-virus interactions necessary for efficient uptake. Strategies to enhance these molecular interactions will be useful to improve the therapeutic efficacy of gene therapy directed to the CF lung.

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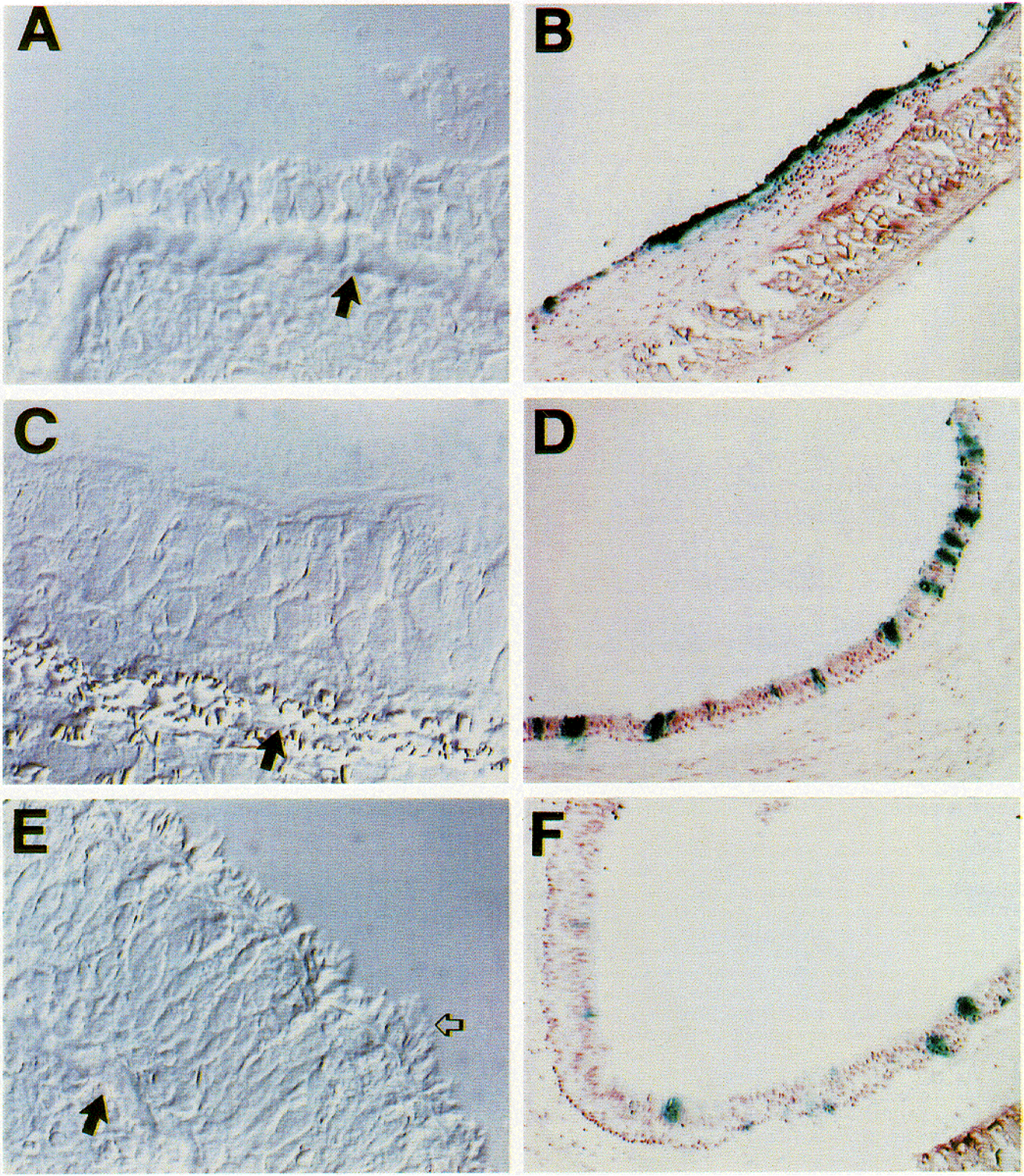
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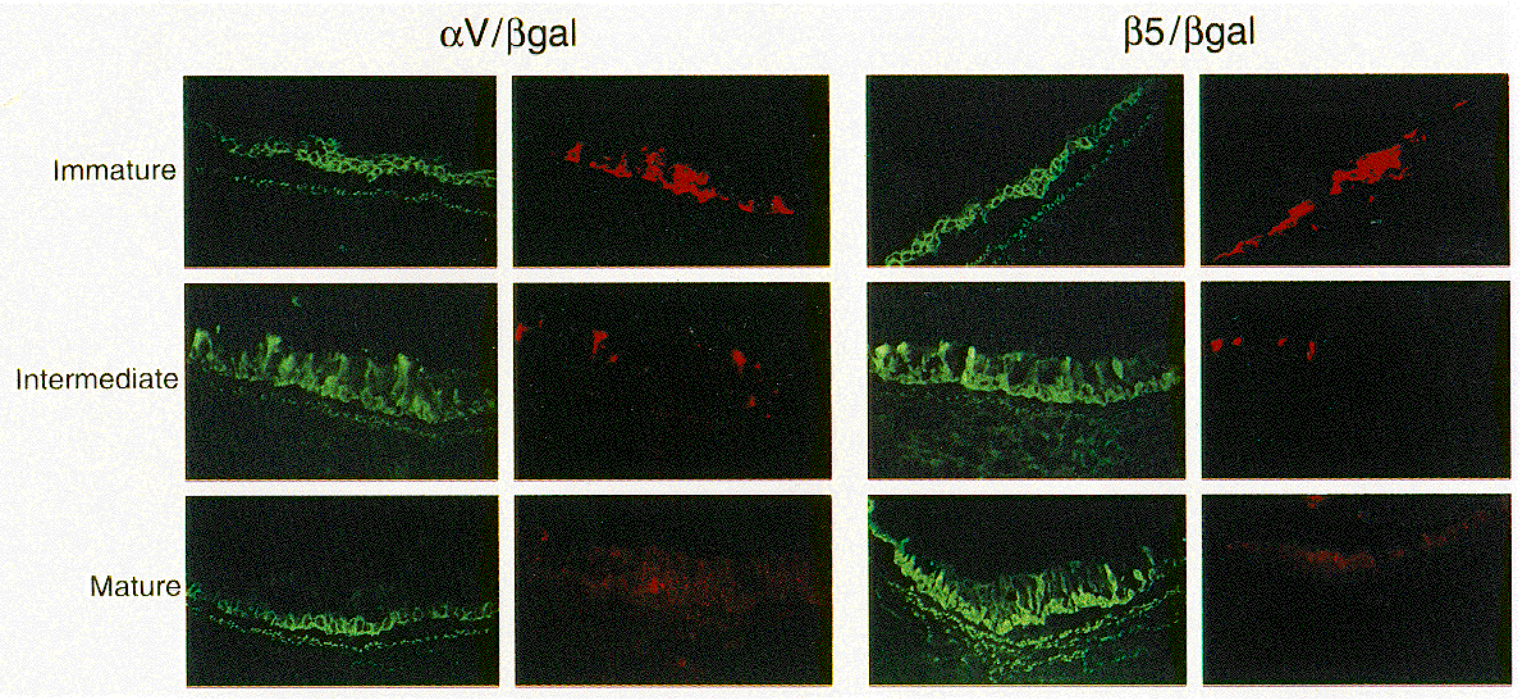
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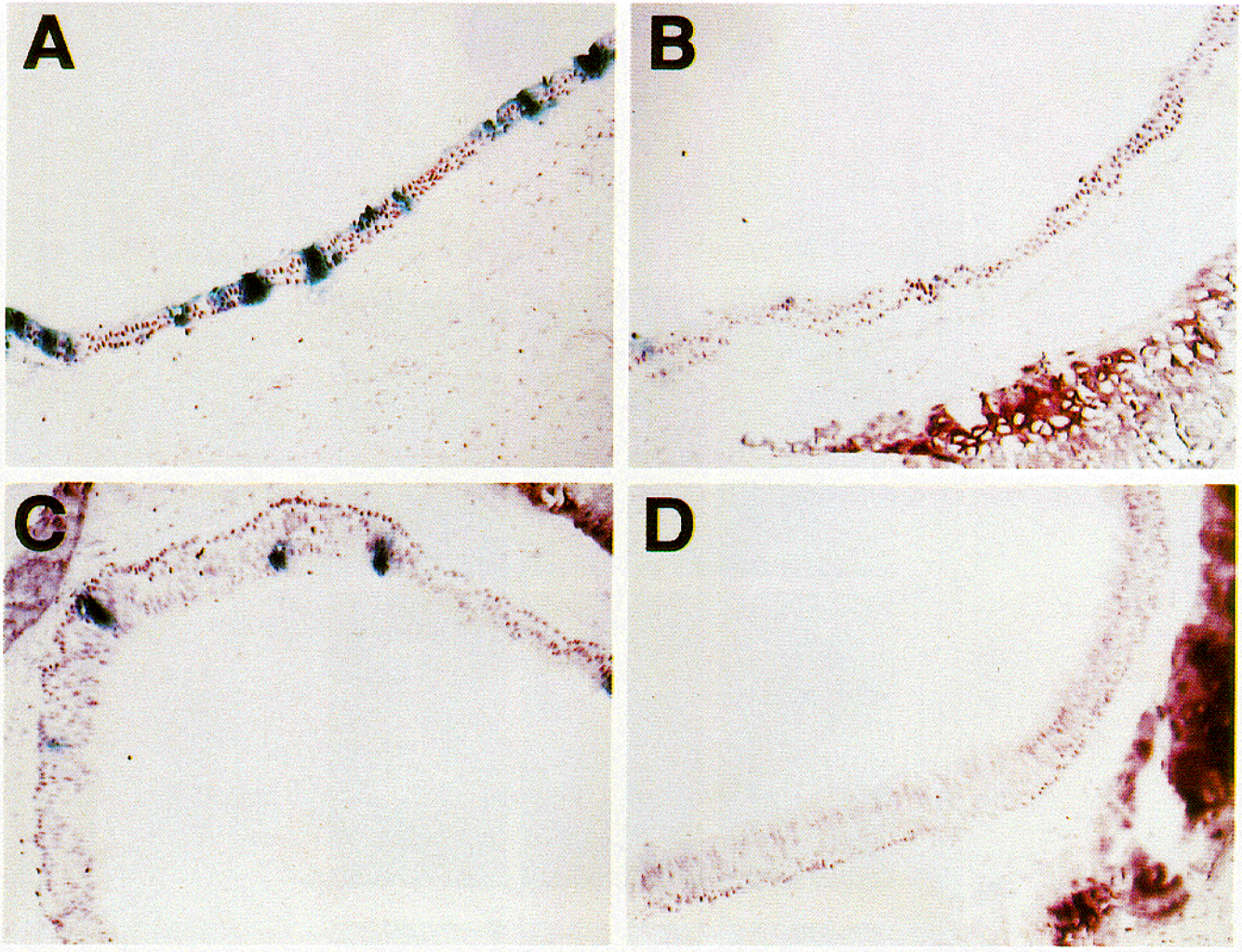
Intermediate
Mature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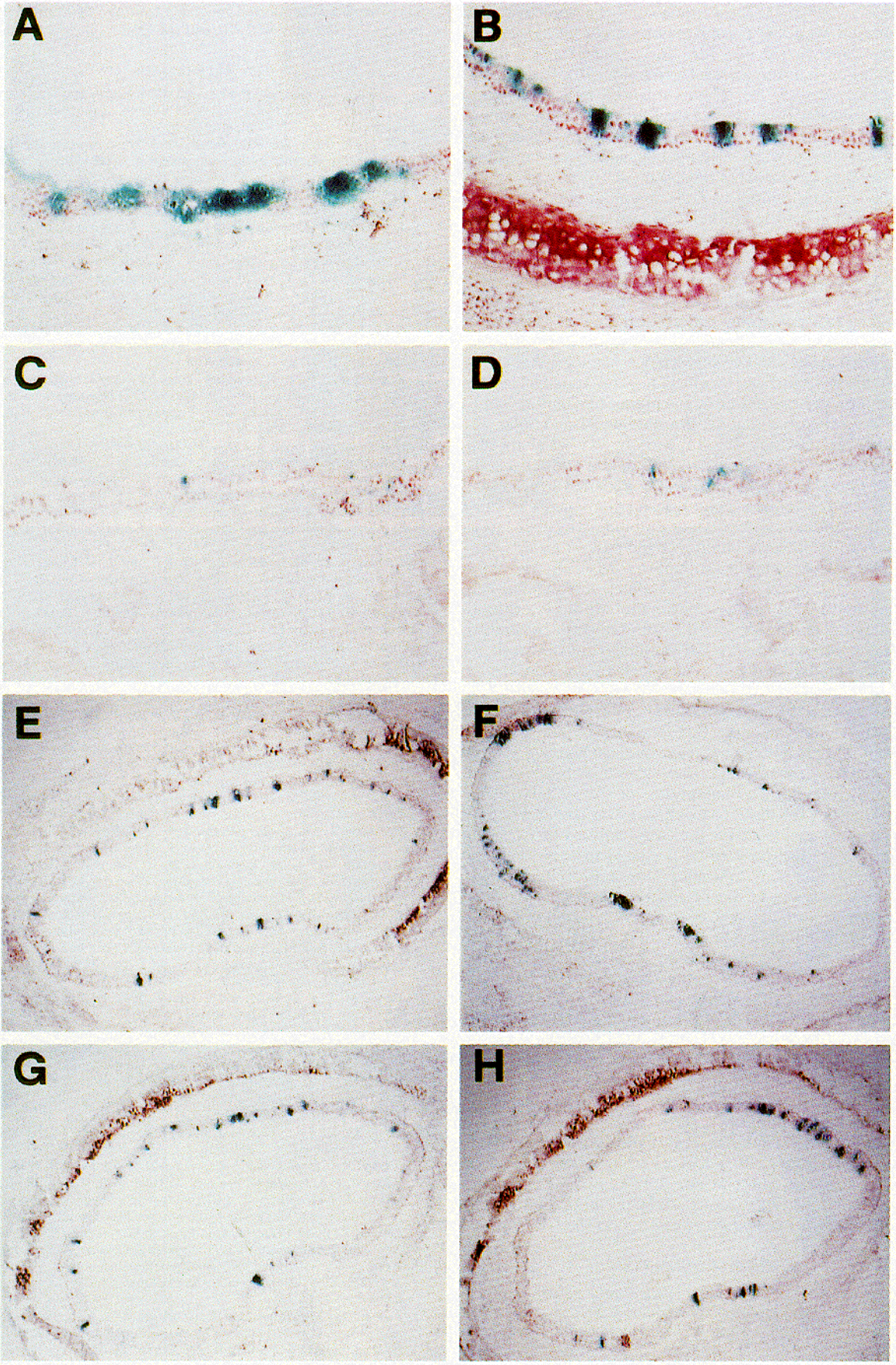
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