HUMAN GENE THERAPY 4:17–24 (1993)
Mary Ann Liebert, Inc., Publishers

Direct *In Vivo* Gene Transfer to Airway Epithelium Employing Adenovirus–Polylysine–DNA Complexes

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ABSTRACT

Adenovirus–polylysine–DNA complexes were evaluated for their capacity to accomplish direct *in vivo* gene transfer to airway epithelium employing a rodent model. Binary complexes containing transferrin or adenovirus, or combination complexes containing both transferrin and adenovirus, were evaluated. The highest *in vitro* gene transfer efficiency in primary cultures of airway epithelial cells was accomplished by the combination complexes. This result was paralleled *in vivo*. Transient gene expression of up to 1 week was observed with localization of the transduced cells to the region of the small airways. These results establish the feasibility of this type of approach for gene therapy applications.

OVERVIEW SUMMARY

Conjugate vectors offer many potential advantages as vehicles to accomplish direct *in vivo* gene transfer. In this study, Gao *et al.* used adenovirus–polylysine–DNA complexes to deliver reporter genes to the respiratory epithelium by the airway route. Transient genetic modification of airway epithelial cells *in situ* was demonstrated. Receptor-mediated gene delivery strategies thus offer a potential means of therapeutic modification of airway epithelium.

INTRODUCTION

GENETIC MODIFICATION of airway epithelium offers a potential therapeutic strategy for a variety of inherited and acquired pulmonary disorders. Because it has not yet been feasible to reimplant airway epithelial cells modified *ex vivo*, delivery of the heterologous genetic material must occur by direct *in vivo* transduction of the target cell *in situ*. The access to airway epithelium offered by the anatomy of the tracheobronchial tree suggests that *in vivo* gene transfer be accomplished by direct delivery via the airway route. The low proliferative rate of the airway epithelium (Bolduc and Reid, 1976) requires that candidate vectors be capable of gene transfer to a nonreplicating cellular target (Engelhardt and Wilson, 1992).

Foreign gene expression in airway epithelium has been demonstrated after direct *in vivo* delivery employing lipofection (Brigham *et al.*, 1989; Hazinski *et al.*, 1991; Yoshimura *et al.*, 1992) and recombinant adenoviruses (Rosenfeld *et al.*, 1991, 1992). For practical application in therapeutic protocols, however, lipofection may be limited by its cellular toxicity (Felgner *et al.*, 1987; Malone *et al.*, 1989). In addition, this vector lacks cell-specific tropism. Nonspecific delivery after topical administration *via* the airway route may be potentially deleterious in settings where cell-specific gene expression is required. Direct *in vivo* delivery employing a recombinant derivative of the respiratory tropic adenovirus offers a more efficient vector system (Rosenfeld *et al.*, 1992). Potential safety hazards, however, derive from the obligatory codelivery of gene elements of the parent virus. In this regard, recombinant adenovirus vectors containing deletions of the early gene regions E1A/E1B have been shown to be associated with expression of viral late genes as well as limited viral replication (Nevins, 1981; Gaynor and Berk, 1983; Imperiale *et al.*, 1984).

As an alternative to these approaches, we have explored the utility of using adenovirus–polylysine–DNA complexes to accomplish direct *in vivo* gene delivery to the respiratory epithelium. This vector system offers several potential advantages for

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in vivo applications (Wu and Wu, 1988; Wagner *et al.*, 1990; Zenke *et al.*, 1990; Cotton *et al.*, 1991). These advantages include a design plasticity that permits the potential to accomplish cell-specific targeting. As entry is *via* a cellular internalization pathway, complexes may be administered on a continuous or repetitive basis. In addition, adenovirus–polylysine–DNA complexes have been constructed that possess extremely high *in vitro* gene transfer efficiencies.

We have previously shown that gene transfer efficiency *via* the receptor-mediated pathway can be dramatically improved by incorporation of an adenovirus moiety into the design of the complex (Curiel *et al.*, 1992; Wagner *et al.*, 1992). In this configuration, the adenovirus functions to allow escape of the conjugate–DNA complex from endosomes. Because this effect of adenovirus is mediated by viral capsid proteins and is independent of viral gene expression (Pastan *et al.*, 1986), it is possible to take measures to inactivate the adenoviral genome. Thus, a combination of genomic deletions and psoralen plus UV-irradiation can be used to minimize the potential safety hazards deriving from the presence of viral gene elements (Cotten *et al.*, 1992).

In the present work, we show that we can accomplish direct *in vivo* gene transfer to the respiratory epithelium in a rodent model using adenovirus–polylysine–DNA complexes. This establishes the feasibility of this approach as a method to accomplish transient gene expression in the respiratory epithelium. The capacity to achieve genetic modification of the airway epithelial cells *in situ* offers a potential strategy to accomplish gene therapy for disorders afflicting the airway epithelium.

MATERIALS AND METHODS

Preparation of gene transfer vectors

Human transferrin–polylysine–DNA complexes (hTfpL) were prepared by combination of (8.0 μg) human transferrin–polylysine (Serva Biochemical) in 150 μl of NaCl 150 m*M*/ HEPES 20 m*M* pH 7.3 (HBS) plus 6.0 μg of plasmid DNA in 350 μl of HBS followed by 30 min incubation at room temperature. The adenovirus–component complexes were of two types: binary complexes that contained adenovirus linked to polylysine–DNA (AdpL) and combination complexes that contained adenovirus plus human transferrin linked to polylysine–DNA (hTfpL/AdpL). The adenoviral component complexes were prepared utilizing the chimeric adenovirus P202 linked to polylysine by an antibody bridge (Curiel *et al.*, 1992) or the replication-incompetent adenovirus *dl*312 linked to polylysine by a chemical bridge consisting of biotin and streptavidin (bAd) (Wagner *et al.*, 1992). In the latter instance, the virus was further inactivated by treatment with psoralen plus UV irradiation (Cotten *et al.*, 1992) prior to complex formation. The reporter plasmid DNA pCLuc4 was used for assays of net gene expression. This plasmid contains the firefly luciferase gene under the transcriptional control of the cytomegalovirus (CMV) enhancer/early promoter. The reporter plasmid DNA pCMVβ was used for assays of localized gene expression. This plasmid contains the bacterial *lacZ* (β-galactosidase expressing) gene under the transcription control of the CMV enhancer/early promoter.

Gene transfer to primary cultures of cotton rat airway epithelial cells

Cultures of cotton rat airway epithelial cells were prepared by described methods (Van Scott *et al.*, 1986). Dissociated cells were harvested, washed three times with F12-7X media, and plated at a density of 5.0×10^5 cells/dish in 3-cm tissue culture dishes. Cells were maintained in F12-7X media and utilized for gene transfer experiments when they achieved 50–75% confluency. This usually required 2–3 days. For gene transfer experiments, the formed complexes were delivered directly to the cells and incubated for 24 hr. Complexes evaluated included human transferrin–polylysine (hTfpL), adenovirus–polylysine (AdpL), and human transferrin–adenovirus–polylysine (hTfpL/ AdpL). After incubation, cells were either lysed and evaluated for luciferase gene expression by described methods (Brasier *et al.*, 1989) or stained for β-galactosidase expression utilizing X-gal (MacGregor and Caskey, 1989). For luciferase assays indicating net gene expression, epithelial cells in primary culture were treated with complexes containing the reporter plasmid DNA pCLuc4 (6.0 μg). For β-galactosidase assays indicating *in situ* gene expression, cells were treated with complexes containing the reporter plasmid DNA pCMVβ (6.0 μg).

Gene transfer to cotton rat airway epithelium in vivo

Formed complexes were delivered to cotton rats *via* the intratracheal route. For analysis of relative *in vivo* transfer effi-

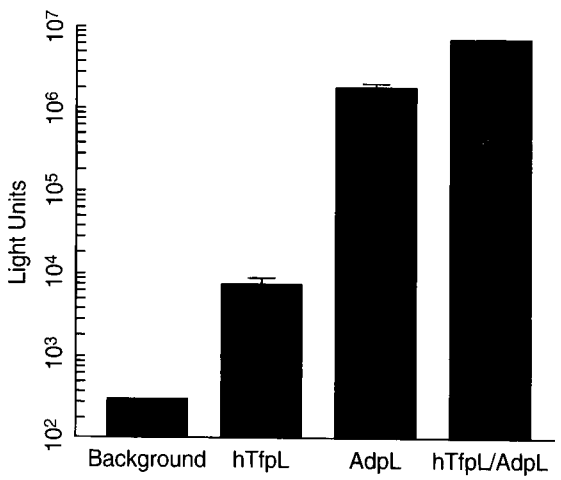

FIG. 1. Relative levels of net gene transfer to cotton rat airway epithelium in primary culture. The firefly luciferase reporter gene containing plasmid pCLuc4 was used to form conjugate–DNA complexes, which were delivered to airway epithelial cells harvested from cotton rat tracheas. Cell lysates were evaluated for luciferase gene expression after 24 hr. The vector species included human transferrin–polylysine–DNA complexes (hTfpL), adenovirus–polylysine–DNA complexes (AdpL), and human transferrin–adenovirus–polylysine–DNA complexes (hTfpL/AdpL). Background indicates evaluation of unmodified cells. Ordinate represents luciferase gene expression as light units per 25 μg of total protein derived from cellular lysates. Experiments were performed three to four times each and results are reported as mean ± SEM.

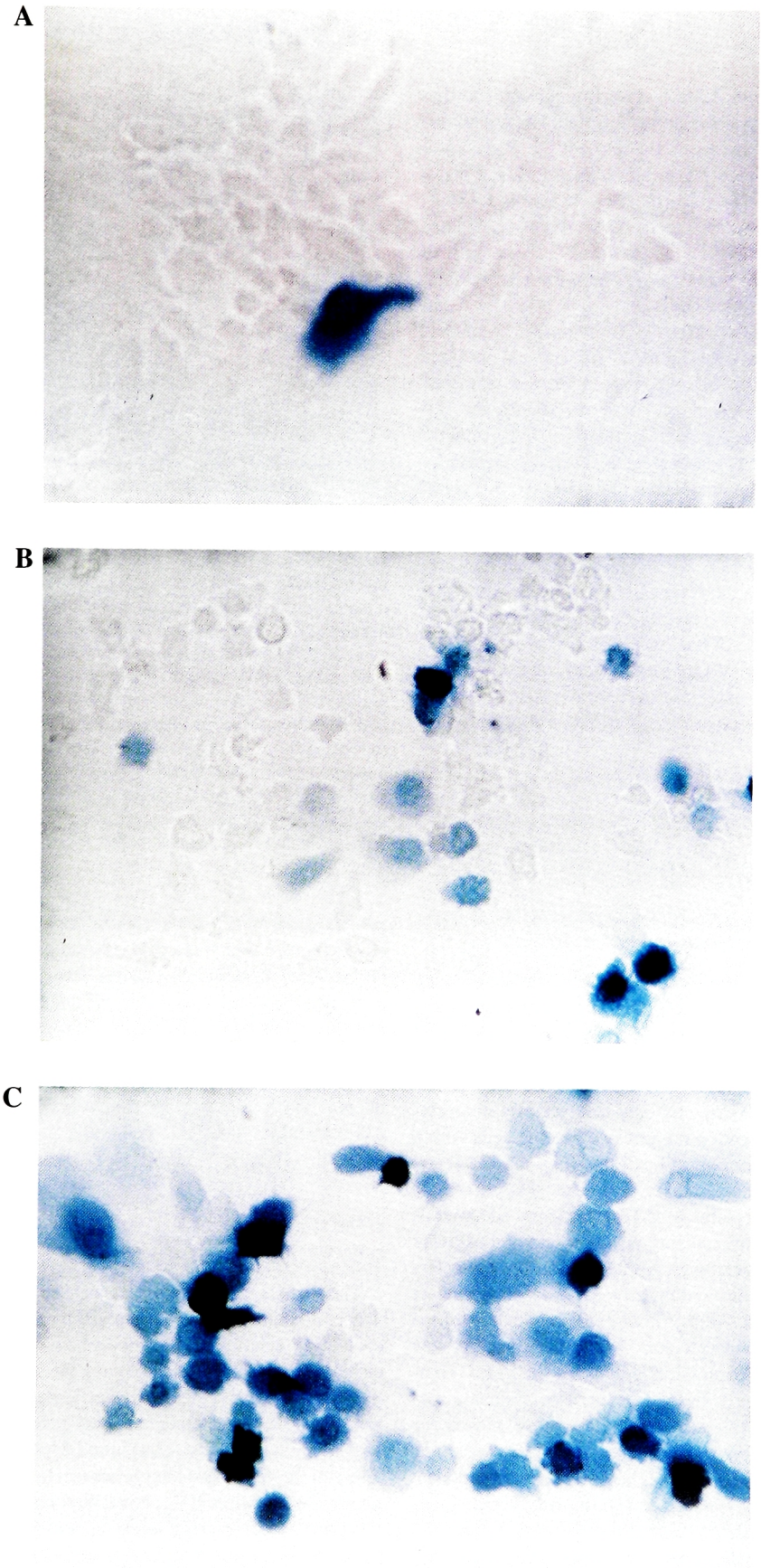

FIG. 2. Relative transduction frequency of cotton rat airway epithelium in primary culture. The *lacZ* histologic reporter containing plasmid pCMVβ was used to form conjugate–DNA complexes and delivered to primary cultures of cotton rat airway epithelia as before. Cells were evaluated for expression of the reporter gene by staining with X-gal at 24 hr. Results are shown for primary cultures of cotton rat epithelial cells transduced with the various complex species: A. hTfpL; B. AdpL; C. hTfpL/AdpL. Magnification, 320×.

ciency, the evaluated complexes included human transferrin–polylysine (hTfpL), adenovirus–polylysine (AdpL), and human transferrin–adenovirus–polylysine (hTfpL/AdpL). These complexes contained the reporter plasmid DNA pCLuc4. For histologic localization of *in vivo* gene transfer, the human transferrin–adenovirus–polylysine complexes (hTfpL/AdpL) contained the *lacZ* reporter plasmid DNA pCMVβ. For evaluation of temporal pattern of *in vivo* gene expression, the complex utilized was the human transferrin–adenovirus–polylysine complex containing replication-defective adenovirus *dl*312 that had been inactivated by psoralen plus UV-irradiation (hTfpL/bAdpL). These complexes contained the reporter plasmid DNA pCLuc4. Animals were anesthetized with methoxyflurane. After a vertical incision in the ventral aspect of the neck, the trachea was isolated by blunt dissection. With the animal inclined at a 45° angle, the complexes (250–300 μl; 3.0 μg of plasmid DNA) were injected directly into the trachea under direct visualization. At indicated times post-injection, the animals were sacrificed by CO_2 inhalation and trachea and lung were harvested *en bloc* after perfusion of pulmonary vessels *in situ* with cold phosphate-buffered saline (PBS). For luciferase assays, the lung blocks were homogenized in extraction buffer, and lysates were standardized for total protein content and evaluated for luciferase gene expression as described (Brasier *et al.*, 1989). For the β-galactosidase assays, frozen sections of intact unperfused lung were prepared and stained with X-gal as described (MacGregor and Caskey, 1989).

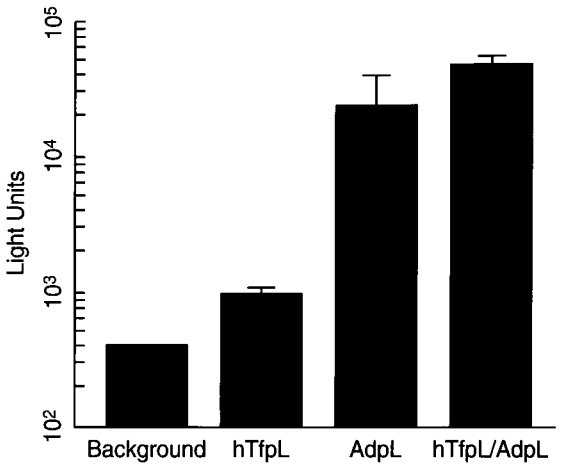

FIG. 3. Relative levels of net gene transfer to cotton rat airway epithelium *in vivo*. The firefly luciferase reporter gene containing plasmid pCLuc4 was used to form conjugate–DNA complexes, which were delivered to cotton rats *via* injection by the intratracheal route. Lungs were harvested and lysates were evaluated for luciferase gene expression after 24 hr. Vector species included human transferrin–polylysine–DNA complexes (hTfpL), adenovirus–polylysine–DNA complexes (AdpL), and human transferrin–adenovirus–polylysine–DNA complexes (hTfpL/AdpL). Background indicates evaluation of lungs from untreated animals. Ordinate represents luciferase gene expression as light units per 1250 μg total protein derived from lung lysates. Experiments were performed three to four times each and results are expressed as mean ± SEM.

RESULTS

Gene transfer to cotton rat airway epithelial cells in primary culture via *receptor-mediated delivery*

The cotton rat (*Sigmodon hispidus*) has been shown to be an animal model of human adenoviral lung disease (Pacini *et al.*, 1984) and therefore was employed as a target for gene transfer to respiratory epithelial cells employing adenovirus–polylysine–DNA complexes. The gene transfer efficiency of the various conjugate designs was initially evaluated by transfecting primary cultures of cotton rat airway epithelial cells with a firefly luciferase reporter plasmid pCLuc4 (Fig. 1). Comparison was made among simple binary complexes that internalize through the transferrin pathway (hTfpL), binary adenoviral-component complexes internalizing via the adenoviral pathway (AdpL), and combination complexes possessing both transferrin and adenoviral domains and thus the capacity to internalize by both pathways (hTfpL/AdpL). In this analysis, the cotton rat airway epithelium in primary culture showed only a very low level of luciferase gene expression employing the hTfpL complexes. This is consistent with the fact that this conjugate species may be entrapped within cellular endosomes, owing to the lack of a specific cell vesicle escape mechanism (Curiel *et al.*, 1991, 1992; Wagner *et al.*, 1992). The adenovirus–component binary complexes (AdpL) exhibited significantly greater gene expression. This was further augmented by the inclusion of a second ligand domain in the combination configuration (hTfpL/AdpL).

To determine if the relative levels of net gene expression correlated with transduction frequency, we next evaluated the percentage of cells transduced with the various complex species employing the *lacZ* histologic reporter plasmid pCMVβ, which encodes the bacterial β-galactosidase gene (Fig. 2). In this analysis, it could be seen that the relative levels of net gene expression observed in the luciferase assay reflected the relative numbers of cells transduced. Thus, the hTfpL-modified airway epithelium in primary culture exhibited <1% transduction frequency, the AdpL complexes on the order of 20–30%, and the hTfpL/AdpL combination complexes greater than 50% modified cells.

Gene transfer to cotton rat airway epithelia in vivo via *receptor-mediated delivery*

The various complex species were next delivered to the airway epithelium of the rodent model by the airway route. Initial evaluation determined the relative *in vivo* gene transfer efficiency of the complexes employing the luciferase reporter (Fig. 3). In this analysis, the relative efficiency of the complexes *in vivo* paralleled the finding in the analysis of primary cultures of airway epithelial cells. Thus, the hTfpL complexes mediated levels of luciferase gene expression in lung extract only slightly above levels observed in unmodified lung. Higher levels were achieved by the AdpL complexes and the highest levels were achieved by the hTfpL/AdpL combination complexes. Whereas the magnitude of net gene expression observed *in vivo* was of a lower order than that observed for the *in vitro* experiments, no conclusions may be drawn as relates to relative efficiency in

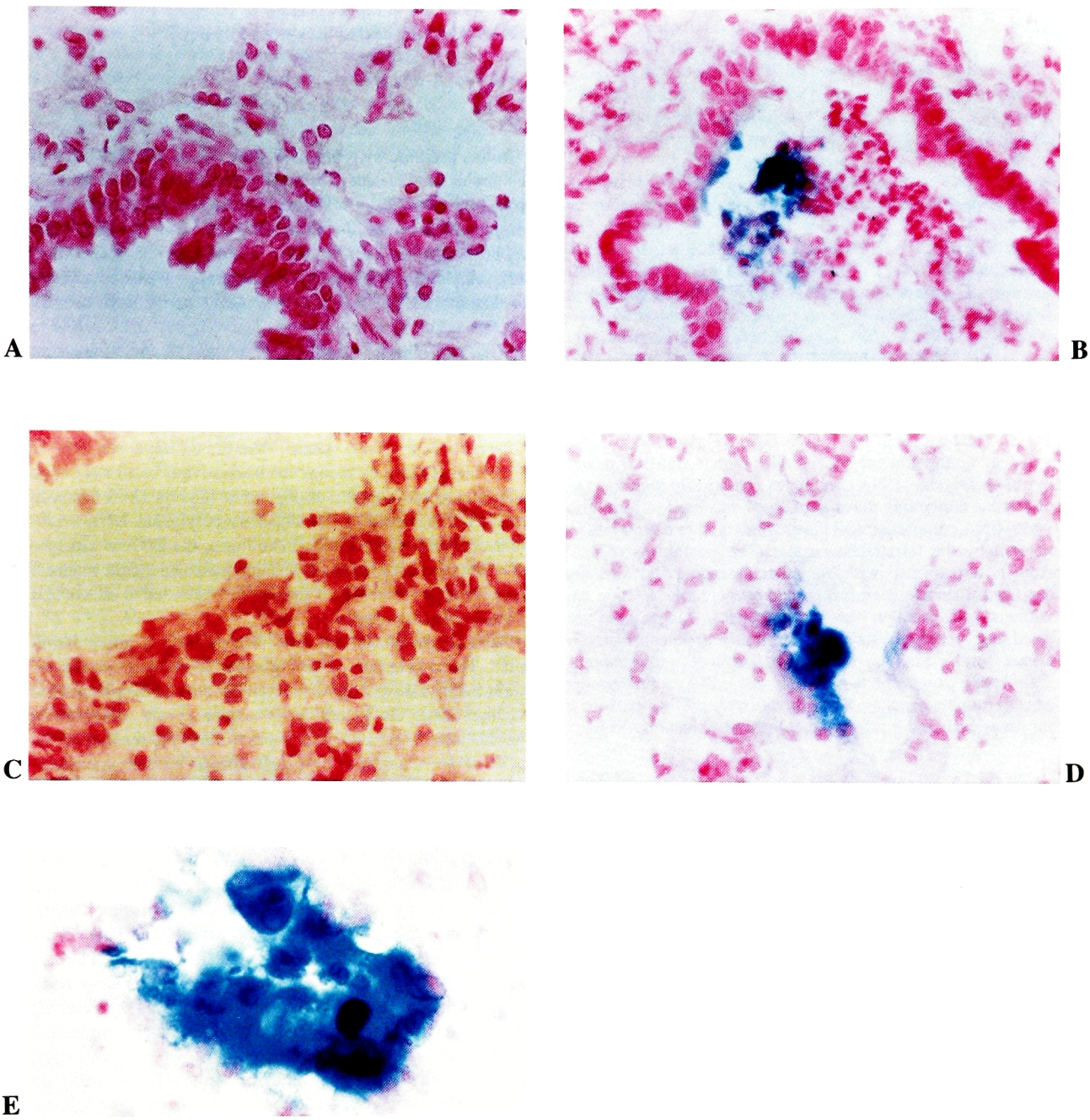

FIG. 4. Localization of heterologous gene expression in cotton rat airway epithelium. The *lacZ* histologic reporter containing plasmid pCMVβ was used to form human transferrin–adenovirus–polylysine-DNA (hTfpL/AdpL) complexes and delivered to cotton rats *via* injection by the intratracheal route. At 24 hr, 14-μm-thick frozen sections of harvested lungs were evaluated for expression of the reporter gene by stain with X-gal and counterstained with Nuclear Fast Red. Results are shown for cotton rats treated with hTfpL/AdpL complexes containing an irrelevant non-*lacZ* plasmid Rc/RSV or pCMVβ containing the *lacZ* reporter plasmid. A. Bronchiolus of cotton rat treated with hTfpL/AdpL complexes containing plasmid DNA pRc/RSV. B. Bronchus of cotton rat treated with hTfpL/AdpL complexes containing plasmid DNA pCMVβ. C. Distal airway region of cotton rat treated with hTfpL/AdpL complexes containing plasmid DNA pRc/RSV. D. Distal airway region of cotton rat treated with hTfpL/AdpL complexes containing plasmid DNA pCMVβ. Magnification, 600×. E. Enlargement of β-galactosidase-positive region from lungs of cotton rat treated with hTfpL/AdpL complexes containing plasmid DNA pCMVβ. Magnification, 1,000×.

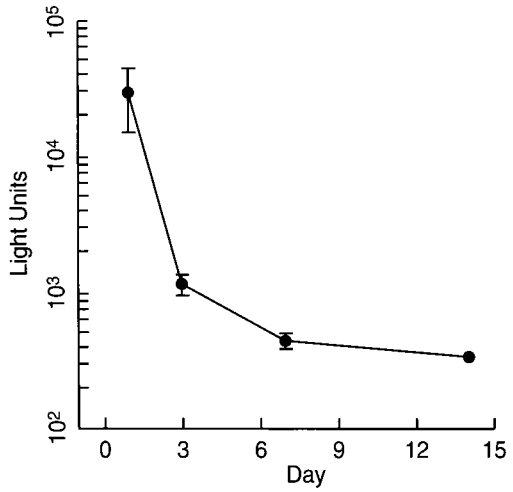

FIG. 5. Time course of heterologous gene expression in cotton rat airway epithelium transduced with human transferrin–adenovirus–polylysine–DNA complexes. The firefly luciferase reporter gene containing plasmid pCLuc4 was used to form conjugate–DNA complexes which were delivered to cotton rats *via* injection by the intratracheal route. The complexes (hTfpL/bAdpL) were formed with human transferrin–polylysine and adenovirus that had been inactivated by genomic deletion and treatment with psoralen plus UV-irradiation. Lungs were harvested and lysates evaluated for luciferase gene expression at various time points post-injection. Ordinate represents luciferase gene expression as light units per 1,250 μg total protein derived from lung lysates. Experiments were performed three to four times each and results were expressed as mean ± SEM.

these two contexts. For the *in vitro* experiments, all of the cells harvested for analysis were accessible to conjugate-mediated gene transfer. For the *in vivo* experiments, the respiratory epithelium accessible to transduction represented only a minor fraction of the harvested lung material evaluated for net gene expression.

We next evaluated *in vivo* transduction efficiency employing the *lacZ* histologic reporter (Fig. 4). This analysis was limited to the hTfpL/AdpL complex species, which exhibited the highest net *in vivo* gene transfer. Evaluation of histologic lung sections of animals treated in this manner demonstrated patchy areas of β-galactosidase activity containing multiple marked cells. As a control, no β-galactosidase activity could be detected in animals transduced with the hTfpL/AdpL complexes containing an irrelevant plasmid DNA. These positive regions were localized to the bronchioles and distal airway region. Specific airway epithelial subsets modified could not be determined in this assay.

The time course of heterologous gene expression in the airway epithelium was evaluated by using the luciferase reporter gene in combination with the hTfpL/bAdpL combination complexes (Fig. 5). For this analysis, the adenovirus had been inactivated by a combination of gene deletion and treatment with psoralen plus UV-irradiation (Cotten *et al.*, 1992). This modification allows prolonged *in vitro* expression consequent to minimized adenoviral replication and/or gene expression. Maximum gene expression was noted at 24 hr post-administration. There was a rapid decrease of net gene expression such that levels diminished to background by day 7.

DISCUSSION

In this preliminary report, we demonstrate the feasibility of accomplishing heterologous gene expression to the respiratory epithelium *in situ* employing adenovirus–polylysine–DNA complexes. Whereas *in vivo* transduction of airway epithelium has been obtained utilizing other vector systems, adenovirus–polylysine–DNA complexes offer several potential advantages for this application. Practical advantages derive from the fact that this vector system transports heterologous DNA bound to the viral capsid exterior rather than incorporated into the parent virus genome as is the case for recombinant adenoviral vectors (Berkner, 1988; Curiel *et al.*, 1992). Thus, the amount of DNA that can be transported is not limited by packaging constraints of the recombinant viral system. Whereas the upper size limit of DNA transportable by recombinant adenoviral vectors is on the order of 6–8 kb (Berkner, 1988), up to 48 kb of DNA has been transferred utilizing the adenovirus–polylysine–DNA complexes (Cotten *et al.*, 1992). In addition, the DNA is incorporated into the complexes in a sequence-independent manner. Gene constructs transferred are thus not restricted to the context of viral regulatory controls.

Potential advantages are also offered from a safety standpoint. The production of recombinant adenoviral vectors requires maintenance of the functional integrity of the parent viral genome, since the heterologous sequences are incorporated therein. Despite genetic maneuvers to limit the replicative capacity of the vectors, the E1A/E1B deletion mutants are associated with late viral gene expression and detectable viral replicative capacity (Nevins, 1981; Gaynor and Berk, 1983; Imperiale *et al.*, 1984; Gregory *et al.*, 1992). In the configuration of the adenovirus–polylysine–DNA complexes, the entry mechanism of the virus is exploited in a selective manner whereby viral gene elements are not an essential feature (Cotten *et al.*, 1992). Thus, it is feasible to inactivate the parent viral genome by using a combination of mechanisms, including viral gene deletions and psoralen plus UV irradiation, as we have done here. By extending this strategy of vector design, it is theoretically possible that viral gene elements may be ultimately eliminated, thereby creating an even safer vector.

The marked plasticity of molecular conjugate design allowed the derivation of a vector with optimized *in vivo* gene transfer efficiency. The low gene transfer capacity of the hTfpL complexes *in vitro* and *in vivo* is consistent with the fact that this species may be entrapped within the cell vesicle system after internalization consequent to the lack of a specific endosome escape mechanism (Curiel *et al.*, 1991, 1992; Wagner *et al.*, 1992). The AdpL complexes make use of the adenovirus as both ligand domain and endosomolysis principle. These complexes could thus be internalized *via* adenoviral receptors and escape cell vesicle entrapment by virtue of adenovirus-mediated endosomolysis. This fact was reflected in the significantly augmented gene transfer capacity of these complexes. The addition of a second ligand to the complexes in the hTfpL/AdpL configuration allowed even greater gene transfer to occur both *in vitro* and *in vivo*. The fact that these complexes contain two

potential ligand domains allows their internalization by both of these pathways. Whereas no direct comparison is made in this study between the *in vivo* gene transfer efficiency of recombinant adenoviral vectors and adenovirus–polylysine–DNA complexes, it is noteworthy that in the case of the adenovirus–polylysine–DNA complexes the conjugate design may be modified such that it possesses the capacity to internalize both by the adenoviral as well as alternate internalization pathways. A more direct comparison can be made to lipofectin whereby gene expression levels obtained after delivery employing the human transferrin–adenovirus polylysine–DNA complexes were two orders of magnitude greater than levels observed in a similar protocol utilizing the cationic liposomes (Yoshimura *et al.*, 1992) (data not shown).

The detectable *in vivo* gene expression mediated by the adenovirus–polylysine DNA complexes was of a transient nature. This closely parallels the expression pattern noted after lipofectin-mediated *in vivo* gene transfer to the respiratory epithelium (Hazinski *et al.*, 1991). This result is not unanticipated because the delivered DNA would be present as a plasmid episome lacking replicative or integrative capacity (Wilson *et al.*, 1992). In the present design, the conjugate system lacks a mechanism to mediate integration and thus the stable transduction frequency would be expected to be low. Alternatively, attrition of the modified cells could explain the extinction of gene transfer in the lung. The fate of individual modified cells was not addressed in this study and thus the possibility of vector-associated cell toxicity could not be excluded.

Transient genetic modification of the airway epithelium may be potentially beneficial in certain therapeutic contexts. For gene therapy of inherited disorders afflicting respiratory epithelium such as cystic fibrosis (Boat *et al.*, 1989), however, permanent correction of cellular targets is desirable. Application of transient expression systems such as molecular conjugates or recombinant adenoviruses to achieve long-term correction would thus require repetitive dosing. It is unclear whether this would be feasible given the potential immunologic sequelae that may derive from this type of treatment. Thus, the incorporation of an integration mechanism within the design of the conjugate vector would likely enhance its utility for therapeutic genetic modification of airway epithelium as well as for a variety of other genetic correction applications.

ACKNOWLEDGMENTS

The authors wish to acknowledge the expert editorial assistance of L. Brown.

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Received for publication October 27, 1992; accepted after revision November 13, 1992.