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Research Article

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Incorporation of Adenovirus in Calcium Phosphate Precipitates Enhances Gene Transfer to Airway Epithelia In Vitro and In Vivo

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Abstract

Adenovirus (Ad)-mediated gene transfer to airway epithelia is inefficient because the apical membrane lacks the receptor activity to bind adenovirus fiber protein. Calcium phosphate (CaPi) precipitates have been used to deliver plasmid DNA to cultured cell lines. However, such precipitates are not effective in many primary cultures or in vivo. Here we show that incorporating recombinant adenovirus into a CaPi coprecipitate markedly enhances transgene expression in cells that are resistant to adenovirus infection. Enhancement requires that the virus be contained in the precipitate and viral proteins are required to increase expression. Ad:CaPi coprecipitates increase gene transfer by increasing fiber-independent binding of virus to cells. With differentiated cystic fibrosis (CF) airway epithelia in vitro, a 20-min application of Ad:CaPi coprecipitates that encode CF transmembrane conductance regulator produced as much CF transmembrane conductance regulator Cl^- current as a 24-h application of adenovirus alone. We found that Ad:CaPi coprecipitates also increased transgene expression in mouse lung in vivo; importantly, expression was particularly prominent in airway epithelia. These results suggest a new mechanism for gene transfer that may be applicable to a number of different gene transfer applications and could be of value in gene transfer to CF airway epithelia in vivo. (*J. Clin. Invest.* 1998. 102:184–193.) Key words: adenovirus • cystic fibrosis • gene transfer • airway epithelia • calcium phosphate

Introduction

A common problem in the development of gene therapy is the limited efficiency of gene transfer by viral and nonviral vectors. This is especially a problem in airway epithelia, a target for gene transfer in the genetic disease cystic fibrosis (CF)[1] (1). Several studies have shown that recombinant adenovirus vectors are able to transfer CF transmembrane conductance regulator (CFTR) cDNA and reporter genes to cultured airway epithelia and to the airways of animals (2–14). Moreover, studies in humans have shown that adenovirus vectors can direct expression of CFTR and at least partially correct the defect in airway epithelial Cl^- transport (15–19). However, when all the data are considered together, they suggest that the efficiency of gene transfer to differentiated, ciliated airway epithelia is low.

Adenovirus (Ad) interacts with cultured cell lines via adenovirus fiber binding to its cell surface receptor (20, 21) and binding of adenovirus penton base with $\alpha\gamma$ integrins (22). We found that infection of airway epithelia is inefficient because differentiated epithelia lack adenoviral fiber receptor activity on their apical surface (23). The lack of apical integrins may also limit gene transfer, although their absence seems to play a less important role than the lack of fiber receptors (23, 24). These limitations can be circumvented by incubating virus with the epithelium for a prolonged period of time (25). We also found that cationic molecules (poly-L-lysine [PLL] or cationic lipids) form a noncovalent complex with negatively charged adenovirus and associate with the negatively charged cell membrane. This increases cell binding via a non–fiber-dependent pathway and enhances adenovirus-mediated transgene expression (26). Arcasoy et al. (27) came to similar conclusions. In a related approach, Wickham et al. (28) found that inclusion of a polylysine tail at the COOH-terminal end of fiber protein enhanced gene transfer to adenovirus-resistant cells.

Although complexes of cationic molecules and adenovirus enhanced gene transfer, there are limitations. First, previous studies have shown that cationic molecules can have direct cell toxicity; this is especially the case for cationic lipids (29–31). Moreover, intrapulmonary delivery of cationic lipids and DNA can generate toxic and inflammatory responses on their own (32). Second, delivery of adenovirus alone to the lung can generate an immune response (10, 33, 34). When a cationic lipid is administered with adenovirus, the lipid might serve as an adjuvant, increasing the immune response to the coadministered adenovirus (35); in addition, PLL and cationic lipids can activate complement (36). Third, in earlier work we showed that complexes of adenovirus and cationic molecules enhanced gene transfer to airway epithelia in mouse nasal mucosa (26). However, in subsequent work we found that intrapulmonary delivery of adenovirus complexed with cationic lipids targeted the alveolar epithelium (our unpublished observations), whereas the airways are the intended target in CF.

While studying complexes of adenovirus and PLL or cationic lipids, we found that they formed aggregates (26). This observation suggested the possibility that an aggregate or precipitate might itself facilitate infection. Therefore, we tested the hypothesis that inclusion of adenovirus in a precipitate

[†]In memory of Dr. Al Fasbender.

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1. *Abbreviations used in this paper:* Ad, adenovirus; CaPi, calcium phosphate; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; EMEM, Eagle's minimal essential media; IU, infectious units; PLL, poly-L-lysine.

would enhance gene transfer to airway epithelia. We investigated calcium phosphate (CaPi) because it readily forms a precipitate. CaPi precipitates have also been used for transfection of plasmid DNA into some cultured cell lines (37, 38). However, it is well known that DNA:CaPi precipitates are not effective in most primary cell cultures or in vivo (also see Fig. 6 below).

Methods

Cell culture. NIH-3T3 and COS-1 cells were cultured on 24-well plates (Corning, 25820) in DMEM (high glucose) supplemented with 10% FCS (Sigma Chemical Co., St. Louis, MO), 100 U/ml penicillin, and 100 μg/ml streptomycin (P/S). HeLa cells were cultured in Eagle's minimal essential media (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% FCS, 10 mM nonessential amino acids (Sigma), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cultured cell lines were infected 18–24 h after seeding when the cells were ~ 70% confluent. Primary cultures of normal and CF human airway epithelia were isolated and grown as described previously (25, 39). The culture media consisted of a 1:1 mix of DMEM/Ham's F12, 5% Ultraser G (Biosepra SA, Cedex, France), 100 U/ml penicillin, 100 μg/ml streptomycin, 1% nonessential amino acids, and 0.12 U/ml insulin. Cells were seeded on Millicell polycarbonate filters (Millipore Corp., Bedford, MA). 24 h after seeding, the cells were switched to the air–liquid interface and then grown with a dry apical surface. Epithelia were infected at least 2 wk after seeding; transgene expression was measured 2–4 d later.

Vectors and vector-related reagents. Recombinant adenovirus vectors expressing β-galactosidase, Ad2/βGal-2, and CFTR, Ad2/CFTR-16, were prepared as described previously (25) by the University of Iowa Gene Transfer Vector Core at titers of ~ 10^{10} infectious units (IU)/ml. Fiber knob protein was a gift of Dr. Paul Freimuth (Brookhaven National Laboratories, Upton, NY). In some studies, adenovirus was complexed with PLL (55 kD; Sigma) or the cationic lipid GL-67 (a gift of Drs. Seng Cheng and David Harris, Genzyme Corp., Cambridge, MA), as previously described (26). Neutralizing antibody (1401; Virostat, Portland, ME) or nonneutralizing antibody (mAb 8052; Chemicon International Inc., Temecula, CA) was applied to virus in some studies. A 1:100 dilution of antibody was incubated with 2×10^9 particles for 30 min at room temperature before formation of coprecipitates.

For most studies CaPi coprecipitates containing adenovirus were formed by placing 8×10^9 particles of recombinant adenovirus in 1 ml of Eagle's minimal essential media (M-0268; Sigma), which contains 1.8 mM Ca^{2+} and 0.86 mM Pi. Then an aliquot of a 2 M $CaCl_2$ ($CaCl_2 \cdot 2H_2O$, E1200; Promega, Madison, WI) solution was added to achieve the desired Ca^{2+} concentration. However, a wide range of adenovirus particles and concentrations of Ca^{2+} and Pi was used as described in the text and figure legends. The solution was mixed by vortex or gentle pipette tip aspiration. Note that the Ca^{2+} and Pi concentrations refer to total rather than free concentrations. Unless otherwise noted, the mixture was allowed to incubate for 20–30 min at room temperature. Then 250 μl was applied to cells for 20 min followed by washing the surface to remove the complex.

To evaluate virus association with cells, adenovirus was labeled with the carbocyanine dye Cy3 (Amersham Inc., Arlington Heights, IL) using methods described to us by Drs. P.L. Leopold and R.G. Crystal (40). Cy3 was covalently conjugated to capsid proteins of Ad2/βGal-2 by mixing 5 nmol of Cy3 with 10^{12} particles of virus in 1.5 ml of Na_2CO_3, pH 9.0, for 2 h at 4°C. The solution was subsequently transferred to a dialysis chamber (Slide-A-Lyzer, 10,000 mol wt cutoff; Pierce Chemical Co., Rockford, IL) and dialyzed against two changes of PBS, 3% sucrose, pH 7.4, at 4°C for 24 h. Expression and infection studies were performed immediately after dialysis of the conjugate. This labeling procedure decreased the IU/particle ratio by 5–35%.

Evaluation of transgene expression and toxicity. β-galactosidase activity was measured 24 h after application of vector as described previously (26). X-gal staining of epithelia was as described previously (25). To evaluate transepithelial electrolyte transport, epithelia were mounted in modified Ussing chambers (25). Short-circuit current (Isc) was measured under baseline conditions, and after addition of amiloride (10 μM), cAMP agonists (10 μM forskolin and 100 μM IBMX), and bumetanide 100 μM. Individual experiments were performed using three sets of cells and all experiments were repeated at least three times. Statistical significance was evaluated using a paired or unpaired *t* test.

Transmission electron microscopy. Ad:CaPi coprecipitates were processed for transmission electron microscopy using a negative stain technique. 15-μl drops of freshly prepared samples were placed on glow-discharged collodion/carbon-coated 400-mesh copper grids for 3 min. The solution was wicked off with filter paper and replaced with 1% aqueous uranyl acetate for 30 s. After removal of this solution, grids were allowed to dry and imaged in a Hitachi H-7000 transmission electron microscope.

Studies in mice. For in vivo analysis, we used 6–8-wk-old C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME). Mice were lightly anesthetized using a methoxyflurane chamber. Ad2/βGal-2 (2×10^8 IU, 5×10^9 particles) was administered alone or as Ad:CaPi coprecipitates intranasally in two 62.5-μl instillations delivered 5 min apart. The experiment was performed twice with six animals in each group. 3 d after vector administration, animals were killed. PBS (10 ml) was instilled into the right ventricle and then the lungs and heart were removed intact. The trachea was intubated and instilled at 10 cm of pressure with the following solutions in order: PBS, 4% paraformaldehyde and 0.2% glutaraldehyde, PBS, and finally X-gal reagent for an overnight incubation at room temperature. After photography, lungs were embedded in paraffin and serially sectioned. Lung sections for each condition ($n = 3$) were analyzed by counting all airways that were cut at a perpendicular angle. For each such airway, the airway diameter and number of blue cells was determined. The percentage of blue cells was calculated as: *number of blue cells* ÷ (*diameter* × π ÷ 4.9 μm). The average width of an airway cell (4.9 μm) was determined in separate experiments using hematoxylin and eosin–stained sections and is in excellent agreement with earlier studies (41).

Results

Ad:CaPi coprecipitates enhance gene transfer to NIH 3T3 cells. To learn whether incorporation of Ad2/βGal-2 in a precipitate would enhance adenovirus-mediated gene transfer, we initially studied 3T3 cells, because they show little fiber receptor activity and are resistant to adenovirus infection (42). In these respects they resemble the apical surface of differentiated airway epithelia. We applied vector for only a short time (20 min), then removed it, and measured transgene expression 24 h later. Fig. 1, *A* and *B*, shows that when we increased Ca^{2+} and Pi concentrations during complex formation, β-galactosidase expression increased, reached a maximum, and then decreased. These data suggest that formation of an effective precipitate or aggregate required an optimal stoichiometry of Ca^{2+} to Pi. Since CaPi precipitation and CaPi-mediated plasmid transfection is pH dependent (37, 38), we asked what role pH would play in gene transfer by Ad:CaPi coprecipitates. Fig. 1 *C* shows that when we attempted to form complexes at acidic pH (below pH 7) there was little enhancement of gene transfer; an acidic pH has similar effects on CaPi-mediated plasmid transfection (37, 38). As a control for the effect of pH on the virus, we infected HeLa cells with adenovirus alone that had been exposed to the same pH as the Ad:CaPi coprecipitates; we found no effect of pH on gene transfer (not shown). The

time during which Ca^{2+}, Pi, and adenovirus complexes were incubated before they were applied to cells also influenced gene transfer. As the duration of the preincubation period increased, transfection efficiency increased and then decreased (Fig. 1 *D*). We also saw a time-dependent effect on precipitate formation, light microscopy and visual inspection showed first the formation of fine precipitates when infection was maximal, and then with long duration preincubation periods, macro-precipitates formed and gene expression decreased. Future studies will be required to evaluate the size of the precipitate that correlates with the greatest enhancement of transgene expression. Fig. 1 *E* shows that when the amount of virus added during complex formation increased (without a change in volume, Ca^{2+}, or Pi), gene expression increased.

The increase in total transgene expression shown in Fig. 1 was paralleled by an increase in the percentage of 3T3 cells expressing transgene, after applying adenovirus alone for 20 min, 3.5% of the cells stained blue (n = 607 cells) after staining for β-galactosidase, whereas with Ad:CaPi coprecipitates 99% of cells stained blue (n = 800 cells). Ad:CaPi coprecipitates also enhanced expression in other cells that are relatively resistant to adenovirus infection (9L gliosarcoma cells, a 130-fold increase compared with adenovirus alone, and primary cultures of human umbilical vein endothelial cells, a 150-fold increase) and in cells that are easily infected (HeLa cells, a 9-fold increase, and COS cells, a 12-fold increase).

After the Ad:CaPi coprecipitates were formed, they could be placed in serum-containing media and added to cells without loss of efficacy (Fig. 2 *A*). This suggests that once the precipitate is generated, it is relatively stable. However, when we added serum to the solution before combining Ca^{2+}, Pi, and adenovirus, the enhancement in gene transfer was abolished (Fig. 2 *B*). This result suggests that serum interferes with formation of the Ad:CaPi coprecipitate. If we first formed CaPi precipitates and then separately applied them and the adenovirus to cells, there was little enhancement of expression (Fig. 2 *C*). This result indicates that enhanced infection efficiency requires that adenovirus be included in the precipitate.

Ad:CaPi coprecipitates increase virus association with cells. We tested the hypothesis that the coprecipitate enhanced gene expression by increasing the amount of virus associated with cells. Fig. 3, *A–D*, shows representative photo-

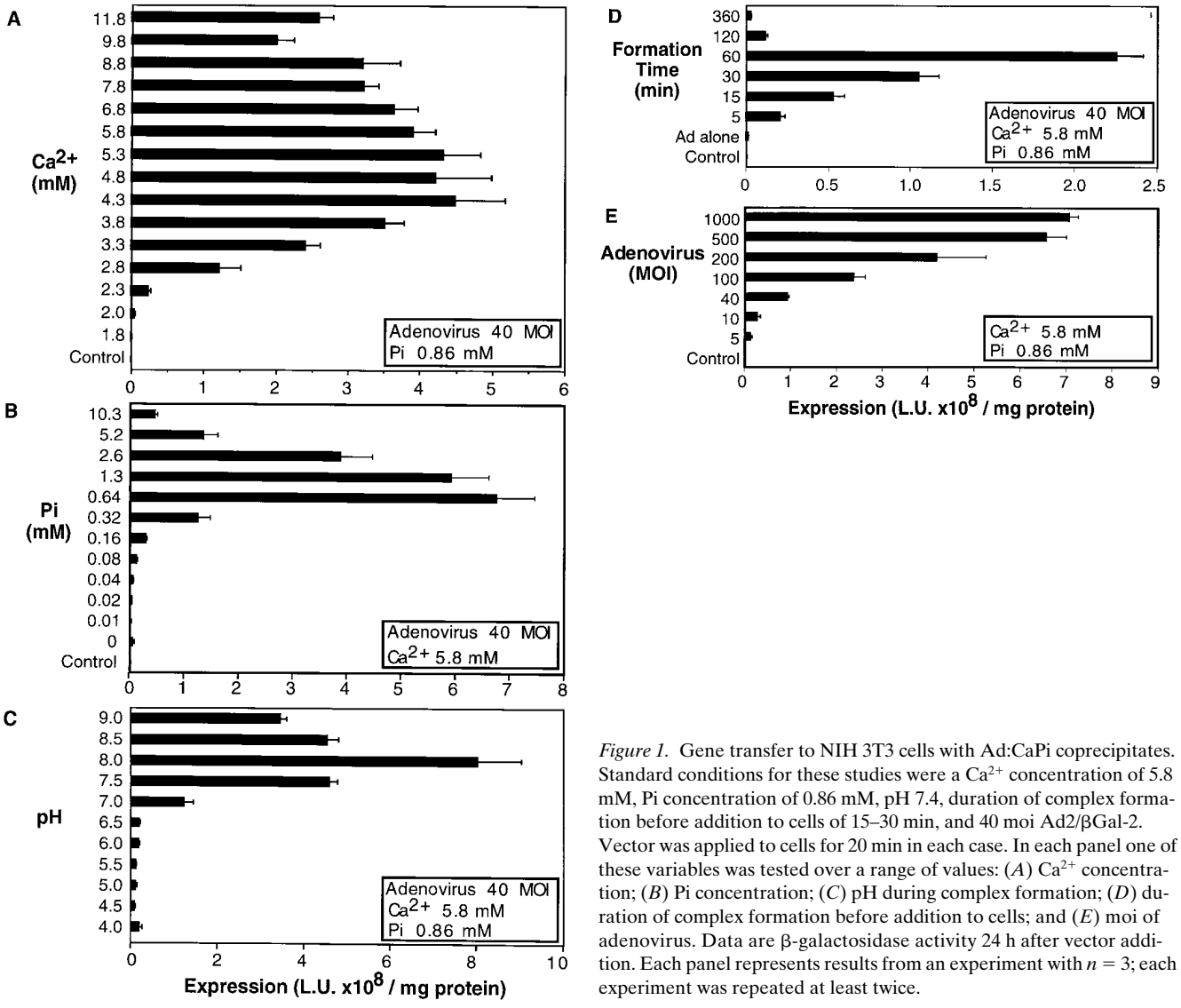

Figure 1. Gene transfer to NIH 3T3 cells with Ad:CaPi coprecipitates. Standard conditions for these studies were a Ca^{2+} concentration of 5.8 mM, Pi concentration of 0.86 mM, pH 7.4, duration of complex formation before addition to cells of 15–30 min, and 40 moi Ad2/βGal-2. Vector was applied to cells for 20 min in each case. In each panel one of these variables was tested over a range of values: (*A*) Ca^{2+} concentration; (*B*) Pi concentration; (*C*) pH during complex formation; (*D*) duration of complex formation before addition to cells; and (*E*) moi of adenovirus. Data are β-galactosidase activity 24 h after vector addition. Each panel represents results from an experiment with n = 3; each experiment was repeated at least twice.

micrographs of NIH-3T3 cells treated with Cy3-labeled adenovirus alone (*A* and *B*) or with Ad:CaPi coprecipitates (*C* and *D*). When virus was incorporated into an Ad:CaPi coprecipitate there was more cell binding and/or uptake than when virus was applied alone. The increase in cell binding was paralleled by an increase in transgene expression by the Cy3-labeled virus (Fig. 3 *E*).

To learn whether the increase in association of virus with cells required uptake, we compared results obtained at 37 and 4°C; incubation at 4°C will block endocytosis. Reduction in temperature had little effect on the amount of cell-associated fluorescence (Fig. 3 *F*). These results suggest that inclusion of virus in a coprecipitate enhances expression by increasing the amount of virus bound to cells. We cannot exclude the possibility that it might also enhance endocytosis.

Gene transfer by Ad:CaPi coprecipitates does not depend on fiber receptor. The ability of Ad:CaPi coprecipitates to enhance gene transfer to NIH 3T3 cells, which express little fiber receptor, suggested that binding of adenovirus fiber to its cell surface receptor was not required. To test this hypothesis directly, we studied COS cells which are readily infected by adenovirus. Fig. 4 *A* shows that fiber knob protein inhibited transgene expression by adenovirus alone by 94%. However, fiber knob protein had no appreciable effect on transgene expression by Ad:CaPi coprecipitates. These results indicate that adenovirus fiber is not required for the enhanced efficiency of infection.

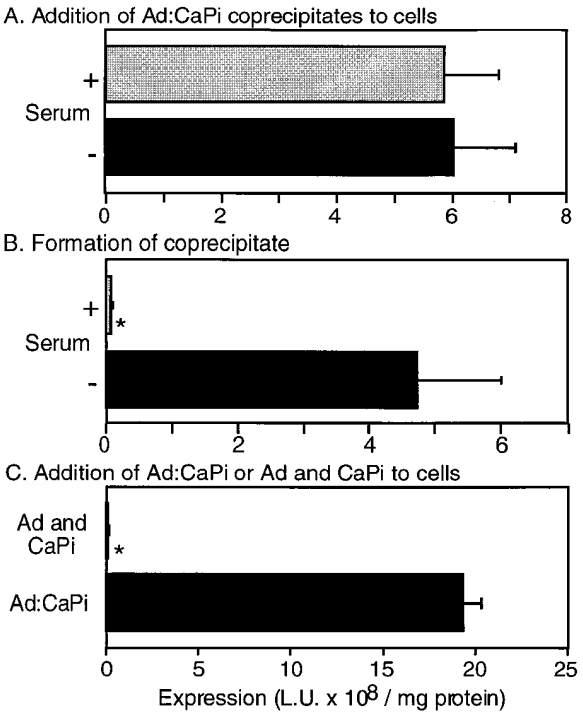

Figure 2. Effect of serum and separate addition of CaPi precipitates and adenovirus. In all experiments NIH 3T3 cells were treated with 40 moi adenovirus for 20 min. (*A*) Ad:CaPi coprecipitates were formed in the absence of serum and then added to cells in the presence (+) or absence (−) of 10% FCS. (*B*) Ad:CaPi coprecipitates were formed in the presence (+) of 10% FCS, or 10% FCS was added after formation of the precipitate (−). (*C*) Ad:CaPi coprecipitates or adenovirus and CaPi precipitates were added separately to cells in the presence of 10% FCS. For *A–C*, the Ca^{2+} concentration was 5.8 mM, Pi concentration was 0.86 mM, and the precipitates were formed for 15–30 min. $*P < 0.05$; data are from one experiment ($n = 3$) and each experiment was repeated at least three times.

When we disrupted viral proteins by heat inactivation, we inhibited transgene expression (Fig. 4 *B*). We obtained similar results when, before complex formation, adenovirus was treated with a neutralizing antihexon antibody that presumably inhibits infection by interfering with steps subsequent to binding such as endosomal escape and traffic of viral DNA to the nucleus. Moreover, when we transfected cells with CaPi coprecipitates containing 2×10^9 plasmids encoding β-galactosidase, there was little expression compared with precipitates formed with 2×10^9 particles of Ad2/βGal-2 (Fig. 4 *B*). These data indicate that although fiber is not necessary for infection with Ad:CaPi coprecipitates, other adenoviral proteins are required to facilitate gene transfer and expression. These results also explain why CaPi precipitates that contain adenovirus produce much more transgene expression than those containing DNA alone (Fig. 4 *B*).

We also formed precipitates with other anions and cations in a preliminary attempt to find a coprecipitate that might be more effective than CaPi. For example, magnesium phosphate, manganese phosphate, cobalt phosphate, calcium carbonate, and calcium sulfide were capable of enhancing infection. However, they were not as effective as CaPi (data not shown). Monovalent cations such as sodium and potassium that do not form coprecipitates did not enhance infection.

Gene transfer with Ad:CaPi coprecipitates to human airway epithelia in vitro. To evaluate gene transfer to airway epithelia, we studied primary cultures of human airway epithelia grown at the air–liquid interface. Under these conditions, the epithelia differentiate and form a ciliated epithelium that is resistant to gene transfer by adenovirus and cationic lipid vectors (25, 43). Fig. 5 *A* shows that when vector was applied to the apical surface for a short exposure time (20 min), Ad:CaPi coprecipitates enhanced transgene expression.

The ability of Ad:CaPi coprecipitates to transfer CFTR cDNA to CF airway epithelia was tested by applying 50 moi Ad2/CFTR-16 for 20 min. To assess CFTR Cl^- current, we inhibited Na^+ current with amiloride (10^{-5} M), applied cAMP agonists, and then measured the current inhibited by bumetanide (100 μM) applied to the basolateral surface. Fig. 5 *B* shows that untreated CF epithelia had no Cl^- current. As a positive control, Ad2/CFTR-16 remained on the mucosal surface for 24 h; this long incubation period allows significant transgene expression and transepithelial Cl^- transport increases into the normal range (25). When we applied adenovirus alone for only 20 min there was little Cl^- current, as previously reported (23, 25). However, when we applied Ad:CaPi coprecipitates for 20 min, the Cl^- current was at least as large as that obtained after a 24-h incubation with adenovirus alone. These data indicate that Ad:CaPi coprecipitates are much more efficient than virus alone for transfer of CFTR cDNA to differentiated airway epithelia and generation of CFTR Cl^- current.

Ad:CaPi coprecipitate–mediated gene transfer to mouse airway epithelia in vivo. To investigate Ad:CaPi coprecipitates in vivo, we administered adenovirus to mouse lungs. We delivered Ad2/βGal-2 (2×10^8 IU) by intranasal administration. This dose is lower than usually applied to obtain significant pulmonary gene transfer because we wished to learn whether delivery as a coprecipitate would enhance gene transfer. Fig.

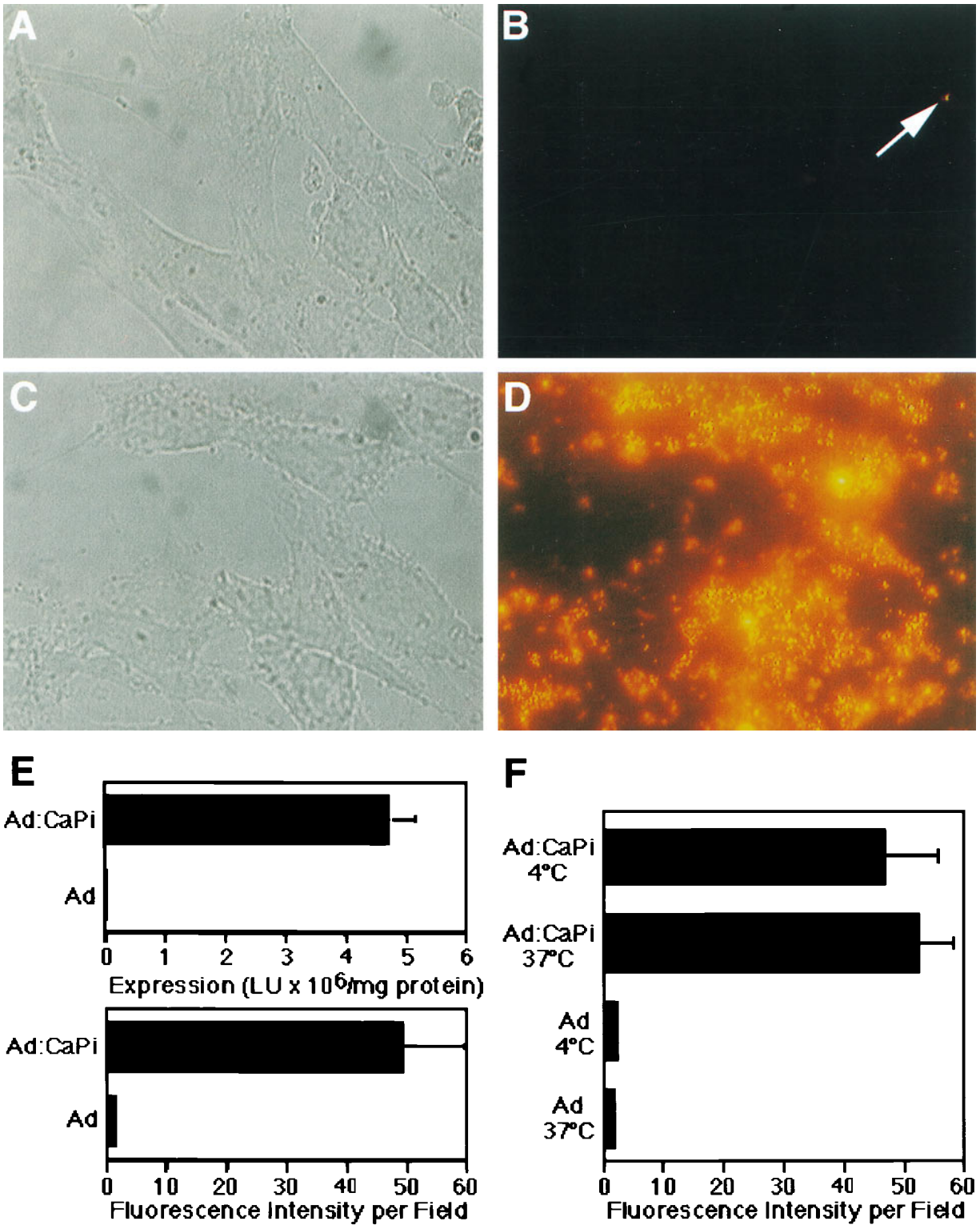

Figure 3. Effect of Ad:CaPi coprecipitates on adenovirus association with cells. To assess vector binding, NIH 3T3 cells were treated with 2×10^9 particles of Cy3-labeled Ad2/βGal-2 for 60 min, rinsed with 3T3 media to remove unbound virus, and fixed with 4% paraformaldehyde. *A* and *C* show examples of light photomicrographs of cells also shown in *B* and *D*, respectively. *C* shows cells treated with Cy3:Ad alone and *D* shows cells treated with Cy3:Ad:CaPi coprecipitates. The arrow in *B* indicates labeled adenovirus. Ad:CaPi coprecipitates were formed with 5.8 mM Ca^{2+} and 0.86 mM Pi and applied to cells at 37°C. *E* shows expression (*top*) and binding (*bottom*) from experiments shown in *A–D*. $n = 3$ for expression and $n = 5$ for binding data. *F* shows binding at 4 and 37°C. In both cases virus was placed on cells for 60 min and then removed by washing. $n = 5$.

6 *B* shows a lung that received Ad2/βGal-2 alone. On gross examination, there was little evidence of staining and the lung appeared similar to lungs not treated with virus (Fig. 6 *A*). In contrast, X-gal staining was readily apparent in the lungs treated with the same dose of virus delivered as Ad:CaPi coprecipitates (Fig. 6 *C*). Particularly striking was the pattern of X-gal staining which traced the airways, rather than the parenchyma. Using direct inspection as in Fig. 6, we examined coprecipitates with a range of Ca^{2+} concentrations (5.8, 12, 18, and 36 mM) and found that precipitates that contained 12 mM Ca^{2+} and 0.86 mM Pi were the most effective; those are the concentrations used in Fig. 6 *C*.

Fig. 7 shows representative photomicrographs from lungs treated with adenovirus alone or Ad:CaPi coprecipitates. In sections from lungs treated with adenovirus alone, there were a few blue-stained cells in airways and in the parenchyma (Fig. 7 *B*). In contrast, Fig. 7 *C* shows a field from a lung treated with Ad:CaPi coprecipitates; most small and medium-sized airways showed positively stained cells. Staining was predominantly in the airways with only rare positive cells in the parenchyma.

Fig. 8 shows a quantitative assessment of expression. We compared the percentage of blue-stained cells in airways of different diameters. In each airway region, more cells expressed the transgene after administration of Ad:CaPi coprecipitates than adenovirus alone. The greatest percentage of positive cells occurred in airways of 81–200 μm in diameter. Of note, the actual percentage of cells transduced may be underestimated because of the limited sensitivity in detecting β-galactosidase activity with X-gal staining. Moreover, only a small number of CFTR Cl^- channels is necessary for transepithelial Cl^- transport (1, 44).

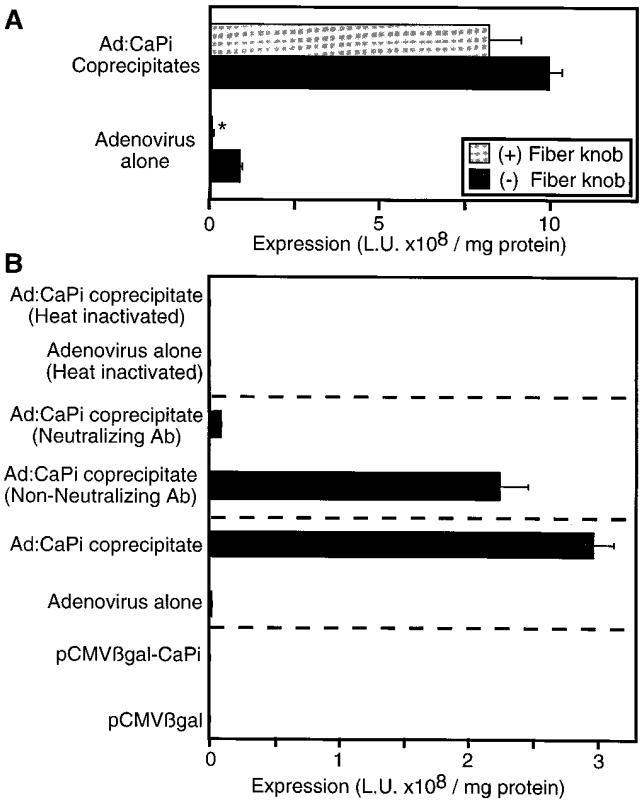

Figure 4. Effect of fiber knob protein, heat, and antibody inactivation on gene transfer by Ad:CaPi coprecipitates. (*A*) COS-1 cells were studied because they express fiber receptor. Fiber knob (0.7 μg/ml) was added to cells for 10 min and then Ad:CaPi coprecipitates were applied in the continued presence of fiber knob (*shaded bars*). Cells studied in absence of fiber knob are shown by black bars. $*P < 0.05$. (*B*) NIH 3T3 cells were treated with all interventions for 20 min, the vectors were then removed, and β-galactosidase activity was measured 24 h later. Virus was heat inactivated at 60°C for 30 min where indicated. Antibodies were incubated with virus for 30 min before coprecipitation. Plasmid DNA (pβGal, 16.6 ng) was prepared in CaPi precipitates with the same conditions used for adenovirus. This amount of plasmid equals $\sim 2 \times 10^9$ plasmids. In both panels, cells treated with adenovirus received 40 moi (2×10^9 particles) and Ad:CaPi coprecipitates were prepared with 5.8 mM Ca^{2+} and 0.86 mM Pi incubated for 15–30 min. Data are from one experiment ($n = 3$) and each experiment was repeated three times.

Discussion

The apical surface of airway epithelia forms a barrier that protects the internal environment from many agents in the external environment. This barrier has also proven to be a major impediment to the viral and nonviral gene transfer vectors currently being developed for CF airway epithelium. Our data show that gene transfer can be significantly enhanced by including adenovirus in a CaPi precipitate. We found enhanced

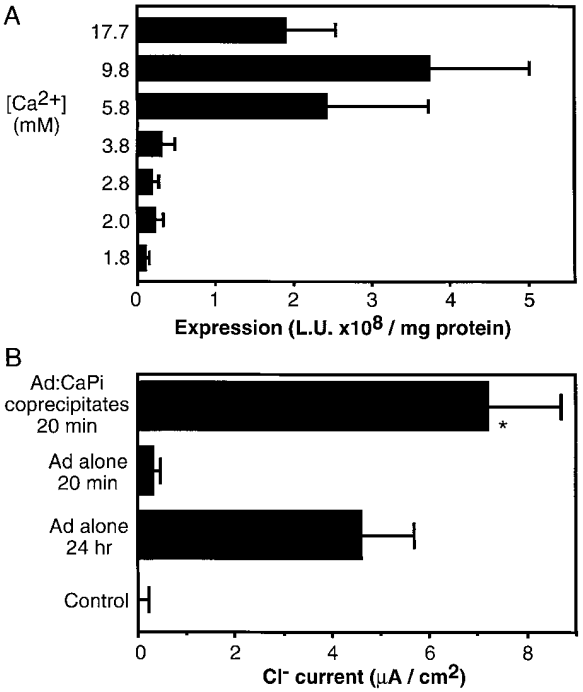

Figure 5. Expression of β-galactosidase and CFTR Cl^- current in normal and CF airway epithelia in vitro. Primary cultures of normal (*A*) and CF (*B*) airway epithelia grown at the air–liquid interface were studied 14–20 d after seeding. Ad2/βGal-2 or Ad2/CFTR-16 (50 moi) was applied to apical surface of epithelia for 20 min and then removed by washing. 3 or 4 d later, β-galactosidase activity (*A*) or transepithelial Cl^- current (*B*) was measured as described in Methods. The Ca^{2+} concentration was varied as indicated in *A* and was 5.8 mM in *B*. Pi concentration was 0.86 mM. $n = 9$ in *A* and 6 in *B*. $*P < 0.05$ compared with adenovirus alone for 20 min.

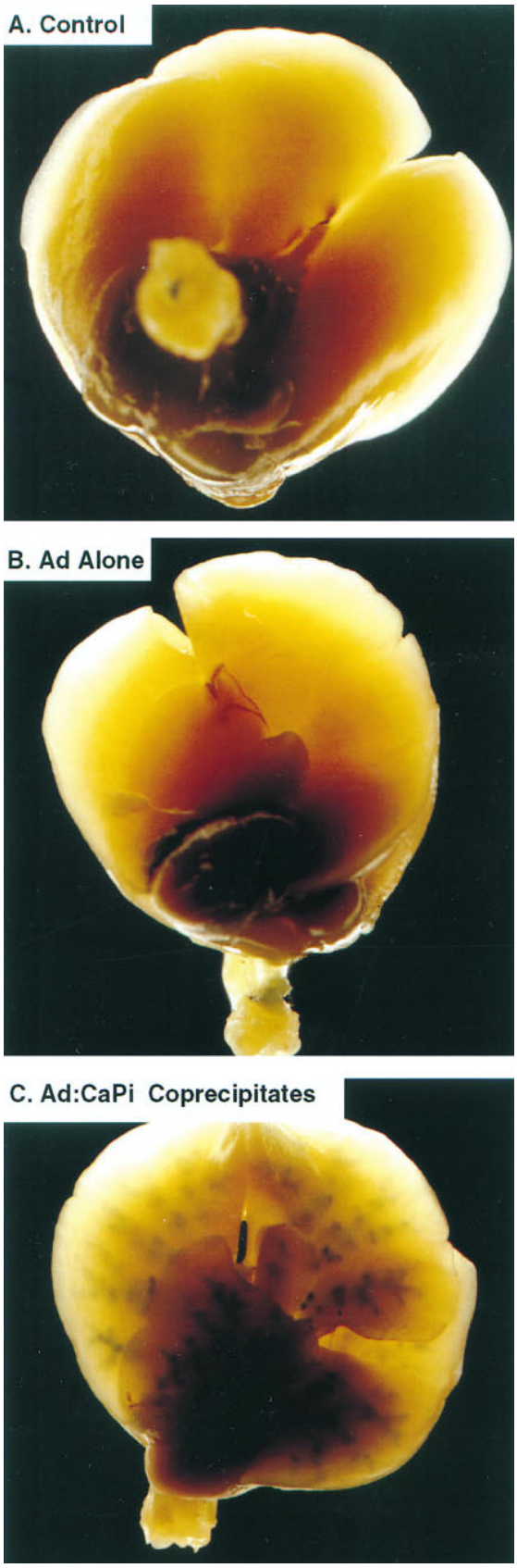

Figure 6. Ad:CaPi coprecipitates administered to mouse lung. Ad2/βGal-2 (2×10^8 IU) were administered as virus alone or as Ad:CaPi coprecipitates. 3 d later whole lungs were stained with X-gal reagent. Photomicrographs are from animals treated with vehicle control (*A*), adenovirus alone (*B*), or Ad:CaPi coprecipitates (*C*). Similar results were obtained with six animals in each group. Ad:CaPi coprecipitates were formed with 12 mM Ca^{2+} and 0.86 mM Pi.

expression in the NIH 3T3 cell line, the 9L gliosarcoma cell line, primary cultures of human umbilical vein endothelial cells, primary cultures of differentiated human airway epithelia, and mouse airway epithelia in vivo. All of these are resistant to adenovirus infection, especially when virus is in contact with the cells for only a short time.

Compared with adenovirus alone, Ad:CaPi coprecipitates increased binding of virus to cells. Although we do not understand the mechanism involved, the increased binding was likely responsible for the increased transgene expression. This conclusion is consistent with several other observations. For example, binding can be specific through receptor targeting with bispecific antibodies (45, 46) or with modified sequences in penton base and fiber (47, 48). Binding can also be nonspecific with complexes of adenovirus with cationic lipids and polymers (26, 27) or with polylysine added to the COOH terminus of adenovirus fiber (28). Even prolonged incubation of virus with cells can markedly increase gene transfer to differentiated airway epithelia (25). Thus, an interaction between adenovirus fiber protein and its specific receptor on the cell surface (20) is not required for infection. Moreover, the fiber–fiber receptor interaction may not always be optimal; we found that Ad:CaPi coprecipitates also increased expression in COS and HeLa cells which express fiber receptor and are easily infected. Our data also suggest that an interaction between viral penton base and cell surface integrins (49) is not required for infection because αv integrins are not present on the apical membrane of airway epithelia (23, 24). Thus we speculate that almost any maneuver that increases the contact time between adenovirus and the cell will facilitate infection.

The results suggest a model by which Ad:CaPi coprecipitates affect gene transfer. First, they increase binding (Fig. 3), and then the cell internalizes Ad:CaPi coprecipitates into endosomes or phagosomes that differ from the usual receptor-mediated pathway used by adenovirus. We speculate that as the pH falls in the endosome, the CaPi precipitate will dissociate, releasing the virus. Viral proteins can then facilitate escape from the endosome and mediate traffic of viral DNA through the cytoplasm, entry into the nucleus, and transcription. It is also possible that Ca^{2+} released from endosomes may activate signal transduction pathways that facilitate gene transfer. In contrast to Ad:CaPi coprecipitates, gene transfer by plasmid DNA complexed with CaPi is inefficient in most cells; such a system lacks the viral proteins that are required to facilitate processes subsequent to binding. The same holds true for cationic lipid-mediated transfection of plasmid DNA (43, 50). Thus, use of a virus that encodes the transgene offers an advantage in terms of efficiency over vector systems in which plasmid DNA encodes the transgene.

In developing gene transfer for CF, a key factor remains unknown; that is the percentage of cells that must be targeted to achieve clinical benefit. However, earlier studies suggest that gene transfer to 6–10% of cells is sufficient to correct the CF defect in transepithelial Cl^- transport (51); and in preliminary studies we found that correction of 10–15% of airway cells is sufficient to completely correct the CF defect in transepithelial Cl^- transport and correction of 5–20% of cells re-

verses the abnormally increased apical NaCl concentration in CF (52). The data with Ad:CaPi coprecipitates are encouraging in a number of respects. Several studies have delivered adenovirus alone to airway epithelia of CF patients in vivo. In most of those studies there was a significant, albeit small, correction of the CF electrophysiologic defect (15–19). In comparison to virus alone, Ad:CaPi coprecipitates produced a 25-fold greater increase in Cl^- transport by differentiated CF airway epithelia (Fig. 5 *B*) and a 17-fold greater increase in the percentage of cells expressing β-galactosidase in mouse conducting airways (81–200 μm diameter; Fig. 8). Thus, the data suggest that Ad:CaPi coprecipitates would markedly increase the degree of correction that has already been observed with adenovirus alone.

Use of Ad:CaPi coprecipitates for gene transfer has several potential advantages. First, for CF they have the distinct advantage that they produce expression in the airways rather than the alveoli, because airway epithelia are the site of disease in CF (1). Second, compared with cationic lipids, they show little direct cell toxicity; our preliminary results suggest they are also less toxic in vitro and in vivo. Third, they may be of value for use with other viral vectors such as adeno-associated virus, which can show limited efficiency in airway epithelia (53, 54). Fourth, they are simple and inexpensive to prepare; thus they could easily be used for a number of experimental applications in the research laboratory. They may be especially effective for cells that are resistant to infection. It is possible that they may also be of value for gene transfer in vivo to sites other than airway epithelia. Fifth, we would not expect that Ca^{2+} and Pi would have substantial toxicity; with our current formulation, we would deliver ~ 200 mg Ca^{2+}. We are not aware of studies evaluating Ca^{2+} delivery to the lung. However, for reference, 273 mg of Ca^{2+} is administered intravenously for hypocalcemia and an oral Tums™ tablet has 200 mg Ca^{2+}. Ad:CaPi coprecipitates have the potential disadvantage that they do not contain a ligand that will target them to a specific cell or tissue type. However, in the airway this limitation can be overcome by delivery through the airway lumen so that the cells with which they first come in contact are the desired target for gene transfer.

The results suggest several experiments for the future. First, it will be important to examine the physical properties of the precipitate and to determine which are most important for gene transfer and expression. Such knowledge may allow the production of even more effective complexes. Second, it will be interesting to learn how Ad:CaPi coprecipitates enter cells. Previous work with DNA delivered by CaPi precipitates suggests that the complex is taken up by endocytosis/phagocytosis (55). The apical surface of differentiated airway epithelia in

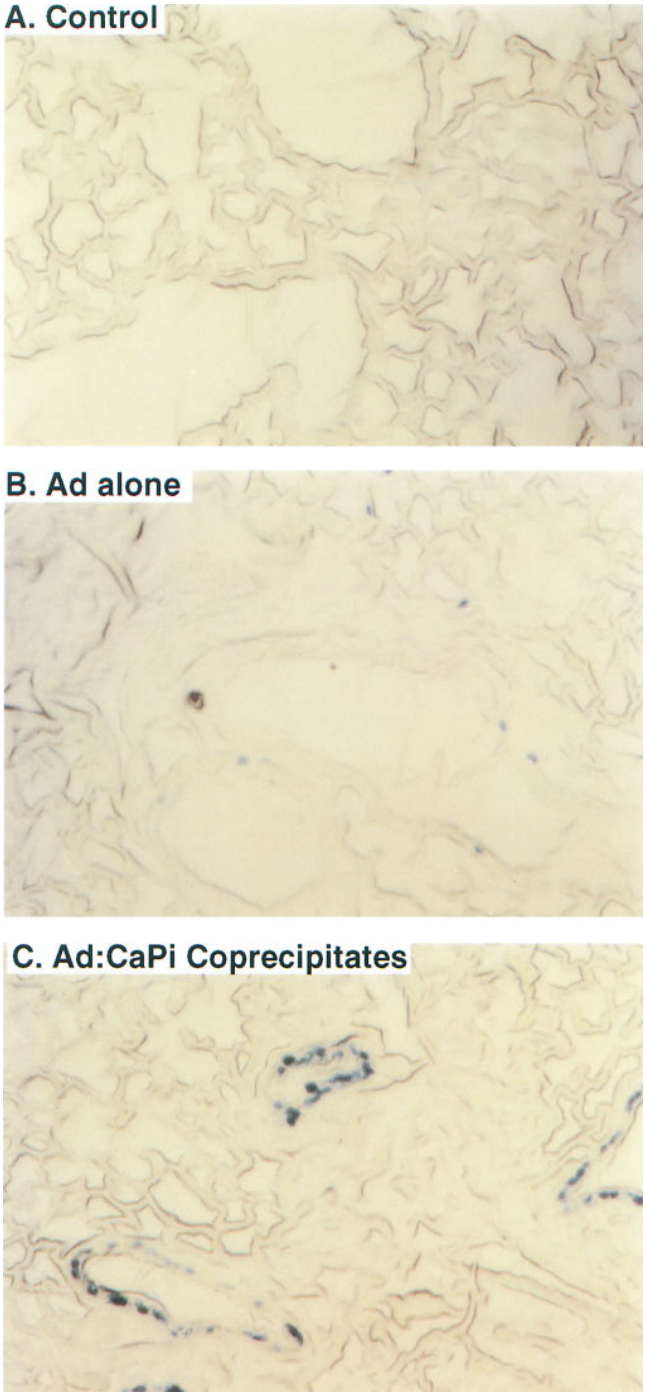

Figure 7. Photomicrographs from sections of mouse lungs treated as described in Fig. 6. (*A*) Control, (*B*) adenovirus alone, and (*C*) Ad:CaPi coprecipitates.

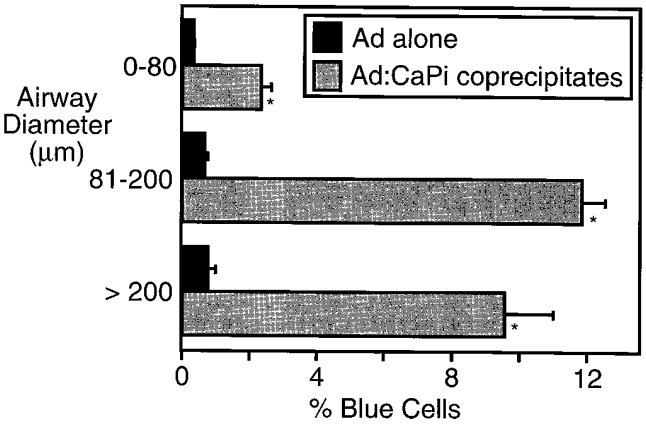

Figure 8. Gene transfer to mouse airway epithelium in vivo. Blue-stained cells were counted in sections of lungs as described in Methods. The percentage of blue cells was determined for airways of the indicated sizes. Each bar indicates data from 50–200 airways. $*P < 0.05$.

vitro and in vivo is not considered to be particularly active at endocytosis. However, a previous study showed that cationic ferritin administered to rat lungs appeared to enter epithelial cells of bronchioles (56). Third, our preliminary data suggest that precipitates produced with CaPi are more effective than those produced with other ions. This may relate to the physical properties of the precipitate. However, it might also be that Ca^{2+} itself may play some role in cell signaling that facilitates gene transfer.

These results are encouraging for developing CF gene transfer because use of Ad:CaPi coprecipitates or related precipitates might lead to a more favorable therapeutic index. Our data suggest that gene transfer and expression can be achieved with a lower viral input dose than with adenovirus alone. This may decrease toxicity and attenuate the immune response while more effectively correcting the CF defect.

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