Gene Transfer of the Na^+,K^+-ATPase β1 Subunit Using Electroporation Increases Lung Liquid Clearance

David Machado-Aranda, Yochai Adir, Jennifer L. Young, Arturo Briva, G. R. Scott Budinger, Anjana V. Yeldandi, Jacob I. Sznajder, and David A. Dean

Division of Pulmonary and Critical Care Medicine and Department of Pathology, Feinberg School of Medicine, Northwestern University, Chicago, Illinois

The development of nonviral methods for efficient gene transfer to the lung is highly desired for the treatment of several pulmonary diseases. We have developed a noninvasive procedure using electroporation to transfer genes to the lungs of rats. Purified plasmid (100–600 μg) was delivered to the lungs of anesthetized rats through an endotracheal tube, and a series of square-wave pulses were delivered via electrodes placed on the chest. Relatively uniform gene expression was observed in multiple cell types and layers throughout the lung, including airway and alveolar epithelial cells, airway smooth muscle cells, and vascular endothelial cells, and this finding was dose- and pulse length–dependent. Most important, no inflammatory response was detected. To demonstrate efficacy of this approach, the β1 subunit of the Na^+,K^+-ATPase was transferred to the lungs of rats with or without electroporation, and 3 days later, alveolar fluid clearance was measured. Animals electroporated with the β1 subunit plasmid showed a twofold increase in alveolar fluid clearance and Na^+,K^+-ATPase activity as compared with animals receiving all other plasmids, with or without electroporation. These results demonstrate that electroporation is an effective method to increase clearance by introducing therapeutic genes (Na^+,K^+-ATPase) into the rat lung.

Keywords: acute lung injury; edema; electroporation; plasmid

Over the past decade, numerous viral and nonviral approaches have been proposed and developed for transferring genes to the lung, but most have significant limitations. The inefficiency of gene transfer, immunologic responses, and nonspecificity of cell targeting are just a few of the problems. For example, although adenovirus appears to be the vector of choice for pulmonary gene therapy in the laboratory, its use can cause inflammation and cell damage (1). Furthermore, immune responses developed against the viral vector limit the success of repeated administration. These drawbacks make its clinical use doubtful. In contrast, much less inflammation and immune response are generated against DNA, either as naked plasmid or when complexed with liposomes or other polymers, such as polyethylenimine (2). Plasmid production is simple and yields high levels of contaminant-free, pure vector. Unfortunately, the efficiency of nonviral gene transfer remains low (2). Thus, for nonviral vectors to be of use in the lung, their ability to transfect cells *in vivo* must be increased.

Recent research from our laboratory and others has demonstrated that electroporation can be used to efficiently deliver genes to various tissues *in vivo* without damage and yields high-level gene expression (3). The application of electric fields to tissues transiently opens pores in the plasma membrane for the lifetime of the pulse, allowing exogenous DNA or other extracellular molecules to enter the cell (4). Electroporation causes up to a 1,000-fold increase in gene expression compared with DNA injection alone (5–9). At the appropriate field strengths, electroporation has proved to be a safe and effective method.

In the current study, we have developed a relatively noninvasive procedure to transfer genes to the lungs of rats using electric fields. The procedure is safe and nontraumatic and results in levels of reporter gene expression that approach those obtained with the latest generation adenoviruses. Moreover, this technique is appropriate for the delivery of therapeutic genes without associated inflammatory responses that are common to most other methods for pulmonary gene delivery. Some of the results of these studies have been previously reported in the form of abstracts (10, 11).

METHODS

Plasmids

The plasmids pEGFP-N1 (Clontech, Palo Alto, CA), pCMV-lux-DTS, pCMV-lacZ-DTS, and pCMV-β1 express green fluorescent protein (GFP), firefly luciferase, β-galactosidase, and the rat Na^+,K^+-ATPase β1 subunit, respectively, from the cytomegalovirus (CMV) immediate early promoter/enhancer (9, 12). pGFP-β1 expresses a GFP-rat Na^+,K^+-ATPase β1 subunit fusion protein from the CMV promoter. Plasmids were purified using Qiagen Giga-prep kits (Qiagen, Chatsworth, CA) and suspended in 10 mM Tris (pH 8.0), 1 mM ethylenediaminetetraacetic acid, and 140 mM NaCl.

In Vivo Gene Transfer to the Lung

Male Sprague-Dawley rats (200–350 g) were anesthetized and hair under the forelimbs was removed. Pediatric cutaneous pacemaker electrodes (Quik-Combo RTS; Medtronic Physio-Control Corporation, Redmond, WA) were cut to 3 × 4 cm and placed on either side of the chest under the forelimbs. A small amount of K-Y jelly (Johnson and Johnson) was placed on the skin, and the electrodes were held in place using surgical tape. A solution of 500 μl of plasmid in saline was administered between breaths over a 2-second period via a 16-gauge Angiocath catheter (BD, Franklin Lakes, NJ) used as an endotracheal tube, and the animal was allowed to recover breathing for 20 seconds. Immediately after this step, a series of eight square-wave electric pulses were administered using an ECM830 electroporator (BTX, San Diego, CA). Unless otherwise stated, a field strength of 200 V/cm was applied (calculated as through the chest, electrode center to electrode center). Later, the lungs were removed and analyzed. All experiments were conducted in accordance with institutional guidelines in compliance with the recommendations of the *Guide for Care and Use of Laboratory Animals* (35).

Adenoviral gene transfer was performed as previously described, using 5×10^8 plaque-forming units (pfu) of either a null virus (InVivogen, San Diego, CA) or a virus expressing the rat Na^+,K^+-ATPase β1 subunit (Ad-β1), delivered with surfactant (Survanta; Abbott Laboratories, Columbus, OH) (13).

(*Received in original form March 9, 2004; accepted in final form October 19, 2004*)

Supported by the Sandler Program for Asthma Research (D.A.D.), and grants HL71643 (D.A.D. and J.I.S.), HL48129 (J.I.S.), and HL67835 (G.R.S.B.), from the NIH.

Correspondence and requests for reprints should be addressed to David A. Dean, Division of Pulmonary and Critical Care Medicine, Feinberg School of Medicine, Northwestern University, 240 East Huron Avenue, McGaw 2300, Chicago, IL 60611. E-mail: dean@northwestern.edu

Am J Respir Crit Care Med Vol 171. pp 204–211, 2005
Originally Published in Press as DOI: 10.1164/rccm.200403-313OC on October 29, 2004
Internet address: www.atsjournals.org

Measurement of Lung Liquid Clearance

The isolated, fluid-filled, perfused lung preparation was performed immediately after brief ventilation of rats as previously described (14–16). Changes in concentration of Evan's blue–tagged albumin instilled into the airspace were used to estimate the volume of fluid cleared from the alveolar airspaces. The unidirectional flux of Na^+ from the alveolar space (i.e., active transport and passive movement) was calculated from the rate of loss of $^{22}Na^+$ from the airspaces. Passive Na^+ flux was calculated by subtracting the active Na^+ flux (calculated from the rate of net fluid clearance) from total Na^+ flux (16). Similarly, the flux of mannitol was calculated from the rate of loss of ^{3}H-mannitol from the airspaces (16). Albumin flux from the pulmonary circulation into the alveolar space was determined from the fraction of fluorescein isothiocyanate (FITC-)–labeled albumin, placed in the perfusate, that appeared in the alveolar instillate during the experimental protocol.

Histologic Analysis

Paraffin-embedded thin sections were cut from lungs inflated to total lung capacity with 3% paraformaldehyde immediately following euthanasia. Lungs were X-gal stained as described (9). Immunohistochemistry was performed using the Vectastain ABC-AP system (Vector Laboratories, Foster City, CA). Hematoxylin and eosin–stained sections (three sections each from three animals per condition) were blinded and reviewed by a pathologist for lung injury.

Measurement of Luciferase Expression

The lungs were frozen in liquid nitrogen immediately after removal, and extracts were prepared as previously described (9). Luciferase activity was measured in duplicate using the Luciferase Assay System (Promega, Madison, WI) in a Turner luminometer (Turner Biosciences, Sunnyvale, CA). Purified recombinant luciferase (Promega) was used to produce a standard curve for each experiment.

Measurement of Interleukin 6

Interleukin 6 (IL-6) was measured in lung extracts by ELISA (R&D Systems, Minneapolis, MN).

Isolation of Alveolar Type II Cells

Three days after lung electroporation, alveolar epithelial type II cells (ATII) were isolated as previously described (17–20). Productively transfected cells were scored for GFP-β1 expression by counting GFP^+ cells in multiple fields by fluorescence microscopy.

Measurement of Rubidium Uptake in Isolated Cells

Ouabain-sensitive $^{86}Rb^+$ uptake was used to estimate Na^+,K^+-ATPase activity in ATII cells isolated from treated animals as previously described (21).

Quantitation of Na^+,K^+-ATPase β1 Subunit

Western blots were performed on basolateral membranes isolated from the lungs of treated animals as previously described (21).

RESULTS

In Vivo Gene Transfer to the Rat Lung Using Electroporation

We have developed a noninvasive electroporation procedure for gene transfer to rat lungs for *in vivo* studies (Figure 1A). Briefly, DNA was delivered to the lungs via an endotracheal tube, the electric field was applied transthoracically using electrodes placed directly on the chest, and gene expression was assessed 3 days later. In our initial studies, we attempted to apply 10-millisecond square-wave pulses at a field strength of 200 V/cm, as we did *ex vivo* and in previous *in vivo* studies in other tissues. However, because the size of the rats necessitated a large gap (4 cm between electrodes requiring 800 V), we were unable to administer the field for more than 10 microseconds using the ECM830 electroporator, because of instrument constraints. The ECM830 will allow the administration of up to 500 V at long pulse lengths (millisecond range), but above this voltage, only

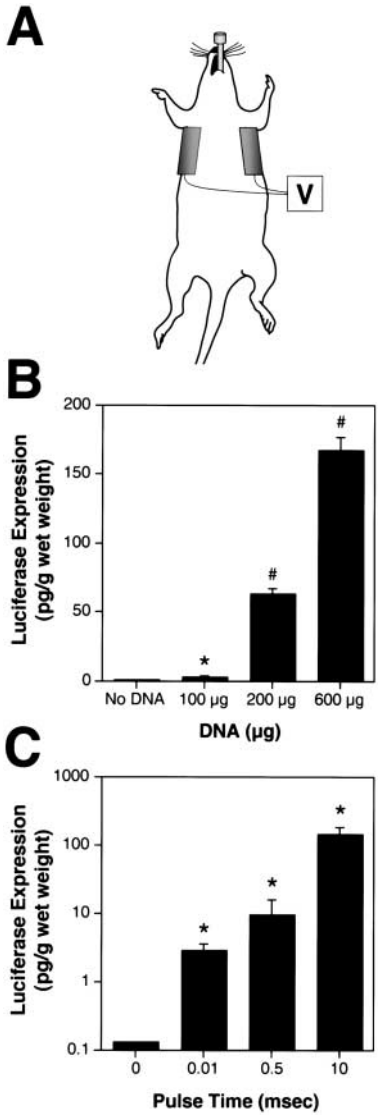

Figure 1. Electroporation-mediated reporter gene transfer to the rat lung. (*A*) *In vivo* plasmid delivery and electroporation for the lungs showing endotracheal tube and electrodes attached to voltage (V) supply. (*B*) Dose-dependent gene transfer and expression *in vivo*. Five hundred microliters of DNA or saline was delivered to the lungs of anesthetized animals at the indicated doses, followed by electroporation (200 V/cm; eight pulses; 10 microseconds each). Three days later, luciferase gene expression was measured (mean ± SEM; n = 6). *$p < 0.01$ and #$p < 0.001$ versus no DNA using the nonparametric Mann-Whitney U test. (*C*) Effects of pulse length on *in vivo* gene transfer. One hundred micrograms of plasmid was delivered to the lungs of anesthetized rats, which were immediately electroporated at 200 V/cm using eight pulses at 10 microseconds or 1 millisecond or at 100 V/cm using eight pulses at 10 milliseconds. Three days later, luciferase expression was measured (mean ± SEM; n = 6). *$p <$ 0.01 compared with DNA only (0 milliseconds) by Mann-Whitney U test.

shorter pulse lengths can be used (microsecond range). Thus, we investigated the ability of this reduced pulse length to mediate gene transfer (Figure 1B). Even at a pulse length of 10 microseconds, statistically significant gene transfer and expression were observed over DNA delivered in the absence of an electric field. Electroporation-mediated gene transfer was dose-dependent, with 170 ± 9 pg of luciferase per gram of wet weight (mean ± SEM; n = 6) being expressed when 600 μg of DNA was electroporated into the lungs. Expression was detected as early as 6 hours post electroporation (but not at 1 hour) and persisted for 3 days; by 5 and 7 days, gene expression could no longer be detected (not shown).

To determine whether longer pulse durations or lower field strengths could be used to increase gene delivery and expression, a set of experiments was performed using small rats (200–225 g) whose chests were small enough to electroporate with higher pulse times (Figure 1C). Pulse times of 10 microseconds, 0.5 milliseconds, and 10 milliseconds were used. The mortality rate using all conditions was very low (<5%). In all cases, death appeared to be caused by fluid delivery, and, on necropsy, there was no evidence of hemorrhaging or bleeding. Essentially, no gene expression was detected in the absence of an electric field when 100 μg of DNA was delivered to the lungs, but the application of an electric

field with all pulse durations promoted gene transfer and expression. Maximal gene transfer and expression was detected using a field strength of 100 V/cm with 8 pulses at 10 milliseconds each. This amount of expression was similar to that obtained using higher doses of DNA, but with the 10-microsecond pulse duration (compare Figures 1B and 1C). Consequently, either approach (i.e., high-dose DNA with low pulse length or low-dose DNA with long pulse length) promotes high-level gene transfer to the rat lung *in vivo* with very little mortality. However, for all subsequent studies, the low pulse length method (10 microseconds) was used to be able to use rats of all sizes.

Localization of Gene Transfer and Expression

To determine the distribution of gene transfer and expression in the lungs, several different reporter plasmids were used. When a plasmid expressing a fusion protein between GFP and the Na^+,K^+-ATPase β1 subunit was electroporated into the rat lung, GFP-β1 expression could be detected throughout multiple lobes of the lung but was absent from surrounding tissues (Figures 2A and 2B). As can be seen, GFP-β1 expression is relatively uniform throughout the lungs. When ATII epithelial cells were isolated from the lungs of electroporated animals 3 days after electroporation and observed by fluorescence microscopy, 54.7 ± 7.4% of cells were GFP-$\beta1^+$. Similarly, 54% of macrophages that were panned during isolation were GFP-$\beta1^+$ as well. To better distinguish what cell types received DNA in the intact lung, plasmids expressing β-galactosidase were electroporated into the lungs of rats, and 3 days later, the lungs were removed and reacted with X-gal to visualize gene expression (Figures 2C and 2D). β-Galactosidase expression was dose-dependent, with increasing amounts of X-gal–reactive tissue in all lobes of the lungs as the dose of DNA was increased. Lungs treated with 600 μg of pCMV-lacZ DNA in the absence of an electric field and naive lungs showed no X-gal reactivity (not shown in whole lungs). To determine which cells in the lungs expressed the gene product, lungs were paraffin-embedded, sectioned, and stained for β-galactosidase expression by immunohistochemistry (Figure 3). In naive lungs (Figure 3A), lungs electroporated without added DNA (200 V/cm,

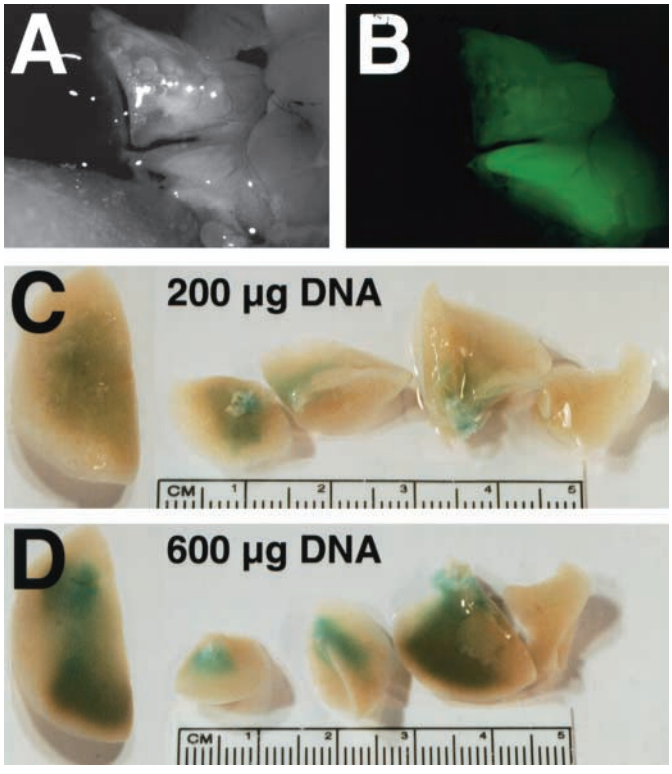

Figure 2. Distribution of gene delivery and expression in electroporated rat lungs. (*A* and *B*) Green fluorescent protein β1 (GFP-β1–)–expressing plasmids (600 μg) were administered to the lungs and electroporated (200 V/cm, eight pulses at 10 microseconds each). Three days later, the lungs were visualized *in situ* (*A*) and GFP-β1 expression was detected by fluorescence microscopy (*B*). (*C* and *D*) Dose-dependency of gene transfer and expression. Two hundred micrograms (*C*) or 600 μg (*D*) of a β-galactosidase–expressing plasmid were transferred to lungs and electroporated (200 V/cm, eight pulses at 10 microseconds each). Three days later, the lungs were removed and reacted with X-gal to visualize β-galactosidase expression.

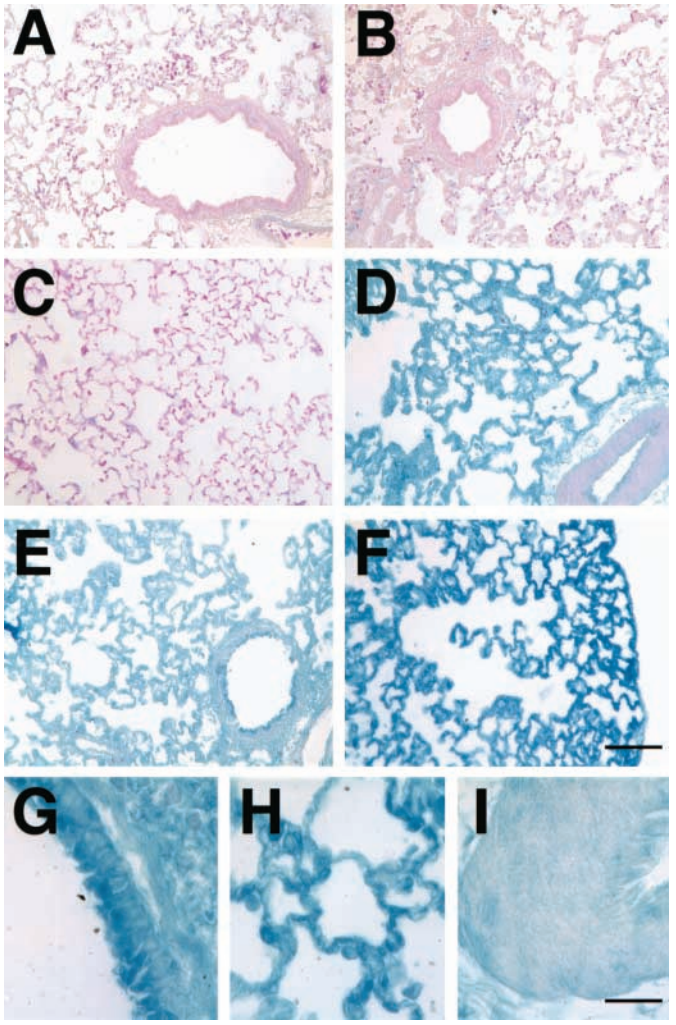

Figure 3. Localization of gene expression in electroporated lungs. β-galactosidase–expressing plasmids were transferred to rat lungs (n = 6) and electroporated (200 V/cm, eight pulses at 10 microseconds each); 3 days later, the lungs were removed, inflated to total lung capacity, fixed, and paraffin-embedded for sections. Immunohistochemistry was performed with antibodies against β-galactosidase using the Vector-blue ABC reagent (Vector Laboratories, Foster City, CA), and sections were counterstained with eosin (*A–F*). (*A*) Naive lung. (*B*) Electroporation only (no plasmid). (*C*) DNA only (no electroporation). (*D–I*) Plasmid with electroporation. At high magnification, airway epithelial and smooth muscle cells (*G*), alveolar type I and type II cells (*H*), and vascular smooth muscle cells (*I*) can be seen to express transgene. *A–F*, *bar* = 100 μm; *G–I*, *bar* = 20 μm.

10 microseconds; Figure 3B) and lungs receiving DNA without electroporation (Figure 3C) did not express any detectable β-galactosidase in any cell type. By contrast, electroporation of the reporter plasmid gave significant gene transfer and expression (Figures 3D–3I). At high magnification, expression is detectable in airway epithelial and smooth muscle cells (Figure 3G), ATI and ATII cells (Figure 3H), and vascular endothelial and smooth muscle cells (Figure 3I). However, less gene expression was consistently detected in the smooth muscle layers of large blood vessels (Figure 3D). This expression pattern was present throughout the lung in all lobes, although some areas showed greater expression than others. Taken together, these results demonstrate that electroporation can be used effectively to target genes to multiple cell types throughout the lung.

Inflammatory Response and Histologic Analysis of Electroporated Lungs

To assess whether electroporation elicited any damage in the rat lung, sections of treated and naive lungs were examined 3 days after treatment for histologic changes and cytokine responses from the procedure (Figure 4 and Table 1). Additional animals were examined at 1, 24, and 72 hours following treatment (Table 1). Blinded pathologic examination could not distinguish between naive lungs (Figures 4A and 4B), lungs from animals that received Tris–ethylenediaminetetraacetic acid–saline with electroporation (Figures 4C and 4D), and those that received DNA (600 μg of pcDNA3) with electroporation using the following four criteria on a 5-point scale: vascular congestion, hyaline membranes, polymorphonuclear cell infiltrates, and interstitial infiltrates. Furthermore, there was no difference between lungs from animals that were electroporated using 10-microsecond (not shown) or 10-millisecond pulses (Figures 4E and 4F). By contrast, animals receiving 5×10^8 pfu of a control null first-generation adenovirus showed profound vascular congestion and polymorphonuclear cell and interstitial infiltrates, as has been previously described (14). As another indicator of inflammation, levels of IL-6 were also measured in treated and naive lungs. Again, no differences in levels of this cytokine were noted between animals that were treated under any of the previously described conditions, with the exception of the adenovirus-treated animals (Table 1).

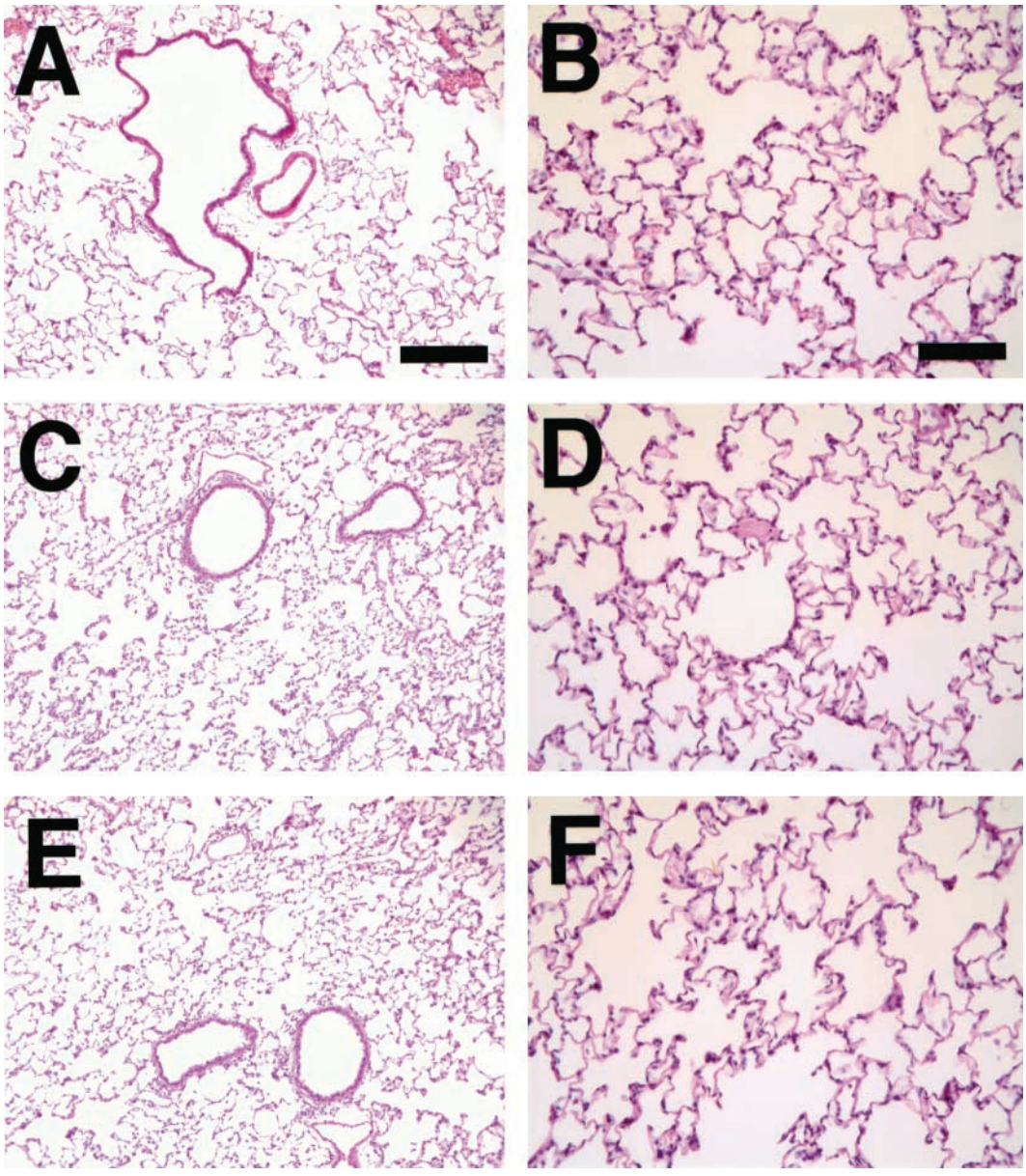

Figure 4. Histologic analysis of electroporated lungs. Lungs from naive animals (*A*), or those that were electroporated (100 V/cm, eight pulses at 10 microseconds each) without added DNA (*C* and *D*), or with 600 μg of pcDNA3 (empty vector; *E* and *F*) were harvested 3 days post electroporation, inflated to total lung capacity, fixed, paraffin-embedded, sectioned, and stained with hematoxylin and eosin. *A, C,* and *E* were taken at the same magnification (*bar* = 200 μm), and *B, D,* and *F* were taken at a higher magnification (*bar* = 100 μm). Representative sections from one of three animals at each condition are shown.

TABLE 1. HISTOLOGIC ANALYSES AND MEASUREMENT OF INTERLEUKIN-6 LEVELS IN ELECTROPORATED AND CONTROL LUNGS

Condition	DNA	Time (*h*)	Vascular Congestion	Hyaline Membranes	Alveolar Infiltrates	Interstitial Infiltrates	IL-6 (*pg/ml*)
Naive	−		0.14 ± 0.1	0	0.43 ± 0.2	0.14 ± 0.14	29 ± 14
100 V/cm 10 ms	−	1	0	0	0	0.33 ± 0.33	25 ± 25
	−	24	0	0	0.33 ± 0.33	0.33 ± 0.33	87 ± 56
	−	72	0	0	0.67 ± 0.33	1 ± 0	0
200 V/cm 10 μs	−	1	0	0	0.33 ± 0.33	0.67 ± 0.33	0
	−	24	0	0	0.67 ± 0.33	0.67 ± 0.33	31 ± 31
	−	72	0.2 ± 0.2	0	0.80 ± 0.49	1.2 ± 0.37†	19 ± 19
100 V/cm 10 ms	+	1	0	0	0	0.67 ± 0.33	44 ± 44
	+	24	0	0	1 ± 0	1.33 ± 0.33*	132 ± 24
	+	72	0.33 ± 0.33	0	0.83 ± 0.39	1 ± 0.26†	73 ± 25
200 V/cm 10 μs	+	1	0	0	0	0	22 ± 22
	+	24	0	0	1 ± 0	1 ± 0	0
	+	72	0.33 ± 0.33	0	1 ± 0.4	1.16 ± 0.33†	0
Adenovirus		72	1 ± 0*	0	3 ± 0.58*	2 ± 0*	161 ± 42*

Definition of abbreviation: IL-6 = interleukin 6.
Results are presented as mean ± SEM. Scale for histopathologic analysis of lung injury is from 0 (healthy) to 5 (severely injured).
* $p < 0.01$ compared with naive by Mann-Whitney U test.
† $p < 0.05$ compared with naive by Mann-Whitney U test.

Because transgene expression was detected throughout the lung in cells within and below the epithelial layer (Figure 3), DNA must be gaining access to cells across the epithelial layer. Thus, we asked whether the process of electroporation caused a transient increase in epithelial barrier permeability. When FITC-labeled albumin was instilled into the lungs together with DNA and electroporated, there was no significant accumulation of the alveolar tracer in the blood of the rats within 15 minutes of the procedure, compared with animals that were instilled with the tracer and DNA but not electroporated (0.011 ± 0.01% vs. 0.09 ± 0.01% of the instilled tracer in the blood, mean ± SEM; n = 5). These results suggest that although the DNA may be gaining access to the subepithelial cells through the epithelial tight junctions, there is no significant increase in epithelial barrier permeability.

Transfer of the Na^+,K^+-ATPase β1 Subunit to Rat Lungs

On the basis of the results that reporter genes could be transferred efficiently to the lungs with little or no inflammation or trauma to the tissue, we evaluated electroporation as a means to transfer the rat Na^+,K^+-ATPase β1 subunit to the lung and measured alveolar fluid clearance (AFC) after gene transfer. Plasmids were transferred to the lungs of animals with or without electroporation; 3 days later, the animals were euthanized and AFC was measured in the isolated lungs (Figure 5A). Electroporation in the absence of DNA, transfer of the β subunit–expressing plasmid (pCMV-β1 subunit) without electroporation, or electroporation of an "empty" plasmid that does not code for a gene product (pcDNA3) did not increase AFC compared with that seen in naive animals. By contrast, electroporation of the pCMV-β1 subunit into lungs increased AFC by 74%. Similarly, a recombinant adenovirus expressing the Na^+,K^+-ATPase β1 subunit (Ad-β1) also increased AFC by almost a factor of 2. However, fluid clearance could not be assessed in the adenovirus-treated lungs until 10 days after transfer because of severe inflammation between Days 2 and 7 (14). Furthermore, electroporation did not alter the permeability of FITC-albumin, mannitol, or sodium across the alveolar–capillary barrier under any of the conditions tested (Table 2).

To study whether the increases in AFC were from the functional expression of the β1 subunit, the activity of the Na^+,K^+-ATPase was measured in cells isolated from treated lungs. Three days after gene transfer by electroporation, ATII epithelial cells were isolated from the lungs and the uptake of $^{86}Rb^+$ was measured as an assay for Na^+,K^+-ATPase activity (Figure 5B). Cells isolated from naive lungs, lungs electroporated without DNA, lungs receiving pCMV-β1 subunit DNA alone (no electroporation), or lungs electroporated with the empty vector or a luciferase-expressing plasmid showed essentially the same levels of ouabain-sensitive $^{86}Rb^+$ uptake. However, cells isolated from lungs electroporated with the pCMV-β1 subunit plasmid showed a twofold increase in ouabain-sensitive $^{86}Rb^+$ transport, confirming that the increased fluid clearance paralleled the increased Na^+ pump activity. Finally, as shown in Figure 5C, the levels of the β1 subunit, quantified by Western blot, from the lungs of rats that were electroporated with the pCMV-β1 subunit plasmid showed a twofold increase in the Na^+,K^+-ATPase β1 subunit protein abundance at the basolateral membranes isolated from the lungs at 3 days after treatment relative to naive lungs. Taken together, these results demonstrate that electroporation can effectively deliver the Na^+,K^+-ATPase β1 subunit, resulting in increased lung liquid clearance.

DISCUSSION

One obstacle to the use of *in vivo* gene delivery is the level of technical difficulty associated with many delivery methods. The production of high-quality, helper-free recombinant viral vectors can be difficult, costly, and time-consuming. Although polyplex and lipoplex formulations are easier to create, as are the plasmid constructs that are delivered with them, effective formulation of these agents can be cumbersome. By contrast, electroporation offers an alternative that only requires purified plasmids and an electric field generator. Large-scale plasmid production and purification is easy and inexpensive. Furthermore, the application of this approach to the lung is fast and relatively noninvasive. Once the animal is anesthetized, the entire procedure takes less than 10 minutes, and recovery is rapid. The use of an endotracheal tube circumvents any incision being made to deliver the DNA to the lungs, as we have reported in mice (9).

Perhaps the major limitation of viral vectors is the potent proinflammatory response. The application of an electric field (200 V/cm) to the lungs, either to animals receiving vehicle or DNA, caused no increases in IL-6 levels and showed no

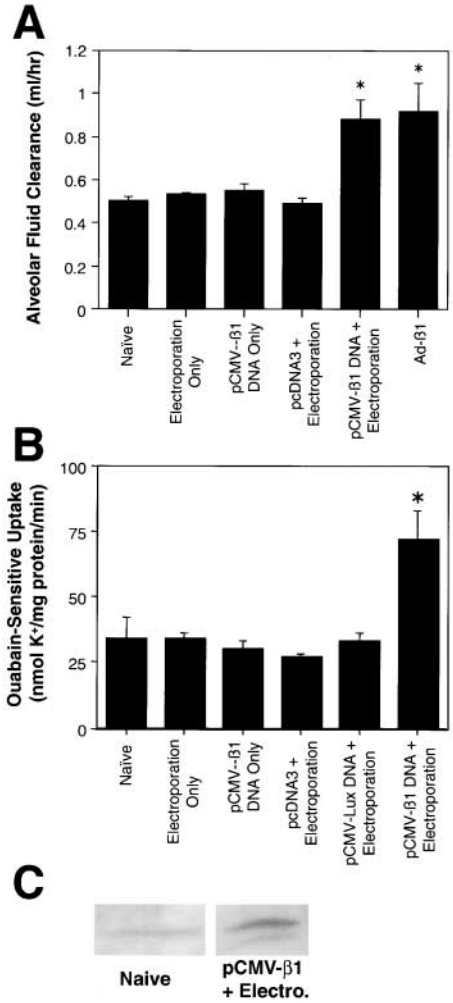

Figure 5. Alveolar fluid clearance and Na^+,K^+-ATPase activity in electroporated rat lungs. (*A*) Alveolar fluid clearance was measured in isolated lungs, 3 days after the indicated nonviral treatment or 10 days after recombinant adenoviral infection. Plasmids were administered at 600 μg per animal, and electroporations were performed at 200 V/cm using eight pulses of 10 microseconds each. Ad-β1 was administered in surfactant at 5×10^8 pfu per animal. Alveolar fluid clearance was measured as described in METHODS (mean ± SEM; n = 6). *$p < 0.003$ versus all conditions except Ad-β1 and pCMV-β1 + electroporation, using the nonparametric Mann-Whitney U test. (*B*) Na^+,K^+-ATPase activity in alveolar type II cells isolated from treated lungs, 3 days after treatment. Plasmids (600 μg) were transferred to rat lungs, with or without electroporation (200 V/cm, eight pulses at 10 microseconds each). Ouabain-sensitive $^{86}Rb^+$ uptake was measured (mean ± SD; n = 3) as an indicator of $Na^+,K+$-ATPase activity. *$p < 0.005$ versus all other conditions using the nonparametric Mann-Whitney U test. (*C*) Western blot of β1 protein abundance in basolateral membranes of naive rats and those electroporated with pCMV-β1 as in *B* (n = 5).

histopathologic changes compared with naive animals, either immediately after electroporation (1 hour) or 1 and 3 days later. By contrast, lungs from animals treated with recombinant adenoviruses show increases in all of these criteria between Days 2 and 7 compared with naive lungs.

We report here that electroporation achieves high levels of gene expression. Furthermore, the physiologic effects after electroporation-mediated delivery of the Na^+,K^+-ATPase β1 subunit were indistinguishable from those using recombinant adenoviruses expressing the β1 subunit, which have been very effective for pulmonary gene transfer (14).

One additional drawback to most viral and nonviral methods of gene delivery is that they are limited in delivery to only those cells with which they come into contact. Thus, delivery from the airways results overwhelmingly in gene transfer to the airway and alveolar epithelial cells only, whereas systemic delivery targets only endothelial cells in the lung (22–26). However, electroporation mediates gene delivery to multiple cell layers within the lung following DNA delivery via the airways. In addition, we detected relatively uniform gene expression throughout the periphery and parenchyma in airway and alveolar epithelial cells, airway smooth muscle cells, vascular endothelial cells, and some vascular smooth muscle cells. Similar distributions of gene transfer and expression were observed using GFP and *lacZ* genes with detection by direct fluorescence and immunohistochemistry, respectively. When lungs that had been electroporated with a *lacZ* construct were reacted with X-gal, gene expression did not appear as evenly distributed as it did in sections from the same lungs using immunohistochemistry. A similar observation was made in the mouse lung where most X-gal reactivity congregated in ATI and ATII epithelial cells, although the gene product appeared uniformly distributed throughout all cell types when immunohistochemistry was performed using antibodies against β-galactosidase (9). The likely reason for this apparent discrepancy is that the use X-gal reactivity to measure β-galactosidase expression resulting from gene transfer routinely underestimates transfection efficiency during transient transfections (27). By contrast, GFP fluorescence or immunohistochemistry using an amplification system such as the Vector ABC system (Vector Laboratories, Foster City, CA) are much more sensitive and more reliable indicators of real transfection efficiency (28). Thus, it is possible that more DNA is delivered to the alveolar epithelium or that these cells may express more protein than other pulmonary cells, but all cells in the lung do receive and express the transgene.

How the DNA is gaining access to the subepithelial cells in the electroporated lungs is currently unclear. Electroporation has both electrophoretic and electropermeabilization components that aid DNA delivery to cells within tissues (4). Thus, the DNA is electrophoresed to and into cells via membrane pores that exist for the lifetime of the electric pulse (29). However, it is unlikely that the DNA moves transcellularly by electrophoresis to access cells on the other side of the epithelium. Rather, it is more likely that the DNA moves across and/or through the epithelial tight junctions with the applied electric field. This would imply that a transient increase in epithelial barrier permeability would be coincident with the electric field. Although we did not detect changes in alveolar epithelial permeability, or see histologic evidence of pulmonary edema, we cannot exclude the possibility of a very transient, but physiologically insignificant, barrier permeability. Furthermore, if electrophoresis and membrane permeabilization occur only during the duration of the electric field that is between 10 microseconds and 10 milliseconds in our experiments, it is unlikely that any significant permeability changes would be detected or detrimental.

It has been demonstrated previously that transfer of the β1 or α2 subunit of the Na^+,K^+-ATPase using recombinant adenoviruses can increase expression of the Na^+,K^+-ATPase in lungs of rats and result in increased AFC (13, 14, 21, 30). A drawback to these studies is that the effects of gene transfer could only be experimentally measured 7 days after infection because of the induction of inflammatory responses by the viral vector. As such, the timing of this therapeutic response corresponds more to the subacute or "proliferative" phase of acute lung injury, rather than the acute phase when therapy may be more meaning-

TABLE 2. NONSPECIFIC PERMEABILITY MEASUREMENTS IN ISOLATED RAT LUNGS AFTER ELECTROPORATION

Condition	Passive Na^+ Flux (*ml/h*)	Mannitol Flux (*ml/h*)	Albumin Flux (*ml/h*)
Control	1.665 ± 0.195	1.230 ± 0.294	0.044 ± 0.012
Electroporation alone	1.274 ± 0.250	1.152 ± 0.138	0.040 ± 0.033
pCMV-β1 alone	1.253 ± 0.374	0.936 ± 0.218	0.054 ± 0.025
pcDNA3 + electroporation	1.220 ± 0.415	0.966 ± 0.212	0.072 ± 0.007
pCMV-β1 + electroporation	1.439 ± 0.262	1.299 ± 0.148	0.025 ± 0.013
Ad-β1	1.500 ± 0.400	0.965 ± 0.060	0.070 ± 0.020

The nonspecific permeability of solutes was measured in the isolated lungs from electroporated, control, or Ad-β1–treated animals shown in Figure 5A. All measurements are mean ± SEM (n = 6).

ful (31). The fact that gene transfer using electroporation causes no inflammatory response greatly widens the therapeutic window of gene expression from between 1 and 5 days after electroporation to cover the entire acute phase of lung injury (9).

Several groups have reported that gene transfer of either the Na^+,K^+-ATPase β1 or α2 subunit can protect from lung injury initiated after gene delivery; in the clinical setting, however, this is not the way that therapy would be administered (13, 14, 21, 30, 32). Rather, the ideal therapy would be administered after lung injury and edema have developed. However, the injured lung presents many additional barriers to gene transfer that are not encountered in the normal lung (33). It has been shown recently that recombinant adenoviruses can be used effectively in edematous lungs after hyperoxia-induced acute lung injury (34). It remains to be seen whether electroporation will work as efficiently in the injured lung as it does in the healthy lung, but because electroporated DNA moves through mucous and multiple cell and extracellular matrix layers to target cells beyond the pulmonary epithelium, it could be effective and warrants further research. Thus, on the basis of the ease, efficiency, and nontraumatic nature of this electroporation method for pulmonary gene transfer, its use may have clinical potential.

***Conflict of Interest Statement*:** D.M.-A. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; Y.A. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; J.L.Y. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; A.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; G.R.S.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; A.V.Y. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; J.I.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; D.A.D. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

***Acknowledgment*:** The authors thank Drs. Karen Ridge, Laura Dada, and Emilia Lecuona for helpful discussions and Dr. Phil Factor for the gift of the Na^+, K^+-ATPase β1 plasmid.

References

1. Liu Q, Muruve DA. Molecular basis of the inflammatory response to adenovirus vectors. *Gene Ther* 2003;10:935–940.
2. Niidome T, Huang L. Gene therapy progress and prospects: nonviral vectors. *Gene Ther* 2002;9:1647–1652.
3. Trezise AE. In vivo DNA electrotransfer. *DNA Cell Biol* 2002;21:869–877.
4. Somiari S, Glasspool-Malone J, Drabick JJ, Gilbert RA, Heller R, Jaroszeski MJ, Malone RW. Theory and in vivo application of electroporative gene delivery. *Mol Ther* 2000;2:178–187.
5. Mathiesen I. Electropermeabilization of skeletal muscle enhances gene transfer in vivo. *Gene Ther* 1999;6:508–514.
6. Mir LM, Bureau MF, Gehl J, Rangara R, Rouy D, Caillaud J-M, Delaere P, Branellec D, Schwartz B, Scherman D. High-efficiency gene transfer into skeletal muscle mediated by electric pulses. *Proc Natl Acad Sci U S A* 1999;96:4262–4267.
7. Martin JB, Young JL, Benoit JN, Dean DA. Gene transfer to intact mesenteric arteries by electroporation. *J Vasc Res* 2000;37:372–380.
8. Blair-Parks K, Weston BC, Dean DA. Gene delivery to the cornea by plasmid injection and electroporation. *J Gene Med* 2002;4:92–100.
9. Dean DA, Machado-Aranda D, Blair-Parks K, Yeldandi AV, Young JL. Electroporation as a method for high-level non-viral gene transfer to the lung. *Gene Ther* 2003;10:1608–1615.
10. Machado-Aranda D, Adir Y, Sznajder JI, Dean DA. Electroporation-mediated transfer of the Na+/K+-ATPase Beta-1 subunit safely increases alveolar fluid clearance in rat lungs [abstract]. *Mol Ther* 2003; 7:S381.
11. Machado-Aranda D, Adir Y, Sznajder JI, Dean DA. Efficient gene transfer of the Na+/K+-ATPase beta-1 subunit in rat lungs using electroporation [abstract]. *Am J Respir Crit Care Med* 2003;167:A122.
12. Factor P, Senne C, Dumasius V, Ridge K, Jaffe HA, Uhal B, Gao Z, Sznajder JI. Overexpression of the Na+,K+-ATPase alpha1 subunit increases Na+,K+-ATPase function in A549 cells. *Am J Respir Cell Mol Biol* 1998;18:741–749.
13. Factor P, Dumasius V, Saldias F, Brown LA, Sznajder JI. Adenovirus-mediated transfer of an Na+/K+-ATPase beta1 subunit gene improves alveolar fluid clearance and survival in hyperoxic rats. *Hum Gene Ther* 2000;11:2231–2242.
14. Factor P, Saldias F, Ridge K, Dumasius V, Zabner J, Jaffe HA, Blanco G, Barnard M, Mercer R, Perrin R, *et al.* Augmentation of lung liquid clearance via adenovirus-mediated transfer of a Na,K-ATPase beta1 subunit gene. *J Clin Invest* 1998;102:1421–1430.
15. Lecuona E, Saldiás F, Comellas A, Ridge K, Guerrero C, Sznajder JI. Ventillator-associated lung injury decreases lung ability to clear edema in rats. *Am J Respir Crit Care Med* 1999;159:603–609.
16. Rutschman DH, Olivera W, Sznajder JI. Active transport and passive liquid movement in isolated perfused rat lungs. *J Appl Physiol* 1993;75: 1574–1580.
17. Dobbs LG, Gonzalez R, Williams MC. An improved method for isolating type II cells in high yield and purity. *Am Rev Respir Dis* 1986;134:141–145.
18. Ridge K, Olivera W, Rutschman DH, Mercer RW, Uhal B, Horowitz S, Hughes F, Factor P, Barnard ML, Sznajder JI. Alpha-2 Na,K-ATPase contributes to lung liquid clearance. *Ann N Y Acad Sci* 1997;834:651–652.
19. Pesce L, Guerrero C, Comellas A, Ridge KM, Sznajder JI. Beta-agonists regulate Na,K-ATPase via novel MAPK/ERK and rapamycin-sensitive pathways. *FEBS Lett* 2000;486:310–314.
20. Bertorello AM, Ridge KM, Chibalin AV, Katz AI, Sznajder JI. Isoproterenol increases Na+-K+-ATPase activity by membrane insertion of alpha-subunits in lung alveolar cells. *Am J Physiol* 1999;276:L20–L27.
21. Ridge KM, Olivera WG, Saldias F, Azzam Z, Horowitz S, Rutschman DH, Dumasius V, Factor P, Sznajder JI. Alveolar type 1 cells express the alpha2 Na,K-ATPase, which contributes to lung liquid clearance. *Circ Res* 2003;92:453–460.
22. Canonico AE, Conary JT, Meyrick BO, Brigham KL. Aerosol and intravenous transfection of human alpha 1-antitrypsin gene to lungs of rabbits. *Am J Respir Cell Mol Biol* 1994;10:24–29.
23. Uyechi LS, Gagne L, Thurston G, Szoka FC Jr. Mechanism of lipoplex gene delivery in mouse lung: binding and internalization of fluorescent lipid and DNA components. *Gene Ther* 2001;8:828–836.
24. Song YK, Liu F, Chu S, Liu D. Characterization of cationic liposome-mediated gene transfer in vivo by intravenous administration. *Hum Gene Ther* 1997;8:1585–1594.
25. Yonemitsu Y, Kitson C, Ferrari S, Farley R, Griesenbach U, Judd D, Steel R, Scheid P, Zhu J, Jeffery PK, *et al.* Efficient gene transfer to

airway epithelium using recombinant Sendai virus. *Nat Biotechnol* 2000;18:970–973.
26. Weiss DJ, Bonneau L, Liggitt D. Use of perfluorochemical liquid allows earlier detection of gene expression and use of less vector in normal lung and enhances gene expression in acutely injured lung. *Mol Ther* 2001;3:734–745.
27. Bebok Z, Abai AM, Dong JY, King SA, Kirk KL, Berta G, Hughes BW, Kraft AS, Burgess SW, Shaw W, *et al.* Efficiency of plasmid delivery and expression after lipid-mediated gene transfer to human cells in vitro. *J Pharmacol Exp Ther* 1996;279:1462–1469.
28. Zhang G, Gurtu V, Kain SR. An enhanced green fluorescent protein allows sensitive detection of gene transfer in mammalian cells. *Biochem Biophys Res Commun* 1996;227:707–711.
29. Zaharoff DA, Barr RC, Li CY, Yuan F. Electromobility of plasmid DNA in tumor tissues during electric field-mediated gene delivery. *Gene Ther* 2002;9:1286–1290.
30. Adir Y, Factor P, Dumasius V, Ridge KM, Sznajder JI. Na,K-ATPase gene transfer increases liquid clearance during ventilation-induced lung injury. *Am J Respir Crit Care Med* 2003;168:1445–1448.
31. Ware LB, Matthay MA. The acute respiratory distress syndrome. *N Engl J Med* 2000;342:1334–1349.
32. Stern M, Ulrich K, Robinson C, Copeland J, Griesenbach U, Masse C, Cheng S, Munkonge F, Geddes D, Berthiaume Y, *et al.* Pretreatment with cationic lipid-mediated transfer of the Na+ K+ -ATPase pump in a mouse model in vivo augments resolution of high permeability pulmonary oedema. *Gene Ther* 2001;7:960–966.
33. Weiss D. Delivery of gene transfer vectors to lung: obstacles and the role of adjunct techniques for airway administration. *Mol Ther* 2002;6: 148–152.
34. Factor P, Mendez M, Mutlu GM, Dumasius V. Acute hyperoxic lung injury does not impede adenoviral-mediated alveolar gene transfer. *Am J Respir Crit Care Med* 2002;165:521–526.
35. Guide for the care and use of laboratory animals. Washington, DC: National Academy Press, 1996.