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Expression of the human cystic fibrosis transmembrane conductance regulator gene in the mouse lung after *in vivo* intratracheal plasmid-mediated gene transfer

Kunihiko Yoshimura, Melissa A.Rosenfeld, Hidenori Nakamura, Eva M.Scherer, Andrea Pavirani[1], Jean-Pierre Lecocq[1] and Ronald G.Crystal
Pulmonary Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892, USA and [1]Transgène SA, Strasbourg 67000, France

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ABSTRACT

As an approach to gene therapy for the respiratory manifestations of cystic fibrosis (CF), *in vivo* plasmid-mediated direct transfer of the normal CF transmembrane conductance regulator (CFTR) gene to the airway epithelium was investigated in mice. To evaluate the feasibility of this strategy, pRSVL, a plasmid composed of a firefly luciferase gene driven by the Rous sarcoma virus long terminal repeat (RSV-LTR), along with cationic liposomes was instilled into the trachea of C57Bl/6NCR mice. With administration of 200–400 μg plasmid DNA, luciferase expression could be detected in the mouse lung homogenates for at least 4 wk. With this background, a CFTR expression plasmid vector (pRSVCFTR) constructed by replacing the luciferase cDNA from pRSVL with the normal human CFTR cDNA was evaluated *in vivo* in mice. Intratracheal instillation of pRSVCFTR with cationic liposomes followed by analysis of mouse lung RNA by polymerase chain reaction amplification (after conversion of mRNA to cDNA) using a RSV-LTR specific sense primer and a human CFTR-specific antisense primer demonstrated human CFTR mRNA transcripts from one day to 4 wk after instillation. Further, *in vivo* evaluation of β-galactosidase activity after intratracheal administration of an *E. coli lacZ* gene expression plasmid vector directed by the cytomegalovirus promoter (pCMVβ) demonstrated that the airway epithelium was the major target of transfer and expression of the exogenous gene. These observations demonstrate successful plasmid-mediated gene transfer to the airway epithelium *in vivo*. This strategy may be feasible as a form of gene therapy to prevent the pulmonary manifestations of CF.

INTRODUCTION

The clinical manifestations of cystic fibrosis (CF), one of the most common lethal hereditary disorders in North America and Europe, are dominated by abnormalities of the airway epithelial surface, including chronic mucus production, infection, neutrophil-dominated inflammation and subsequent airway derangement and eventual respiratory failure (1). The gene responsible for CF is a 27 exon gene spanning a 250 kb segment of chromosome 7 at q31 (2,3). The predicted protein, termed the 'cystic fibrosis transmembrane conductance regulator' (CFTR), is a 1480 residue glycosylated molecule composed of two membrane-associated domains each with six membrane-spanning segments and intracytoplasmic regions including two nucleotide binding folds and the one R domain (3–7). *In vitro* studies suggest that the CFTR protein functions as a Cl^- channel capable of conducting Cl^- permeability across the apical membrane of epithelial cells in response to elevations of intracellular cAMP (5–12). A variety of mutations of the CFTR gene renders epithelial cells unable to carry out this function (13–21). Importantly, transfer of the normal CFTR cDNA to epithelial cell lines derived from individuals with CF can override this abnormality, and permit the cells to modulate the permeability of Cl^- in a normal fashion in response to increased intracellular cAMP (22,23).

Based upon these *in vitro* studies and the knowledge that the fatal consequences of mutations of the gene occur predominantly in the lung (1), it may be feasible to correct the pulmonary manifestations caused by mutations of the CFTR gene by directly transferring a normal CFTR cDNA to airway epithelial cells *in vivo*. In this context, the target cells will be the pseudostratified layer of epithelium lining the airways (24). *In vivo* studies in humans have shown the CFTR gene is actively transcribed in these cells but at a low rate, with CFTR mRNA levels averaging 1–2 copies per cell (25,26).

One of the major hurdles for gene therapy for CF is the architecture of the lung. Because the epithelial cells of the human airway surface cover the successively branching tree-like structure of the lung (27), it is not possible to complement the abnormal CFTR gene expression of the epithelial cells of CF individuals by removing them for *in vitro* correction and subsequent reimplantation. Further, the majority of human respiratory cells are terminally differentiated and even those that can proliferate do so at a slow rate (24). Thus, it is necessary to consider *in vivo* methodologies capable of transferring the normal recombinant gene to non-proliferating epithelial cells.

One possible solution to this problem is to transfer a DNA plasmid containing a constitutive promoter and the normal CFTR cDNA directly to the respiratory epithelium *in vivo* via the air route. The present study evaluates this strategy in C57Bl/6NCR mice utilizing a plasmid containing the Rous sarcoma virus-long terminal repeat (RSV-LTR) as the promoter (28) and the normal human CFTR cDNA in combination with cationic liposomes.

MATERIALS AND METHODS

Sources of cells, cell culture and experimental animals

COS-7, an SV40 virus transformed African Green monkey kidney cell line, was obtained from American Type Culture Collection (ATCC; CRL 1651) and maintained in Dulbecco's modified Eagle's medium (DMEM, Whittaker Bioproducts) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin (all from Biofluids). The T84 colon carcinoma cell line (ATCC; CCL 248), which is known to express the CFTR gene, was maintained in DMEM with the identical supplementation except for 5% FBS. Animals used in *in vivo* studies were C57Bl/6NCR mice [all female, age 4–6 wk; body weight, 18.1 ± 0.2 g (mean ± standard error of the mean); Division of Cancer Treatment, NCI].

Plasmid constructs

pRSVCFTR, a CFTR expression plasmid vector utilizing the RSV-LTR promoter, was constructed from pRSVL, a firefly luciferase expression vector, developed by de Wet et al. (Figure 2A) (29). The CFTR cDNA, including the entire human CFTR protein coding sequences, was derived from pTG4991 (25). Briefly, the plasmid pTG4991 was linearized at the unique *Pst*I site located at the 3′ end of CFTR cDNA, and the *Pst*I site was blunted using T4 DNA polymerase (both enzymes from New England Biolabs). The CFTR cDNA was then released by partial digestion with *Hind*III (New England Biolabs) [there is one *Hind*III site at the 5′ end of the CFTR cDNA in pTG4991 in addition to two internal sites in the CFTR cDNA sequence (3)]. pRSVCFTR was generated by ligating the subsequent CFTR cDNA fragment with the remaining pRSV vector after the firefly luciferase cDNA had been excised by *Hind*III and *Sma*I (New England Biolabs) digestion from pRSVL. The validity of the pRVSCFTR construct was confirmed by restriction digestion and direct sequencing. pCMVβ, an *E.coli lacZ* (β-galactosidase) gene expression plasmid vector driven by the human cytomegalovirus immediate early gene promoter (30), was obtained from Clontech Laboratories.

In vitro transfection of cultured cells

For *in vitro* transfection studies, COS-7 cells were detached by trypsin, counted, and seeded at $2-3\times10^5$ cells/ml in 10 cm plates. Cells were then transfected by calcium-phosphate co-precipitation method using 10 μg of pRSVCFTR, or as controls, 10 μg of an irrelevant plasmid, pBluescript II SK+ (pBS, Stratagene), or water (31). To achieve efficient transfection of COS-7 cells, glycerol (15%) was applied for 2 min after 5 h of transfection. The presence of CFTR mRNA transcripts, and as a control, β-actin transcripts was evaluated 48 h after transfection as described below.

In vivo gene transfer to mouse airways

For *in vivo* studies, pRSVL was first utilized to evaluate the optimal conditions for gene transfer. C57Bl/6NCR mice were anesthetized by methoxyflurane inhalation (32). The trachea was exposed by an anterior midline incision, and a total volume of 200 μl of plasmid DNA solution was injected into the airways. To establish the method for *in vivo* gene transfer using plasmid vectors, pRSVL was administered with or without complexing with 100 μg of cationic liposomes [Lipofectin Reagent; Bethesda Research Laboratory (BRL)] (33). At various times, the animals were sacrificed and the lungs homogenized and evaluated for luciferase activity as described below. Irrelevant plasmids pUC19 or pBS served as negative controls. To investigate a dose-response relationship between the amount of pRSVL administered and luciferase expression, 0 to 1,400 μg of pRSVL preincubated with liposomes were instilled intratracheally into the lung and the animals evaluated after 48 h. Chronicity of luciferase expression was evaluated for 1 d to 4 wk after intratracheal instillation with 200 or 400 μg of pRSVL.

To evaluate the feasibility of CFTR gene transfer to mouse lung, 200 μg of pRSVCFTR were incubated with 100 μg of liposomes in a total volume of 200 μl, and instilled into the mouse trachea as described above. Human CFTR mRNA transcripts were evaluated in mouse lung after 1 d to 4 wk (see below).

To demonstrate that the transferred genes could be expressed in airway epithelium after intratracheal plasmid-mediated gene delivery *in vivo*, 400 μg of pCMVβ were instilled (after combining with liposomes) into the mouse airways and the animal lungs were evaluated for β-galactosidase expression 2 or 4 d later (34,35). Control mice received liposomes alone or pRSVL plus liposomes.

Detection of luciferase activity

To detect luciferase activity, the mice were sacrificed and the lungs and trachea exposed. Blood was removed by cardiac puncture, lungs lavaged with PBS twice, and the pulmonary arteries perfused and rinsed with PBS. The lungs and trachea were then homogenized in 200 μl of lysis buffer [100 mM potassium phosphate, pH 7.8, and 1 mM dithiothreitol (Sigma)] using a polypropylene micropestle (Pellet Pestle; Kontes), followed by 3 cycles of freezing and thawing, centrifugated (16,000 g) at 4°C for 4 min, and supernate recovered (29). Luciferase activity was assayed with 100 μl of lung lysate supernate, 5 mM ATP (Pharmacia-LKB), 15 mM $MgCl_2$ and 1 mM D-luciferin (Analytical Luminescence Laboratories) using a Monolight 2010 luminometer (Analytical Luminescence Laboratories) (29). The total protein concentration of the lung homogenate was measured by the Bradford method (36) using a Bio-Rad protein assay kit (Bio-Rad) with bovine serum albumin as a standard. Luciferase activity in the mouse lung was expressed as relative light units/mg lung lysate protein after subtracting background.

Analysis of CFTR mRNA transcripts

For *in vitro* transfection experiments, total RNA was isolated from COS-7 cells 48 h after transfection, or as a control, from subconfluent T84 cells (not transfected) using the guanidine thiocyanate-CsCl technique (37). CFTR mRNA transcripts in transfected COS-7 cells were evaluated by Northern analysis (38) using 10 μg of total RNA with a ^{32}P-labeled 4.5 kb CFTR cDNA probe generated by the random priming method (39), and

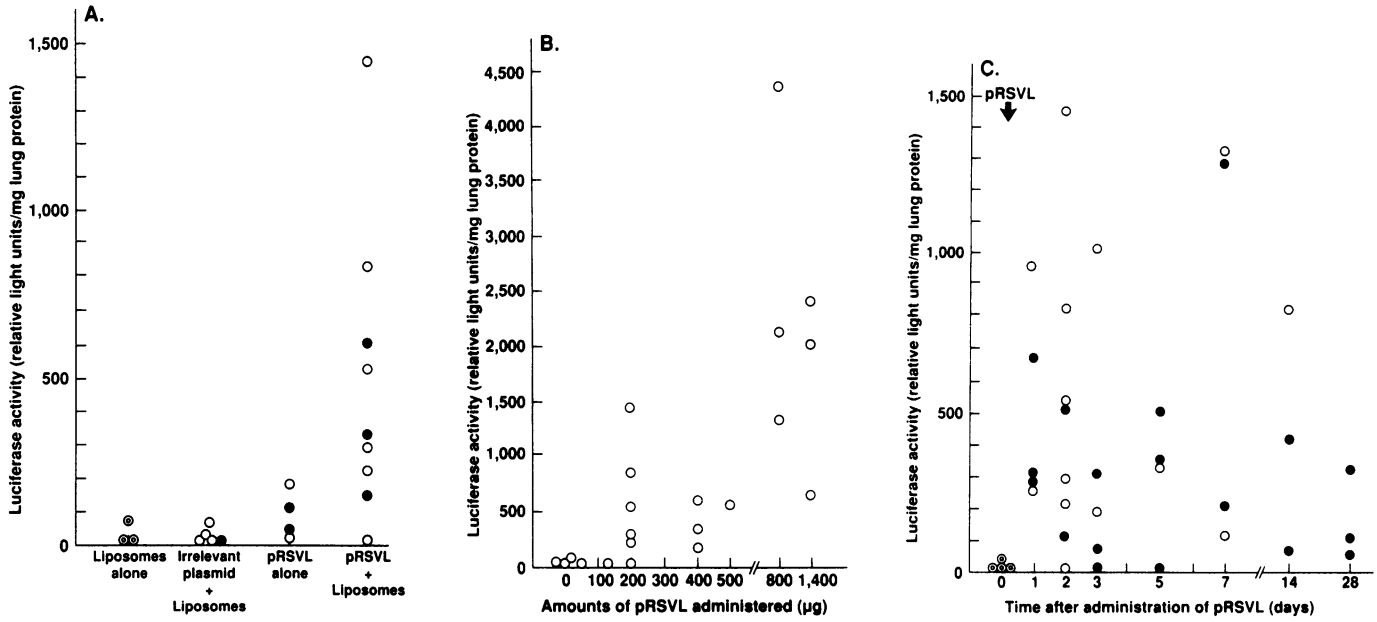


Figure 1. Luciferase expression in transfected mouse lung after intratracheal instillation of the firefly luciferase expression vector pRSVL. Luciferase activity was determined in lung homogenates and expressed as relative light units/mg lung protein. A. *In vivo* luciferase expression in mouse lung under different conditions. Shown are data from mice 2 d after receiving cationic liposomes (Lipofectin) alone, mice receiving irrelevant plasmids [pBluescript II SK+ (pBS) or pUC19] with liposomes, mice receiving pRSVL alone, or mice receiving pRSVL complexed with liposomes. The different symbols refer to the amount of plasmid instilled (O, ○ μg; , 200 μg; ●, 400 μg). B. Dose-response relationship between the amount of pRSVL administered and luciferase activity in mouse lung. Shown are data from mice 2 d after receiving a variety of amounts of pRSVL complexed with liposomes. Each circle represents a value from an individual animal. C. Time-course of luciferase activity in mouse lung. Shown are data from mice before (O) and after administration of pRSVL with liposomes at the indicated time point (days). Each circle represents a value from an individual animal with 200 μg (○) or 400 μg (●) of pRSVL.

autoradiography as previously described (25,26). As a control, the same membrane was subsequently hybridized with a human β-actin cDNA probe (pHFβA-1) (40).

For analysis of *in vivo* expression of human CFTR, C57Bl/6NCR mice were evaluated from 1 d to 4 wk after intratracheal administration. The lungs and trachea were isolated as described above, and total lung RNA was extracted (32,37). pRSVCFTR-directed human CFTR mRNA transcripts in mouse lung RNA were evaluated using polymerase chain reaction (PCR) amplification (after conversion of mRNA to cDNA) and Southern hybridization analysis (32,41,42). Total RNA was first treated with DNase (10 units/μg RNA; RNase-free RQ1 DNase, Promega) to eliminate possible residual plasmid DNA. RNA was then converted to cDNA by Moloney murine leukemia virus reverse transcriptase (RT; BRL) with random hexanucleotide primers (Promega) (43) and amplified by PCR (25 cycles) with *Taq* DNA polymerase (Perkin-Elmer Cetus) (44). To evaluate RSV-LTR-driven human CFTR mRNA, an RSV-LTR specific sense primer (RSVS2; 5'-CATTCACCACATTGGTGTGC-ACCTC-3') (28,45) and a human CFTR-specific antisense primer in CFTR exon 5 (HCF60; 5'-CATCAAATTTGTTCA-GGTTGTTGG-3') were used for PCR amplification. As a control, each DNase-treated RNA sample was used as a PCR template in parallel without conversion to cDNA to eliminate the possibility that amplification of potentially contaminating plasmid DNA had occurred. The PCR amplification products were evaluated by agarose gel electrophoresis followed by Southern hybridization using a nested ^{32}P-labeled human CFTR cDNA probe [a 462 bp *Pvu*II-*Xba*I fragment spanning exons 2 to 5 (3)] (Figure 3A). As a further control, luciferase mRNA transcripts expressed by pRSVL were amplified under the identical conditions except that the 3' primer was luciferase cDNA specific antisense primer [LUCAS2; 5'-AGTTCTATGCGGAAGGGCC-ACACC-3', corresponding to the firefly luciferase cDNA residues 670 to 693 (29)]. In addition, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts corresponding to nucleotide residues of mouse GAPDH cDNA 439 to 993 were amplified under similar conditions using GAPDH transcript-specific primers [(GAPDH-1; 5'-AATGCATCCTGCACCACC-AACTGC-3') and (GAPDH-2; 5'-GGAGGCCATGTAGGC-CATGAGGTC-3') (46)]. Each DNase-treated RNA sample was also used as a PCR template in parallel without conversion to cDNA. PCR amplification products were evaluated by agarose gel electrophoresis followed by Southern hybridization as described above. The ^{32}P-labeled, nested or internal cDNA probes used were a 540 bp *Xba*I-*Eco*RI fragment for luciferase transcripts (29), or a 281 bp *Ban*II fragment within the amplified GAPDH transcript region derived from rat GAPDH cDNA sequence for mouse lung GAPDH mRNA transcripts [the amplified mouse GAPDH transcripts region (555bp) is 97% homologous to the rat counterpart (46,47)].

Localization of transferred gene expression using *E.coli lacZ* gene in the mouse lung

After animals were sacrificed, blood was removed by cardiac puncture, the lungs lavaged twice with PBS, and the pulmonary vasculature perfused as described above. The lungs and trachea were removed, washed with PBS, injected slowly with 2 ml of cold fixing solution (2% formaldehyde, 0.2% glutaldehyde in PBS), and immersed in the same fixing solution at 4°C for 1.5

h (34,35). The lungs were then washed with PBS, and stained by intratracheal infusion of and immersion in staining solution [5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$ (both from Sigma), and 2 mM $MgCl_2$ in PBS containing 1 mg/ml of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, Boehringer Mannheim Biochemicals) dissolved in N,N-dimethylformamide (Sigma)] for 5 h at 37°C (34,35). To obtain sections of the proximal and distal airways, both lungs were then sliced longitudinally along the axis of the main bronchi. The lungs and trachea were then embedded in glycomethacrylate using standard techniques. Four μm sections (both with and without nuclear fast red counterstaining) were analyzed under light microscopy.

RESULTS

Luciferase expression in mouse lung after transfection by pRSVL *in vivo*

Luciferase activity was detectable in mouse lung homogenate after intratracheal instillation of the pRSVL plasmid (Figure 1). The expression of luciferase was exclusively derived from the pRSVL vector since luciferase activity was undetectable in the control mice administered either liposomes alone or irrelevant plasmids (panel A). More efficient *in vivo* gene transfer was accomplished when the pRSVL plasmid was complexed with cationic liposomes than with the pRSVL vector alone. The levels of luciferase expression increased with increasing amounts of the pRSVL (with above 200 μg, panel B). Based on this observation, 200–400 μg of the pRSVL vector were used for subsequent studies. Importantly, luciferase activity was detected as early as 1 d after intratracheal administration of pRSVL and remained detectable at similar levels for at least 4 wk, although there were mouse-to-mouse variations in the levels of luciferase expression (panel C).

Expression of pRSVCFTR-directed human CFTR mRNA transcripts in COS-7 cells *in vitro*

The *in vitro* evaluation of the pRSVCFTR vector (Figure 2A) demonstrated that it directed the expression of human CFTR mRNA transcripts (panel B). As has been previously observed (3,25,26,48), Northern analysis of T84 human colon carcinoma cells demonstrated 6.5 kb CFTR mRNA transcripts (lane 1). In contrast, untransfected COS-7 cells or cells transfected with pBS did not contain detectable CFTR mRNA transcripts (lanes 2, 3, respectively). However, after *in vitro* transfection of COS-7 cells with pRSVCFTR, 5.0 kb transcripts (the expected size of pRSVCFTR-directed human mRNA transcripts) were observed (lane 4). The levels of control β-actin mRNA transcripts were similar in all samples (lanes 5–8).

Expression of pRSVCFTR-directed human CFTR mRNA transcripts in mouse airways *in vivo*

Following intratracheal administration of pRSVCFTR in C57Bl/6NCR mice *in vivo*, human CFTR mRNA transcripts directed by pRSVCFTR could be detected in lung tissue (Figure 3B,C). pRSVCFTR-directed human CFTR transcripts were detected in the mouse lung after pRSVCFTR transfection (panel B, lanes 6, 8), but not in untransfected animals (lane 2) or after pRSVL transfection (lane 4). In the absence of RT, none of the samples demonstrated amplified CFTR mRNA sequences (lanes 1, 3, 5, 7). Specificity of the PCR analysis was also

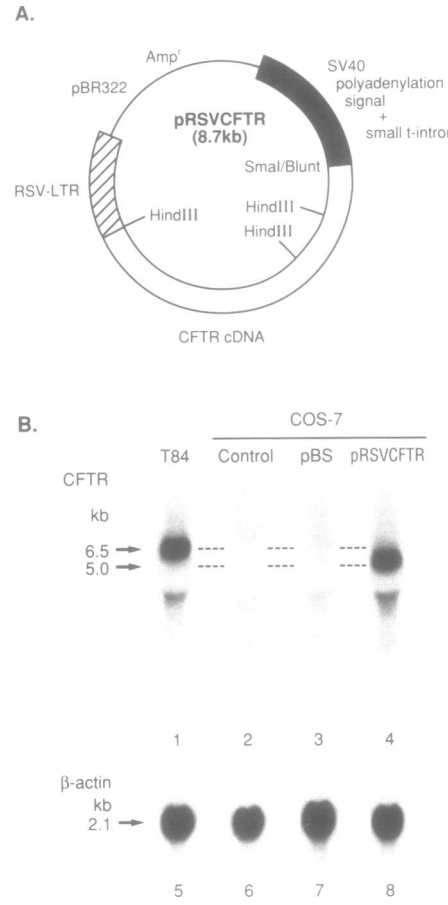


Figure 2. Evaluation of human CFTR mRNA transcripts after transfection of COS-7 cells with pRSVCFTR *in vitro*. A. Schematic of the plasmid vector pRSVCFTR. The vector was constructed from pRSVL by replacing the luciferase cDNA with the CFTR cDNA at *Hind*III and *Sma*I sites. B. Shown are Northern analyses (10 μg total cellular RNA/lane) evaluated with a 4.5 kb human CFTR cDNA probe (*top*, lanes 1–4), and subsequent stripping and rehybridization of the same filter with a 2.0 kb human β-actin cDNA probe (pHFβA-1; *bottom*, lanes 5–8). Lane 1—untransfected T84 cells; lane 2—untransfected COS-7 cells; lane 3—COS-7 cells transfected with pBluescript II SK+ (pBS); lane 4—COS-7 cells transfected with pRSVCFTR. The 6.5 kb endogenous human CFTR mRNA transcripts are indicated, as are the 5.0 kb pRSVCFTR-directed CFTR mRNA transcripts. The latter is expected to be smaller than the endogenous human CFTR transcript due to the deletion of 5′ and 3′ untranslated sequences from the CFTR cDNA in the construction of pRSVCFTR (see panel A). Lanes 5–8–same as lanes 1–4, but with the β-actin probe.

demonstrated by the presence of RSV-LTR-luciferase mRNA transcripts shown only in the animal transfected with pRSVL (compare lane 13 with lanes 10–12, 14–17). However, as a control, glyceraldehyde-3-phosphate dehydrogenase mRNA transcripts were detected in mouse lung total RNA in all samples after incubation with RT (lanes 20, 22, 24, 26), but not in the absence of RT (lanes 19, 21, 23, 25).

The chronicity of pRSVCFTR-directed CFTR mRNA expression was demonstrated in C57Bl/6NCR mouse lung evaluated at 1, 4, 7 d, 2 and 4 wk after *in vivo* transfection with pRSVCFTR (panel C). RSV-LTR-directed CFTR transcripts were observed at all time points with the PCR amplification albeit with minor variations in the levels of expression (lanes 29, 31, 33, 35, 37). In the absence of RT, no samples showed amplification of CFTR sequences (lanes 28, 30, 32, 34, 36).

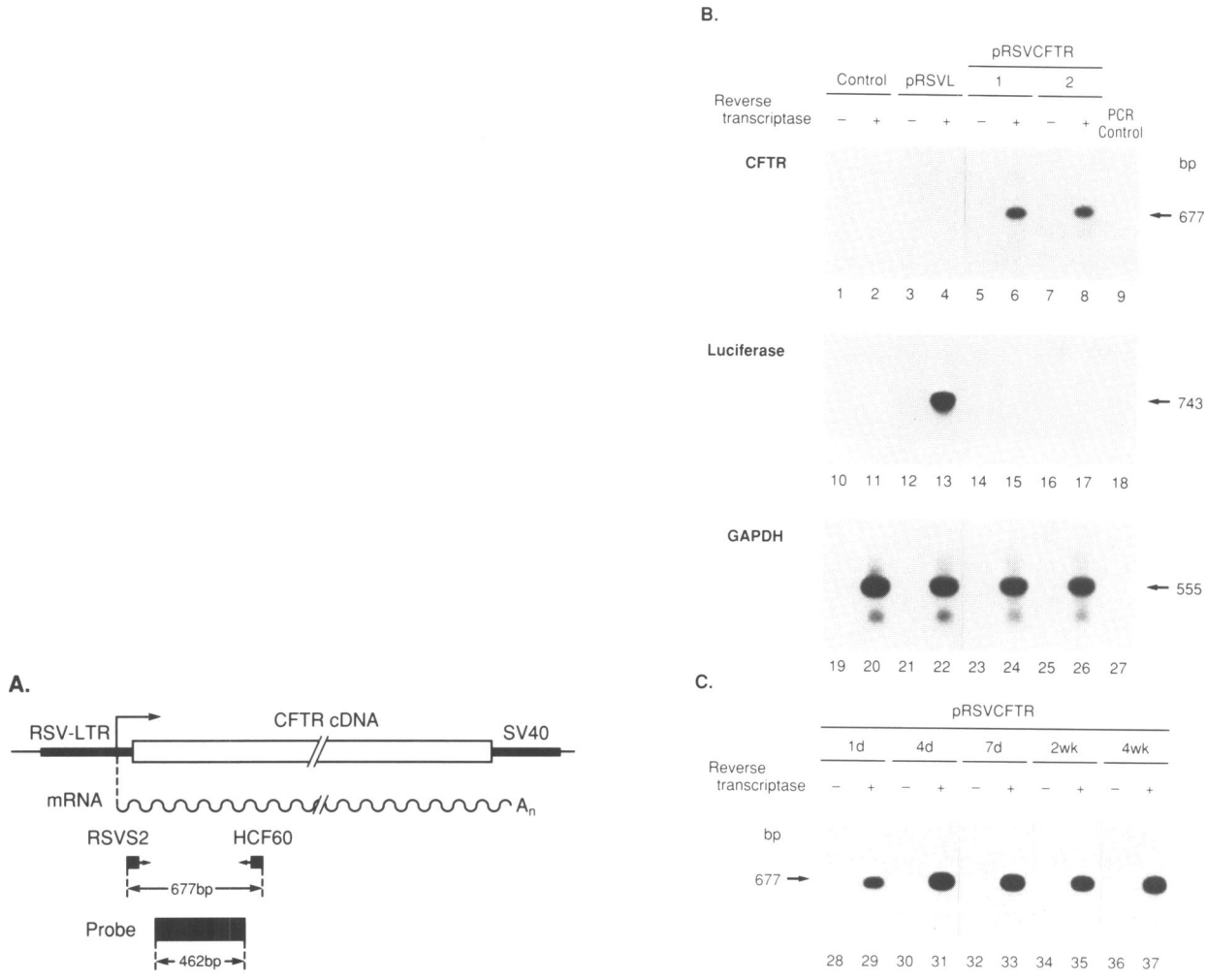
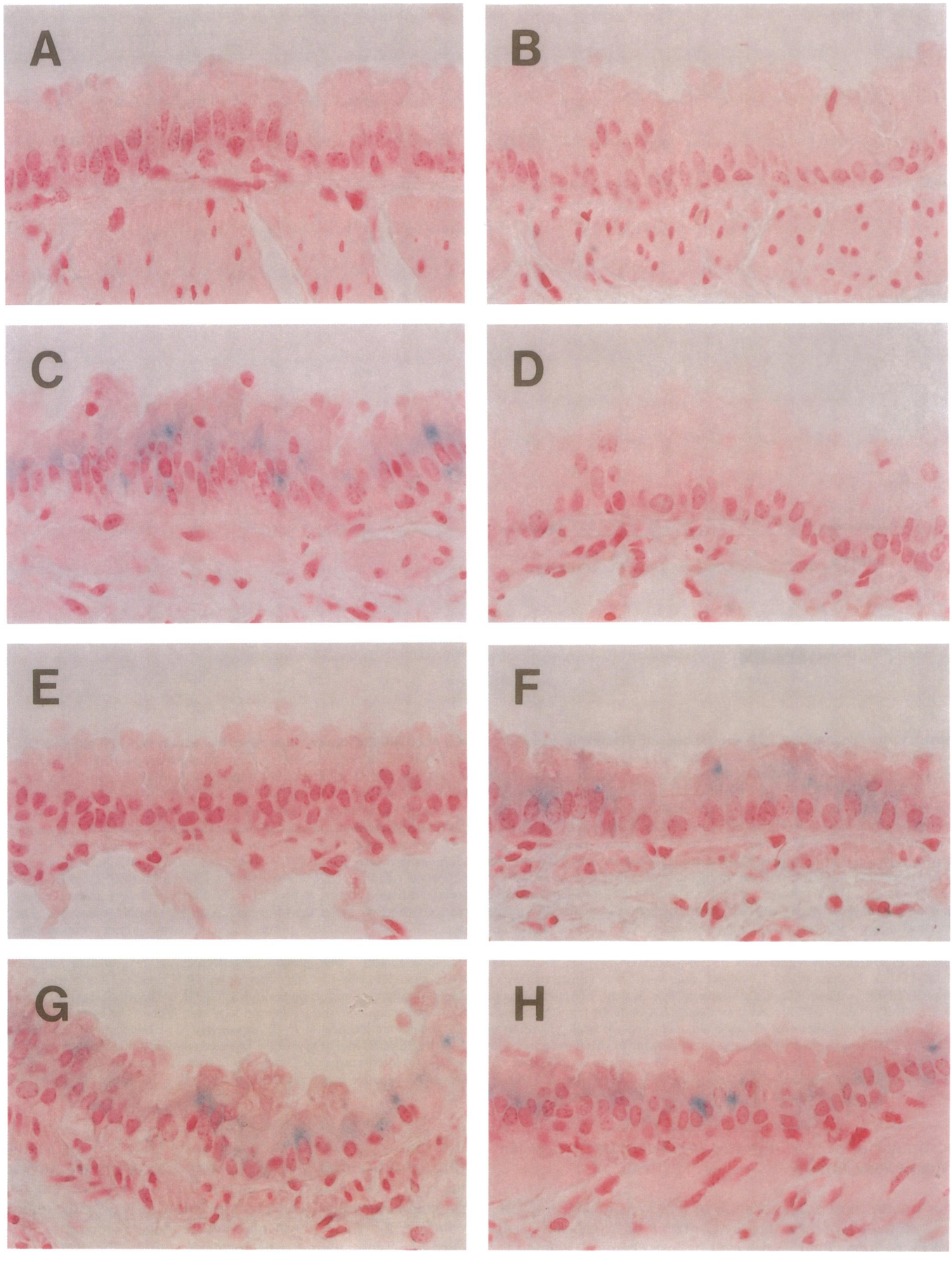


Figure 3. Evaluation of pRSVCFTR-directed human CFTR mRNA transcripts in lungs of C57Bl/6NCR mice following *in vivo* transfection with pRSVCFTR. A. Schematic of a portion of pRSVCFTR showing the expected pRSVCFTR-derived CFTR mRNA transcript and the location of the primer pair used to specifically identify the pRSVCFTR-directed mRNA transcripts. 'A_n' indicates the poly(A) tail of the transcripts. The pRSVCFTR transcript amplification primer pair consists of a 5′ RSV-LTR-specific sense primer (RSVS2) and a 3′ human CFTR cDNA-specific antisense primer located in exon 5 (HCF60). Also shown are the sizes of the expected amplification products (677 bp) and the nested probe (462 bp) used to detect specifically amplified pRSVCFTR-directed transcripts. B. mRNA transcripts in mouse lung following *in vivo* transfection with either water (control), pRSVL, or pRSVCFTR, all in the presence of liposomes. mRNA was converted to cDNA and amplified as described in Methods. '−' or '+' indicates the absence or presence of reverse transcriptase (RT), respectively, in the cDNA synthesis reactions. The sizes of expected amplification products are indicated on the right in each panel. *Top*—evaluation of the pRSVCFTR-directed human CFTR mRNA transcripts. *Middle*—evaluation of pRSVL-directed firefly luciferase mRNA transcripts. The same 5′ primer (the RSV-LTR sense primer) was used for both CFTR and luciferase mRNA transcripts. The 3′ primers were either a human CFTR or a luciferase cDNA-specific antisense primer, respectively. The probes used were internal sequences within the amplified cDNA regions (see Methods). *Bottom*—evaluation of mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA transcripts. The 5′ primer was a sense primer corresponding to mouse GAPDH cDNA nucleotide residues 439 to 462, and the 3′ primer was an antisense primer corresponding to nucleotide residues 970 to 993 (46). The probe was a 281 bp rat GAPDH cDNA fragment which is 97% homologous to the mouse counterpart within the amplified mouse GAPDH transcript region. Lanes 1−9—evaluation with the ^{32}P-labeled CFTR probe. Lane 1—control mouse lung RNA 2 d after incubation with water, without RT; lane 2—same as lane 1, with RT; lane 3—2 d after transfection with pRSVL, without RT; lane 4—same as lane 3, with RT; lane 5—2 d after transfection with pRSVCFTR, without RT; lane 6—same as lane 5, with RT; lane 7—another example 2 d after transfection with pRSVCFTR, without RT; lane 8—same as lane 7, with RT; lane 9—PCR control (without RNA or DNA template). Lanes 10−18—evaluation with the ^{32}P-labeled luciferase probe. The lanes are identical to 1−9, respectively. Lanes 19−27—evaluation with the ^{32}P-labeled GAPDH probe. The lanes are identical to 1−9, respectively. C. Evaluation of the chronicity of human CFTR gene expression in mouse lung following *in vivo* transfection with 200 μg of pRSVCFTR. The size of the expected amplification product is indicated. Lane 28—1 d after transfection, without RT; lane 29—same as lane 28, with RT; lane 30—4 d after transfection, without RT; lane 31—same as lane 30, with RT; lane 32—7 d after transfection, without RT; lane 33—same as lane 32, with RT; lane 34—2 wk after transfection, without RT; lane 35—same as lane 34, with RT; lane 36—4 wk after transfection, without RT; lane 37—same as lane 36, with RT.

Localization of pCMVβ-directed β-galactosidase expression in mouse lung *in vivo*

After intratracheal administration of pCMVβ to C57Bl/6NCR mice, expression of the transduced *E. coli lacZ* (β-galactosidase) gene in the lung could be detected by histochemical staining at 2 d (not shown) and 4 d as shown by blue staining of cells (Figure 4). Animals received liposomes alone (panels A,D), or the control plasmid pRSVL (panels B,E) did not show any blue staining of the lung architecture including airway epithelium. In contrast, the tracheobronchial tree of mice received pCMVβ appeared blue, with β-galactosidase activity localized to the


A
B
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airway epithelium (panels C,F,G,H). This was especially true for the proximal bronchi (panel C) and, to a lesser extent, for the intermediate and distal bronchi (panels F,G,H).

Examination of the airway epithelium of animals received pCMVβ demonstrated that the majority of airway epithelial cells including ciliated cells expressed the *lacZ* gene, albeit with the cell-to-cell variation in intensity of staining. Other types of cells including alveolar macrophages did not exhibit β-galactosidase activity with the staining conditions used.

DISCUSSION

The present study demonstrates the possible therapeutic application of plasmid-mediated gene transfer for the respiratory manifestations of CF by the direct transfer of a normal human recombinant CFTR gene to the respiratory tract *in vivo* utilizing a plasmid vector complexed with cationic liposomes. The rationale underlying this approach is suggested by a variety of evidence including: 1) the fatal manifestations of CF involve the respiratory epithelium (1); 2) the CFTR gene is normally expressed at low levels in airway epithelial cells (25,26); 3) the transfer of a normal human CFTR cDNA into the epithelial cells derived from individuals with CF can restore the defective cAMP-regulated Cl^- permeability in these cells to normal (22,23); 4) the slow rate of turnover of respiratory tract epithelial cells makes it difficult to utilize a vector that requires host cell proliferation for recombinant gene expression such as retroviral vectors (24,49); 5) the complex anatomy of the human airway epithelial surface precludes the approach to gene therapy of removing defective cells for *in vitro* transfer of a normal gene and reimplantation of these cells (27); and 6) although the genes in non-linearized plasmid vectors likely are not integrated into cellular chromosomal DNA *in vivo* (50), plasmid vectors have the advantage that they could be administered repetitively via the airways whenever necessary, thus offering the possibility of keeping transferred gene expression above required threshold levels despite transient expression.

Several observations in the present study support the feasibility of this direct *in vivo* approach using plasmid DNA-liposome complexes. First, following *in vivo* administration of pRSVCFTR to the respiratory tract, PCR amplification and subsequent Southern analysis demonstrated pRSVCFTR-directed human CFTR mRNA expression in the lungs, the major site of the lethal manifestations of CF (1). Second, although the low level of expression of CFTR in the lung makes it difficult to demonstrate which type(s) of cell in the respiratory tract express the CFTR gene, the available evidence suggests all airway epithelial cells express CFTR and the expression of the abnormal CFTR gene in these cells is responsible for the respiratory manifestations of CF (2,16,26). In this regard, although the relatively low level of expression of the human CFTR cDNA and the limitation of the methodology to detect transduced human CFTR protein in mouse lung precludes exact identification of the expressing cells, most likely they are epithelial cells, the initial cell type exposed to the vector. Importantly, direct evidence for this has been provided by transferring the β-galactosidase reporter gene to the mouse lung *in vivo*. These studies clearly demonstrated that the majority of cells expressing the exogenous gene after plasmid-mediated gene transfer are airway epithelial cells. No cell types other than airway epithelium showed β-galactosidase activity including alveolar macrophages [cells abundant on the airway surface and known to express the endogenous CFTR gene at very low levels (51)]. These *in vivo* observations are consistent with the observations by us and others that macrophages are not very susceptible to transfer and expression of foreign genes (52). Third, human CFTR mRNA transcripts could be detected in C57Bl/6NCR mouse lungs for at least 4 wk after transfection, implying that the corrected phenotype in Cl^- permeability may be restored using this method for a considerable period of time.

While the present study demonstrates that it is possible to utilize a plasmid vector to transfer a recombinant normal CFTR gene to the lung, and have it expressed for at least 4 wk, most likely in the airway epithelial cells, there are several issues that have to be addressed for the application of this therapeutic approach to individuals with CF. For example, further studies will have to be conducted regarding the threshold levels of expression of the transferred CFTR gene required for function. It is known that the normal human CFTR gene is expressed at low levels in human airway epithelial cells at the transcriptional and mRNA levels, with an average of only 1−2 copies/cell (25,26). It is also known that individuals heterozygous for the normal and common abnormal Û ÛF508 CFTR alleles are clinically well and express both alleles in the respiratory epithelium equally at the mRNA level (26). Together, these observations suggest that gene transfer for respiratory manifestations of CF will require only low level expression of the transferred normal CFTR to correct the defective Cl^- permeability in the airway epithelium.

The safety of the plasmid vector system also needs to be considered. While this approach in theory may be safer than viral vector systems to deliver CFTR to the lung epithelium, plasmid vectors may have drawbacks due to the high viscosity of the DNA solution (53,54).

Finally, it remains to be addressed whether or not the newly introduced gene would need to be regulated. Because the structural features of the CFTR gene promoter categorize it in the housekeeping gene class, the expression may be constitutive (25). However, the presence of multiple potential transcriptional regulatory elements suggests that it may also be regulated, and modulation of CFTR gene expression has been demonstrated *in vitro* (25,48). If regulation of transferred CFTR gene expression is essential in *in vivo* situations, a plasmid vector with the CFTR promoter (or at least partial sequences) may be utilized.

Figure 4. Evaluation of β-galactosidase expression in lungs of C57Bl/6NCR mice following *in vivo* intratracheal instillation of pCMVβ. The lungs were removed 4 d after injection and stained for β-galactosidase activity prior to sectioning as described in Methods. Sections were made from plastic (glycomethacrylate) embedded tissues and were counterstained by nuclear fast red. The blue color indicates the expression of β-galactosidase activity. All panels, 630x. Panel A. Airway epithelium of a proximal bronchus from a control animal received liposomes alone. Panel B. Same as A, but an animal received pRSVL. Panel C. Same as A, but an animal received pCMVβ. Note that cells with blue stain in cytoplasm are all bronchial epithelial cells, not mesenchymal cells or macrophages. Panel D. Airway epithelium of a distal bronchus from a control animal received liposomes alone. Panel E. Same as D, but an animal received pRSVL. Panel F. Airway epithelium of an intermediate size bronchus from an animal received pCMVβ. Note that β-galactosidase activity (blue color) is observed only in cytoplasm of airway epithelial cells, not in other cell types. Panel G. Another example of airway epithelium of a distal bronchus from a mouse received pCMVβ. Panel H. Same as G, but from a different section of the distal bronchus.

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