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# The effect of synthetic surfactant Exosurf on gene transfer in mouse lung in vivo

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*Gene transfer in the lung holds promise for the treatment of diseases such as pulmonary fibrosis, cystic fibrosis and asthma. Pulmonary surfactant has been reported to enhance expression from endobronchial, adenovirus-mediated gene transfer in experimental animals. This study examines the effect of exogenous synthetic surfactant (Exosurf) on gene expression from naked plasmid DNA administered endobronchially to adult mice. Transfection efficiency was evaluated by quantifying the expression of chloramphenicol acetyltransferase (CAT) and luciferase (Luc) genes in the lung. Endobronchial administration of either CAT or Luc expression plasmid DNA resulted in detectable concentrations of each reporter protein. CAT expression from plasmid DNA was monitored after endobronchial administration with the maximal expression observed at 3–5 days after administration and decreasing for 5 days thereafter. When DNA was delivered in a 50% suspension of Exosurf, the expression of either CAT or Luc was significantly reduced by 89.6 ± 1.4% and 82.7 ± 10.5%, respectively. The decrease in Luc expression was closely correlated ($r = 0.99$, $P < 0.001$) to log concentration of surfactant in the plasmid buffer solution ($IC_{50} = 8.6\%$). CAT expression was not altered when surfactant was administered either 2 h before or after plasmid DNA instillation. Examination of the components of Exosurf revealed that two compounds, DPPC and tyloxapol, showed inhibitory effects on CAT expression. However, the inhibition caused by Exosurf appeared greater than that of either component. Our results suggest that the lung surfactant is a barrier to transfection of the endobronchial airway and may be partly responsible for the low expression of exogenous DNA in vivo in the bronchial tree.*

**Keywords:** *transfection; surfactant; Exosurf; lung; gene delivery*

## Introduction

Gene transfer to the lung is under evaluation for the treatment of a variety of inherited or acquired pulmonary disorders. The respiratory epithelium is a primary target for cystic fibrosis gene therapy because a deficit or absence of CFTR function in this region leads to the gradual destruction of the lung tissue. This destruction is a major cause of morbidity and mortality in afflicted patients.[1] The delivery of genes to the airway is aided by the relative accessibility of the bronchial tree through inhalation, and this route of administration essentially limits transgene expression to the bronchial epithelium and alveoli. Animal models of bronchial gene transfer have proved crucial to the evaluation of the feasibility, efficiency and safety of various therapeutic strategies.[2] In these models, *in vivo* transfection of respiratory epithelium through the airways has been demonstrated after delivery of either adenovirus–polylysine-DNA complexes,[3] replication-deficient adenovirus,[4–8] DNA complexed with cationic lipids[9–13] or naked plasmid DNA under the control of CMV promoter/enhancer.[14] A major advantage of transfection with naked plasmid DNA is that it avoids the now recognized safety and inflammatory problems associated with either viral vectors,[15,16] or gene transfer using liposomes or cationic polymers.[17–19] However, the only low levels of transfection and expression are observed in the lung as the result of the administration of naked plasmid DNA.[3,11,14]

Exogenous pulmonary surfactant has been reported to enhance adenovirus-mediated luciferase gene expression in rabbits[20] and improve the uniformity of β-galactosidase expression of replication-deficient adenovirus in rats.[21] Natural pulmonary surfactant is a combination of lipids, proteins and carbohydrates that provide alveolar stability at low lung volumes.[22] A synthetic, protein-free form of surfactant has also been developed and used for replacement therapy in the course of respiratory distress syndrome (RDS).[23] This material is functionally identical to the natural product, but is made of three defined chemicals: cetyl alcohol, tyloxapol and colfosceril palmitate (DPPC). We have hypothesized that endobronchial transgene expression might be enhanced in the presence of synthetic surfactant because of its dispersion capabilities.[24,25] This material might aid the rapid and uniform distribution of plasmid DNA throughout the respiratory epithelium.

Animal models of cystic fibrosis have now been developed in mice[26–28] and therefore it is important to establish the effect of pulmonary surfactant on *in vivo* plasmid DNA transfection in the mouse respiratory epithelium. We have evaluated the transfection of two different DNA expression plasmids coding for chloram-

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phenicol acetyltransferase (CAT) and luciferase (Luc), *in vivo* in mice after nasal administration. Transgene expression was also evaluated after the administration of plasmid DNA in the presence of protein-free synthetic surfactant Exosurf (Glaxo Wellcome, Research Triangle Park, NC, USA), as well as the three components that form this material. In this report, we show that the presence of synthetic surfactant during *in vivo* delivery of plasmid DNA markedly decreases the efficiency of gene transfer into the mouse lung.

## Results

### In vivo expression of CAT gene in murine lung

The transfection of naked pCF1CAT plasmid DNA produced significant amounts of CAT protein (4–20 ng/g tissue protein) after the intranasal delivery of 10 to 100 μg of DNA solution in water (Figure 1). Comparison of intranasal with intratracheal delivery gave similar results (data not shown). Maximal expression of CAT (about 20 ng/g tissue protein) was observed after instillation of 50 μg of plasmid and did not increase when larger amounts of DNA were used (Figure 1). The expression of CAT protein was observed over a period of 10 days after nasal instillation of 66 μg of pCF1CAT plasmid (Figure 2). The maximal expression of CAT was detected at 3–5 days after the plasmid DNA was administered and decreased thereafter to about 6 ng/g tissue protein by day 10 after administration (Figure 2).

### Inhibitory effect of Exosurf on expression of CAT and Luc

In contrast with the results obtained when pCF1CAT plasmid was administered in water, significantly reduced amounts of CAT protein were detected when different doses of plasmid were administered in 50% (v/v) suspension of Exosurf (Figure 1). Delivery of DNA in the presence of Exosurf caused significant inhibition of CAT levels ($P < 0.01$) both at the peak time-points and throughout the 10-day period of expression (Figure 2). To clarify that the inhibitory effect of Exosurf on CAT expression was due to inhibition of plasmid uptake and/or expression and not an alteration in the processing or the detection of CAT protein by immunoassay, transfection studies were also carried out with Luc expression plasmid (Figure 3). Similarly to CAT, luciferase activity detected in the lungs was reduced at 5 days after intranasal delivery when the plasmid DNA was suspended in surfactant ($P < 0.01$). The CAT and Luc plasmid DNA expression when administered in Exosurf, as compared with these plasmids administered in water, showed

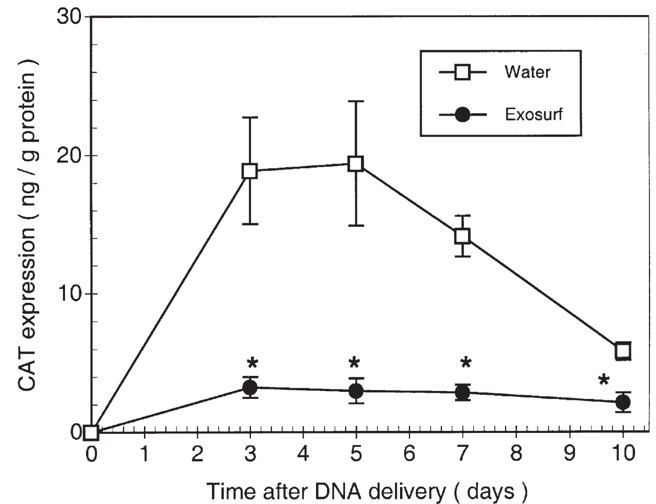


***Figure 2*** *Time course of CAT gene expression in the mouse lung. Plasmid pCF1CAT DNA (66 μg) was delivery in 100 μl of 50% (v/v) Exosurf or water. The protein expression in the presence of Exosurf was significantly decreased compared with the control group (* at least $P < 0.01$) for all of the time-points tested (3, 5, 7, 10 days). The values are the means ± s.e.m. of seven mice.*

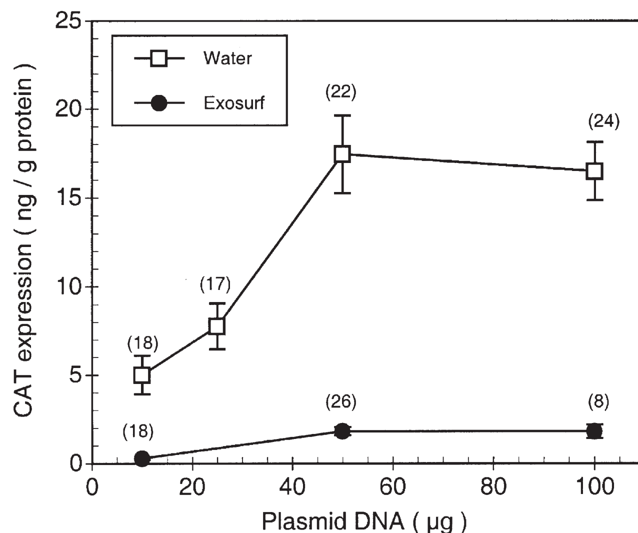


***Figure 1*** *Plasmid DNA concentration optimization for in vivo delivery. Expression of CAT protein in the mouse lung tissue 5 days after intranasal delivery of indicated amounts (10, 25, 50, 100 μg) of pCF1CAT plasmid DNA delivered in 100 μl of 50% (v/v) Exosurf or water. Groups of eight to 26 mice (shown in parentheses) were used. For different doses the mean values and s.e.m. are shown. The values for the Exosurf groups are significantly lower than the corresponding water groups ($P < 0.001$).*

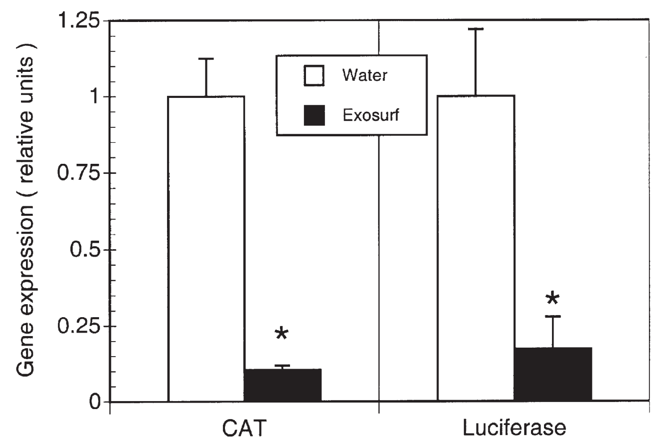


***Figure 3*** *Inhibitory effect of Exosurf on gene expression in the lung. Comparison of the relative inhibition of expression after transfection with pCF1CAT and pCF1Luc plasmids measured 5 days after intranasal DNA delivery. One unit corresponds to the expression obtained in the absence of Exosurf. The expression was significantly reduced in the presence of 50% Exosurf for both marker genes (* at least $P < 0.01$). Eight to 10 mice per group were employed.*

reductions by 89.6 ± 1.4% or 82.7 ± 10.5%, respectively (Figure 3).

### *Dose-dependent inhibition of gene expression by Exosurf*

Exosurf inhibited gene expression when used at concentrations up to 50% (v/v) in water in a dose-dependent manner. As shown in Figure 4, the decrease in expression of the transfected CAT gene is closely correlated ($r = 0.99$, $P < 0.01$) to the log concentration of surfactant used for plasmid suspension ($IC_{50} = 8.6\%$).

### *CAT expression from the plasmid DNA administered either before or subsequent to nasal instillation of Exosurf*

When Exosurf in a volume of 50 μl at 50% (v/v) concentration was administered intranasally either 2 h before or 2 h after plasmid DNA was delivered in water (volume of 50 μl at 1 μg/μl), transgene expression did not differ from that obtained in the absence of surfactant (Figure 5). Also, when 50 μl of water was instilled 2 h after plasmid DNA delivery transfection efficiency was not altered (data not shown).

### *Detection of CAT gene by PCR*

The survival of transgenic CAT DNA in the lung was monitored by PCR. The 248 bp fragment of CAT gene was amplified from the DNA fraction isolated from lung tissue. The reporter gene DNA was easily detected in total lung DNA in both Exosurf treated as well as untreated lungs 10 days after delivery (Figure 6).

### *Localization of CAT protein expression in mouse lung*

Enzymatically active CAT protein was detected *in situ* in lung sections using a histochemical staining technique.

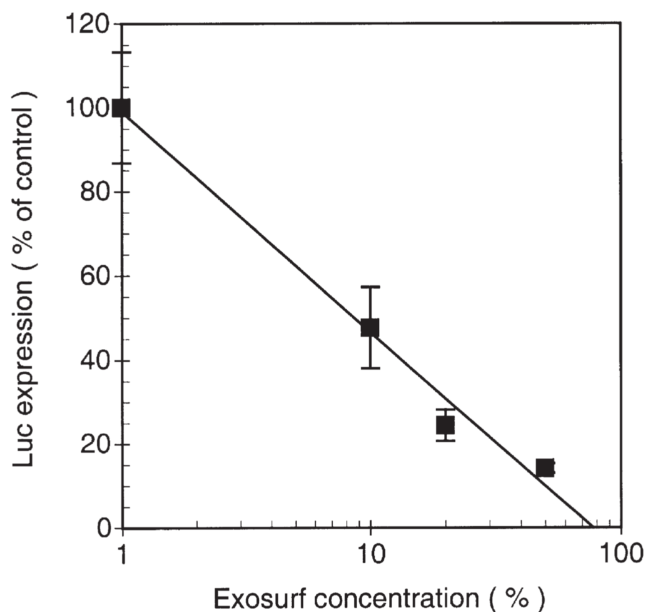


***Figure 4*** *Dose effect inhibition of Luc expression by Exosurf. The values for Luc expression in Exosurf are represented as the percentage of Luc plasmid delivery in water. The values for Exosurf concentration are presented on the logarithmic scale. The mean ± s.e.m. of eight mice are presented. The correlation between Exosurf concentration and gene inhibition expression is highly significant (r = 0.99, P < 0.001).*

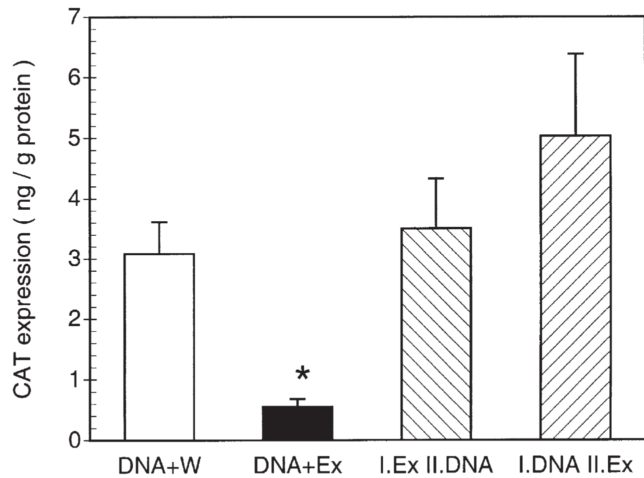


***Figure 5*** *The effect of sequential administration of pCF1CAT plasmid and Exosurf. Plasmid DNA delivered in 50 μl of water (white column) and 50 μl of 50% (v/v) Exosurf (black column) were used as controls. Dashed columns represent CAT expression when 50 μl of plasmid DNA was delivered either 2 h after (I.Ex II.DNA) or 2 h before (I.DNA II.Ex) the administration of Exosurf (50 μl). The total volume of 100 μl delivered was divided into two instillations of 50 μl. The values are the means ± s.e.m. of eight mice.*

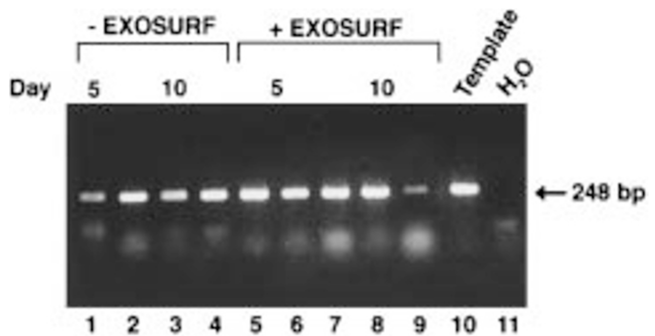


***Figure 6*** *PCR amplification of CAT sequences in the DNA fraction of lung homogenate. The 248 bp of CAT gene was detectable at the time-points tested (5 and 10 days) in Exosurf-treated (lanes 5–9) and Exosurf-untreated (lanes 1–4) lungs. Plasmid pCF1CAT (20 fg of template DNA) and $H_2O$ were used as controls.*

Hatchett's Brown precipitate revealed transfection which was primarily localized to the ciliated epithelium of respiratory bronchiole (Figure 7a) and was also seen occasionally in the pneumocytes of alveoli (Figure 7b).

### *The effect of the components of Exosurf on the expression of CAT*

The three components of Exosurf, cetyl alcohol, tyloxapol and DPPC, were tested independently for their ability to inhibit the expression of CAT. As compared with plasmid DNA delivered in water, plasmid DNA administered in cetyl alcohol had slightly decreased expression (15.9 ± 21.4%) (Figure 8). In contrast, plasmid DNA administered in either tyloxapol or DPPC inhibited expression by 39.2 ± 18.1% and 43.9 ± 15.1%, respectively (Figure 8), and this difference approached statistical significance. However, administration of plasmid DNA in Exosurf reduced expression by 72.9% ± 3.9%, a decrease significantly greater than either tyloxapol or DPPC alone ($P \leqslant 0.001$) (Figure 8).

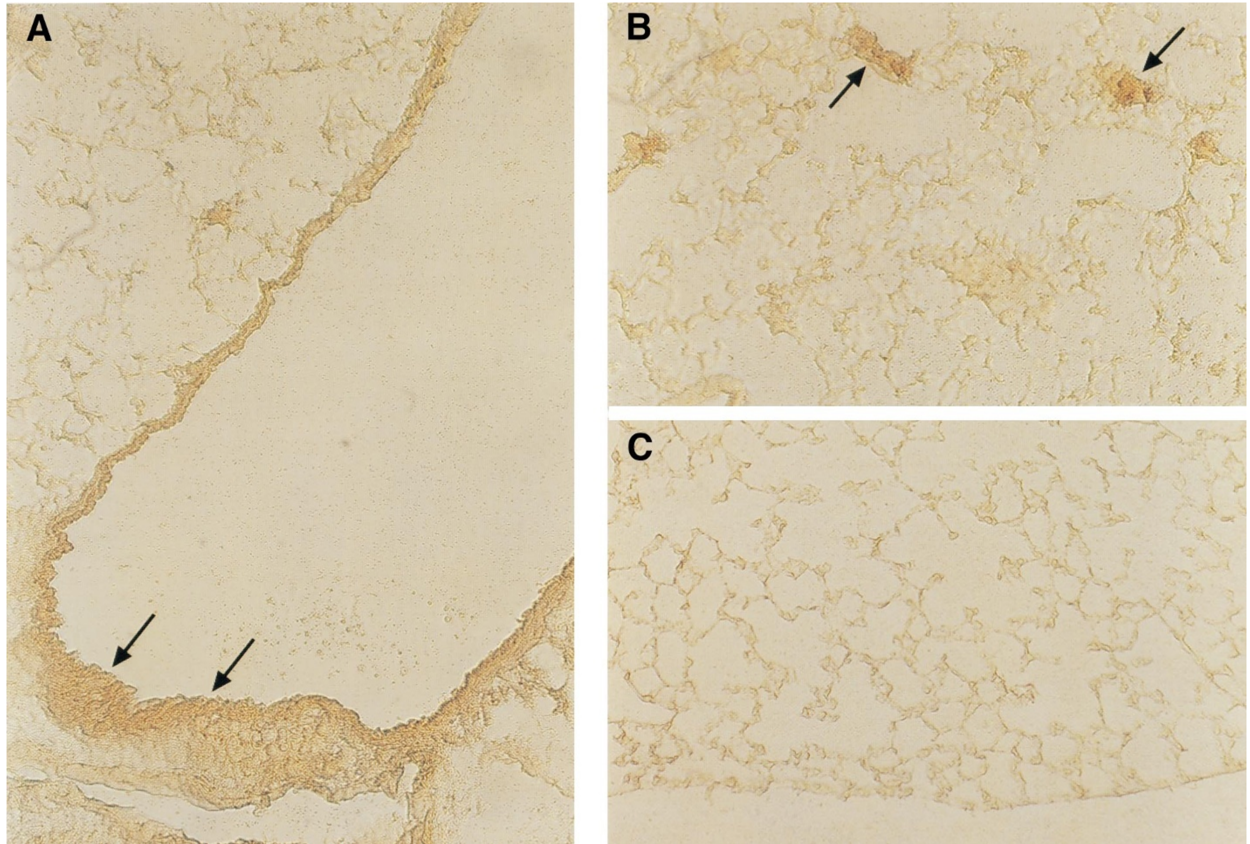


***Figure 7*** *In situ detection of CAT protein in mouse lung sections after intranasal delivery of 100 μg of naked pCF1CAT DNA. Hatchett's Brown precipitate is present in ciliated epithelium showing columnar cells of respiratory bronchiole of peripheral lung (A) and in pneumocytes of alveoli (B) compared with a control mouse which received 100 μg of naked pCF1Luc DNA (C).*

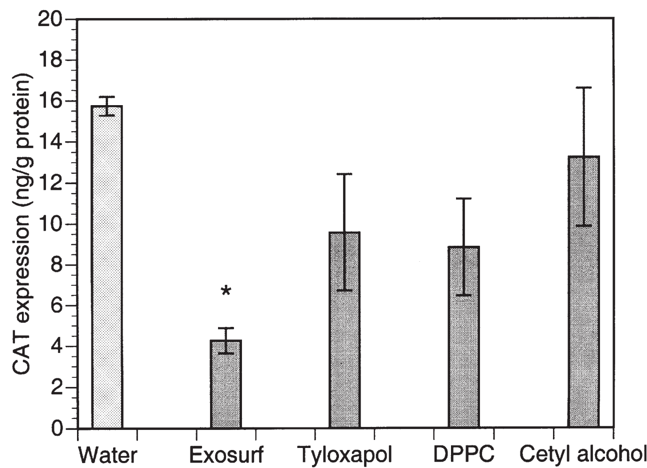


***Figure 8*** *Comparison of effect of Exosurf and its individual components on gene expression in the lung. The expression of CAT 4 days after intranasal delivery of pCF1CAT plasmid DNA (100 μg) in 100 μl of water, 50% Exosurf (v/v), tyloxapol (0.5 mg/ml), DPPC (6.75 mg/ml) and cetyl alcohol (0.75 mg/ml). The protein level was significantly decreased with Exosurf ($P < 0.001$) compared with the control group. The values are the means ± s.e.m. of 17, 13, 6, 6 and 6 mice, respectively.*

## Discussion

The process of gene transfer and the achievement of efficient levels of expression remains a major obstacle to human gene therapy. While naked plasmid DNA delivery avoids the safety issues associated with viral vectors,[15,16] transfection efficiency in the lung from plasmid DNA is relatively low.[3,11] However, it has been suggested that certain applications of pulmonary gene therapy might require only low levels of expression for therapeutic effect. For example, in cystic fibrosis there is evidence both *in vitro*[29] and *in vivo*[30] indicating that low levels of CFTR expression could re-establish physiological ion transport. Thus some form of enhanced transfer of naked DNA may be a viable therapeutic approach for certain disorders afflicting the respiratory epithelium.

The *in vivo* results presented in this study demonstrate that the naked plasmid DNA expressing either *CAT* or *Luc* genes delivered endobronchially can mediate significant levels of transfection in the mouse lung. Initial reports examining the transfer of naked plasmid DNA to the respiratory epithelium failed to show transgene expression after either intratracheal injection of CAT plasmid into rat lung[11] or after aerosol delivery into mice.[12] Subsequently, the expression of CAT in mouse lung extract was reported after plasmid DNA instillation

through an intranasal catheter.[17] The levels of CAT expression for the naked plasmid DNA in that study were comparable to the ones presented in our work. The present results are also similar to those achieved by other investigators using the pCF1CAT plasmid, and it is likely that the different levels of expression can be attributed to the different plasmid constructs, their promoters, regulatory elements and the relation of these elements to the specific biology of the respiratory epithelial cells.[4,31] Gene transfer with naked plasmid DNA was also described in rat lung after tracheal insufflation.[14] Quantitative comparison of those results with our data is difficult since the expression was measured in an acetylation assay of $^{14}$C-labeled substrate and the CAT activity was presented in milliunits per whole organ. Our studies also localized the expression from plasmid to the respiratory epithelium in the bronchial epithelium, rather than restricting this expression to the alveoli as is observed with adenovirus-mediated gene transfer. Thus, our findings support the ability of naked plasmid DNA to express transgenes significantly in the respiratory epithelium.

We were interested in assessing the possible enhancement of gene expression from plasmid DNA in the lung using the synthetic pulmonary surfactant Exosurf. The surface active properties of surfactant can lead to rapid dispersion of substances in the air spaces and greater volumes of material administered to the airways in surfactant are better tolerated by mice than DNA delivered in either saline[21] or sterile water (personal observation). Improved distribution and an approximate 50% increase in the expression of β-galactosidase gene has been reported after endobronchial transfer of replication-deficient adenovirus resuspended in either bovine surfactant (Survanta Beractant; Ross Products Division of Abbott, Abbott Park, IL, USA) or Exosurf when compared with the same virus suspended in saline.[21] These surfactant preparations also were reported to facilitate adenovirus-mediated expression of luciferase gene in the peripheral lung in rabbits.[20] However, there has been no data analyzing the effect of a surfactant on the expression of a gene delivered with a naked plasmid DNA *in vivo*. We have shown that the synthetic lung surfactant Exosurf inhibits the expression of two different reporter genes (*CAT* and *Luc*) from the naked plasmid DNA administered into the mouse lung. The inhibition depends on the Exosurf concentration and persists for the duration of transgene expression. The time course of expression from water and surfactant delivered plasmid DNA are similar, so processing and compartmentalization of the plasmid within the cell after uptake is likely no altered by the Exosurf. The inhibition is also independent of the reporter gene or assay employed, which suggests it is not the result of alterations in translation, protein stability or the expression assay. Neither does it depend on the delivery technique since direct administration of the plasmid DNA by intratracheal intubation did not yield significantly greater levels of expression as compared with nasal administration (data not shown). This suggests that the uptake of the plasmid DNA into the cells was the parameter altered by the surfactant.

Given our findings, the inhibition of expression mediated by surfactant may be the result of decreased DNA uptake by endobronchial cells. However, the reasons for decreases in DNA uptake by the cells are not obvious. Exosurf most likely inhibits the adhesion and subsequent endocytosis of plasmid DNA into airway epithelial cells, since surfactant reduces the permeability of the alveolar capillary membrane and overcomes the adhesive properties of alveolar proteins. It has also been reported to stimulate phagocytosis and enhance the clearance of foreign particles and alveolar fluid.[32] This could lead to the accelerated breakdown or clearance of the plasmid. However, there is no evidence of this since the reporter gene is easily detected by PCR amplification of DNA fraction regardless of Exosurf treatment of the lungs, and the administration of additional amounts of plasmid DNA did not reverse the inhibition of expression (data not shown). Further studies are needed to elucidate the exact process by which Exosurf inhibits the uptake of the plasmid.

Analysis of each individual component of Exosurf revealed that the inhibition is mainly due to the presence of nonionic surfactant tyloxapol and DPPC. Neither component would be expected to interact on charge basis with DNA, but both could stabilize the plasmid in suspension. This could partition the plasmid away from the membrane, thus preventing the interaction required for uptake. On the other hand, the inhibition of transfection observed with complete surfactant was greater than either component. Since the increasing concentrations of each component did not further decrease expression (data not shown), it is possible that each of them inhibited transfection in a different manner, thus leading to the additive inhibition observed with the compound as a whole.

In conclusion, synthetic surfactant appears significantly to inhibit the expression of plasmid DNA *in vivo*. While it is functionally identical to natural surfactant, the synthetic formulation we employed is protein-free. The observed effects are entirely due to the nonionic surfactant tyloxapol and the lipid component (DPPC). Endogenous surfactant may be more potent in altering transgene expression due to the presence of naturally occurring surfactant-specific proteins which play the same role as tyloxapol in the Exosurf preparation. Further studies on the interaction of the surfactant components with plasmid DNA and with respiratory epithelium, possibly in organ culture, would be important to help to increase the efficiency of naked plasmid DNA gene transfer in the bronchial epithelium *in vivo*.

## Materials and methods

### *Synthetic lung surfactant*

The synthetic surfactant, Exosurf Neonatal was used in these studies. It is an aqueous solution of protein-free synthetic surfactant consisting of lipid component: colfosceril palmitate (dipalitoylphosphatidylcholine = DPPC; 13.5 mg/ml), spreading agent for DPPC: cetyl alcohol (1.5 mg/ml) a polymeric long-chain repeating alcohol and tyloxapol (1.0 mg/ml), a nonionic surfactant which acts to disperse both DPPC and cetyl alcohol. The lyophilizate of Exosurf was resuspended in water according to the manufacturer's instructions. Tyloxapol, DPPC, and cetyl alcohol were purchased from Sigma Chemical, St Louis, MO, USA.

### *Plasmids*

The pCF1CAT plasmid used in this report was provided by Drs R Scheule and S Cheng (Genzyme, Framingham,

MA, USA). The construction and optimization of this plasmid has been previously described.[31,33] Plasmid pCF1Luc was constructed by inserting blunt ended *Hin*dIII–*Xba*I fragment of luciferase gene from pGL3 plasmid (Promega, Madison, WI, USA) into blunt ended *Not*I fragment of pCF1CAT vector with deleted *CAT* gene.

### Animals

Female Balb/c mice from Charles River Laboratories (Wilmington, MA, USA) were used weighing 18–20 g. All of the experiments performed followed the guidelines of the University of Michigan Committee on the Use and Care of Animals.

### Delivery of plasmid DNA

Mice were anesthetized with methoxyflurane (Metofane; Mallinckrodt Veterinary, Mundelein, IL, USA) inhalation. For intranasal delivery mice were held in a slightly inclined supine position with the mouth shut. The transfection mixture in a 100 μl volume was delivered quickly from the pipette tip, directly through the nasal orifices as the mouse was breathing. In one experiment two instillations of 50 μl were delivered 2 h apart since the introduction of larger than 100 μl volume could cause respiratory distress in mice.

### Harvest and processing of lung tissue

At time-points after plasmid delivery, mice were deeply anesthetized with Metofane the abdominal cavity was opened and the mice were exsanguinated by cutting the abdominal aorta. The chest was then opened and the pulmonary vascular bed was flushed by slowly injecting 3 ml of PBS into the right ventricle of the heart. The trachea was excised and carefully removed together with the lungs, then washed in PBS and weighed. An appropriate cold lysis buffer was added at 1 μl per 1 mg tissue (lysis buffer for CAT Elisa proprietary to Boehringer Mannheim (Indianapolis, IN, USA) was supplemented with 5 μg/ml aprotinin, 5 mm DTT, 0.2 mm PMSF, and cell culture lysis reagent from Promega was used for the luciferase samples). The tissue was homogenized using a tissue homogenizer (Biospec Products, Bartlesville, OK, USA) for 30 s and frozen in liquid nitrogen.

### CAT ELISA

To quantify chloramphenicol acetyltransferase (CAT), 50 μl of lung tissue homogenate was analyzed in a CAT ELISA (Boehringer Mannheim). The amount of CAT protein was adjusted for the protein concentration of the sample.

### Luciferase assay

Luciferase activity was quantified in a chemiluminescense assay. Light emission from lung tissue homogenate (10–20 μl) incubated with $2.35 \times 10^{-2}$ μmol of luciferin substrate (Promega), was measured in a chemiluminometer (LB96P; EG & G/Berthold, Gaithersburg, MD, USA) and adjusted for the protein concentration of the sample.

### Protein assay

The protein concentration in the lung lysates was measured using the BCA protein assay reagent kit (Pierce, Rockford, IL, USA) with bovine serum albumin as a standard.

### Isolation of DNA fraction from lung tissue

Total cellular DNA was isolated using DNAzol/DNA isolation reagent according to manufacturer's protocol (MRC, Cincinnati, OH, USA).

### Polymerase chain reaction

PCR amplification of 248 bp fragment of CAT region of pCF1CAT vector was performed with DNA fraction (20–100 ng) of lung homogenate at a final concentration of 200 μm dNTPs, 0.3 μm of each 5′ (gcatcg-taaagaacattttgagg) and 3′(gtttgctcatggaaaacggt) primers and 0.4 U of AmpliTaq (Perkin-Elmer/Roche, Jersey City, NJ, USA) in a total volume of 20 μl for 35 cycles at 60°C annealing temperature (GeneAmp PCR System 2400/Perkin Elmer). Plasmid pCF1CAT DNA (20 fg of template) and $H_2O$ were used as positive and negative controls, respectively.

### Histochemical staining of CAT activity

Lung tissue for cryostat sections was prepared by washing the pulmonary vascular bed with PBS, then injecting with OCT compound (Sakura Finetek, Torrance, CA, USA) and frozen in liquid nitrogen. Histochemical staining of CAT activity in tissue slides was performed using CAT staining kit from Boehringer Mannheim. The Hatchett's Brown precipitate is produced by acetyltransferase activity which generates free sulfhydryl group of CoA, resulting in the reduction of ferricyanide to ferrocyanide. The ferrocyanide then complexes with copper ions to form the precipitate.

### Statistical analysis

Statview 4.5 was used for the statistical analysis. Between eight and 26 mice were used for each data point. All measurements were performed in duplicates or triplicates on indiviual lung homogenates and presented as averages. Data were compared by one-way analysis of variance (ANOVA), and if significant differences occurred by pair-wise comparison with Bonferroni's correction. All values were expressed as a mean ± s.e.m., and $P$ values of less than 0.05 were considered significant.

## Acknowledgements

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