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Transfer of a constitutive viral promoter – cystic fibrosis transmembrane conductance regulator cDNA to human epithelial cells conveys resistance to down-regulation of cAMP-regulated Cl^- secretion in the presence of inflammatory stimuli

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ABSTRACT

The expression of the cystic fibrosis transmembrane conductance regulator (CFTR) gene can be down-regulated by inflammatory stimuli such as phorbol myristate acetate (PMA). Since the respiratory manifestations of cystic fibrosis (CF) are characterized by intense chronic airway inflammation very early in life, successful gene therapy for CF will require that expression of the transferred normal CFTR gene be resistant to down-regulation by inflammatory mediators. To evaluate the concept that a viral promoter – human CFTR cDNA unit would be resistant to this form of down-regulation, a retrovirus promoter (5′ long terminal repeat of the Moloney murine leukemia virus) – human CFTR cDNA unit was transferred to T84 human colon carcinoma cell line using a retrovirus vector. Exposure of the retrovirus-modified T84 cells to PMA resulted in down-regulation of the endogenous CFTR mRNA transcripts (6.5 kb), but did not affect the level of exogenous CFTR transcripts (8.0 kb). Importantly, in parallel with the persistence of the exogenous CFTR transcripts, the modified cells still maintained cAMP-regulated Cl^- secretion in the presence of PMA. These *in vitro* data suggest that a constitutive viral promoter – CFTR cDNA unit should be resistant to modulation by inflammatory stimuli, a likely requirement for successful gene therapy for CF.

INTRODUCTION

Cystic fibrosis (CF), a common lethal hereditary disorder of Caucasians, has its major manifestations on epithelial surfaces, particularly the lung, pancreas and intestinal tract (1,2). The disease is caused by mutations of the cystic fibrosis transmembrane conductance regulator (CFTR) gene, a 250 kb, 27 exon gene on chromosome 7 (3–5). A major consequence of these mutations of the CFTR gene is that epithelial cells are unable to secrete Cl^- in response to increases in intracellular cAMP (6–8). Current evidence indicates that the CFTR gene product is a cAMP-regulated Cl^- channel on the apical surface of epithelial cells and the channel activity is regulated by two processes: phosphorylation of the R domain by protein kinase A and protein kinase C and the interaction of ATP with the nucleotide-binding folds (9–18).

Several lines of evidence suggest that the respiratory manifestations of mutations of the CFTR gene might be prevented by *in vivo* transfer of the normal CFTR gene to epithelial cells of organs at risk for the disease. First, while cultured epithelial cells derived from individuals with CF are unable to secrete Cl^- in response to elevated levels of cAMP *in vitro*, transfer of the normal human CFTR cDNA to these epithelial cells complements the abnormal endogenous CFTR genes, conveying to the cell the ability to secrete Cl^- in response to cAMP in a normal fashion (19–21). Second, the normal human CFTR cDNA can be transferred to the airway epithelium of living animals, with consequent expression of the normal human gene at the mRNA and protein levels (21,22). Finally, ion transport defects in the trachea of transgenic mice that are homozygous for a null mutation in the endogenous CFTR can be corrected by *in vivo* delivery of the normal human CFTR cDNA to the airways (23).

In developing a strategy for *in vivo* human gene therapy for CF, one critical decision is whether to design the gene to be transferred as a constitutive gene expressing the normal human CFTR mRNA in a constant fashion, or to use the normal human CFTR promoter to drive the CFTR cDNA. Expression of the CFTR gene can be modulated at the transcriptional level in cell culture by a variety of model stimuli, including phorbol myristate acetate (PMA) and Ca^{2+} ionophores (24,25). Importantly, CFTR gene expression can be sufficiently down-regulated by

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stimuli such as PMA to convert a normal cell to one that has the characteristics of a 'CF phenotype' e.g., unable to secrete Cl^- in response to increases in cAMP (24). Further, recent evidence suggests that, despite the disparity of PMA and Ca^{2+} ionophores as cell surface stimuli, they modulate CFTR gene transcription through protein kinase C (26), suggesting that activation of protein kinases by any mechanism may down-regulate CFTR gene expression.

The fact that the CFTR promoter can be down-regulated to a degree that results in the inability of the cell to secrete Cl^- in response to increases in cAMP has important implications for the design of a gene construct for gene therapy purposes. In this regard, the airway epithelial milieu in CF is that of an intense, chronic neutrophil-dominated inflammatory process which begins early in childhood (27). In the context of gene therapy, if transcription of the new gene is lower than expected and, as recently demonstrated by Breuer *et al.* (28), inflammatory stimuli may also decrease the half-life of the CFTR protein, it is conceivable that the use of the normal CFTR promoter would not be optimal for gene therapy, because its ability to respond to inflammatory stimuli would render the newly transferred normal CFTR gene susceptible to chronic down-regulation. Thus, the transferred CFTR cDNA would be unable to complement the abnormal endogenous CFTR genes.

To evaluate the concept that a constitutive promoter–CFTR cDNA unit is the most rational design for gene therapy for CF, we compared the expression of a constitutive promoter–CFTR cDNA unit to the expression of the endogenous CFTR promoter–CFTR gene by transferring a constitutive promoter–cDNA unit via a retrovirus to the genome of T84 cells, human colon carcinoma cells known to express an endogenous CFTR gene that can be down-regulated by phorbol esters. The data demonstrate that, despite the down-regulation of the endogenous CFTR gene, the constitutive viral promoter–CFTR cDNA unit is resistant to down-regulation by inflammatory stimuli, maintaining expression of its mRNA product and allowing the cell to maintain cAMP-regulatable Cl^- secretion.

MATERIALS AND METHODS

Cell cultures

The retrovirus packaging cell line, PA317 cells [American Type Culture Collection (ATCC), CRL 9078] and NIH 3T3 cells were maintained in improved minimal essential medium (IMEM) with 10% fetal calf serum (FCS), 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin (all from Biofluids, Rockville, MD). T84 colon carcinoma cells (ATCC, CCL 248) were maintained in Dulbecco's modified Eagle's medium (DMEM, Whittaker Bioproducts, Walkersville, MD) supplemented with 5% FCS, 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin (24). CFPAC-1 cells (ATCC, CRL 1918), derived from a pancreatic adenocarcinoma of an individual with CF (29), were cultured as for T84 cells except with 10% FCS. Transfected PA317 cells and infected T84 and CFPAC-1 cells were selected in medium containing 1 mg/ml of neomycin (G418, GIBCO-BRL, Gaithersburg, MD).

Construction of a recombinant retroviral vector

The vector containing the human CFTR cDNA (pG1CFSVNA) was based on the retroviral vector pG1XSVNA, derived from Moloney murine leukemia virus (MoMLV) (provided by P.Tolstoshev, Genetic Therapy, Inc., Gaithersburg, MD). pG1CFSVNA was designed to include (5′ to 3′): the 5′ long terminal repeat (LTR; used to drive the CFTR cDNA), a human CFTR cDNA [the 4.7 kb *Pst*I fragment of pBQ4.7 (provided by Drs J.Rommens and L.-C.Tsui, The Hospital for Sick Children, Toronto, Canada) containing the open reading frame of the human CFTR cDNA devoid of all but 60 bp of the 5′ untranslated sequence and 150 bp of the 3′ untranslated sequence] (4), SV40 early promoter, the neomycin resistance gene, and the 3′LTR (see Figure 1A for details of the construct).

Modification of T84 and CFPAC-1 cells

Infectious amphotropic recombinant retrovirus containing the CFTR cDNA was produced as follows. The retroviral vectors, pG1XSVNA or pG1CFSVNA, were transfected into the amphotropic packaging cell line PA317 by calcium phosphate coprecipitation (30). Individual clones of transfected PA317 cells were isolated in the presence of 1 mg/ml of neomycin. Recombinant retroviruses (RV_{mock} or RV_{CFTR}) were harvested from PA317 cells transfected with pG1XSVNA or pG1CFSVNA, respectively. Titers of RV_{mock} and RV_{CFTR} were determined using NIH 3T3 cells (31). CFPAC-1 cells or T84 cells were then infected with the recombinant amphotropic retroviruses in the presence of 5 μg/ml of polybrene (Sigma, St Louis, MO) and G418-resistant clones of these cells were selected for further study.

Southern and Northern analysis

The presence and expression of the exogenous CFTR gene in the G418-selected CFPAC-1 cells and T84 cells were evaluated by Southern and Northern analyses. High molecular weight DNA was isolated from parental and infected CFPAC-1 cells and T84 cells. The DNA (20 μg) was digested with the restriction enzyme *Pst*I, and was evaluated by Southern hybridization (32) with a ^{32}P-labeled human CFTR cDNA probe (Figure 1A) labeled by random primer method (33). Total cellular RNA was isolated by the guanidine thiocyanate–cesium chloride gradient method (34). RNA (10 μg/lane) was analyzed by Northern hybridization with the CFTR probe as described above. As a control, the same RNA was also evaluated for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA transcripts using ^{32}P-labeled human GAPDH cDNA probe (pPB312). To examine the stability of transferred CFTR transcripts, modified T84 cells were incubated with actinomycin D (5 μg/ml, Calbiochem, San Diego, CA) for up to 24 h. CFTR mRNA transcripts were evaluated by Northern analysis as described above.

Immunoprecipitation

The CFTR protein in modified CFPAC-1 and T84 cells was evaluated by immunoprecipitation followed by phosphorylation using protein kinase and [γ-^{32}P]ATP essentially as described by Cheng *et al.* (35). Equal amounts of protein (determined with a Pierce protein assay reagent kit) were incubated with 1 μg of a mouse monoclonal anti-human CFTR antibody (M-13; Genzyme, Cambridge, MA) at 4°C for 2 h. *Staphylococcus aureus* cells (Pansorbin, Calbiochem) precoated with rabbit anti-mouse immunoglobulin G (Cappel, Durham, NC) were added and incubated at 4°C for an additional 1 h. Immunoprecipitated proteins were phosphorylated with 10 μCi of [γ-^{32}P]ATP [6000 Ci/mmol (NEN Research Products, Boston, MA)] plus 100 ng of the catalytic subunit of cAMP-dependent protein kinase (Sigma) at 30°C for 1 h. Protein samples were analyzed by 6%

sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by autoradiography.

Measurement of Cl^- efflux

The ability of the cells to secrete Cl^- in response to an elevation in cAMP was evaluated with a $^{36}Cl^-$ efflux assay (24,25). Briefly, CFPAC-1 or T84 cell monolayers in 35 mm culture plates were loaded with $^{36}Cl^-$ (2.5 μCi/ml; >5 mCi/g [NEN]; 2 h, 37°C), and then washed 6 times with 3 ml aliquots of Ringer's buffer to remove extracellular $^{36}Cl^-$. After washing, Cl^- efflux was measured every 15 to 60 s for 5 min by sequentially removing and replacing aliquots of the buffer with or without 20 μM forskolin. The amount of $^{36}Cl^-$ in the efflux aliquots and remaining in the cells at the end of the efflux period were quantified by liquid scintillation counting. The rate constant of chloride efflux (r) for each sampling interval was calculated as described by Venglarik *et al.* (36) using the following formula: $r = [\ln(R_1) - \ln(R_2)]/(t_1 - t_2)$, where R_1 and R_2 are the percent of $^{36}Cl^-$ counts remaining in the cells at time t_1 and t_2. The data were expressed as the rate constant of basal Cl^- efflux (r_{basal}) and of forskolin-stimulated Cl^- efflux (r_{forsk}) for each sampling interval. The ratio of forskolin-stimulated over basal efflux rates (r_{forsk}/r_{basal}) was calculated 2 to 3 min after addition of forskolin to determine the CAMP-stimulated Cl^- efflux from the intracellular component of the monolayer (36).

CFTR expression in the presence of PMA

To evaluate the effect of PMA on CFTR expression from the endogenous and exogenous CFTR genes, modified monoclonal T84 cells and parental T84 cells were exposed to various amounts of PMA for 12 h and to 100 nM PMA for up to 24 h. After incubation, total RNA was isolated and Northern analysis was carried out as described above. CFTR and GAPDH mRNA transcript levels were quantified by phosphor imaging (PhosphorImager, Molecular Dynamics, Sunnyvale, CA). To evaluate the effect of PMA on cAMP-regulatable Cl^- efflux, subconfluent parental and modified T84 cells were incubated alone or with 100 nM of PMA for 24 h, conditions in which the PMA-induced down-regulation of CFTR function in T84 cells is maximal (24).

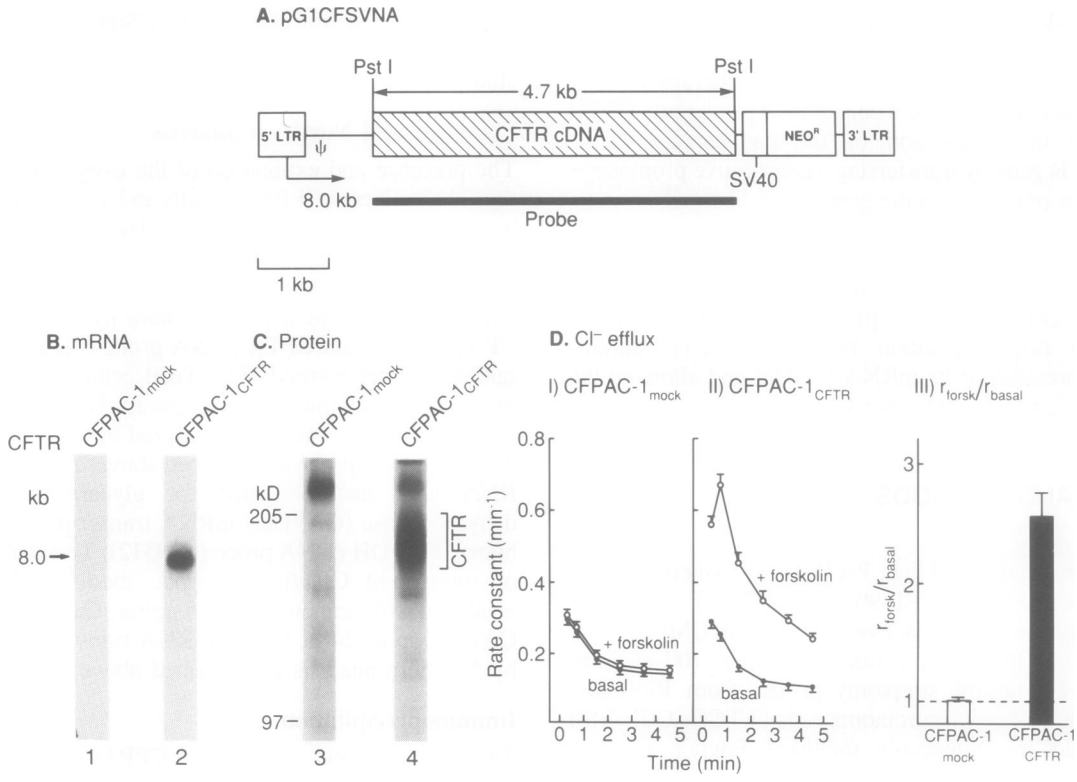

Figure 1. Retrovirus transfer of a constitutive promoter–human CFTR cDNA unit into the genome of CFPAC-1 cells. (**A**) Structure of the recombinant retrovirus vector, pG1CFSVNA. The vector, based on Moloney murine leukemia virus, consists of the 5′ long terminal repeat (LTR; used as the constitutive promoter for the CFTR cDNA), the packaging signal (ψ), CFTR cDNA, the SV40 early promoter (SV40), the neomycin resistance gene (Neo^R) and the 3′ LTR. Theoretically, transcription from the 5′ LTR promoter (arrow) should result in 8.0 kb CFTR mRNA transcripts. The restriction enzyme sites for *PstI* are indicated. The CFTR cDNA probe used in the DNA and RNA analyses is indicated below the vector. (**B**) Northern analysis demonstrating the expression of the transferred CFTR gene in retrovirus-modified CFPAC-1 cells. Total RNA (10 μg/lane) was evaluated with a ^{32}P-labeled CFTR probe. Lane 1: mock-infected CFPAC-1 clone ($CFPAC\text{-}1_{mock}$). Lane 2: RV_{CFTR}-infected CFPAC-1 clone ($CFPAC\text{-}1_{CFTR}$) showing 8.0 kb exogenous CFTR transcripts. (**C**) Immunoprecipitation demonstrating the transduced CFTR protein in retrovirus-modified CFPAC-1 cells. Proteins immunoprecipitated with an anti-CFTR antibody were incubated with protein kinase A and [γ-^{32}P]ATP. Lane 3: mock-infected CFPAC-1 clone. The endogenous CFTR protein is expressed at levels too low to be detected. Lane 4: RV_{CFTR}-infected CFPAC-1 clone showing 180 kDa CFTR protein. (**D**) Effect of forskolin on Cl^- efflux in retrovirus-modified CFPAC-1 cells. Time course of the basal (r_{basal}) and forskolin-stimulated (r_{forsk}) rate constants of Cl^- efflux in: I) mock-infected CFPAC-1 clones and II) RV_{CFTR}-infected CFPAC-1 clones. III) Ratio of forskolin-stimulated to basal rate constant (r_{forsk}/r_{basal}) of Cl^- efflux 2 to 3 min after addition of forskolin. Values are means ± SEM for mock-infected CFPAC-1 clones (n = 3) and RV_{CFTR}-infected CFPAC-1 clones (n = 4).

RESULTS

Evaluation of the normal CFTR cDNA transferred to CFPAC-1 cells

Of the five G418-resistant PA317 clones evaluated, three expressed 8.0 kb CFTR mRNA transcripts expected from the design of the vector. Each of these clones yielded virus titers of 10^3-10^4 colony forming units (cfu)/ml. One clone (titer 10^4 cfu/ml) which showed cAMP-regulated Cl^- secretion was used to infect CFPAC-1 cells. Following selection in G418-containing medium, six G418-resistant CFPAC-1 clones were isolated for further evaluation. Expression of the transduced CFTR gene was studied by Northern analysis using the CFTR probe shown in Figure 1A. As previously described (21,22), CFTR mRNA transcripts were not detected by Northern analysis, in parental CFPAC-1 cells (not shown) or in mock-infected CFPAC-1 clones (Figure 1B, lane 1). Four of six RV_{CFTR}-modified clones demonstrated MoMLV−LTR-directed CFTR transcripts with the expected size (8.0 kb); one clone is shown as an example (Figure 1B, lane 2). Expression of the transduced CFTR protein was evaluated using immunoprecipitation with an anti-CFTR antibody and phosphorylation by protein kinase A. No CFTR protein was detectable in mock-infected CFPAC-1 clones (Figure 1C, lane 3). CFTR protein with a size of approximately 180 kDa was detected in RV_{CFTR}-modified clones; one clone is shown as an example (Figure 1C, lane 4).

To confirm the physiological function of the CFTR protein directed by the CFTR cDNA transferred by the retrovirus, cAMP-stimulated Cl^- efflux was evaluated in modified CFPAC-1 cells (Figure 1D). The ratio of efflux rate constants (r_{forsk}/r_{basal}) was determined 2 to 3 min after addition of forskolin to eliminate the contribution of efflux from the extracellular compartment which occurs during the first min of efflux (36). As expected, mock-infected CFPAC-1 clones did not show forskolin-stimulated Cl^- efflux (r_{forsk}/r_{basal} = 1.01 ± 0.02, n = 3). In contrast, RV_{CFTR}-modified CFPAC-1 clones demonstrated forskolin-stimulated Cl^- permeability (r_{forsk}/r_{basal} = 2.52 ± 0.19, n = 4), indicating that the retrovirus-directed CFTR product functions to establish cAMP-regulated Cl^- permeability in CF epithelial cells. This is consistent with the observation of Drumm *et al.* (20) for retrovirus-mediated transfer of the normal CFTR cDNA into CFPAC-1 cells, and confirmed that the retrovirus promoter−CFTR cDNA unit functioned in a normal fashion.

Evaluation of T84 cells modified with the recombinant retrovirus

After infection with RV_{CFTR} or RV_{mock}, retrovirus-modified clones of T84 cells were isolated in G418-containing media. As an example, with *Pst*I digestion of genomic DNA and Southern hybridization, endogenous CFTR signals were observed in parental, RV_{mock}- or RV_{CFTR}-modified T84 cells. However, a 4.7 kb DNA band derived from the exogenous CFTR gene was detected in only RV_{CFTR}-modified T84 cells, thus confirming integration of the retroviral vector DNA (Figure 2A). Transcription from RV_{CFTR} should produce a CFTR mRNA transcript of 8.0 kb (Figure 1A). Of fifteen clones of RV_{CFTR}-modified T84 cells, Northern analysis demonstrated expression of the transferred exogenous (8.0 kb) CFTR transcripts in three clones ($T84_{CFTR}$) in addition to the endogenous (6.5 kb) CFTR transcripts. In contrast, parental and mock-infected T84 ($T84_{mock}$) cells expressed only the endogenous CFTR transcripts (Figure 2B).

Stability of the endogenous and exogenous CFTR mRNA transcripts was evaluated in modified T84 cells in the absence of PMA using actinomycin D (data not shown). The half-life of the exogenous CFTR transcripts was 12.2 ± 1.1 h (mean ± SEM, n = 3), similar to that of endogenous CFTR transcripts, 11.9 ± 1.6 h (mean ± SEM, n = 3; $p > 0.1$, two-tailed Student's t test).

Evaluation of modulation of endogenous and exogenous CFTR gene expression by PMA

As has been observed previously for T84 cells (24), exposure of parental or mock-infected T84 cells to 100 nM of PMA for

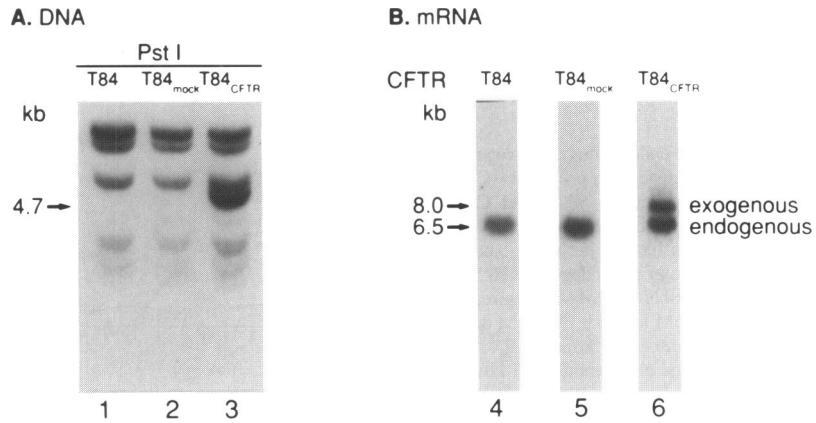

Figure 2. Presence and expression of the exogenous promoter−CFTR cDNA unit in T84 cells modified by the retrovirus vector, pG1CFSVNA. (**A**) Southern analysis demonstrating integration of the exogenous promoter−CFTR cDNA unit into the genome of T84 cells. Genomic DNA (20 μg) was digested with *Pst*I and hybridized with the ^{32}P-labeled CFTR probe. Lane 1: parental T84 cells; lane 2: mock-infected T84 cells ($T84_{mock}$); and lane 3: RV_{CFTR}-modified T84 cells ($T84_{CFTR}$) demonstrating an additional 4.7 kb band corresponding to the retroviral construct. (**B**) Northern analysis demonstrating expression of the endogenous CFTR gene as well as the exogenous transferred CFTR cDNA. Total RNA (10 μg/lane) were analyzed with a ^{32}P-labeled CFTR probe. Lane 4: parental T84 cells show the endogenous (6.5 kb) CFTR mRNA transcripts; lane 5: mock-infected T84 cells ($T84_{mock}$) show the endogenous CFTR transcripts; and lane 6: RV_{CFTR}-modified T84 cells ($T84_{CFTR}$) demonstrate the exogenous (8.0 kb) CFTR mRNA transcripts in addition to the endogenous 6.5 kb transcripts.

12 h caused down-regulation of the endogenous 6.5 kb CFTR transcripts (Figure 3A, lanes 1–4). Likewise, in $T84_{CFTR}$ cells, PMA caused a decrease in the endogenous CFTR transcript level (lanes 5–6). In marked contrast, the level of the 8.0 kb CFTR transcripts expressed by the LTR–CFTR cDNA unit inserted into the genome by retrovirus transfer did not change after addition of PMA (lanes 5–6). GAPDH mRNA transcript levels were not modified by PMA in any of the cell lines.

In $T84_{CFTR}$ cells, quantitative analysis demonstrated that PMA concentrations of 10 nM and above decreased the endogenous CFTR transcript levels after 12 h (Figure 3B), in a fashion similar to the down-regulation of CFTR transcripts by PMA in parental T84 cells as observed previously (24). In contrast, the exogenous CFTR transcript levels did not change with any PMA concentration evaluated. Following exposure to a fixed amount of PMA, the endogenous CFTR mRNA transcript levels declined to its nadir after 8 to 12 h and rose slowly thereafter (Figure 3C). In contrast, the exogenous CFTR transcript levels did not change during this 24 h period.

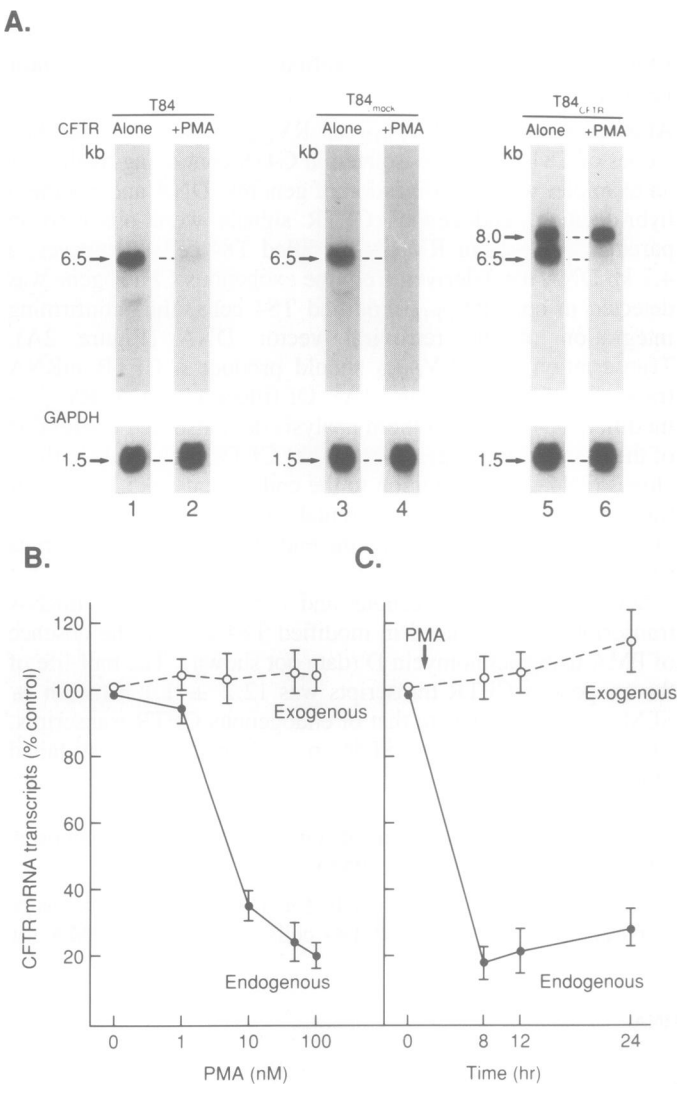

Figure 3. Effect of PMA on the expression of the endogenous and exogenous CFTR genes in T84 cells modified by retrovirus transfer of a promoter–CFTR cDNA unit. (**A**) Northern analysis. Top: analysis with a ^{32}P-labeled CFTR probe. Bottom: analysis with a ^{32}P-labeled GAPDH probe. Lane 1: parental T84 cells before PMA exposure; and lane 2: similar to lane 1, but after exposure to 100 nM PMA for 12 h. Note the down-regulation of the endogenous (6.5 kb) CFTR transcripts. Lane 3: mock-infected T84 cells before PMA exposure; and lane 4: similar to lane 3, but after exposure to 100 nM for 12 h. Note the down-regulation of the endogenous (6.5 kb) CFTR transcripts. Lane 5: RV_{CFTR}-modified T84 cells before PMA exposure. Note the endogenous (6.5 kb) and exogenous (8.0 kb) CFTR mRNA transcripts. Lane 6: same as lane 5, but after exposure to 100 nM PMA for 12 h. Note the down-regulation of the endogenous (6.5 kb) CFTR transcripts, but not the exogenous (8.0 kb) mRNA transcripts. The levels of GAPDH mRNA (1.5 kb) transcripts were not affected by PMA in either cell line. (**B**) Dose-dependent effect of PMA on the endogenous (●) but not exogenous (○) CFTR mRNA transcript levels in $T84_{CFTR}$ cells. The $T84_{CFTR}$ cells were exposed to a various concentrations of PMA for 12 h. (**C**) Time course of the endogenous (●) and exogenous (○) CFTR mRNA transcript levels in $T84_{CFTR}$ cells after exposure to 100 nM of PMA for up to 24 h. For panels B and C, the endogenous and exogenous CFTR mRNA transcript levels were quantified by Northern analysis and phosphor imaging.

Effect of PMA on functional CFTR

In parental T84 cells, addition of forskolin caused a rapid increase in the rate constant (r_{forsk}) of Cl^- efflux that gradually decreased, probably due to depletion of loaded $^{36}Cl^-$ from the responsive cells (Figure 4). In the absence of forskolin, the basal rate constants (r_{basal}) of Cl^- efflux were greater during the initial

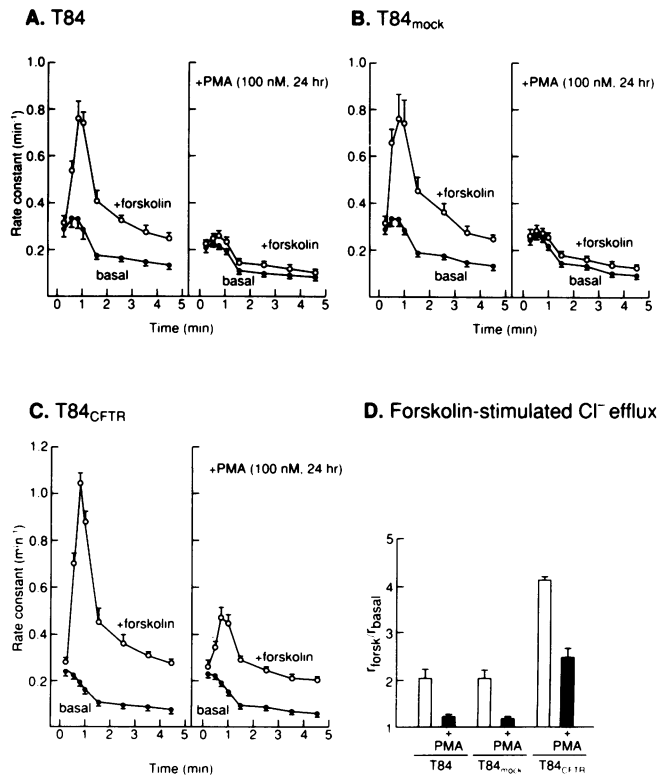

Figure 4. Effect of PMA on forskolin-stimulated $^{36}Cl^-$ efflux in parental and retrovirus-modified T84 cells. Panels A–C. Time course of the basal (r_{basal}) and forskolin-stimulated (r_{forsk}) rate constants of Cl^- efflux. Values are means ± SEM of four experiments in each group. (**A**) Parental T84 cells incubated with or without 100 nM of PMA for 24 h. (**B**) Mock-infected T84 cells incubated with or without 100 nM of PMA for 24 h. (**C**) RV_{CFTR}-modified T84 cells incubated with or without 100 nM of PMA for 24 h. (**D**) Ratio of forskolin-stimulated over basal rate constants (r_{forsk}/r_{basal}) of Cl^- efflux 2 to 3 min after addition of forskolin. Values are means ± SEM, n = 4 in each group.

1 min than those after 2 min, suggesting that the initial increase in the basal efflux rate, and also in the forskolin-stimulated efflux rate, might reflect, as postulated by Venglarik *et al.* (36), a rapid depletion of extracellular $^{36}Cl^-$ (panel A). To evaluate the intracellular forskolin-stimulated efflux, we calculated the ratio of forskolin-stimulated to basal efflux rates (r_{forsk}/r_{basal}) 2 to 3 min after forskolin addition; the basal rate constant was relatively constant after 2 min, suggesting that little extracellular $^{36}Cl^-$ remained after 2 min (panel D). Addition of forskolin increased the Cl^- efflux rate (r_{forsk}/r_{basal} 2 to 3 min after forskolin addition) 2-fold in parental T84 cells (r_{forsk}/r_{basal} = 2.02 ± 0.20, n = 4). As described previously (24), continuous exposure to 100 Nm PMA for 24 h reduced the forskolin-stimulated Cl^- secretion (r_{forsk}/r_{basal} = 1.24 ± 0.05, n = 4; $p < 0.01$).

PMA exposure produced a similar reduction in the cAMP-regulated Cl^- secretion in mock-infected T84 cells (panels B and D). Interestingly, forskolin elicited a greater increase in the efflux rate from $T84_{CFTR}$ cells (r_{forsk}/r_{basal} = 4.14 ± 0.08, n = 4) than in parental T84 cells ($p < 0.01$), suggesting that higher expression of the CFTR gene within $T84_{CFTR}$ cells conveyed to them higher cAMP-regulated Cl^- permeability (panels C and D). In $T84_{CFTR}$ cells, PMA exposure reduced forskolin-stimulated Cl^- efflux by 38% (r_{forsk}/r_{basal} = 2.56 ± 0.21, n = 4; $p < 0.01$), probably due to a decrease in the amount of endogenous CFTR. However, a 2.6-fold increase in the Cl^- efflux rate by 2 to 3 min after forskolin stimulation compared well with the 2.0-fold increase in parental T84 cells in the absence of PMA, suggesting RV_{CFTR}-modified T84 cells could maintain CFTR function after PMA exposure.

DISCUSSION

Successful gene therapy for the respiratory manifestations of CF requires the ability to transfer the normal CFTR coding sequence to the airways in a fashion that will permit restoration of CFTR function to the airway epithelium. The present study focuses on one of the many obstacles to achieving this goal—the design of a CFTR gene expression cassette that is resistant to down-regulation by signals from the intense, chronic inflammatory milieu that characterizes the airway epithelial surface from an early age (27,37,38). In this regard, inflammatory signals are known to induce down-regulation of CFTR gene expression at the transcriptional level and to induce the 'CF Cl^- secretory phenotype' in epithelial cells that otherwise have normal CFTR function (24). Even though use of the CFTR promoter would convey appropriate regulation to the transferred gene (a stratagy that has not been proposed for human gene therapy), this would still present a problem for gene therapy for CF, since ever present inflammation on the airway epithelial surface has the potential to down-regulate the expression of the newly transferred CFTR gene unless the promoter−CFTR gene expression cassette is designed in a fashion that makes the newly transferred CFTR gene 'inflammatory stimuli-resistant'. The data in the present study demonstrate that transfer of a CFTR gene expression cassette that includes the MoMLV−LTR as the promoter driving the normal CFTR cDNA to the genome of human epithelial cells conveys to these cells the ability to maintain a normal CFTR phenotype in the presence of phorbol esters, despite the down-regulation of the endogenous CFTR, and presumably any construct using the CFTR promoter, by the inflammatory signal.

Susceptibility of normal CFTR gene expression to modulation by inflammatory stimuli

There is ample evidence that the airway epithelial surface in CF is chronically exposed to a neutrophil-dominated inflammatory process. In this context, analysis of airway epithelial fluid obtained from individuals with CF shows neutrophil-dominated inflammation as early as 1 year of life, and is universal by ages of 5 to 10 (27). Further, CF epithelial fluid contains a variety of inflammatory mediators including neutrophil elastase (37), interleukin-8 (38,39) and interleukin-1β (40). There are at least two examples of model inflammatory stimuli that will down-regulate the CFTR gene at the transcriptional level.

First, exposure of human epithelial cells for 18 to 24 h with PMA, a model inflammatory stimulus that activates protein kinase C, reduces the amount of CFTR mRNA and protein, with concomitant loss of the ability of the cells to exhibit cAMP-stimulated Cl^- secretion (24). This phenomenon does not appear to be related to the ability of PMA to inhibit cAMP-stimulated Cl^- secretion in epithelial cells via inhibition of basolateral K^+ channels, a short term (1 to 2 h) process that is not associated with a change in the amount of CFTR protein (41). Recently, Breuer *et al.* (28) have shown that PMA stimulates degradation of the CFTR protein in the HT29 colon carcinoma cell line. Thus, at least for PMA-type stimuli, both down-regulation of transcription of the CFTR gene and increased degradation of the CFTR protein are likely important.

Second, agents that increase intracellular Ca^{2+} levels have effects similar to PMA, with suppression of CFTR gene transcription and concomitant reduction of CFTR mRNA levels and CFTR protein (25). Thus, it is likely that mediators that act via a protein kinase C pathway or cause an increase in intracellular Ca^{2+} levels will have similar effects—suppression of CFTR gene expression. One possible mechanism by which PMA down-regulates transcription of the endogenous CFTR gene is through AP-1 or AP-2 binding elements in the CFTR 5′-flanking region (42).

In the context that CFTR gene expression is down-regulated by inflammatory stimuli *in vitro* and that there is intense chronic inflammation present on the airway epithelial surface of individuals with CF, it is reasonable to conclude (but not directly proven) that expression of a newly transferred CFTR gene could be down-regulated *in vivo*. In that regard, it is reasonable to hypothesize that a CFTR gene expression cassette used for gene therapy for CF should include a promoter, although not necessarily of viral origin, that will be resistant to such stimuli.

Consequences of addition of a viral promoter−CFTR cDNA to the genome of human epithelial cells

Analysis of mRNA transcript levels clearly demonstrated that exposure of the cells to PMA caused a dose- and time-dependent decrease in the CFTR mRNA transcripts from the endogenous, but not the exogenous, CFTR genes. Concomitant with this, cells containing the exogenous CFTR expression cassette maintained the ability to secrete Cl^- in response to cAMP despite exposure of the cells to PMA. Interestingly, these modified cells had a higher Cl^- secretory response to cAMP than the parental (or control modified) cells; a similar result was observed by Anderson *et al.* (43). This was presumably caused by the additional density of CFTR protein on the epithelial apical surface from expression of the newly transferred CFTR cDNA.

Choice of an 'inflammation-resistant' promoter for gene therapy for CF

The use of constitutive promoters for gene therapy for CF is based on the concept that sufficient expression is needed to correct the defect in cAMP-mediated Cl^- secretion that characterizes the CF-phenotype at the biologic level, and that over-expression is not detrimental to the epithelial cells (44). Use of constitutive promoters must be done with caution, however, as the normal CFTR promoter can be up- and down-regulated (24,25), and it is possible such regulation is important *in vivo*.

The choice of the MoMLV−LTR was based on studies by Speck *et al.* (45) and Elsholtz *et al.* (46) demonstrating that phorbol ester causes an increase in expression from MoMLV promoter; there is no evidence that PMA causes a decrease in expression from this promoter. Interestingly, the MoMLV−LTR contains two putative AP-1 binding elements (analyzed using the GenBank DNA database), but our data suggest that this constitutive promoter/enhancer (at least in T84 cells) is resistant to signals induced by PMA. It is likely that a variety of constitutive promoters could be used in its place, such as the adenovirus major late promoter−tripartite leader or the adenovirus E1a promoter (21,47). However, even viral 'constitutive' promoters will have to be specifically evaluated for their 'inflammation resistant' properties.

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