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RESEARCH ARTICLE

Optimisation of real-time quantitative RT-PCR for the evaluation of non-viral mediated gene transfer to the airways

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Naked plasmid DNA and DNA/liposome complexes are currently being considered as gene therapy treatments for cystic fibrosis (CF) pulmonary disease. Current methods of gene delivery to the airways result only in transient correction of the CF ion transport defect, and disease treatment is likely to require repeated administrations of vector. However, it is unclear if repeat administration will be tolerated by CF individuals. Technologies including TaqMan (Applied Biosystems) real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) can be used to determine the efficacy of gene transfer formulations. TaqMan RT-PCR assays were designed and optimised to detect plasmid vector-derived and endogenous gene expression. Subsequently, these assays were used to quantify vector-derived mRNA after delivery of naked DNA and DNA/liposome formulations expressing human and murine cystic fibrosis transmembrane conductance regulator (CFTR) to the mouse airways. Vector-derived mRNA was detected in samples following the delivery of naked DNA or DNA/liposomes to the mouse airways, and no reduction in vector-derived mRNA was observed upon repeat administration, a finding that is consistent with the murine and human CFTR being tolerated by the mouse. Although it remains to be seen if CF patients can tolerate long-term expression of wild-type CFTR, these data demonstrate that TaqMan RT-PCR is an effective tool to accurately quantify transgene expression in the airways.
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Introduction

Cystic fibrosis (CF) is a lethal autosomal recessive disease that affects children and young adults, and typically causes death in early adult life. CF is characterised by abnormal epithelial chloride and other ion conductances (reviewed Ref. 1) with subjects suffering from pulmonary, intestinal, pancreatic and other tissue disease. Pulmonary disease is the primary cause of CF morbidity and mortality. Although it is currently unclear how pulmonary disease develops, it is generally thought that altered mucus and/or mucociliary clearance associated with CF airways permits infection by pathogenic bacteria, including *Pseudomonas aeruginosa*.[2] The gene mutated in CF individuals encodes the cystic fibrosis transmembrane conductance regulator (CFTR) protein, which is a member of the ATP-binding-cassette (ABC) transporter family[3] and functions as a cAMP-dependent epithelial chloride channel.[2] The murine *cftr* gene (mCFTR) has also been cloned,[4,5] and the mCFTR protein, which is 78% identical to human CFTR,[4,5] also functions as a cAMP-dependent epithelial chloride channel.[6]

A proposed treatment for CF pulmonary disease is gene augmentation therapy, where the CFTR gene is introduced into the airways of CF individuals. Several aspects of CF pulmonary disease suggest that it may be a good candidate to be treated with gene therapy. Firstly, the air-borne, topical delivery of gene therapy vectors is possible using aerosolisation, and secondly, the expression of only modest amounts of CFTR may alleviate pulmonary disease. Only 6–10% of the cells comprising a polarised CF epithelial monolayer need to express CFTR to correct the CF cAMP-dependent chloride conductance defect.[7] In addition, individuals that express as little as ~10% functional CFTR do not develop CF pulmonary disease.[8] Together, these data support the notion that the expression of modest amounts of CFTR in appropriate target lung cells would be sufficient to alleviate the CF cAMP-dependent chloride conductance defect.

Several viral and non-viral gene transfer vectors have been tested in clinical studies with CF individuals. Recombinant adenoviral and adenoviral-associated virus (AAV) vectors have successfully expressed CFTR in the airway surface epithelia, although concerns over inflammatory and immune responses against these vectors, particularly upon repeat administration, may ultimately preclude their use in the clinic.[9,10] Non-viral vectors are potentially less inflammatory and immunogenic

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due to the absence of viral coat proteins and endogenous viral protein expression. In our clinical studies we have investigated single and multiple doses of a non-viral (DNA/liposome) vector and have shown that efficacy is similar on repeated administration.[11,12]

Regardless of the gene therapy vector system used, the expression of transgenes in the airway following gene transfer is typically transient, with peak expression detected several days following vector delivery.[9,10] In animal models, the loss of transgene expression does not appear to correlate with the loss of vector DNA in the lung, at least in the case of recombinant adenoviral[13] and plasmid DNA vectors.[14] Loss of expression may be due to the inactivation of the transgene promoter through histone deacetylation and/or *de novo* methylation.[15,16] Encouragingly, longer-term transgene expression in the airways may be achieved by the judicious use of transcription signals.[14,17]

Transient expression may also result from immune responses directed against the transgene product, which is a potential antigen, if not endogenous to the host. Given the transient nature of transgene expression, multiple deliveries of vector are likely to be required for long-term transgene expression in target tissues. Although DNA/liposome formulations have successfully directed the expression of CFTR upon repeat administration to the airways of CF transgenic mice[18] and CF subjects,[12] under certain circumstances the efficacy of DNA/liposome formulations may be reduced upon repeat administration. The level of bacterial reporter gene expression detected in the mouse lung following repeated administration of plasmid DNA complexed with the cationic lipid GL-67 was reduced in comparison to a single administration, due to immune responses directed against the transgene product.[19] Thus it remains a formal possibility that CF individuals could be immunologically intolerant of normal CFTR resulting in reduced efficacy upon repeated administration of gene transfer vectors.

The efficacy of gene transfer formulations following vector administration can be determined by measuring the level of transgene mRNA expressed, using reverse transcription-polymerase chain reaction (RT-PCR) assays. However, the large amounts of plasmid DNA used in non-viral formulations necessitates the development of assays which are sensitive enough to detect very low levels of mRNA, whilst at the same time being insensitive to plasmid DNA contamination. An innovative approach to quantifying (RT)-PCR products utilising the 5′ nuclease assay[20] has been developed. During PCR, *Taq* polymerase cleaves a probe labelled with two fluorescent dyes, which are released into solution, and the resulting fluorescence is recorded in real-time using an ABI PRISM 7700 TaqMan sequence detector (Applied Biosystems, Warrington, UK).[20–22] Quantification is achieved by determining when the observed fluorescence first reaches a designated threshold (C_T), which is directly proportional to the number of target copies present in the starting sample.[23] The C_T value is therefore a quantitative measurement of the amount of target in a sample.

Previously, we have used TaqMan technology to quantify vector DNA and vector-specific mRNA in clinical samples following gene transfer.[12] In the current study we used TaqMan technology to quantify vector-specific mRNA after delivery of naked DNA and DNA/liposome formulations expressing human and murine CFTR to the mouse airways. A reduction in vector-derived mRNA upon repeat administration would be consistent with immune responses destroying cells that express the CFTR transgene product, and might suggest that long-term gene augmentation therapy in CF subjects would be limited by similar responses.

Results

Detection of pCI vector-derived mRNA

A TaqMan RT-PCR assay was designed to quantify transgene mRNA expressed from non-viral vectors based on plasmid pCI in which transgene expression is directed by the immediate–early CMV enhancer/promoter. All pCI-derived vectors contain a recombinant (hybrid) intron juxtaposed between the promoter and transgene, and the assay was designed to quantify mRNA produced from any pCI-derived plasmid that maintained this intron (pCI RT-PCR). Figure 1 shows the positions of the TaqMan primers and probe, which hybridise to sequences flanking the hybrid intron splice junction in order to minimise the detection of contaminating plasmid DNA. Derivatives of plasmid pCI expressing a variety of transgenes were constructed, including human and murine CFTR (pCIKCFTR and pCIKmCFTR, respectively). Fluorescence microscopy[24] was used to demonstrate the expression of functional human and murine CFTR from these plasmids (data not shown). Plasmid pCIKCFTR was used to transiently transfect HEK293T cells, and total RNA was prepared and treated with DNaseI to eliminate plasmid and genomic DNA contamination. The sensitivity and dynamic range of the TaqMan pCI RT-PCR assay was examined in one-step reactions (RT and PCR performed in the same tube) containing between 0.64 and 2000 pg of treated total RNA. Quantification using this assay was linear over the range of RNA dilutions tested (Figure 2). Similarly, the one-step pCI RT-PCR assay readily detected *in vitro* synthesised RNA molecules containing the pCI RT-PCR assay target sequence. Quantification of pCI target RNA molecules with the one-step pCI RT-PCR assay was linear between 500 and 10^7 RNA molecules (Figure 2). The use of such synthetic target molecules allows the absolute quantification of pCI mRNA levels and thus they were used as reference standards in the following studies.

Optimisation of pCI vector-derived mRNA detection in murine lung samples

Whereas the detection of vector-specific mRNA from cells grown in culture is facile, the detection of the lower levels often present in pre-clinical or clinical samples is more difficult, requiring further optimisation to maximise assay sensitivity. Thus the assay conditions required for the optimal quantification of pCI vector-derived mRNA in total RNA extracted from the murine lung and trachea were investigated. Firstly the maximal amount of total RNA that could be added to the TaqMan one-step pCI RT-PCR assay was determined. For assays containing 100 ng total RNA (in a final volume of 50 μl) there was a linear relationship between the logarithmic amount of input target RNA and the C_T value as long as reactions contained $\geq 10^4$ target mRNA molecules (Figure 3). In reactions containing $\leq 10^4$ target molecules, the relationship between input target RNA and C_T was not linear

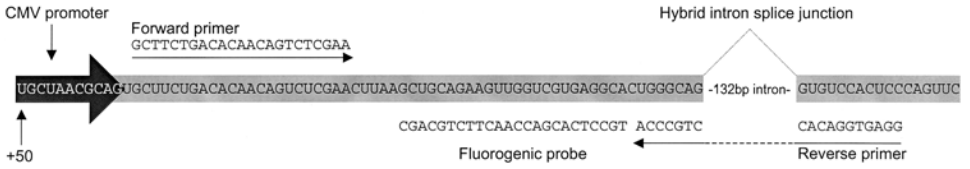

Figure 1 *Schematic of TaqMan probe and primer positions within plasmid pCI. Boxed sequence (shaded) indicates the transcribed mRNA from the pCI vector sequence (GenBank accession number U47119) starting 50 bases beyond the transcription start site (position 732 of pCI). The arrow indicates the 3′ end of the CMV enhancer/promoter. Primers and fluorogenic probe sequences for the pCI RT-PCR assay are shown relative to their homology to the pCI vector sequence.*

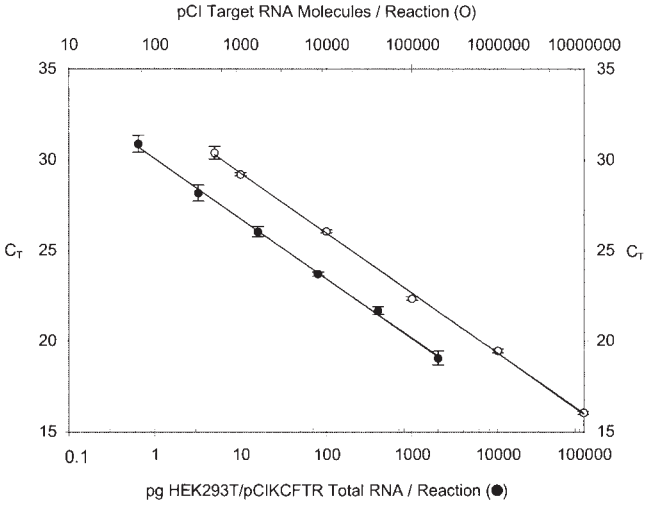

Figure 2 *Detection of pCI-derived mRNA. The efficiency of the one-step pCI RT-PCR assay to detect pCI-derived mRNA was evaluated using total RNA isolated from HEK293T cells transfected with pCIKCFTR (filled circles) and pCI synthetic target RNA molecules (open circles). The relationship between C_T and the amount of target RNA is indicated using a double x-axis plot. Assay linearity was observed between 640 fg and 2 ng of HK293T/pCIKCFTR total RNA and between 10^7 and 500 pCI synthetic target RNA molecules. Assays were performed in triplicate, mean values and s.e.m. are indicated.*

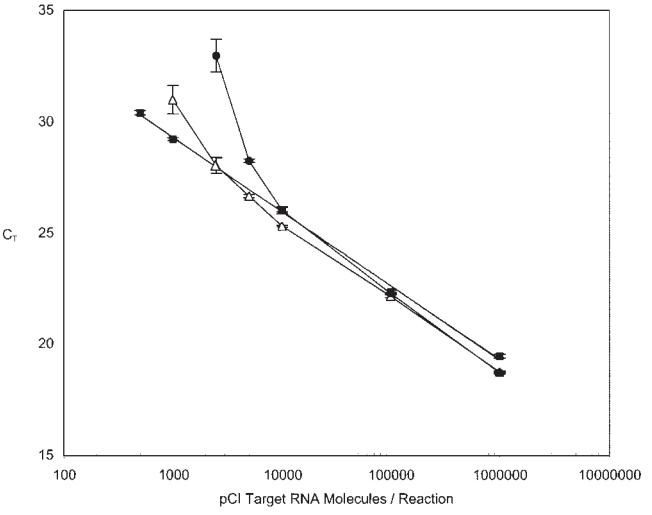

Figure 3 *Detection of pCI-derived mRNA in total lung RNA. The effect of mouse lung and trachea total RNA on the efficiency of the pCI RT-PCR assay was evaluated by diluting total RNA isolated from HEK293T cells transfected with pCIKCFTR with naive mouse lung and trachea total RNA. HEK293T cells were transfected using DC-Chol/DOPE liposomes. Between 10^3 and 10^6 molecules of pCI-derived mRNA, in water (filled squares) or either 100 ng (open triangles), or 500 ng (filled circles) mouse lung and trachea total RNA, were assayed using the one-step pCI RT-PCR assay. Amount of target RNA assayed is plotted against C_T. Assays were performed in triplicate, mean values and s.e.m. are indicated.*

(C_T was increased) and quantification was unreliable. As the amount of total RNA per reaction was increased to 500 ng, this inhibitory phenomenon was more pronounced (Figure 3).

The pCI RT-PCR one-step assay readily detected pCI vector-derived mRNA in total RNA isolated from the airways of mice dosed with DNA/liposomes (Figure 4a). However, control assays in which reverse-transcriptase was omitted (−RT), showed that the contribution of plasmid DNA to this signal was large (up to 10%) (Figure 4a), despite the assay being designed to span an intron–exon boundary (Figure 1). Interestingly, the $\triangle R_n$ (an indication of PCR product accumulation) after 40 cycles in the RT-PCR assays was approximately 50% lower than in the −RT control assays, suggesting that the accumulation of PCR product was being inhibited. Thus, detection of vector-specific mRNA in mouse airways was possible, although further optimisation was required.

To increase the sensitivity of the assay, subsequent experiments aimed to reduce the levels of contaminating DNA and to enhance the detection of specific mRNA. Total RNA isolated from mice treated with DNA/liposome complexes was treated with DNaseI and amplified in the pCI RT-PCR assay. Treatment with DNaseI effectively removed contaminating DNA from total RNA samples, such that no amplification was observed in the absence of reverse transcriptase (Figure 4b). However, DNaseI treatment also tended to result in an increased C_T (decreased assay sensitivity) and decreased $\triangle R_n$ (decreased PCR product accumulation) (compare with Figure 4a). Surprisingly, total RNA samples subjected to the DNaseI treatment temperature profile (37°C for 30 min and 75°C for 5 min) in the absence of DNaseI had both a robust $\triangle R_n$ and the lowest C_T of all samples assayed (Figure 4b). Thus the heating of total RNA appeared to specifically enhance the detection of pCI-derived mRNA, without a pronounced effect on the detection of contaminating DNA.

To investigate the effect of heating on the detection of pCI-derived mRNA, total RNA samples from a group of DNA/liposome treated mice were subjected to various heating regimens (Figure 4c) and then assayed using the pCI RT-PCR assay. All heated samples had decreased C_T and increased $\triangle R_n$ values compared with the unheated sample (Figure 4c). Heating the total RNA at 75°C for 5 min, increased detection of pCI vector-derived mRNA approximately four-fold compared with no heat treatment ($P = 0.0431$; $n = 5$). Interestingly, detection of the pCI-derived mRNA species was enhanced regardless of

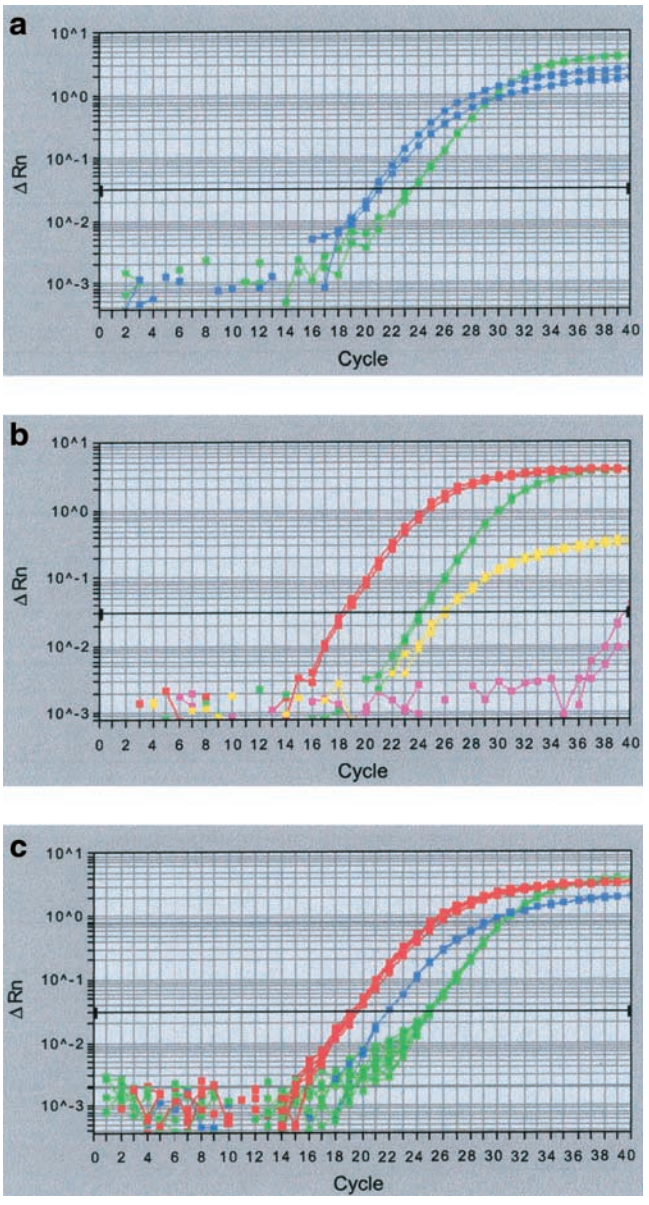

Figure 4 *Detection of pCI-derived mRNA in total lung RNA is enhanced by heating. Plot of PCR cycle number against $\triangle R_n$ (change in TaqMan probe derived fluorescence) for total RNA isolated from a DNA/liposome treated mouse amplified using the one-step pCI RT-PCR assay (a) Cycle number against $\triangle R_n$ for a representative treated mouse sample; +RT (blue) and −RT (green) assays are illustrated. (b) As panel (a), following DNaseI treatment of the same total RNA sample; +RT (yellow) and −RT (pink) assays are shown. The same sample in the absence of DNaseI was incubated in parallel with the DNaseI treated sample; +RT (red) and −RT (green) assays are also shown. (c) Samples heated at (i) 37°C for 30 min and then 75°C for 5 min; (ii) 37°C for 30 min; (iii) 75°C for 5 min; or (iv) 75°C for 15 min and amplified in RT-PCR assays are all shown in red. A sample that was incubated on ice for 35 min and amplified in RT-PCR assays is shown in blue. All samples were also assayed −RT (green). Reactions contained 250 μg total mouse lung and trachea RNA and were assayed in 25 μl in duplicate.*

the heating strategy adopted (Figure 4c). The contribution of contaminating DNA, as measured in −RT assays, was unaltered by the heating process and amounted to a negligible amount (1.17 ± 0.03%; $n = 5$); consequently, it was ignored for ease of calculation throughout the study. The detection of endogenous murine CFTR mRNA and other RNA species in appropriate TaqMan RT-PCR assays was essentially unaffected by heating (data not shown). As heating improved the detection of pCI-derived mRNA and had no deleterious effects on detection in other assays, all subsequent TaqMan RT-PCR assays utilised total RNA that had been heated to 75°C for 5 min.

Detection of pCI vector-derived mRNA in naked DNA treated mice

Following quantification of pCI-derived mRNA in mice treated with DNA/liposomes, the conditions required for the detection of the target mRNA in mice treated with naked DNA were investigated. Reliable quantification of pCI-derived mRNA from such mice required a further increase in assay sensitivity due to the reduced level of transgene expression in comparison to DNA/liposomes.[19] Although the one-step assay described above could readily detect pCI-derived mRNA in mice treated with naked DNA (data not shown), samples typically contained $\leq 10^4$ target molecules, and consequently could not be quantified accurately. However, the linear range of a two-step pCI RT-PCR assay (RT and PCR steps performed separately) was determined to be between 32 and $\geq 3.2 \times 10^5$ target RNA molecules (Figure 5). This increase in assay sensitivity over the one-step approach made the two-step approach the preferred method for quantifying pCI-derived mRNA in samples containing low abundance of targets. Detection of very low quantities (≤80) of pCI-derived mRNA was occasionally problematic using the two-step approach, with 20% of samples in this range failing to amplify. This phenomenon is reflected in the increased errors associated with target detection in such samples. These observations are consistent with the reported efficiency and sensitivity of similar RT reactions.[25]

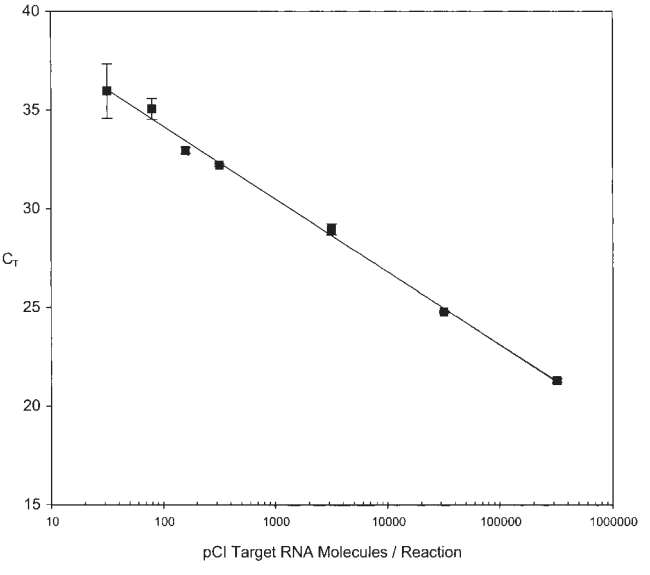

Figure 5 *Detection of pCIKCFTR-derived mRNA in lung total RNA is enhanced using a two-step RT-PCR assay. The TaqMan two-step pCI RT-PCR was used to assay total RNA extracted from the lungs and trachea of a DNA/liposome treated mouse diluted with total RNA isolated from naive mice. Amount of target RNA assayed is plotted against C_T. Assays were performed in triplicate, mean values and s.e.m. are indicated.*

Detection of endogenous mCFTR mRNA

To control for possible variability in RNA sample loading, the RNA expression levels from a second 'normalising' gene were routinely analysed in parallel. Endogenous mCFTR mRNA was chosen since it is expressed in the airway,[26] and a ratio of vector-derived to endogenous mRNA levels has been accepted as an appropriate surrogate end point marker in CF gene therapy clinical trials.[12,27] Thus a TaqMan assay was designed to detect endogenous mCFTR (EmCFTR) mRNA. In comparison to the one-step pCI RT-PCR assay, detection by the one-step EmCFTR RT-PCR assay was less sensitive to inhibition in the context of high total RNA concentrations. Detection of between 10^4 and 10^6 molecules of EmCFTR synthetic target RNA (in up to 500 ng total RNA) was linear (data not shown). The EmCFTR assay was designed to be insensitive to mCFTR sequences within the pCIKmCFTR vector, and the detection of endogenous mCFTR mRNA was not affected by the presence of an excess of pCIKmCFTR transcripts (data not shown). The suitability of endogenous mCFTR mRNA to normalise for RNA loading was also confirmed by showing that expression of the mCFTR gene did not vary across the experimental conditions studied (data not shown).

Single administrations of murine and human CFTR expression vectors to the mouse airway

Following the elucidation of suitable RT-PCR assay conditions, the levels of pCI vector-derived mRNA expressed from plasmids pCIKmCFTR or pCIKCFTR in the murine lung and trachea were investigated following a single administration of naked DNA or DNA/liposome complexes. Total RNA was extracted from lungs and tracheae 2 days after vector delivery and assayed using the TaqMan pCI and EmCFTR RT-PCR assays (Figure 6). As expected, DNA/liposome delivery resulted in far higher vector-specific mRNA levels than naked DNA delivery ($P = 0.0090$ for both vectors). Expression directed by pCIKmCFTR or pCIKCFTR following naked DNA administration was modest (0.08 and 0.12% of endogenous mCFTR mRNA, respectively). However, pCIKmCFTR and pCIKCFTR liposome complexes resulted in expression 333% and 1874% of endogenous mCFTR mRNA levels, respectively, far exceeding the theoretical 5% of endogenous mCFTR level predicted by some investigators to be therapeutic.[7,28]

Repeat administrations of murine and human CFTR expression vectors to the mouse airway

Mice were treated with two doses of pCIKmCFTR or two doses of pCIKCFTR, separated by a period of 42 days. Vectors were delivered either as naked DNA or DNA/liposomes. Total RNA was extracted from mouse lungs and tracheae 2 days after the final vector administration, and RNA levels quantified using TaqMan pCI and EmCFTR RT-PCR assays. There was no statistical difference between normalised vector-specific mRNA levels from pCIKmCFTR ($P = 0.4647$; Figure 7a) or pCIKCFTR ($P = 0.9168$; Figure 7b) following a single or repeat administration of DNA/liposomes, or naked DNA (pCIKmCFTR, $P = 0.2207$ and pCIKCFTR, $P > 0.9999$; Figure 7c and d). These data show that under the circumstances tested, there was no reduction in vector-specific mRNA expression upon repeat delivery of either naked DNA, or DNA/liposome formulations.

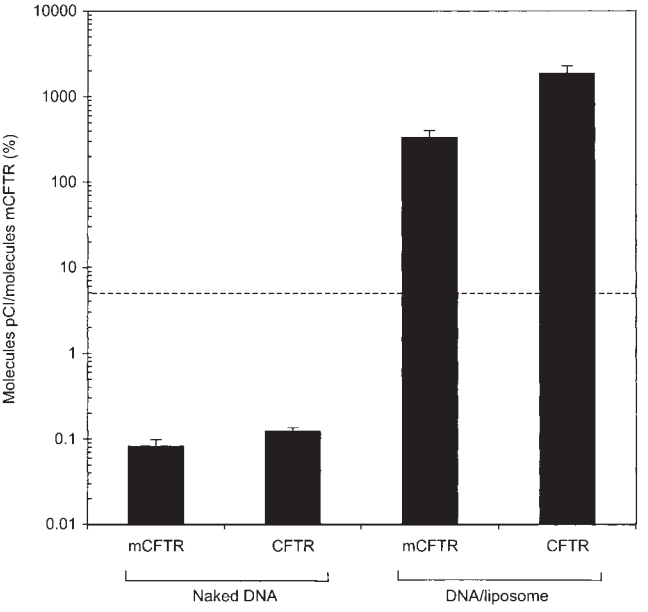

Figure 6 *Quantification of pCI-vector specific mRNA following non-viral in vivo airway gene transfer. Total lung and trachea RNA extracted from mice treated with naked DNA or DNA/liposomes was assayed using the TaqMan one-step or two-step pCI RT-PCR assays, respectively. The number of pCI-derived mRNA molecules expressed by pCIKmCFTR (mCFTR) and pCIKCFTR (CFTR) was normalised to and expressed as a percentage of the number of endogenous mCFTR mRNA molecules (determined using the one-step TaqMan EmCFTR RT-PCR assay) in the sample (eg One molecule of pCI-derived mRNA measured for every one molecule of endogenous mCFTR mRNA, represents a normalised expression level of 100%). Mean data are displayed (n = 5); error bars indicate s.e.m. The dashed line indicates 5% of endogenous mCFTR mRNA expression.*

Discussion

We wished to exploit TaqMan technology to accurately and reproducibly quantify transgene expression *in vivo*. Quantitative TaqMan assays were designed and optimised to detect vector-specific mRNA and endogenous mCFTR mRNA in murine lungs and tracheae. Vector-specific mRNA was detected in samples following the delivery of naked DNA or DNA/liposomes to the mouse airways. Following delivery of DNA/liposomes, the detection of pCI vector-derived mRNA was enhanced by heating total RNA samples. The mechanism by which heating enhances the detection of pCI vector-derived mRNA is currently unknown, but is likely to be a result of increasing the efficiency of the reverse transcription reaction. Possible explanations for this increased efficiency include an increase in available mRNA, perhaps by the disruption of inhibitory secondary structure, or by the denaturation of mRNA/DNA heteroduplexes. Consequently, all RNA samples were routinely heated to 75°C for 5 min before assay. Fortuitously, HEK293T total RNA used in earlier experiments had been heated for this length of time during DNaseI treatment.

At the beginning of the study, the required concentration of input RNA sample was unclear. We wished to maximise the sensitivity of the assays to detect low levels of vector-specific mRNA. Thus, the optimal level of total RNA added to the one-step pCI RT-PCR assay was determined, and in general, it was found that increasing the amount of total RNA per reaction inhibited mRNA

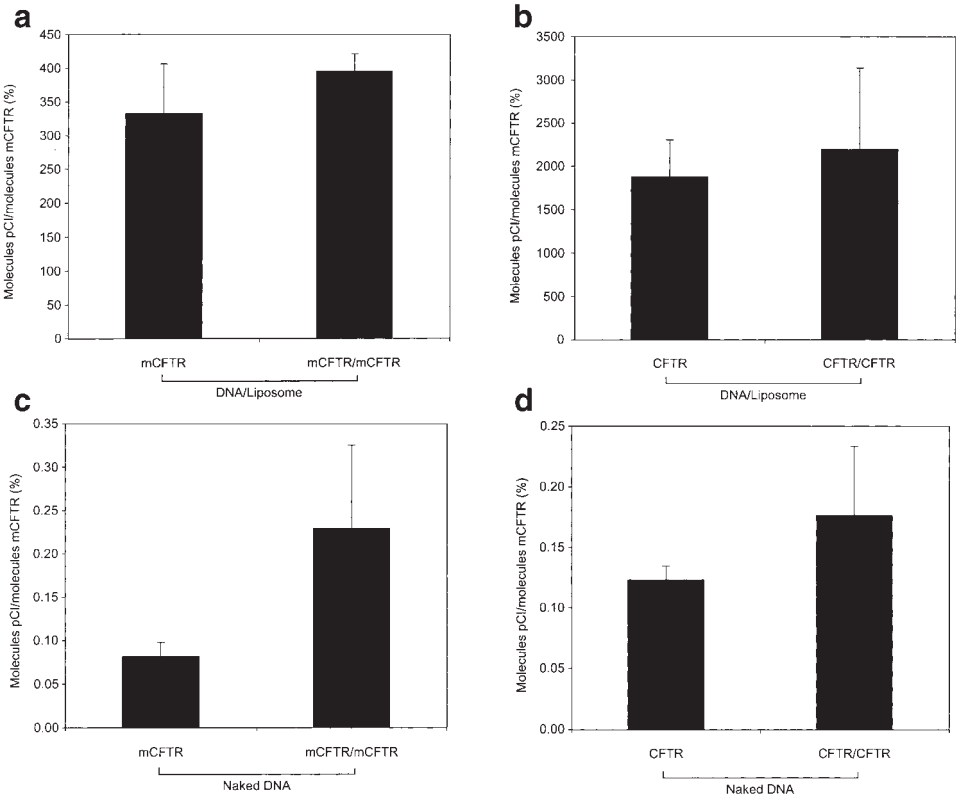

Figure 7 *Quantification of pCI-vector specific mRNA following repeated non-viral in vivo airway gene transfer. Total lung and trachea RNA was extracted from mice treated with either DNA/liposomes (a, b) or naked DNA (c, d), and assayed using the TaqMan one-step or two-step pCI RT-PCR assays, respectively. The number of pCI-derived mRNA molecules was expressed as a percentage of the number of endogenous mCFTR mRNA molecules in the sample. (a, c) Mice treated with pCIKmCFTR (mCFTR), (b, d) mice treated with pCIKCFTR (CFTR). Mean data are displayed (n = 5); error bars indicate s.e.m.*

detection, particularly at low levels of target mRNA. It is possible that this may be due to the components of the RT-PCR assay being sequestered non-specifically by total RNA. Despite this observation, it was found that $\geq 10^4$ molecules of pCI vector-derived mRNA could be reliably quantified in 100 ng of total RNA using the one-step pCI RT-PCR assay. The one-step EmCFTR RT-PCR assay could also reliably quantify as few as 10^4 target mRNA molecules in either 100 or 500 ng total RNA.

The relative abundance of vector-derived mRNA produced following naked DNA or DNA/liposome administration was markedly different. The lower levels of vector-derived mRNA observed in naked DNA treated animals, required the use of the more sensitive two-step pCI RT-PCR assay. Although the two-step assay was more laborious and potentially more prone to error due to the increased number of manipulations involved, one advantage was that the RT step could be performed under optimal RT conditions, which is likely to lead to the more efficient conversion of target RNA to cDNA.

Low inter- and intra-assay coefficient of variance percent values (CV%) confirmed the precision of the assays. For both the one- and two-step pCI assays, and for the EmCFTR assay, triplicate measurements of a single sample determined the intra-assay variability to be similar, ranging from 0.05 to 2.01% (>95% of measurements were <1.70%). The inter-assay variability for these assays was also similar; CV% ranged from 0.48 to 2.24% (>95% of measurements were <2%). Together, these measurements confirm that quantitative TaqMan RT-PCR can be a reliable and precise methodology for the determination of gene expression.

Once the TaqMan assays were optimised, they were used to quantify vector-derived expression in murine airways. Following a single administration of DNA/liposomes expressing murine or human CFTR, the number of vector-derived mRNA molecules was found to be well in excess of the number of endogenous mCFTR mRNA molecules. Published studies have indicated that the expression of as little as 5% of the normal number of endogenous mCFTR mRNA molecules can correct a large proportion of the CF ion transport defect in the mouse intestine and prevent fatal intestinal disease.[28] It is currently unclear if data presented in the latter study could be extrapolated to the murine or human airways; would 5% of the normal level of CFTR mRNA cure fatal pulmonary disease in CF individuals? A problem with this kind of extrapolation is that it does not account for the distribution of CFTR mRNA molecules. As TaqMan RT-PCR is a solution-based assay, these studies cannot assess the location of vector expression in the airways, and it is likely that cell specific expression of CFTR is critical for the amelioration of CF pulmonary disease. Results from other studies have determined that a large proportion of transgene expression following instillation of DNA/liposomes is located in the lung parenchyma, rather than in the conducting airways where CFTR expression is likely to be required.[29,30]

The presence of mRNA does not necessarily indicate a comparable level of functional protein. In a clinical study evaluating DNA/liposome gene transfer to the nasal epithelium in CF subjects,[12] greater than 5% of the level of endogenous CFTR mRNA, was expressed, yet the CF chloride conductance defect was only partially corrected.[12] Thus it is important to correlate CFTR mRNA expression levels with correction of the cAMP-dependent chloride conductance defect, or other functional endpoint assays. In addition, differences can be observed in host–vector interactions with different gene transfer reagents. With DNA/liposomes, similar vector-specific mRNA levels were observed after each of three doses,[12] whereas the repeated administration of adenovirus expressing CFTR to the bronchi of CF subjects led to a decrease in vector-specific mRNA on repeated administration.[27] Therefore we support the inclusion of a combination of endpoint assays in clinical CF gene transfer studies, and would not recommend reliance on quantitative RT-PCR alone to determine efficacy as some studies have.[27] Measuring a range of functions is likely to result in a more useful assessment of CFTR correction in the airways.

The delivery of naked DNA plasmids expressing murine and human CFTR resulted in less than 0.1% of the level of mRNA expression directed by DNA/liposomes, and was well below the theoretical 5% level of endogenous mCFTR mRNA. However, clinical studies with naked DNA vectors have indicated that they are as effective as GL-67 DNA/liposomes at correcting the CF chloride conductance defect after delivery to the human nasal epithelium.[31] The difference between the levels of naked DNA-mediated expression observed in the murine airways and human nasal epithelia could result from either species or cell-type differences between the epithelia. These data highlight the problems of using the mouse as a model for the human lung and trachea, and support the notion that multiple models should be used when evaluating gene therapy formulations.

Following the repeat administration of naked DNA or DNA/liposome formulations expressing murine and human CFTR to the murine airways, the levels of vector-derived mRNA expression were not reduced in comparison to the first administration. These data are consistent with the hypothesis that the expression of both murine and human CFTR are well tolerated in the BALB/c mouse, and concur with studies showing that multiple doses of DNA/liposomes expressing human CFTR in the airways of CF mice can correct CF ion transport defects upon repeat administration.[18] As murine CFTR is an endogenous protein, we assumed that its expression would be immunologically tolerated by mice. Human CFTR may have been tolerated due to its high similarity to murine CFTR, although for other genes, such as the human EPO and Factor IX, this is not the case.[32] Alternatively, murine and human CFTR expression levels may have been below the threshold required by the immune system to trigger a potent immune response. Although no statistically significant reduction in vector-derived mRNA levels was observed following two administrations of vector in the current study, repeated delivery of many doses to the mouse airway might stimulate an immune response resulting in a significant reduction in mRNA expression. Studies involving extended numbers of doses, perhaps at higher transgene expression levels may clarify this further. Thus it remains to be seen if wild-type CFTR can be tolerated upon very long-term gene expression in human CF subjects.

This study demonstrates that quantitative TaqMan RT-PCR can be used to accurately quantify transgene expression in the murine airways. It is particularly suited to non-viral gene transfer studies where high levels of vector DNA can interfere with the detection of low levels of mRNA in RT-PCR assays. The principles employed in this study will be extended to quantify gene expression in other organs and other animal models, and used to monitor gene transfer in pre-clinical and clinical studies.

Materials and methods

Preparation of plasmid DNA

Endotoxin-free plasmid DNA was purified using EndoFree Plasmid Mega kit (Qiagen, Crawley, UK).

Plasmid expression vectors

The plasmid pCI (Promega, Madison, WI, USA; GenBank accession No. U47119), containing the human CMV immediate–early gene promoter/enhancer and a hybrid intron (with intronic regions from β-globin and immunoglobulin heavy chain variable region genes) was used to express transgenes in this study. Plasmid pCIKCFTR was constructed by removing the 4530 bp *Kpn*I–*Xho*I fragment containing the CFTR cDNA from plasmid pTrial10-CFTR2,[33] and inserting it into pCI digested with *Kpn*I and *Sal*I. Plasmid pCIKmCFTR was constructed as follows. The murine CFTR cDNA, isolated from the C57/B6 mouse strain (obtained from B Wainwright and D Lunn, University of Queensland, Brisbane, Australia), was supplied in plasmid pEFmCFTR.[6] The 5′ region of the mCFTR cDNA was amplified by PCR from pEFmCFTR using Vent DNA polymerase (New England Biolabs (NEB), Hitchin, UK), and 500 nM forward (GGCTAGCCACCATGCAGAAGTCGCCTTGG) and reverse (CCAGTACTTTATACTCTTGTTTCTGCAGG) primers, and ligated into the plasmid pBluescript SK(−) (pBSKM) digested with *Sma*I, to produce pBSKM5′ mCFTR. A 290 bp *Hin*dIII–*Sal*I fragment of the plasmid pSL301 (Invitrogen, Leek, The Netherlands) multiple cloning site was isolated, and ligated into the low copy number plasmid pBR322 (NEB) digested with *Hin*dIII and *Sal*I, to produce pBRSL. A 1048 bp *Nhe*I–*Sna*BI fragment containing the 5′ end of the mCFTR cDNA was isolated from plasmid pBSKM5′mCFTR, and ligated into pBRSL digested with *Nhe*I and *Sna*BI, to produce pBRSL5′mCFTR. A 6395 bp *Sna*BI–*Nsi*I fragment containing the 3′ region of the mCFTR cDNA was isolated from plasmid pEFmCFTR, and ligated into pBRSL5′mCFTR digested with *Sna*BI and *Nsi*I, to produce pBRSLmCFTR. A 7468 bp *Nhe*I–*Not*I fragment containing the reconstituted mCFTR cDNA was then isolated from plasmid pBRSLmCFTR, and ligated into pCI digested with *Nhe*I and *Not*I, to produce pCIKmCFTR.

Transfection of cultured epithelial cells

The human embryonic kidney cell line HEK293T[34] were transiently transfected with plasmid DNA and the cationic liposome 3′β[N-(N′,N′-dimethylaminoethane)-carbomoyl] cholesterol and dioleoylphosphatidylethanolamine (DC-Chol/DOPE)[35] essentially as described.[14] Total RNA was prepared 48 h after transfection.

Vector administration to the mouse airways

For gene delivery to the lungs, female BALB/c mice (6–8 weeks old) were anaesthetised with Metofane (Mallinckrodt Veterinary, Mundelein, IL, USA) and instilled intranasally essentially as described.[19] For delivery of naked plasmid DNA, 100 μg of DNA was instilled in a total volume of 150 μl water for injection. For DNA/liposomes, plasmid DNA was mixed with the cationic lipid, GL-67:DOPE:DMPE-PEG(5000) (Genzyme, Framingham, MA, USA)[19] and 100 μl of the DNA/liposome solution (0.5 mM GL-67:2 mM plasmid DNA (660 ng/μl)) was delivered to the mouse airway by instillation. Repeat administrations of vectors were separated by a period of 42 days. All animals were killed by rising CO_2 and the lungs and tracheae removed *en bloc*, rinsed in PBS to remove excess blood, submerged in >5 volumes (3 ml) RNALater (Ambion, Austin, TX, USA) and stored at 4°C until processed (typically <48 h later).

Preparation of total RNA

Total RNA was extracted from HEK293T cells using RNeasy Mini kit (Qiagen), as recommended by the supplier. Total RNA (2 μg) was treated with 2 U DNaseI (Ambion) in a volume of 20 μl to remove contaminating DNA (37°C for 30 min, 75°C for 5 min). The RNeasy Midi kit (Qiagen) was used to prepare total RNA from mouse lungs and tracheae, as recommended by the supplier. Only half of the lung and trachea lysate from a single mouse was purified on a single RNeasy Midi column, as greater quantities of lysate tended to reduce RNA yields (data not shown). Where required, 5 μg total RNA was incubated with 2 U DNaseI in a final volume of 100 μl (as above).

Real-time quantitative TaqMan RT-PCR

Quantitative RT-PCR assays were performed using the ABI PRISM 7700 TaqMan sequence detector and Sequence Detector v1.6.3 software (Applied Biosystems). Oligonucleotide primers and fluorogenic probe combinations for TaqMan assays were designed using Primer Express V.1.0 (Applied Biosystems). The TaqMan pCI RT-PCR assay forward primer (5′-GCTTCTGACA CAACAGTCTCGAA), reverse primer (5′-GGAGTGGAC ACCTGCCCA) and fluorogenic probe (5′-TGCCTCAC GACCAACTTCTGCAGC) detected pCI vector specific mRNA (Figure 1). The TaqMan EmCFTR RT-PCR assay forward primer (5′-TCGTGATCACATCAGAAATT ATTGATAAT), reverse primer (5′-CCACCTCTCTCAA GTTTTCAATCAT) and fluorogenic probe (5′-CGCTGATTCCCAACAATATGCCTTAACAGAATA) detected endogenous murine CFTR mRNA. The EmCFTR RT-PCR forward primer hybridised to the region of the mCFTR cDNA previously mutated to remove a cryptic bacterial promoter,[6] in order to eliminate hybridisation to mCFTR sequences in the vector pCIKmCFTR.

Typically, RNA was heated to 75°C for 5 min before being assayed using either one-step or two-step reactions, with reagents supplied by Applied Biosystems, except where stated. The RT step of two-step pCI RT-PCR assays utilised the TaqMan Reverse Transcription Reagents kit (Applied Biosystems) and contained 400 nM reverse primer. The one-step pCI RT-PCR assay and the two-step pCI PCR step were performed with the TaqMan PCR Core Reagent kit (Applied Biosystems). One-step pCI RT-PCR assays contained 300 nM forward and reverse primers and 200 nM TaqMan probe in a final volume of 50 μl. The PCR step of the two-step RT-PCR assay contained 5 μl of the appropriate RT reaction, 300 nM forward and reverse primers and 200 nM TaqMan probe in a final volume of 50 μl. The one-step EmCFTR RT-PCR assay contained 100 or 500 ng total RNA (DNA/liposome or naked DNA samples, respectively), 50 nM forward primer, 300 nM reverse primer and 100 nM TaqMan probe in a final volume of 50 μl. All samples were assayed in triplicate.

Absolute quantification of vector-specific and endogenous mCFTR expression levels

Absolute quantification of vector-specific mRNA was achieved by comparison of sample C_T values with dilution curves of known numbers of synthetic target RNA molecules. Synthetic target RNA molecules were synthesised *in vitro* using DNA templates containing T7 promoter sequences at the 5′ of the pCI and EmCFTR assay target sequences, and the RT-PCR Competitor Construction kit (Ambion), as recommended by the supplier. The final concentrations of synthetic target RNA molecules were determined using the RiboGreen RNA quantitation kit (Molecular Probes, Eugene, OR, USA). Absolute standard curves for the pCI and EmCFTR assays were prepared by dilution of between 1 and 1×10^9 molecules/5 μl with 10 ng/μl yeast total RNA (inert carrier molecule). The detection of pCI and EmCFTR synthetic target RNA molecules was linear for at least three logarithmic decades in appropriate TaqMan assays, with target RNA to C_T correlation coefficients (r^2) ≥0.99. Control −RT reactions demonstrated that >99.999% of the PCR amplification signal observed with the purified RNA molecules was dependent on reverse transcriptase (data not shown).

Statistical analysis

Data presented with an error indicates sample group mean ± standard error of the mean (s.e.m.). The Wilcoxon Sign Rank test was used to compare two paired sample groups. The Mann–Whitney *U* test was used to compare two unpaired sample groups. The null hypothesis was rejected at $P \leq 0.05$.

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