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RESEARCH ARTICLE

Detection of plasmid DNA vectors following gene transfer to the murine airways

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Non-viral gene therapy is being considered as a treatment for cystic fibrosis. In clinical studies and in studies using the mouse airways as a model, current formulations result in only transient transgene expression. A number of reasons for this have been proposed including the loss of plasmid DNA from cells. The aim of these studies was to investigate why transgene expression from non-viral vectors is transient in the mouse lung. Plasmid DNA encoding the luciferase reporter gene was complexed with the cationic lipid GL67 and delivered to the mouse airways. The persistence of plasmid DNA in the mouse lungs was investigated using quantitative PCR and Southern hybridization. Results showed that intact plasmid DNA persisted in the mouse lung in the absence of any detectable luciferase activity. The de novo methylation of plasmid DNA in vivo was investigated as a potential cause of this transient gene expression but results suggested that plasmid DNA does not become de novo methylated in the mouse lung. Therefore processes other than the loss of plasmid DNA from the lung or the de novo methylation of plasmid DNA vectors must be responsible for the transient transgene expression.
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Introduction

The lung is a major gene therapy target for the treatment of a number of inherited and acquired disorders such as emphysema, asthma, cancer and cystic fibrosis (CF).[1] CF gene therapy in particular has attracted much attention since the cloning of the human CF transmembrane conductance regulator (*CFTR*) gene which encodes a cAMP-regulated chloride channel.[2–5] One of the major obstacles for the development of gene therapy for chronic lung conditions such as CF has been the inability of many viral and non-viral vectors to direct sustained expression of a therapeutic transgene.

The CF chloride transport defect has been partially, but transiently, corrected in the trachea of CF transgenic mice by delivery of liposome/plasmid DNA complexes to the airways as a liquid bolus[6] and aerosol.[7] In clinical studies, we have administered single and multiple doses of plasmid DNA complexed with cationic liposomes to the nasal epithelia of CF patients, demonstrating transient correction of the CF chloride ion transport defect in the nose.[8,9] Short-lived correction was also observed in similar clinical studies undertaken by other groups.[10,11]

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The continued development of cationic liposomes has led to improved levels of gene expression; when liposomes of Genzyme lipid 67 (GL67): DOPE are complexed with plasmid DNA (GL67/plasmid DNA), reporter gene expression in the mouse lung has been observed at levels up to 1000-fold greater than naked plasmid DNA, or 100-fold greater than other plasmid DNA/liposome formulations.[12] The quantification of human CFTR mRNA in the mouse lung 2 days after instillation of GL67/plasmid DNA encoding CFTR showed 10-fold higher levels of human CFTR than endogenous murine Cftr.[13] GL67/plasmid DNA complexes expressing CFTR have partially corrected CF ion transport defects in the nasal tissue of mice[14] and the nose and lungs of CF subjects.[10] However, as with other plasmid DNA/liposome formulations, gene expression from GL67/plasmid DNA complexes is transient, lasting only a few days after administration.[15]

Naked plasmid DNA has also been evaluated as a potential lung gene transfer vector and in spite of low levels of reporter gene expression[12] correction of the chloride defect in the nasal epithelium of CF patients was as effective as GL67/plasmid DNA.[16] Improved duration of gene expression was observed when viral promoters were replaced by endogenously expressed human promoters, such as elongation factor 1α or polyubiquitin B or C, resulting in reporter gene expression for up to 6 months post-administration to the murine lung.[17,18]

This study uses a mouse model to investigate the persistence of plasmid DNA vectors in the lung and to correlate these with the duration of reporter gene

expression from naked plasmid DNA and GL67/plasmid DNA complexes. Southern hybridization was used to study the structural fate and degradation of plasmid DNA in the mouse lungs following administration with GL67/plasmid DNA. Plasmid DNA in the mouse lungs was also studied for evidence of *de novo* methylation to determine if this phenomenon plays a role in the silencing of gene expression from plasmid DNA vectors in the lungs.

Results

Luciferase activity in mouse lungs after administration of naked plasmid DNA and GL67/plasmid DNA

To measure the duration of transgene expression in the murine lung, plasmid DNA expressing luciferase under the control of the cytomegalovirus (CMV) promoter (pCIKLux), or the human poly-ubiquitin C promoter (pUbLux) was delivered to the mouse lung either as naked plasmid DNA or GL67/plasmid DNA complexes. Luciferase activity was measured at various time-points post-administration (Figure 1). For pCIKLux delivered as naked plasmid DNA, or complexed with GL67, luciferase activity was high at day 2 falling to background levels by day 7 (Figure 1a), although expression from GL67 complexes was 50-fold greater than naked DNA at day 2 ($P = 0.009$). As shown previously,[17] luciferase activity from naked plasmid pUbLux remained at initial high levels for up to 2 months falling to background levels by 6 months post-administration (Figure 1b). When pUbLux was delivered complexed with GL67, luciferase activity was higher initially than naked pUbLux ($P = 0.014$), but fell to below background levels at days 7 and 14. At day 28 the level rose above the assay sensitivity and remained detectable at day 56 post-dosing but at these time-points luciferase activity was lower than naked pUbLux ($P < 0.04$).

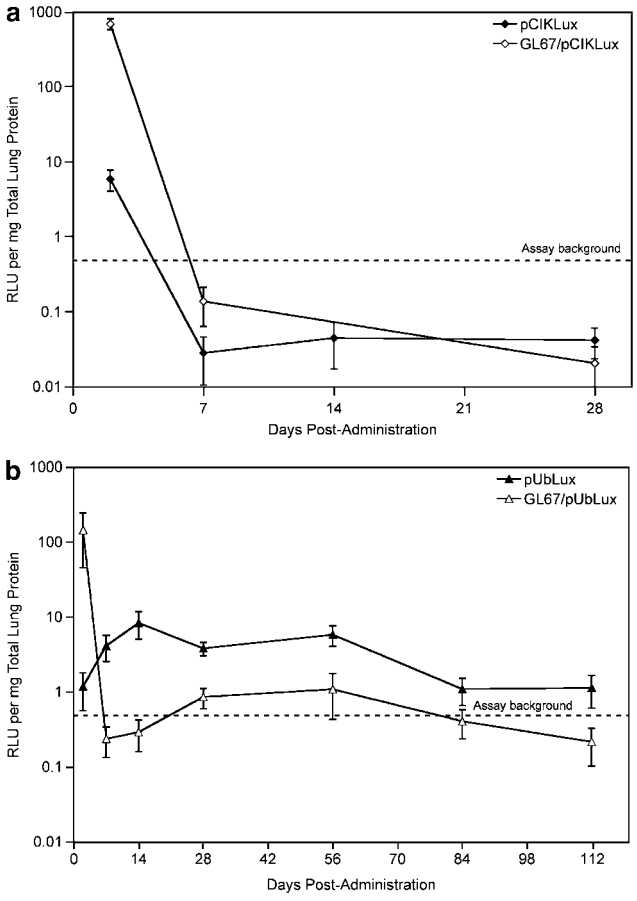

Figure 1 *Duration of luciferase gene expression in mouse lung tissue. (a) Duration of luciferase activity in the lungs of mice instilled with naked pCIKLux and GL67/pCIKLux. (b) Duration of luciferase activity in the lungs of mice instilled with naked pUbLux and GL67/pUbLux. Lungs and tracheas were harvested at the time-points indicated and assayed for luciferase activity expressed as relative light units (RLU) per mg of total lung protein. Sensitivity of the assay is based on naïve lung lysates. Mean ± s.e.m. (n = 6) for each time-point are shown.*

TaqMan PCR analysis of plasmid DNA extracted from mouse lung tissue

Previously we have used quantitative TaqMan PCR to study the persistence of naked plasmid DNA *in vivo* following delivery to the mouse lung,[17] and showed that although only a small fraction of the applied plasmid DNA (approximately 1 ng per lung) was detectable on day 2, plasmid DNA levels were essentially stable for at least 14 days post-administration. Here, a similar study was carried out to determine plasmid DNA persistence in the mouse lung, following delivery of GL67 complexed with plasmids pCIKLux, pUbLux and another plasmid pCIKLux.IO.[17] At 2, 7, 14 and 28 days post-administration, total DNA was prepared from the lungs and TaqMan PCR analysis was performed to quantify plasmid DNA present in the lung (Figure 2). Only a small proportion of the original 66 μg dose was ever detected but levels of plasmid DNA in the mouse lung were similar for each of the three plasmids at all time-points. At day 2, there was a mean value of 7 ng pCIKLux/μg ribosomal DNA (rDNA) present in the lungs of mice, which equates to approximately 1 μg pCIKLux per lung. This value was 1000-fold greater than the level of plasmid DNA detected when mice were

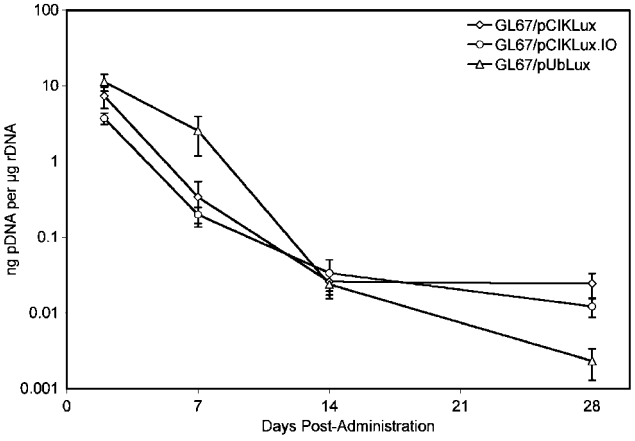

Figure 2 *Quantification of plasmid DNA in lungs of mice instilled with GL67/plasmid DNA. Mice were instilled with pCIKLux, pCIKLux.IO or pUbLux complexed with liposomes of GL67. Lungs and tracheas were harvested at the time-points indicated and assayed for plasmid DNA and rDNA content by real-time quantitative TaqMan PCR. Mean ± s.e.m. (n = 6) for each time-point are shown.*

dosed with naked plasmid DNA.[17] Mean values of 11 ng pUbLux/μg rDNA and 4 ng pCIKLux.IO/μg rDNA were also detected although there was no significant difference between the detection of the three plasmids at day 2 post-administration ($P = 0.92$). By days 7 and 14 post-administration, the levels of GL67/plasmid DNA in the lungs had fallen, but still remained around 10-fold higher than when delivered as naked plasmid DNA.[17]

TaqMan PCR analysis of plasmid DNA extracted from murine bronchoalveolar lavage fluid (BALF)

Quantitative TaqMan PCR analysis was also used to determine if the majority of the plasmid DNA detected in the mouse lungs following administration is present in the lung tissue or in mobile cell populations accessible by BAL such as neutrophils and macrophages. At 1, 2 and 7 days following delivery of GL67/pCIKLux, BALF was collected and centrifuged to separate the cells and supernatant. Total DNA was prepared from the cells, supernatant and remaining lung tissue, and quantified by TaqMan PCR (Figure 3). The results show that the majority of the pCIKLux detected in the mouse lung following delivery of GL67/plasmid DNA was present in the lung tissue at all time-points, although plasmid DNA was also readily detected in the BALF supernatant and the cellular component of the BALF throughout the study.

Analysis of plasmid DNA structure in the mouse lung using Southern hybridization

A limitation of using TaqMan PCR to determine the persistence of plasmid DNA in the mouse lung is that the assay detects a short (72 bp) fragment of DNA and does not discriminate between intact and partially degraded plasmid. Consequently the TaqMan assay may overestimate the level of intact plasmid DNA in the lung. Southern hybridization was therefore used to visualize the topological forms of plasmid DNA present in the lung following delivery of GL67/pUbLux complexes, 2 and 7 days post-administration. Circular and linear plasmid DNA standards were prepared to allow the identification of supercoiled (SC), open circle (OC) and linear forms of plasmid DNA following Southern hybridization (Figure 4). The circular standards also contain high molecular weight bands, representing concatenations of the plasmid DNA in OC and SC forms. At day 2 post-administration, comparison of the pUbLux lung samples with the pUbLux standards showed that both OC and linear plasmid DNA were present in the total DNA purified from the mouse lungs in five of the six samples (Figure 4). These bands represented approximately 10 and 20% of total signal detected, respectively, taking into consideration the large amount of specific signal present as a smear below the linear band representing smaller linear fragments of plasmid DNA. In addition, a faint band was visible that correlated with the position of SC plasmid DNA in the standards, but was largely obscured by the degradation of the linear plasmid DNA. At day 7 post-administration OC and linear forms of plasmid DNA could be detected in seven out of nine samples (Figure 4). However, despite a 5000-fold reduction in reporter gene expression, the percentage composition of plasmid DNA was the same at day 7 as it was at day 2 ($P > 0.05$) with the OC and linear bands representing approximately 10 and 20% of total signal detected, respectively. Together these data indicate that intact OC plasmid DNA is present in the lungs of mice for at least 1 week following administration of GL67/pUbLux.

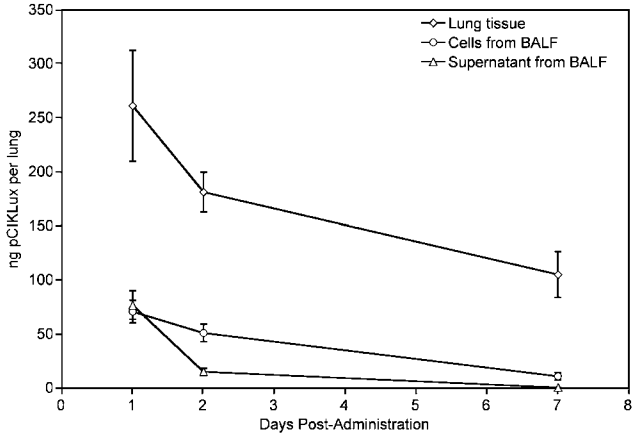

Figure 3 *Quantification of plasmid DNA in lungs and BALF of mice instilled with GL67/pCIKLux. Mice were instilled with GL67/pCIKLux and at the time-points indicated subjected to bronchoalveolar lavage to collect the cellular and liquid contents of the lung lumen. DNA was purified from these samples and analysed for plasmid DNA content per lung by quantitative TaqMan PCR. Mean ± s.e.m. (n = 6) for each time-point are shown.*

Analysis of plasmid DNA for evidence of de novo methylation in the mouse lung

To determine if plasmid DNA persisting in the mouse lung was transcriptionally silenced due to methylation, lung DNA was purified 2 days following instillation of GL67/pUbLux and analysed for changes in CpG methylation by digestion with methylation-sensitive and methylation-dependent restriction endonucleases. The methylation-sensitive endonucleases *Hha*I and *Hpa*II will not cut if the CpG dinucleotide in their respective recognition sequence is methylated. In addition, the methylation-dependent restriction system *Mcr*BC cleaves DNA close to one of the methylcytosines in its complex recognition sequence and therefore cuts only when the recognition sequence is methylated.[19,20] Positive control plasmid DNA was generated by *in vitro* methylation of a sample of day 2 GL67/pUbLux lung DNA with the methylase M.*Sss*I, to convert every CpG to Me-CpG. Positive control plasmid DNA was resistant to digestion with *Hha*I and *Hpa*II and sensitive to digestion with *Mcr*BC.

When six GL67/pUbLux day 2 samples were digested with *Hha*I and *Hpa*II there were no visible OC or linear bands (Figure 5), and the digests were indistinguishable from the digestions with *Msp*I which is insensitive to Me-CpG. Furthermore, digestion of these samples with *Mcr*BC resulted in the detection of both OC and linear plasmid DNA, similar to the signal observed from the undigested controls. Together, these results suggest a lack of *de novo* methylation of pUbLux in the lungs of mice at 2 days post-instillation. Similar results were obtained for plasmid DNA harvested 7 days following administration of GL67/pUbLux (Figure 5).

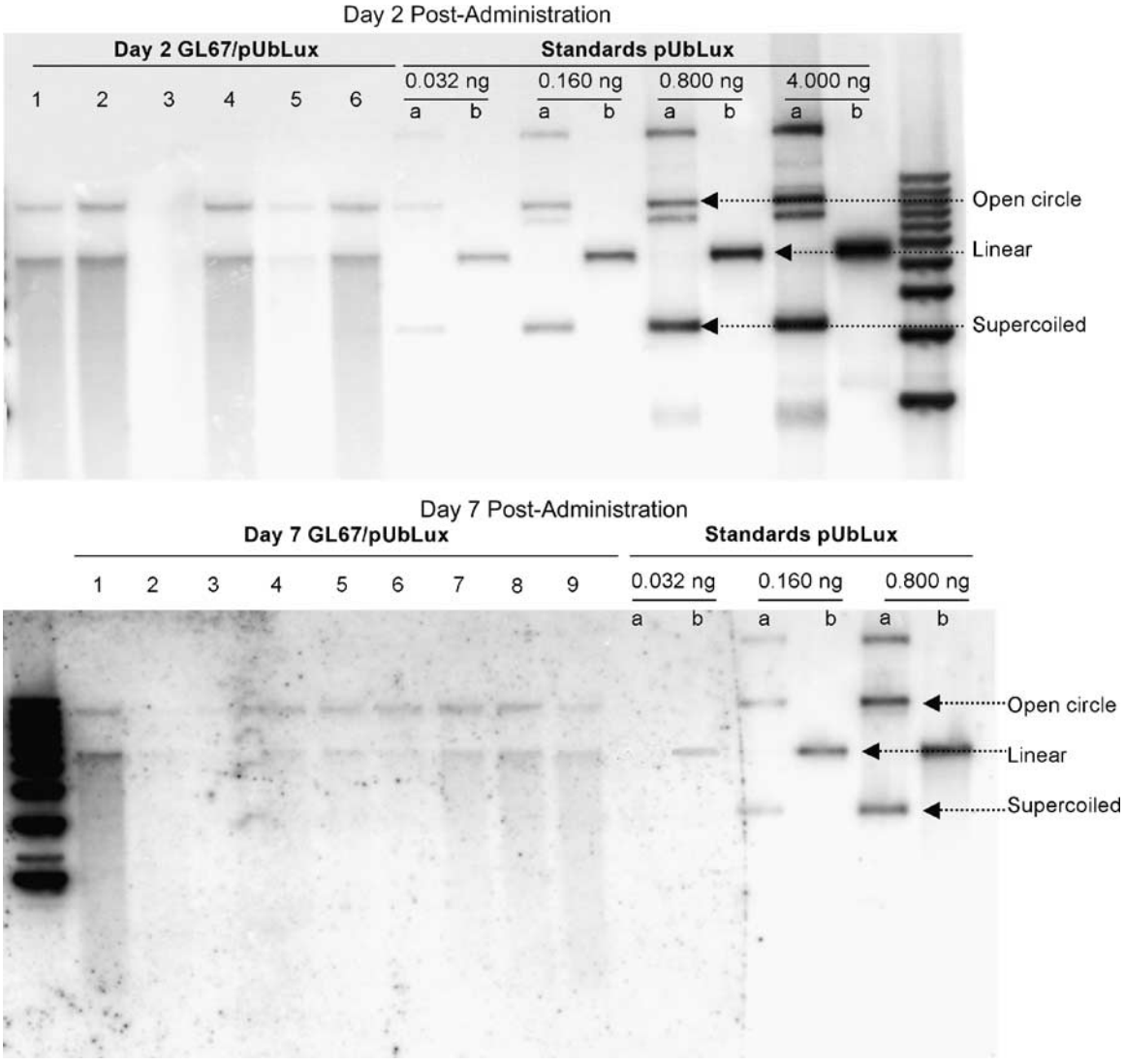

Figure 4 *Detection of intact plasmid DNA in the lungs of mice instilled with GL67/pUbLux. Mice were instilled with GL67/pUbLux and DNA was purified from mouse lungs and tracheas 2 days post-administration (top, lanes 1–6) and 7 days post-administration (bottom, lanes 1–9) and analysed by Southern hybridization. Circular (a) and linearized (b) positive control pUbLux samples were electrophoresed at known concentrations to facilitate the correct identification of SC, OC and linear forms of pUbLux purified from the mouse lungs.*

Discussion

For gene therapy of chronic lung diseases such as CF, the target cell population is the terminally differentiated airway epithelial cells of the lung. Therefore for long-term therapeutic effect it is likely that repeated delivery of any gene transfer agent (GTA) will be required. This places two fundamental restrictions on the choice of GTA for CF gene therapy. In the first instance the GTA must not elicit an immunological response that would diminish its effectiveness following subsequent delivery. Secondly the duration of expression from the GTA must be maximized in order to reduce the frequency of patient treatments. The first of these two requirements means that it is unlikely that recombinant viral vectors will be a viable option for CF lung gene therapy without a significant advance in the manipulation of the host–vector interaction.[21] The second requirement demands improvements in the efficiency of non-viral gene transfer and the duration of expression from non-viral vectors. Of key importance is to understand the factors that limit the duration of expression from current non-viral formulations. In this study, three factors affecting the duration of expression from the non-viral cationic lipid formulation GL67/plasmid DNA were investigated; DNA persistence was measured by TaqMan PCR, DNA integrity was analysed using Southern hybridization and DNA methylation was investigated using differential restriction digestion.

Quantitative TaqMan PCR was used to determine the persistence of plasmid DNA in the lungs of mice and showed that the levels of plasmid DNA detected when mice were instilled with GL67/plasmid DNA were up to 1000-fold higher than previously found for naked plasmid DNA at day 2 (Figure 2).[17] At later time-points, the amount of plasmid DNA falls but was still substantially higher in GL67/plasmid DNA dosed animals at 7, 14 and 28 days post-administration.

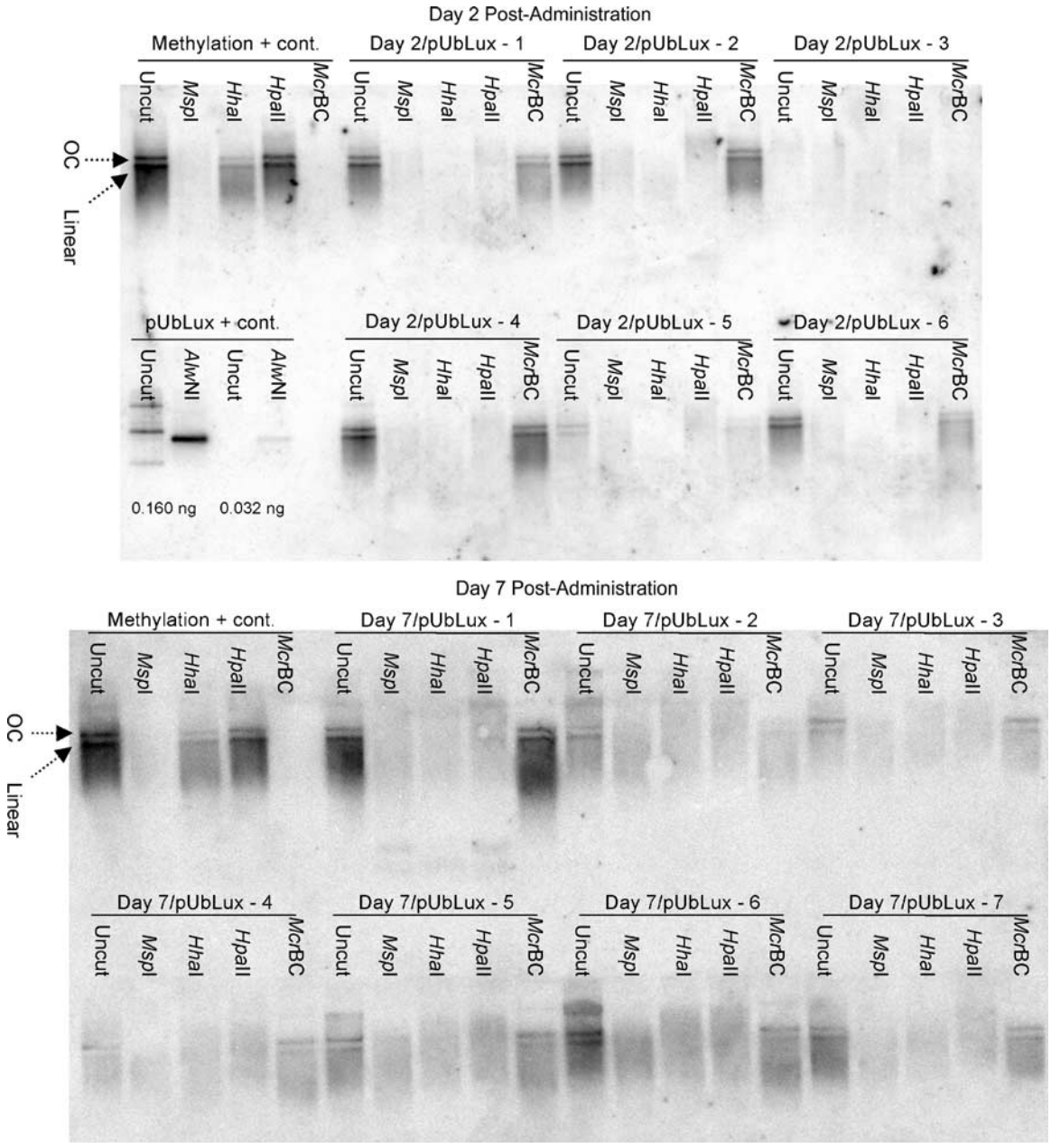

Figure 5 *Detection of de novo methylation of plasmid DNA in the lungs of mice instilled with GL67/pUbLux. DNA was purified from mouse lungs and tracheas 2 and 7 days post-administration. A sample of DNA, purified from mouse lungs dosed with GL67/pUbLux, was methylated with M.SssI to produce a methylation positive control. DNA samples and the positive control were digested with the methylation sensitive restriction endonucleases HhaI and HpaII and with the methylation-dependent endonuclease McrBC. Completion of digestion was confirmed by digestion of all samples with MspI, an isoschizomer of HpaII, which is not affected by the presence of Me-CpG. Following digestion, positive control and lung DNA samples were analysed using Southern hybridization.*

Intriguingly, despite similar reductions in lung plasmid DNA levels, the profile of reporter gene expression following administration of GL67/pCIKLux and GL67/pUbLux are different. Luciferase activity from GL67/pCIKLux is high at day 2 post-administration but falls to below background levels by day 7 (Figure 1a), where it remains for at least 6 months (data not shown). Luciferase activity from GL67/pUbLux shows a similar precipitous fall; however, 14 days post-administration luciferase activities rise (albeit to modest levels) above background for a further 2 months. Thus although the fall in luciferase transgene expression observed with GL67/pCIKLux correlates reasonably well with the fall in lung plasmid DNA content, the same cannot be said for pUbLux. Collectively, these observations suggest that simple monitoring of total lung plasmid DNA content is not a good predictor of the duration of transgene expression.

As the efficacy of plasmid DNA based (non-viral) gene therapy in the airways may be compromised by the degradation of plasmid DNA *in vivo*, a process that cannot be quantified by real-time TaqMan PCR, we chose

to investigate the structure of plasmid DNA found in the mouse lung by Southern hybridization. Nuclease digestion of the delivered plasmid DNA is one simple explanation for the fall in plasmid DNA levels post-administration. *In vivo* studies in mice have shown that following tail-vein injection of plasmid DNA/liposome complexes, the majority of the plasmid DNA was degraded within 2 h[22] and that degradation of naked plasmid DNA in plasma is due to the presence of nucleases.[23,24] Endogenous nucleases are also present in the airways but complexing of plasmid DNA with liposomes has been shown to reduce its susceptibility to nuclease degradation *in vivo*.[25] Following Southern hybridization analysis of DNA from mouse lungs instilled with GL67/plasmid DNA, approximately 10% of the plasmid DNA detected from total lung samples was in the OC form, while approximately 20% was linear suggesting that some degradation had occurred. A modest amount of plasmid DNA was observed in the SC form from total lung DNA but it was too small a proportion to be quantified. These proportions *in vivo* represent a shift from the starting composition of the plasmid DNA used, where 60–70% was found to be SC and around 30% OC (data not shown). Southern hybridization of plasmid DNA extracted from murine BALF indicated that there was no difference in the rate or pattern of DNA degradation in the lung lumen compared with the rest of the lung tissue (data not shown). The degradation rates of the SC, OC and linear forms of plasmid DNA have been studied *in vitro* in plasma, and were shown to vary with $t_{1/2}$ of approximately 1.2, 11 and 21 min for SC, Linear and OC, respectively.[23] Visualization of this process demonstrated a progressive reduction of SC plasmid DNA over time and an increase in the OC form. The short $t_{1/2}$ of SC plasmid DNA in plasma compared to OC, may explain the low levels of SC plasmid found in the lungs following administration. While the detection of OC plasmid DNA at 2 days post-administration is consistent with the high levels of reporter gene expression at day 2 from GL67/plasmid DNA, the presence of intact plasmid DNA at 7 days shows that degradation of plasmid DNA is unlikely to be responsible for the short duration of reporter gene expression observed with these complexes. Southern hybridization has been used to study the persistence of plasmid DNA in the liver and high molecular weight plasmid concatenations have been observed.[26] In this study the sensitivity of the Southern hybridization assay was only sufficient to study the fate of plasmid DNA in mouse lungs 2 and 7 days after delivery of GL67/plasmid DNA complexes. At later time-points and following naked plasmid DNA delivery, insufficient levels were present to allow for detection by Southern hybridization. The levels of plasmid DNA detected by TaqMan analysis and the signal intensity from initial Southern hybridizations were compared and it was estimated that the detection limit of the Southern hybridization was 0.1 ng plasmid DNA. However, at the two time-points that were studied, high molecular weight plasmid concatenations were not detected in the mouse lung.

Having demonstrated that intact plasmid persisted in the lungs of mice dosed with GL67/plasmid DNA, the plasmid DNA was assayed for evidence of *de novo* methylation as a possible cause of transient transgene expression. Integrating viral vectors have been shown to be susceptible to *de novo* CpG methylation which results in transient gene expression *in vivo*.[27–29] *De novo* methylases have been detected in most tissues[30] and *de novo* methylation of plasmid DNA has been observed in human cell culture studies.[31] Lung plasmid DNA samples were incubated with methylation-sensitive and methylation-resistant endonucleases, but no evidence of *de novo* methylation was observed (Figure 4). Incubation of lung plasmid DNA samples with *Mcr*BC, which can potentially cut at 50% of the Me-CpG motifs, did not result in digested plasmid DNA. These findings indicate that the widespread *de novo* methylation of plasmid DNA did not occur in the mouse airways. It is possible that very low levels of *de novo* methylation may have been overlooked using this strategy, but if the levels of methylation were below the detection of this assay, then they may be too low to have a profound effect on reporter gene expression.[32] Bisulphite modification and subsequent PCR/sequencing of DNA has been widely used to quantify changes in methylation patterns in genomic DNA.[33,34] However, given the result of the Southern hybridization assay for *de novo* methylation and the heterogeneous state of the plasmid DNA purified from the mouse lung, this method is unlikely to provide a more definitive result than that achieved herein. Whereas the *de novo* methylation of integrating viral vectors may result in subsequent transient gene expression, these results suggest that alternative mechanisms may be involved in the silencing of non-viral vectors.

Thus in summary, we have analysed the persistence of plasmid DNA following delivery of GL67/plasmid DNA complexes to the mouse lungs, following the observation that the duration of expression from these complexes is limited. We found that even in the absence of reporter gene expression abundant levels of intact plasmid DNA could be detected in the mouse lungs (Figures 2 and 4) and that this plasmid DNA had not undergone *de novo* methylation (Figure 5). There are a number of possible explanations for the observation that the level of plasmid DNA present in the lungs of mice dosed with GL67/plasmid DNA does not correlate with the level of transgene expression observed. For example, as the total DNA is prepared from the entire lung there is no way to determine what proportion of plasmid DNA detected is transcriptionally active in the nucleus. TaqMan analysis of the plasmid DNA content of murine BALF following administration of GL67/plasmid DNA indicated that at least 70% of the total plasmid DNA detected was present in the lung tissue and not the lumen or cells in the BALF (Figure 3). One could employ cellular fractionation methodologies to purify nucleus-associated plasmid DNA, but such methods are more readily applied to cultured cells than intact organs.[35,36] Alternatively, some of the plasmid DNA that persists in the lung may be in a form that cannot be expressed. While the use of Southern hybridization to characterize plasmid DNA *in vivo* can overcome some of the limitations of TaqMan PCR analysis, it still shares a common failing; the assay is performed on DNA prepared from the entire lung with no way of determining in which cellular fraction of which cell types the intact plasmid DNA is found, nor how much of the OC plasmid DNA is transcriptionally active. The relationship between the delivery of vector DNA and gene expression is an important one in models

of gene therapy as the detection of vector DNA has been used a positive outcome marker in gene therapy clinical trials.[9] There are methods available that allow the fine mapping of transcriptionally active regions of DNA,[37] but these methods do not have the required sensitivity to quantify the actively transcribed component of plasmid DNA in a potentially excessive background of untranscribed plasmid DNA. Therefore if these relationships are to be made meaningful, more sensitive assays perhaps coupled with methods to purify specific cell populations (and even subcellular compartments) will have to be developed.

Several studies also show transient transgene expression despite successful delivery and persistence of both viral[38,39] and non-viral vectors *in vivo*.[17,26] An important factor in the silencing of transgene expression may be the phenomenon of promoter attenuation.[40] Commonly used viral promoters such as CMV are transcriptionally regulated by cellular factors that may be affected by vector-induced inflammatory cytokines.[41,42] The use of endogenous or tissue specific promoters may avoid attenuation for some vectors; the use of the endogenous poly-ubiquitin C promoter resulted in longer-term expression when plasmid DNA was delivered to the mouse lung as naked plasmid DNA[17] than when complexed with GL67 in this study (Figure 1b). This difference in the duration of transgene expression with naked plasmid DNA and GL67/plasmid DNA may be due to a difference in host inflammatory response;[43] suggesting that even an inefficient delivery vehicle like naked plasmid DNA can have some significant advantages over more efficient GTAs. Reducing the host inflammatory response to non-viral GTAs by minimizing the CpG content of the plasmid DNA results in a pronounced extension of the duration of transgene expression.[44] Therefore, to treat chronic lung diseases such as CF, non-viral gene transfer must be optimized at the level of plasmid and formulation in order to be as invisible as possible to the host innate immune surveillance systems.

Materials and methods

Procedures involving mice

Female BALB/c mice aged 6–8 weeks were used throughout the current study. Mice were housed in accordance with UK Home Office ethical and welfare guidelines and fed on standard chow and water *ad libitum*. Mice were anaesthetized by exposure to the volatile anaesthetic Metofane (methoxyflurane) (Mallinckrodt Veterinary Inc., Mundelein, IL, USA) until a balanced state of anaesthesia was achieved, as determined by a level of response to foot pad pinch. Plasmid DNA was delivered to the nose of the mouse following anaesthesia, while it was held vertically with closed mouth. A single continuous droplet was maintained by pipetting the dose volume with a Gilson P200 pipette (Gilson Inc., Middleton, WI, USA), the liquid being drawn into the nasal cavity and lungs of the mouse as it inhaled a breath.[12] When required, mice were killed by exposure to a rising concentration of CO_2 in an enclosed chamber or by intraperitoneal injection of 0.3 ml Sagatal (Pentobarbitone sodium) (Rhône Mérieux, Harlow, UK) diluted 1:5 with 1 × PBS. To collect BALF, the trachea was exposed and cannulated, lungs were lavaged four times with 1 ml BALF solution (1 × PBS, 0.1% w/v BSA, 0.05 mM EDTA) recovered and kept on ice. Cells from the BALF were concentrated by centrifugation at 400 r.c.f. for 10 min and re-suspended in 1.0 ml BALF solution. The BALF supernatant was retained for separate analysis.

Plasmid DNA delivery formulations and luciferase reporter gene assays

The plasmid pCIKLux contains the luciferase gene under the control of the immediate early human CMV promoter and the poly-adenylation signal from human SV40. It is derived from the mammalian expression vector pCI[45] (Promega, Southampton, UK) (GenBank accession number U47119). The plasmid pCIKLux.IO[17] is identical to pCIKLux but contains the coding sequence for the adenovirus type 2 E4 gene, open reading frame 3 (Ad2E4ORF3) positioned 3′ to the luciferase gene. Co-expression of luciferase and Ad2E4ORF3 is facilitated by an internal ribosome entry site positioned between the coding sequences. The plasmid pUbLux[17] contains the essential prokaryotic sequences from pCI but the luciferase gene is under the control of the promoter from the human ubiquitin C (Ub) gene (GenBank accession number D63791). Plasmid DNA was prepared using the Qiagen EndoFree Plasmid Purification kit (Qiagen, Crawley, UK), and maintained in endonuclease-free water (Promega) at −80°C. For naked DNA delivery, each mouse received 100 μg of plasmid diluted to a final volume of 150 μl with water for injection (B Braun Medical, Aylesbury, UK). Previous studies have identified this combination of concentration and volume as being the optimal dose for naked plasmid DNA instillation to the lung (data not shown).[17,46] For GL67/plasmid DNA, plasmid DNA was mixed with preformed liposomes as follows. Liposomes were generated by hydrating GL67 (Genzyme, Framingham, MA, USA) to 1 mM with water for injection, vortexed for 30 s and incubated for 10 min at 4°C followed by a final vortex for 2 min. In all, 385 μl of 1 mM liposome solution was heated to 30°C for 5 min in sterile polystyrene Bijou tubes (Bibby Sterilin, Stone, UK). Plasmid DNA (385 μl) was added and the combined solutions incubated for a further 15 min at 30°C to allow the formation of the GL67/plasmid DNA complexes. In all, 100 μl of the final complex at concentrations 0.5 mM GL67 containing 66 μg plasmid DNA was delivered to the airway of mice as described above. Subsequently, luciferase reporter gene activity in mouse lung homogenates was determined as described previously.[17]

Quantitative analysis of DNA by real-time TaqMan PCR

Total DNA was prepared from mouse lungs and BALF using the Qiagen DNeasy Kit (Qiagen). Plasmid DNA present in total DNA extracted from mouse lungs was quantified using an ABI PRISM™ 7700 Sequence Detector (TaqMan) (Applied Biosystems, Warrington, UK). Oligonucleotide primers and fluorogenic probe combinations for TaqMan assays were designed using the Primer Express V.1.5 (ABI) software package. The forward primer (5′-CCGCCTGAAGTCTCTGATTAAGTAC), reverse primer (5′-TGGAGCAAGATGGATTCCAAT) and

fluorogenic probe (5′-FAM-TTCCGATAGTCCACCGA GGGCGAC-TAMRA) hybridize across a 72 bp region of the luciferase reporter gene sequence and were used to quantify the plasmid vector DNA. These results were normalized for genomic DNA input relative to genomic rDNA, using the TaqMan Ribosomal RNA Control Reagents Kit (ABI). Reactions were performed using the TaqMan Universal PCR Master Mix (ABI) containing the thermally stable DNA polymerase AmpliTaq Gold. In all, 5.0 μl of template or standard was used per reaction with 12.5 μl TaqMan Universal PCR Master Mix, 1.5 μl of each primer (5.0 μM) and 0.5 μl probe (100.0 μM) in a final volume of 25.0 μl per reaction. Reactions were performed in 96-well optical reaction plates (ABI) and subjected to the following thermal cycler conditions, 50°C for 2 min followed by 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. Data was collected and the quantities of DNA in samples determined using the ABI PRISM™ Sequence Detection Systems Version 1.6.3 software package (ABI).

Southern hybridization of plasmid DNA purified from mouse lungs

Following agarose gel electrophoresis, lung DNA samples were transferred onto Hybond-N+ membranes (Amersham-Pharmacia, Amersham, UK) using a Posiblot apparatus (Stratagene, Cambridge, UK). DNA was cross-linked to the membrane in a Stratalinker UV Crosslinker (Stratagene) by exposure to 120 μJ UV (254 nm) and the membranes were stored at 4°C. DNA was radioactively labelled by incorporating Redivue α^{32}P-dCTP (3000 Ci/mmol) (Amersham-Pharmacia) into probes made by the Rediprime II Random Prime Labelling System (Amersham-Pharmacia). The plasmids pCIKLux and pUbLux were linearized with the restriction endonuclease *Alw*NI (New England Biolabs, Hitchin, UK) to create a template for the random primed probe and diluted to a concentration of 25 ng in 45 μl of TE buffer. Southern hybridization[47] was carried out at 42°C in 50% v/v formamide, 1 × SSC, 1 × Denhardts, 6% w/v dextran sulphate, 20 μg/ml salmon sperm DNA, 0.01% v/v SDS for 1 h after which the solution was replaced with an equal volume of pre-heated hybridization solution (as above, minus the salmon sperm DNA) with the addition of ^{32}P-labelled probe and incubated overnight. The membrane was removed from the hybridization solution, rinsed in 5 × SSC, 0.01% w/v SDS at RT to remove excess unbound probe and then washed in 2 × SSC, 0.01% w/v SDS then 1 × SSC, 0.01% w/v SDS each for 15 min at 65°C. Radioactive membranes were placed in storage phosphor cassettes (Molecular Dynamics, Wokingham, UK) for between 1 and 24 h. The storage cassettes were laser-scanned at a resolution of 50 μM with a Storm Phosphorimager (Molecular Dynamics) and hybridizations were analysed using ImageQuant version 1.2 for Macintosh (Molecular Dynamics). The Peak Finder analysis tool in ImageQuant was used to quantify the percentage of each isoform of plasmid DNA present. The accuracy of this analysis was validated by performing a separate Southern hybridization, with a DNA ladder (Stratagene) containing known concentrations of each DNA band. Using the Peak Finder tool, experimentally determined DNA concentrations were ±3% of the known amount (data not shown).

De novo methylation analysis of plasmid DNA extracted from mouse lungs

DNA prepared from mouse lungs dosed with GL67/plasmid DNA complexes was digested using endonucleases according to manufacturer's instructions (NEB). To generate a fully methylated positive control, a sample of mouse lung DNA was methylated with M.*Sss*I methylase (NEB) using 1 U of methylase per 1 μg of DNA, in 160 μM *S*-adenosylmethionine (SAM), 1 × reaction buffer. An equal volume of SAM was added after 0, 4 and 8 h and the entire reaction lasted for 18 h at 37°C. Lung DNA and positive control samples were digested with *Msp*I, *Hha*I, *Hpa*II and *Mcr*BC and subjected to agarose gel electrophoresis and Southern hybridization (above).

Statistical analysis

Distribution-free, Mann–Whitney *U*-tests were performed to determine statistical significance. Where appropriate, the Bonferroni correction for group analysis was applied to multiple group comparisons.

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