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RESEARCH ARTICLE

Increased persistence of lung gene expression using plasmids containing the ubiquitin C or elongation factor 1α promoter

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For effective gene therapy of chronic disease, persistent transgene expression at therapeutic levels is required. Clinical studies of airway gene transfer in patients with cystic fibrosis (CF) have resulted in short-lived transgene expression. We used intra-nasal dosing of naked plasmid DNA to the murine lung as a model for investigating the duration of airway gene transfer from a series of reporter expression plasmids. Transgene expression was transient when mediated by the viral promoters CMV, RSV and SV40, falling to less than 10% of peak expression after 2 weeks, although the presence of the adenoviral E4ORF3 gene in cis, resulted in extended duration of reporter activity from the CMV promoter. Transient expression from these promoters was not due to loss of the vector as determined by quantitative TaqMan PCR analysis. However, use of the promoters from the human polybiquitin C (UbC) and the elongation factor 1α (EF1α) genes resulted in persistent gene expression in the mouse lung. The UbC promoter directed high-level reporter activity which was maintained for up to 8 weeks and was still detectable 6 months after a single administration. Such persistent airway transgene expression from a nonviral vector without the concomitant expression of a potential antigen has not been reported previously. Thus, despite the persistence of vector DNA in vivo, attenuation of promoter function may lead to silencing of transgene expression and careful selection of promoter sequences is recommended for in vivo gene transfer. Gene Therapy (2001) **8,** 1539–1546.

Keywords: *gene transfer; naked DNA; lung gene expression; ubiquitin C promoter; elongation factor 1α promoter*

Introduction

The lung is a major gene therapy target for the treatment of inherited and acquired disorders such as emphysema, asthma, cancer and cystic fibrosis (CF). One of the main obstacles to the development of gene therapy for the airways is the inability of current viral and nonviral gene transfer vectors to direct sustained expression of a therapeutic transgene. In clinical studies, we have administered single and multiple doses of pDNA/liposomes to the nasal epithelia of CF patients demonstrating short-lived correction of the CF ion transport defect in the nose.[1,2] The transient nature of the correction was also observed in similar clinical studies undertaken by other groups.[3] In animal models, nonviral gene transfer mediated either by naked plasmid DNA (pDNA) or by pDNA/cationic liposome complexes, showed transgene expression to be maximal 1–2 days following administration, declining to background levels after 2–4 weeks.[4–6]

The loss of transgene expression may be explained in one of several ways: loss of the vector, transcriptional silencing of the transgene promoter, loss of the transfected cell through cell turnover, or the generation of an immune response to the transgene product or the transfected cell itself. The majority of pre-clinical and clinical airway gene transfer studies have used strong viral promoters in order to achieve high-level gene expression in the airways. The human immediate–early cytomegalovirus (CMV) enhancer/promoter which is one of the most widely used, is known to undergo transcriptional inactivation in several tissues[7,8] and does not direct persistent transgene expression following nonviral-mediated gene transfer to the airways.[4]

The persistence of CMV-directed transgene expression in the context of adenovirus-mediated gene transfer vectors is markedly affected by deletions of the E4 region of the adenoviral genome; adenoviral vectors which retain the E4 region direct more persistent *in vivo* expression than those in which the region is deleted.[9,10] This effect is mediated by open reading frame 3 (ORF3). Persistent CMV-mediated gene expression is observed if the entire E4 region is supplied *in trans* from either a second adenoviral vector[9] or from a pDNA vector.[11] However, a potential disadvantage of this approach is that the E4 ORF3 product is likely to be immunogenic and may induce a cellular immune response against transduced cells, thereby limiting its usefulness *in vivo*.

In this study we investigated the persistence of transgene expression mediated by the promoter elements commonly used in clinical studies for CF lung gene transfer.

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We confirmed that the adenoviral E4 ORF3 gene product can extend the duration of CMV-directed airway gene transfer albeit at levels significantly lower than those obtainable with the CMV promoter, and that this was not due to improved stability of the vector. As the improved persistence was promoter-specific,[11] we reasoned that it might be possible to identify promoter sequences capable of persistent lung gene expression without the co-expression of additional factors. Thus, we have identified two constitutive human promoters that offer increased persistence of lung gene expression when compared with strong viral promoters. The human elongation factor 1α (EF1α) promoter directed lung transgene expression similar to that achieved with the CMV promoter in combination with the E4ORF3 gene. The human polybiquitin C (UbC) promoter resulted in peak reporter expression at levels greater than the CMV promoter, 2 weeks after dosing. Transgene expression from the UbC promoter in the airways began to decline after 8 weeks, but was still detectable at 6 months after a single administration. To our knowledge, persistent airway transgene expression from a nonviral vector without the concomitant expression of a potential antigen has not been reported previously.

Results

Comparison of vectors containing commonly used viral promoters

All published clinical studies of nonviral vector-mediated gene transfer in CF patients have utilised constitutive viral promoter elements to direct transgene expression, including the immediate–early enhancer/promoter from CMV, the 3′ long terminal repeat of the Rous sarcoma virus (RSV) and the enhancer/early promoter from simian virus 40 (SV40). Three luciferase plasmid expression vectors pCIKLux, pRIKLux and pSIKLux were constructed by inserting the CMV, RSV or SV40 promoters, respectively, into a vector encoding a hybrid intron and the SV40 late polyadenylation signal. All three vectors were identical apart from their respective promoter sequences. To investigate gene expression from these vectors, BALB/c mice were instilled intra-nasally with 100 μg of pDNA in 150 μl of water and the lungs harvested on various days after administration. This formulation was chosen because it resulted in maximal transgene expression with naked DNA in the murine lung (data not shown). Reporter gene levels were plotted over time relative to CMV-mediated expression on day 2 (Figure 1). Reporter gene activity observed with the SV40 and CMV promoters was similar (8673 ± 1933 and 7455 ± 2352 relative light units per μg lung protein (RLU/μg) respectively, $P = 0.602$; 51 ± 6 RLU/μg for lungs receiving an irrelevant plasmid), with gene expression levels falling rapidly to less than 10% of peak expression after 14 days. Maximum reporter gene activity observed with the RSV promoter (2874 ± 1901 RLU/μg lung protein) was approximately three-fold lower than that observed with the SV40 promoter ($P = 0.047$). Similar levels and duration of gene expression were obtained when pDNA/DC-Chol:DOPE cationic liposome complexes were used instead of naked pDNA (data not shown). These results confirm that airway gene expression is transient when directed by these widely used viral promoters.

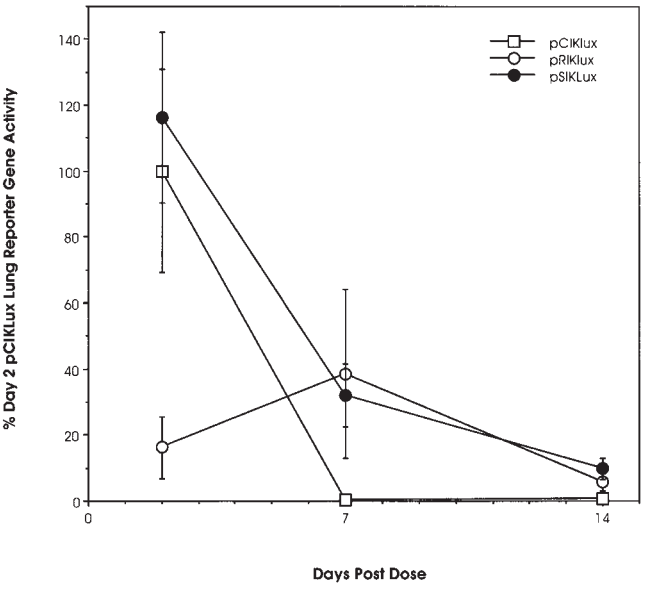

Figure 1 *Persistence of reporter gene expression in transfected mouse lungs. Mice were instilled with 100 μg of pCIKLux, pRIKLux or pSIKlux in 150 μl of water as indicated. Lungs and tracheas were harvested at the time-points indicated and assayed for reporter gene activity. The results are expressed as a percentage of that achieved with pCIKLux at 2 days after administration. Mean ± s.e.m. (n = 5) for each time-point are shown.*

The effect of mouse strain on persistence of gene expression

To investigate whether the reduction in airway gene expression following nonviral vector-mediated gene transfer was due to clearance of transfected cells by an immune-mediated response against the firefly luciferase gene product, the duration of CMV-directed reporter gene expression was compared in an immunocompromised SCID strain of mice, the inbred BALB/c strain and in the outbred MF1 strain. The overall profile of expression was very similar in all three mouse strains, although absolute transgene expression levels were lower in SCID mice (2038 ± 1053 RLU/μg lung protein) than in BALB/c mice ($P = 0.047$; Figure 2). These data suggest that loss of expression in the BALB/c mice is unlikely to be the result of an immune response against the reporter gene product.

Bicistronic pDNA vectors expressing reporter gene and adenoviral E4ORF3 have increased duration of expression

Airway gene transfer using adenovirus results in increased duration of transgene expression when the E4 region is retained in the viral backbone, or when the E4 region (specifically the E4ORF3 polypeptide) is supplied *in trans*.[9,10] Similarly, the duration of CMV-directed transgene expression following pDNA/cationic liposome-mediated airway gene transfer is greatly extended if the entire E4 region is supplied *in cis*.[11] In order to compare the effects of supplying E4ORF3 *in trans* and *in cis* on the duration of pDNA mediated expression, we constructed (1) pCIKORF3 in which the E4ORF3 gene is under the transcriptional control of the CMV promoter, and (2) a bi-cistronic expression plasmid pCIKLux.IO in which the CMV promoter directs the expression of both firefly luciferase and E4 ORF3 utilising an internal ribosome

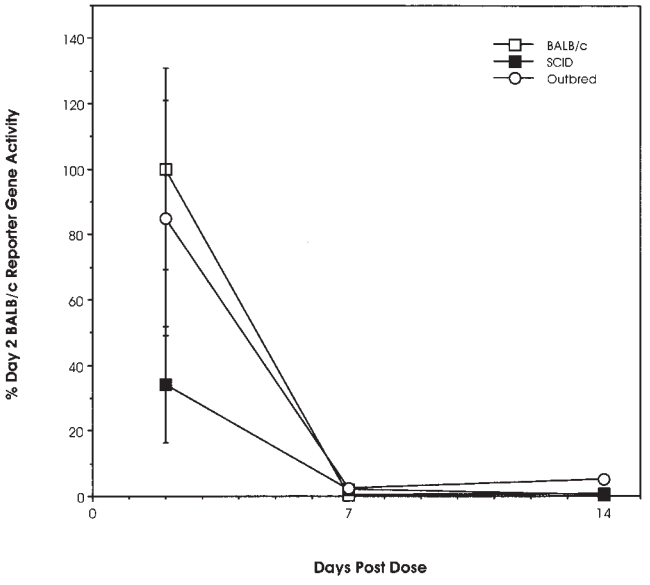

Figure 2 *Persistence of reporter gene expression is independent of mouse strain. Two inbred strains (BALB/c and SCID) and one outbred strain (MF1) of mice were instilled with 100 μg of pCIKLux in 150 μl of water. Lungs and tracheas were harvested at the time-points indicated and assayed for reporter gene activity. The results are expressed as a percentage of that achieved in BALB/c mice 2 days after administration. Mean ± s.e.m. (n = 5) for each time-point are shown.*

entry site. Plasmids pCIKLux and pCIKLux.IO were transiently transfected into HEK293 cells and after 48 h, cell lysates were prepared and assayed for luciferase activity. Figure 3a shows that both plasmids directed similar levels of reporter activity, indicating that incorporation of the 950 bp *Not*I IRES-E4ORF3 (IO) fragment into pCIKLux did not significantly reduce transgene expression in cell culture. Western blotting of HEK293 cells transiently transfected with pCIKLux.IO and pCIKORF3 confirmed the expression of the adenoviral E4ORF3 gene product from these plasmids (Figure 3b).

Formulations containing pCIKLux, pCIKLux.IO or pCIKLux/pCIKORF3 (an equi-mass mixture of pCIKLux and pCIKORF3) were instilled into the lungs of BALB/c mice. Reporter gene activities with pCIKLux and the pCIKLux/pCIKORF3 mixture were similar 2 days after administration ($P = 0.754$) while reporter gene activity with pCIKLux.IO (1999 ± 770 RLU/μg lung protein) was approximately three-fold lower than the levels observed with pCIKLux ($P = 0.028$; Figure 4). However, while reporter gene activity in mice instilled with pCIKLux declined to approximately background levels by 7 days after administration, co-expression of E4ORF3, either *in cis* (pCIKLux.IO) or *in trans* (pCIKLux/pCIKORF3), greatly extended the period in which reporter gene activity persisted (Figure 4), such that after an initial decline, reporter gene expression directed by pCIKLux.IO remained constant for 12 weeks after dosing.

Transient lung gene expression is not mediated by loss of pDNA

One explanation for the increased persistence of airway reporter gene expression observed with pCIKLux.IO compared with pCIKLux (Figure 4) is that co-expression of the E4 ORF3 protein stabilises the plasmid expression vector and prevents its loss or degradation. To assess this

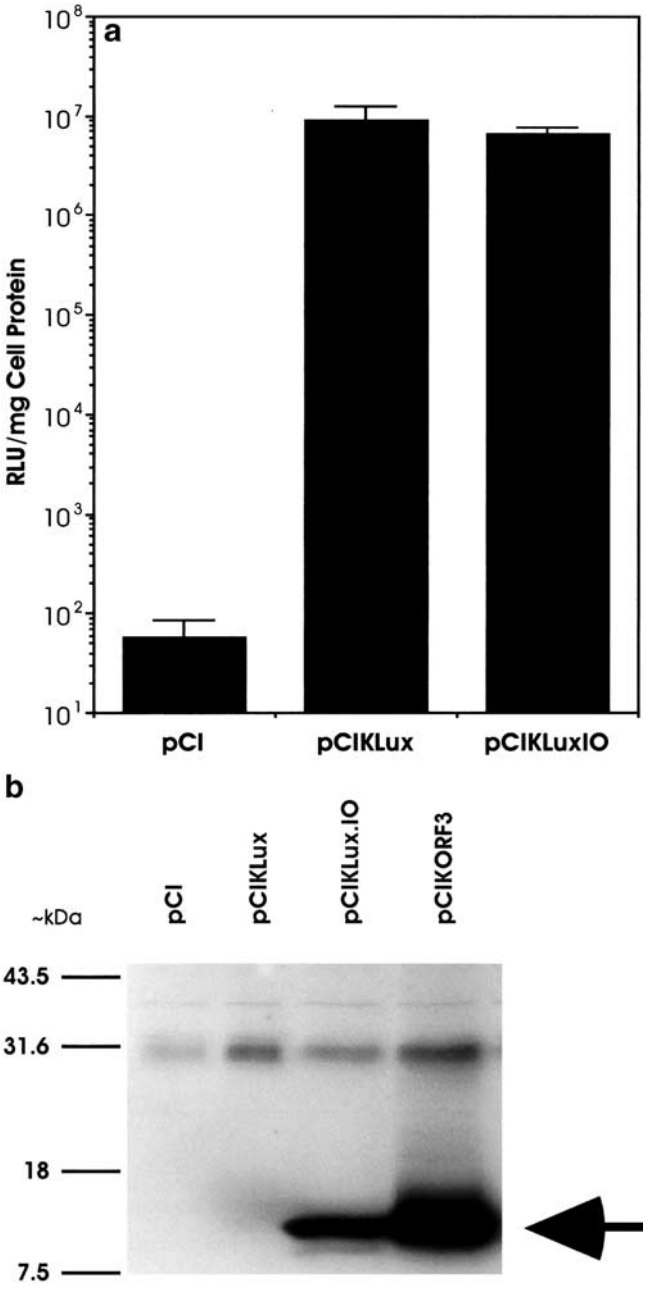

Figure 3 *Co-expression of luciferase and Ad2 E4 ORF3 from pCIKLux.IO. (a) In vitro luciferase reporter gene activity. HEK293T cells were transiently transfected with plasmids pCI, pCIKLux and pCIKLux.IO as indicated. Reporter gene activity, normalised to cell protein concentration, was assayed 48 h after transfection (n = 3). (b) Western blot of HEK293T cells transiently transfected with pCI, pCIKLux, pCIKLux.IO and pCIKORF3 as indicated. Total cell lysates were prepared 48 h after transfection. The arrow indicates the Ad2 E4 ORF3 protein migrating at approximately 11 kDa. Positions of molecular mass markers are indicated.*

possibility we determined the absolute levels of pCIKLux and pCIKLux.IO DNA in airway samples 2 to 14 days after administration. Total DNA was extracted from homogenised mouse lungs and plasmid DNA quantified using quantitative TaqMan PCR analysis. The results showed that only a very small fraction of the applied dose of either plasmid was detectable 2 days after administration, and that pDNA levels for the two vectors were

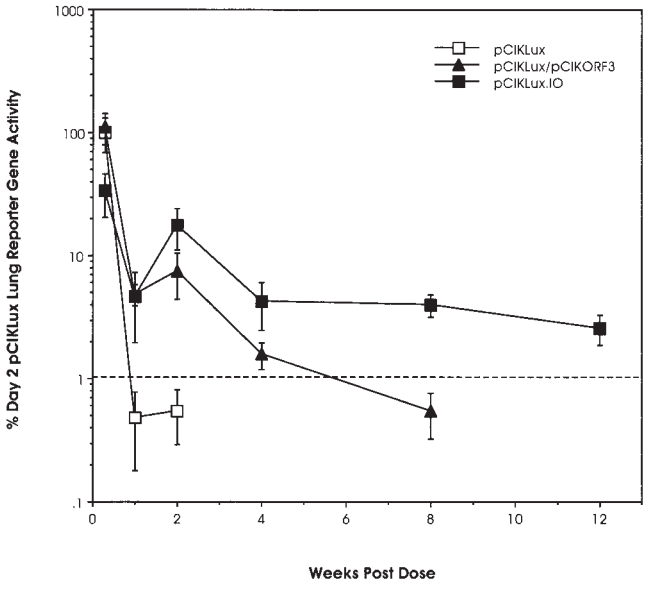

Figure 4 *Persistence of reporter gene activity is enhanced by co-expression of Ad2 E4 ORF3. Mice were instilled with 100 μg of pCIKLux, pCIKLux/pCIKORF3 (an equi-mass mixture of pCIKLux and pCIKORF3) or pCIKLux.IO in 150 μl of water as indicated. Lungs and tracheas were harvested at the time-points indicated and assayed for reporter gene activity. The dashed line represents the approximate sensitivity of the assay. Mean ± s.e.m. (n = 5) for each time-point are shown.*

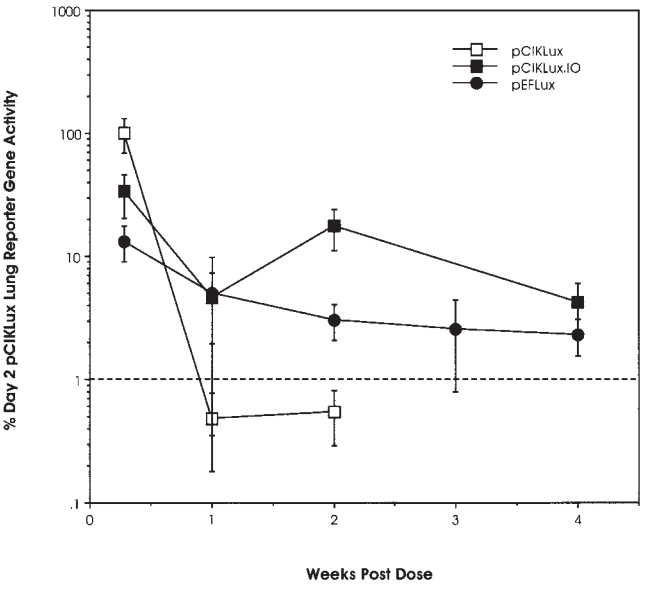

Figure 6 *Persistent reporter gene expression from pEFLux. Mice were instilled with 100 μg of pCIKLux, pCIKLux.IO or pEFLux in 150 μl of water. Lungs and tracheas were harvested at the time-points indicated and assayed for reporter gene activity. The dashed line represents the approximate sensitivity of the assay. Mean ± s.e.m. (n = 5–6) for each time-point are shown.*

essentially identical (Figure 5). Thus, despite an approximately 20-fold greater level of reporter gene expression from pCIKLux.IO at day 14 compared with pCIKLux ($P = 0.008$), no differences in the quantity of vector DNA present in the lung tissue were detected at this time ($P = 0.624$).

Human promoters can result in persistent lung gene expression

Since the E4 ORF3 polypeptide can act to increase the persistence of transgene expression in a promoter-specific manner,[11] we hypothesised that it may be possible to identify alternative promoter elements that by themselves direct persistent airway expression. We replaced the CMV enhancer/promoter and the hybrid intron present in pCIKLux with enhancer/promoter/5′ untranslated elements from the constitutively expressed human genes, elongation factor 1α[12] and polyubiquitin C,[13] to generate plasmids pEFLux and pUbLux, respectively. Thus the plasmid backbone sequence and luciferase transgene were identical to each other and to pCIKLux. After administration to BALB/c mice, both plasmids directed significantly more sustained airway reporter gene expression than the CMV promoter (Figures 6 and 7) without co-expression of the E4 ORF3 protein.

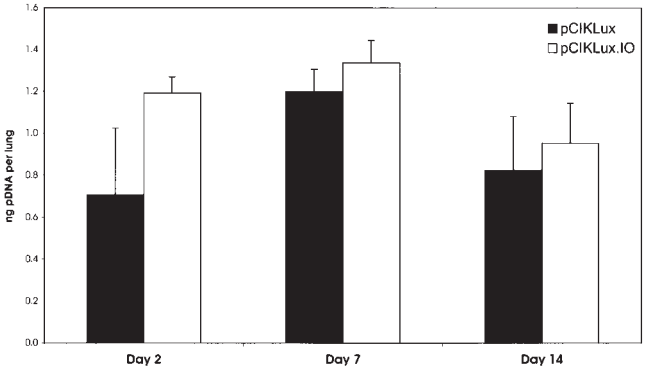

Figure 5 *Transient lung gene expression from pCIKLux is not mediated by loss of pDNA. Mice were instilled with 100 μg of pCIKLux or pCIKLux.IO in 150 μl of water as indicated. Lungs and tracheas were harvested at the time-points indicated and assayed for pDNA content. Mean ± s.e.m. (n = 5) for each time-point are shown.*

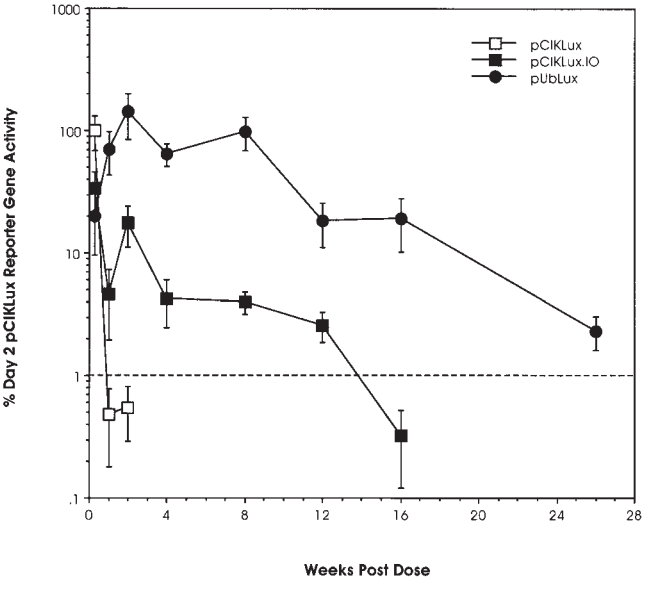

Figure 7 *Persistent reporter gene expression from pUbLux. Mice were instilled with 100 μg of pCIKLux, pCIKLux.IO or pUbLux in 150 μl of water. Lungs and tracheas were harvested at the time-points indicated and assayed for reporter gene activity. The dashed line represents the approximate sensitivity of the assay. Mean ± s.e.m. (n = 5–6) for each time-point are shown.*

Reporter gene expression from pEFLux at 2 days after administration was approximately five-fold lower than that observed for the CMV promoter (Figure 6; $P = 0.016$), but was substantially more sustained, remaining essentially unchanged from day 7 to day 28 after administration (Figure 6; $P = 0.28$). During this period, absolute levels of airway reporter gene expression from pEFLux were very similar to those obtained with pCIKLux.IO (Figure 6; $P = 0.916$ at day 28).

At 2 days after administration, levels of airway reporter gene expression from pUbLux were also approximately five-fold lower than with the CMV promoter ($P = 0.028$), but levels increased at subsequent time-points reaching a maximum approximately 14 days after administration (Figure 7). Crucially, gene expression from pUbLux was similar to levels observed 2 days after administration with the CMV promoter ($P = 0.602$) and were maintained at this level for up to 8 weeks after administration. Between 8 and 26 weeks after dosing, reporter gene expression decreased slowly towards background levels (Figure 7) which may reflect normal cell turnover in the murine lung. No reporter gene expression was observed at 12 months after dosing (data not shown).

Discussion

Although therapeutic benefit in gene therapy does not always rely on long-term gene expression, it is assumed that the treatment of chronic lung disease will require significant persistence of gene expression. Airway gene expression in both pre-clinical and clinical studies for CF has been short-lived.[3] The reasons for this have been difficult to determine from published studies, due to differences in the animal models, gene transfer vectors, transcription signals and transgenes studied. To minimise the variables and determine the causes of transient reporter gene expression in the lung, we focused on the simplest of gene transfer vectors, naked pDNA in water, and systematically investigated the persistence of reporter gene expression in the mouse lung.

The precise rate of turnover of lung epithelial cells is unknown but is likely to be in the region of months rather than weeks. Thus, the observed loss of transgene expression with viral promoters in the mouse lung (Figure 1) is unlikely to be explained by normal lung cell turnover. We have shown that the transient gene expression observed in the mouse lung is unlikely to be due to the loss of transfected cells resulting from an anti-transgene immune response, because transient reporter gene expression was also observed in SCID mice (Figure 2). We also excluded the possibility that transient gene expression might be due to the loss of vector DNA from the transfected cells *in vivo*. Quantitative TaqMan PCR was used to measure the persistence of plasmid DNA in the lung at various time-points after dosing and showed that only a very small fraction of the plasmid (less than 0.001% of the applied dose) was detectable 2 days after administration, indicating that either the majority of plasmid DNA did not reach the lung, or that significant degradation of the plasmid must have occurred within 48 h of dosing. However, there was no significant difference between the persistence of the pCIKLux and pCIKLux.IO vectors in the lung between 2 and 14 days (Figure 5) even though the duration of reporter gene expression from these plasmids was very different due to the presence of E4 ORF3 in pCIKLux.IO (Figure 4). Thus, differential loss of plasmid DNA is unlikely to be responsible for differences in reporter gene expression at similar time-points. Studies designed to measure the stability of naked pDNA in mouse liver have also shown that circular pDNA persists *in vivo*, and that transient transgene expression in the liver cannot be attributed to the instability of vector DNA in transfected cells.[14] Similarly using adenoviral vectors, gene expression from the CMV promoter is lost despite persistence of vector DNA in the liver[8] and the lung.[9] Thus, there are several reports of transient transgene expression despite successful delivery and persistence of viral and nonviral vector DNA *in vivo*.

Several lines of evidence suggest that attenuation of promoter function may be the most significant factor in the lack of persistence of transgene expression.[15–17] Many viral promoters are transcriptionally regulated by cellular factors; the human CMV promoter contains sequences for binding of NFκB required for early gene transcription.[18] Viral promoters are also sensitive to inflammatory-mediated cytokines. An early host response to infection by adenoviral vectors is the production of cytokines including TNFα and IFNγ, which have been shown to inhibit CMV-mediated adenoviral transgene expression *in vivo*.[19] These cytokines have also been shown to inhibit reporter gene expression from CMV, RSV and SV40 promoters in myocytes, when adenoviral, retroviral or plasmid vectors were employed.[16] Furthermore, the presence of CpG motifs in pDNA[20] can lead to elevated levels of IL-12, TNFα, IFNγ and other cytokines after *in vivo* delivery of pDNA and pDNA/liposome vectors to the airways.[21,22] Thus, the complex interaction of transcription factors in the target tissue and the cellular response to vector delivery, are likely to impact on promoter function and the duration of transgene expression. While this may go some way towards explaining contradictory findings in the literature, more importantly, they emphasise that attention should be paid to the specific host–vector interaction if persistent *in vivo* transgene expression is required.

One possible approach to solving promoter attenuation *in vivo*, might be to map the transcription factor binding sites in strong viral promoters and to modify vector sequences accordingly. Incorporation of the 950 bp *Not*I IRES-E4ORF3 (IO) fragment into viral and nonviral gene transfer vectors could theoretically be used to extend transgene expression from the CMV promoter *in vivo* since expression of the adenoviral E4ORF3 gene *in cis* resulted in improved persistence of gene expression (Figure 4). Interestingly, the effects appear to be promoter-specific; co-expression of E4ORF3 *in trans* with the UbC or EF1α promoter plasmids inhibited reporter gene expression (data not shown). However, extended use of the E4ORF3 gene product in the clinic is likely to be antigenic and may be complicated by the variety of effects it mediates in the cell, including the induction of changes in host cell nuclear structure.[23] Although the precise mechanism is unknown, the E4ORF3 protein may act directly or indirectly on the promoter[9,11] to increase persistence. Together these observations are consistent with promoter attenuation being responsible for transient gene expression in the mouse lung.

Promoter attenuation is only one example of the general phenomenon of gene silencing, which is diverse and

found in a wide array of species.[24,25] Although little is known about the precise mechanism of gene silencing, the processes involved include DNA methylation, the recruitment of binding proteins that preferentially recognise methylated DNA and subsequent chromatin re-modelling.[17,26,27] It is perhaps unsurprising that strong viral promoters such as CMV may become attenuated in tissues, such as the lung, that are not naturally infected by the virus.[7] We therefore chose to investigate the expression profile of constitutive human promoters arguing that these promoters might be less sensitive to attenuation *in vivo*. Specifically, we incorporated the EF1α and UbC promoters into our plasmid vectors. Studies in transgenic mice have shown that the UbC promoter directs transgene expression in a wide range of tissues compared with promoter/enhancer sequences that are routinely used for such experiments.[28] Here, we show that both plasmids can direct persistent reporter gene expression in the mouse lung (Figures 6 and 7). Reporter gene activity from the EF1α promoter persists initially compared with CMV, but is relatively low and therefore gene expression declines to background levels within a few weeks (Figure 6). If persistent low-level transgene expression is required, then EF1α may be the promoter of choice. However, reporter activity directed by the UbC promoter at 2–8 weeks after dosing was at a level similar to that observed from CMV at day 2, and is still detectable at 6 months after a single administration (Figure 7). This may suggest that airway cells can exist within the mouse lung for up to 6 months, or that at least some of the transfected cells may be dividing, depending on the cell type(s) transfected in these experiments. Identification of the specific cell populations directing persistent transgene expression in the lung is now underway. If similar persistent gene expression can be observed in human airways, then incorporation of the UbC promoter in gene transfer vectors might be helpful for lung gene therapy in the clinic.

The reasons for the EF1α and UbC promoters being more resistant to gene silencing than the viral promoters examined are unknown. It is possible that as constitutive, endogenous promoters they are relatively insensitive to cellular cytokines in the mouse lung and analysis of promoter function in the context of local host inflammation may help elucidate this. Alternatively, it is possible that a small proportion of the airway cells may have a copy of the plasmid molecule integrated into the genome permitting long-term expression of the transgene, although this would have to occur preferentially for plasmids carrying the UbC and EF1α promoters as opposed to CMV. However, we expect that the majority of plasmid DNA delivered to the lung will remain episomal in the absence of any selection and will eventually be diluted from the airway cell population as cell division in the lung proceeds. It is also possible that linear concatamers of the plasmid are present in the cell, which have been shown to result in significant persistence of transgene expression in the liver,[14] and these possibilities are being investigated.

Pre-clinical and clinical studies have shown that both viral and nonviral airway gene transfer is very short-lived, lasting for only a few weeks at best. We have systematically investigated the reasons for these observations using a series of pDNA vectors in the mouse lung. We have found that in the absence of an immune response, where the gene transfer vector is stable, attenuation of promoter function may be the primary factor responsible for transient transgene expression. If persistent gene expression is required for successful gene therapy then particular attention must be paid to understanding gene silencing mechanisms in the target organ, and to the design of vectors containing promoter sequences which are resistant to gene silencing.

Materials and methods

Preparation of plasmid DNA

Endotoxin-free plasmid DNA was purified using Qiagen-tip Q2500 MEGA columns (Qiagen, Hilden, Germany) and resuspended in sterile water for injection (Sigma, Poole, UK).

Plasmid expression vectors

The plasmid expression vector pCI (Promega, Madison, WI, USA; GenBank accession No. U47119), containing the human CMV immediate–early gene promoter and enhancer, a hybrid intron (5′ splice donor site from the first intron of the human β-globin gene and the branch and 3′ splice acceptor site from the intron of an immunoglobulin heavy chain variable region gene) and the SV40 late polyadenylation signal, was used as the primary backbone for all other plasmids. Fragments for sub-cloning into pCI were amplified using PCR such that they contained a consensus Kozak sequence[29] and ATG start codon, and could be inserted into pCI digested with *Nhe*I and *Not*I. A 1675 bp fragment containing the luciferase cDNA was amplified from pGL3 (Promega) to generate plasmid pCIKLux. Plasmid pCIKORF3 contained the adenovirus E4ORF3 gene[30] amplified from adenovirus type 2 DNA (Gibco BRL, Paisley, UK). The bi-cistronic vector, pCIKLux.IO was constructed from pCIKLux with an internal ribosome entry site (IRES) and the E4ORF3 gene inserted downstream of the luciferase gene as follows. A 601 bp (5′-*Not*I, 3′-blunt ended) PCR fragment containing the encephalomyocarditis virus IRES, amplified from plasmid pCITE-1 (Novagen, Madison, WI, USA) and a 349 bp (5′-blunt ended, 3′-*Not*I) PCR fragment containing E4ORF3 (34 707–34 326 bp of the adenovirus genome[31]), amplified from adenovirus DNA, were ligated into plasmid pCIKLux digested with *Not*I. The IRES-E4ORF3 (IO) sequence could be released from pCIKLux.IO on a 900 bp *Not*I fragment. Plasmid pSIKLux was constructed from pSI (Promega) by insertion of the *Nhe*I-*Not*I luciferase fragment (above). Plasmid pRIKLux was constructed from pCIKLux, by replacing the CMV immediate–early promoter and enhancer with a *Bgl*II–*Pst*I PCR fragment containing the RSV 3′ LTR promoter derived from base pairs 453–1068 of plasmid pREP8 (Invitrogen, Leek, The Netherlands). Plasmid pEFLux was constructed by replacement of the *Bgl*II–*Nhe*I fragment containing the CMV enhancer/promoter and hybrid intron from pCIKLux, with a PCR fragment containing the human elongation factor 1α promoter, exon 1, intron A and exon 2 sequences derived from base pairs 376–1687 of plasmid pEF1/VS-HisA (Invitrogen). Plasmid pUbLux was constructed by replacing the same *Bgl*II–*Nhe*I fragment from pCIKLux with a PCR fragment containing the human polyubiquitin C promoter, exon 1, intron 1 and exon 2 sequences (GenBank D63791; base

pairs 3561–4771 or –333 to +877 relative to the putative transcription start site).

Transfection of cells grown in vitro

Plasmid DNA was introduced into human embryonic kidney (HEK293T) cells[32] using cationic liposomes formulated from 3β[N-(N′,N′-dimethylaminoethane)-carbomoyl] cholesterol and dioleoylphosphatidylethanolamine (DC-Chol/DOPE).[33] Cells were seeded into 60-mm dishes previously coated with poly-L-lysine (Sigma) at a density of 2.5×10^5 cells per dish and the cells grown for 20 h, after which they were transfected with 2.5 μg plasmid DNA and 25 nmol DC-Chol/DOPE liposomes in Opti-MEM (Gibco BRL). Growth medium was replaced after 4 h and cells were harvested after 2 days. Where purification of transfected cells was required, 0.5 μg of plasmid πH3-CD8 (a gift from B Seed, Harvard Medical School, Boston, MA, USA) was included in the DNA for transfection and cells harvested for protein extraction after 24 h.

Preparation of total cell extracts for Western blotting

Transfected cells were mixed with 100 μl of magnetic CD8 Dynabeads (Dynal, Bromborough, UK) and rocked for 15 min. After washing, cells were rocked for 45 min in the presence of 10 μl of DetachaBead CD4/CD8 (Dynal). After removal of the beads, the cells were lysed (PBS containing 2 mM EDTA, 2% SDS, 0.4 mM leupeptin, 0.02 mM pepstatin, 4 mM benzamidine) and the viscous material passed down a Qiashredder column (Qiagen), centrifuged and the eluate collected. Total cell protein was measured using a Biorad detergent compatible protein assay (BioRad, Hemel Hempstead, UK). Samples were incubated in SDS-PAGE loading buffer at 37°C for 30 min before SDS-PAGE on 15% gels and blotted on to ECL nitrocellulose membrane (Amersham, Little Chalfont, UK). Membranes were incubated in PBS 5% skim milk powder and incubated overnight in anti-E4ORF3 antibody (a gift from G Ketner, Johns Hopkins Public School of Health, Baltimore, MD, USA) at a dilution of 1/100. After washing with PBS 0.1% Tween, the membrane was incubated in goat anti-rabbit antibody conjugated with horseradish peroxidase (Dako, High Wycombe, UK). Signal was detected using enhanced chemi-luminescence (Amersham).

Lung dosing and analysis of reporter activity

For gene delivery to the lungs, female BALB/c mice (6–8 weeks old) were anaesthetised with Metofane (Mallinckrodt Vetinary, Mundelein, IL, USA) and instilled intranasally essentially as described.[4] Animals were killed and the lungs and tracheas harvested and stored at –80°C in Reporter Lysis Buffer (Promega). After thawing, tissues were homogenised using a Ultra-Turrax T8 tissue homogeniser (IKA Labortechnik, Staufen, Germany) and reporter activity measured using the Luciferase Assay System (Promega). Reporter enzyme activity for both *in vitro* and *in vivo* samples was determined using standard curves of purified recombinant Luciferase (Promega). Total lung protein concentrations were determined using a detergent-compatible Protein Assay (BioRad). Reporter enzyme activity was normalised for protein content before graphing.

Quantification of plasmid DNA in mouse lungs using TaqMan PCR

Total DNA was prepared using a modified version of the Qiagen DNeasy protocol. Mouse lungs were stored at –80°C until required, thawed, minced and processed according to the manufacturer's instructions, except that whole lung samples were centrifuged through a Qiashredder column to reduce viscosity before processing. Plasmid DNA present in the total DNA extracted from mouse lungs was quantified using an ABI PRISM 7700 Sequence Detector (TaqMan) (Applied Bioystems, Warrington, UK). Oligonucleotide primers and fluorogenic probe combinations for TaqMan assays were designed using the Primer Express V.1.0 (Applied Biosystems) software package and purchased from Applied Biosystems. The quantitative plasmid DNA assay was based on the pCI expression vector such that the target sequence amplified was common to both pCIKLux and pCIKLux.IO. The reaction utilised the forward and reverse primers, pCIRT-PCR-62F, (5′-GCTTCTGACACAACAGT CTCGAA, hybridising between base pairs 797 and 819 of pCI) and pCIDNA-148R (5′-AACCTGTCTTGTAA CCTTGATACTTACCT, base pairs 883 and 855), respectively. The fluorogenic probe pCIRT-PCR-86T (5′-TTAAGCTGCAGAAGTTGGTCGTGAGGC) hybridised between bases 821 and 847 of pCI. A stock dilution series of plasmid vector was used to generate a quantitative standard curve. The results were normalized for genomic DNA using the commercially available TaqMan 18S Ribosomal RNA Control Reagents Kit (Applied Biostems) and a stock dilution series of mouse genomic DNA (Clontech, Basingstoke, UK). Reactions were performed using TaqMan Universal PCR Master Mix with DNA polymerase AmpliTaq Gold. (Applied Biosystems) and thermal cycler conditions: 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. Data were collected and analysed using the ABI PRISM Sequence Detection Systems Version 1.6.3 software package (Applied Biosystems).

Statistical analysis

Values shown are mean ± standard error of the mean. Comparison between means were performed using the Mann–Whitney *U* test with a significance level of 5%. Analyses were performed using StatView for Macintosh (SAS Institute, Cary, NC, USA).

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