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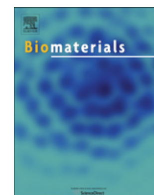

CpG-free plasmid expression cassettes for cystic fibrosis gene therapy

Ian A. Pringle [a,b,1], Stephen C. Hyde [a,b,1], Mary M. Connolly [a,b,1], Anna E. Lawton [a,b,2], Bihui Xu [a,b,3], Graciela Nunez-Alonso [a,b], Lee A. Davies [a,b], Stephanie G. Sumner-Jones [a,b], Deborah R. Gill [a,b,*]

[a] Gene Medicine Research Group, NDCLS, John Radcliffe Hospital, University of Oxford, Oxford OX3 9DU, UK
[b] The UK Cystic Fibrosis Gene Therapy Consortium, UK

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ABSTRACT

Clinical studies are underway for the aerosol delivery of plasmid DNA complexed with Genzyme Lipid GL67A to the lungs of patients with cystic fibrosis (CF). Plasmid vectors contain several functional elements all of which play a role in determining the efficacy of the final clinical product. To optimise the final plasmid, variations of CpG-free 5′ enhancer elements and 3′UTR regions were inserted into a common CpG-free, plasmid backbone containing Luciferase or CFTR transgenes. Plasmids were compared in immortalised cell culture, human airway liquid interface primary cell cultures, and mouse lung models to determine which design directed optimal transgene expression. Following aerosol delivery to mouse lung, plasmids containing the murine CMV enhancer showed higher peak Luciferase activity than the human CMV enhancer, but the human version resulted in persistent expression. In cell culture, the SV40 3′UTR and a novel BGH2 3′UTR exhibited up to 20-fold higher Luciferase activity than the commonly used BGH 3′UTR, but in mouse lung aerosol studies the activity and duration was greater for BGH 3′UTR. Systematic evaluation of each functional component of the plasmid has resulted in an improved design, exhibiting superior levels and duration of lung gene expression.

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1. Introduction

Since the discovery of the cystic fibrosis (CF) transmembrane conductance regulator (CFTR) gene [1], replacement of CFTR in the airways of CF patients has been investigated as a potential treatment for the lung aspects of the disease [2]. Plasmid DNA encoding the CFTR protein can be delivered to cells in the lung when complexed with a gene transfer agent (GTA) such as a cationic lipid, protein or polymer [3]. Representative GTAs from each of these categories have been evaluated pre-clinically following aerosol delivery of plasmid DNA to sheep, a large animal lung model with characteristics similar to the human lung [4]. These studies led to the selection of cationic liposome GL67A as our lead non-viral GTA for CF gene therapy. Early clinical studies delivering plasmid DNA complexed with GL67A liposomes to the lungs of CF patients established proof of principle for GL67A non-viral gene therapy, but effects in the nose [5] and lung [6,7] were transient, and in addition, patients experienced transient flu-like symptoms shortly after delivery [6,7].

The role of the plasmid DNA in generating the observed inflammatory response has been investigated in a mouse lung model. Specifically, the presence of CG dinucleotides (CpGs) in the plasmid DNA can lead to a transient neutrophilic infiltration of the lung and an elevation in pro-inflammatory cytokines in bronchoalveolar lavage fluid collected 24 h after delivery [8,9]. Subsequently, a series of plasmids utilising the R6K origin of replication was developed, that were completely free of CpGs. When GL67A was complexed with CpG-free plasmid DNA and delivered to the mouse lung, no inflammation was detected [10], suggesting that the elimination of CpGs from the plasmid could be an important step in minimising the inflammatory consequences of GL67A liposome delivery to patients in the clinic.

Previously we have shown that the choice of promoter sequence used for plasmid-mediated transgene expression can affect both the level and duration of reporter gene expression in the mouse lung [11] and promoter selection is now understood to be crucial for achieving the desired expression profile [12]. Replacement of commonly used viral promoters with promoters from endogenous ‘housekeeping’ genes, such as the human polyubiquitin C promoter, is a successful strategy in the mouse lung [13]. However, the requirement for CpG-free plasmids, in order to minimise

* Corresponding author. Gene Medicine Research Group, NDCLS, John Radcliffe Hospital, University of Oxford, Oxford OX3 9DU, UK. Tel.: +44 1865 221845; fax: +44 1865 221834.
E-mail address: deborah.gill@ndcls.ox.ac.uk (D.R. Gill).
[1] These authors contributed equally.
[2] Present address: Department of Zoology, University of Oxford, South Parks Road, Oxford OX13PS, UK.
[3] Present address: Cell and Molecular Biology Graduate Group, University of Pennsylvania, PA 19104, USA.

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inflammation, does constrain the choice of promoter and other sequence elements that can impact upon gene expression. We have previously demonstrated that a CpG-free version of the Elongation Factor 1 alpha promoter (EFI) can direct sustained long-term expression in the mouse lung when used in conjunction with a CpG-free version of the human CMV enhancer element (hCEFI) [10]. Enhancer elements can boost gene expression when placed adjacent to promoter sequences, where they bind transcription factors and help recruit RNA Polymerase II [14,15]. The human CMV immediate/early enhancer is effective at elevating transgene expression from the polyubiquitin B [16] and cytokeratin K18 promoters in the mouse lung [17]; and it is also used in gene therapy models in other organs to boost expression from the chicken β-actin promoter (CBA/CAG) [18,19]. Plasmids containing the murine CMV enhancer can direct long-term expression of IFN-γ following high pressure tail vein delivery [20] and persistent expression of Luciferase (Lux) in the liver of immune deficient mice [21]. Therefore, in this study we compared the relative activities of the CpG-free human CMV enhancer (hC) with an analogous murine version (mC).

Another major functional component of plasmids that can be optimised is the 3′ un-translated region (3′UTR) beyond the stop codon. The 3′UTR of the transcribed mRNA is important for RNA stability and regulation [22]. Their composition in mammalian cells

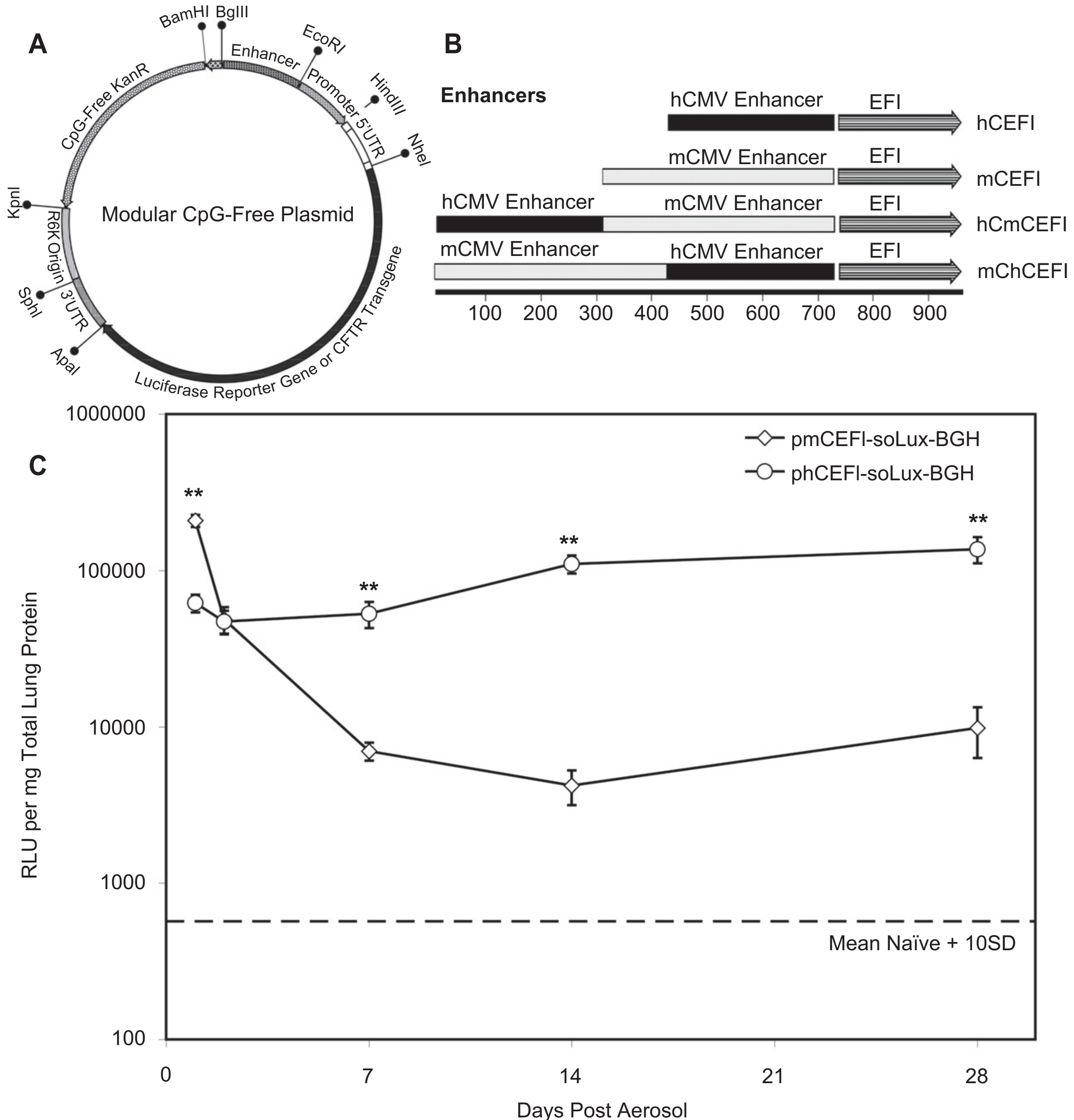

Fig. 1. Effect of enhancer choice on reporter gene expression. (A) All plasmids used in this study are based on a modular CpG-free design with unique restriction sites separating the main structural components (A) and allowing for the creation of variant plasmids with multiple enhancer/promoter options (B). The single human CMV (hCMV) enhancer (black) and the murine CMV (mCMV) enhancer (white) were inserted upstream of the EFI promoter as either single or double enhancer constructs. (C) Duration of expression from phCEFI-soLux-BGH (hCMV) and pmCEFI-soLux-BGH (mCMV). Mice were exposed to a pDNA/25 kDa PEI aerosol and lungs were harvested to measure Lux activity (RLU per mg total lung protein) at the time-points indicated. $n = 6$ per time-point, error = 1 SEM. ★★ indicates $p < 0.01$ by Mann–Whitney *U*-test.

is variable but typically includes the polyadenylation (AAUAAA) signal, which is a binding site for the RNA cleavage complex, and binding sites for proteins and small RNAs that influence RNA stability [22]. In plasmid vectors, short 3′UTR sequences are often used, such as the SV40 3′UTR or the Bovine Growth Hormone (BGH) 3′UTR, each of which has three components: (i) a UTR following the stop codon, (ii) a polyadenylation site (AAUAAA) and (iii) gene regulatory elements (GRE) containing multiple protein binding sites. In addition to evaluating the commonly used, naturally CpG-free SV40 3′UTR sequence and a CpG-free version of the BGH 3′UTR [23], we also designed an element based on the BGH 3′UTR termed BGH2.

In this study we evaluated the main functional elements of an expression plasmid in the context of a minimal, pharmacopoeia-compliant plasmid backbone [24]. In addition to measuring transgene expression in two cell culture models, we also investigated plasmid performance in mouse models of lung gene therapy to facilitate selection of an optimal plasmid design.

Table 1
Names and description of plasmids. All plasmids used in these studies were based on the minimal, modular, CpG-free expression vector (Fig. 1a), incorporating the functional elements as indicated. hCEFI: human CMV enhancer human elongation factor 1α; GZB: synthetic, CpG-free, cytomegalovirus promoter; soLux: synthetic codon, optimised cDNA for firefly luciferase; soCFTR: synthetic, CpG-free, codon-optimised cDNA for human cystic fibrosis transmembrane conductance regulator; 3′ UTR: 3′ un-translated region; BGH: Bovine Growth Hormone; SV40: Simian virus 40.

Plasmid Name	Enhancer/promoter	Transgene	3′UTR
phCEFI-soLux-BGH	hCEFI	soLux	BGH
pmCEFI-soLux-BGH	mCEFI	soLux	BGH
phCmCEFI-soLux-BGH	hCmCEFI	soLux	BGH
pmChCEFI-soLux-BGH	mChCEFI	soLux	BGH
phCEFI-soLux-SV40	hCEFI	soLux	SV40
phCEFI-soLux-BGH2	hCEFI	soLux	BGH2
pGZB-soLux-BGH	GZB	soLux	BGH
pGZB-soLux-SV40	GZB	soLux	SV40
pGZB-soLux-BGH2	GZB	soLux	BGH2
phCEFI-soCFTR-BGH	hCEFI	soCFTR	BGH
phCEFI-soCFTR-BGH2	hCEFI	soCFTR	BGH2

2. Materials and methods

2.1. Plasmid construction

The construction of a modular CpG-free plasmid containing the R6K origin of replication (Invivogen, Toulouse, France), the EM7 promoter and aminoglycoside 3′-phosphotransferase (kanamycin resistance) gene has been described previously [10]. A minimal (1154 bp) modular plasmid was created such that all the major elements of the plasmids are separated by unique restriction sites (Fig. 1A). The human CMV immediate early enhancer from pGZB [23] (Genbank acc# JQ439994) and the murine CMV immediate early enhancer from pCpG-free-LacZ (Invivogen, Toulouse, France)(Genbank acc# GQ853402.1) were amplified by PCR (Fig. 1B) and inserted into a modular CpG-free vector to express codon-optimised luciferase reporter gene (soLux) from the EFI promoter (CpG-free version of the human elongation factor 1α from pCpG-free-LacZ) [10] to create phCEFI-soLux-BGH and pmCEFI-soLux-BGH. In addition the entire human CMV IE enhancer and promoter from pGZB was amplified and inserted directly into the modular plasmid upstream of the 5′UTR to create pGZB-soLux-BGH. Dual enhancer fragments (Fig. 1B) (murine-human Genbank acc# JQ439996 and human-murine Genbank acc# JQ439995) were constructed synthetically by GeneArt (Regensburg, Germany), digested with *Bgl*II and *Eco*RI and inserted into the modular vector in the same manner as the single enhancers to create phCmCEFI-soLux-BGH and pmChCEFI-soLux-BGH. The BGH2 3′UTR (Genbank acc# JQ439998) fragment was made by joining two PCR fragments: PCR A amplified the entire BGH 3′UTR (3′UTR-pA-GRE) (Genbank acc# JQ439997) (Forward (F) primer: GGAAGCTTGGGCCCTGTGCCTTCTAGTT & Reverse (R) primer: CCGCTGAGCCTGC TATTGTCTTCCCAATCC) adding a *Blp*I site to the 3′ end and PCR B amplified the pA-GRE region adding a *Blp*I site at the 5′ end (F:ATGCTCAGCAATAAAATGAGGAAATTGCAT and R:GGCTCGAGACTAGTGGTACCAGGATAACCTAGGAAACC). These PCR fragments were digested with *Blp*I and ligated to form the BGH2 3′UTR sequence. Subsequently this was digested with *Apa*I and *Kpn*I and inserted into the phCEFI-soLux-BGH and pGZB-soLux-BGH plasmids to replace the BGH 3′UTR, creating plasmids phCEFI-soLux-BGH2 and pGZB-soLux-BGH2, respectively (Table 1). The SV40 3′UTR sequence was amplified by PCR from pCpG-LacZ to add appropriate restriction sites (F:GGGCCCCCAGACATGATAAGATAC & R:GCATGCCATACCACATTTGTAGAG) and inserted into the phCEFI-soLux-BGH and pGZB-soLux-BGH plasmids to replace the BGH 3′UTR, creating plasmids phCEFI-soLux-SV40 and pGZB-soLux-SV40, respectively (Table 1). Plasmids were propagated in the *Escherichia coli* strain GT115 (Invivogen) as described previously [10]. Plasmids for *in vivo* use were prepared by Qiagen Endofree Mega prep (Qiagen, Crawley, UK) as described previously [10].

2.2. Cell culture transfections

HEK293T cells (ATCC® number CRL-11268) were seeded at a cell density of 250,000 cells/ml in 2 ml D-MEM (Life Technologies, Paisley, UK) plus 10% Foetal Calf Serum (Sigma–Aldrich, Poole, UK) and cultured overnight at 37 °C in 5% CO_2. Cells were transfected with 3 μg plasmid complexed with JetPEI (Polyplus-Transfection, Illkirch, France) at N/P ratio of 3 and diluted in a final volume of 200 μl physiological saline per well. Cells were incubated for 24 h, before harvesting by aspirating the medium, washing once with Phosphate Buffered Saline and resuspending in 300 μl/well 1× Reporter Lysis Buffer (RLB) (Promega, Southampton, UK).

2.3. Procedures involving mice

Female BALB/c mice aged 6–8 weeks were used throughout the study. Mice were housed and treated in accordance with UK Home Office ethical and welfare guidelines and fed on standard chow and water *ad libitum*.

2.4. Aerosol delivery to the mouse lung

For aerosol delivery to the mouse lung, plasmid DNA (pDNA) was complexed with 25 kDa branched polymer polyethylenimine (PEI) (Sigma–Aldrich) and aerosolised to groups of mice as described [25] or complexed with GL67A and aerosolised as described [10]. Mice were sacrificed at indicated time-points post-aerosol by cervical dislocation. Lungs were placed in Lysing Matrix D Tubes (MP Biomedicals, Oakbank, UK) containing 300 μl 1× RLB and homogenised using a FastPrep FP120 machine (MP Biomedicals)(45 s @ 4.0 m/s).

2.5. Instillation of pDNA/GL67A to the mouse lung

Complexes of pDNA/GL67A were prepared and instilled to groups of mice under IsoFlo (isoflurane) (Abbott Laboratories Ltd., Maidenhead, UK) anaesthesia as described previously [13].

2.6. Luciferase reporter assay

Luciferase (Lux) activity in cell culture and mouse lung lysates was quantified using the Promega Luciferase assay reagent system as described [11,13]. The level of Lux activity, expressed in arbitrary relative light units (RLU), for each sample was normalised against total protein concentration determined using a detergent-compatible protein assay (Bio-Rad, Larne, UK). The background detection limit of the mouse lung Lux assay was determined by calculating the Lux activity in untreated mouse lung lysates. To aid in comparison with other studies, standards of recombinant Lux protein (Promega) were also routinely analysed. For comparative purposes 100 RLU per mg total lung protein corresponds to 2467 ng recombinant luciferase (Promega) per mg total lung protein.

2.7. Transfection of human airway liquid interface cultures

Fully differentiated human airway liquid interface (hALI) cultures (Epithelix SàRL, Genève, CH) were cultured as described previously [26]. Plasmid DNA complexed with Lipofectamine 2000 (Life Technologies) in Opti-MEM (Life Technologies) was applied to the apical surface of the cells as described previously [26]. After 6 h, the transfection solution was removed and the basolateral medium was replaced. Cells were harvested/lysed *in situ* by the addition of 150 μl RLT (Qiagen) with a further 200 μl RLT added following removal of the material from the hALI filter.

2.8. Absolute quantification of mRNA

Total RNA was purified from human ALI cell cultures using the RNeasy Mini Kit (Qiagen). Total RNA was extracted from mouse lung tissue as described previously [10]. Absolute levels of vector-derived mRNA, human endogenous *CFTR* mRNA and murine *Cftr* mRNA were quantified by TaqMan, as described previously [26,27].

2.9. Statistics

Group sizes were selected to achieve >0.8 statistical power for the relevant statistical comparisons using G*Power [28]. Differences in lung Lux activity were determined using Bonferroni corrected Mann–Whitney U tests (MWU) after significant Kruskal–Wallis (KW) analyses where appropriate. ★ indicates $p < 0.05$, ★★ indicates $p < 0.01$, and ★★★ indicates $p < 0.001$.

3. Results

3.1. Comparison of plasmids containing murine and human CMV enhancers

Using a minimal, pharmacopoeia-compliant plasmid backbone [24], a modular CpG-free plasmid was constructed such that each major functional element was separated by a unique restriction site (Fig. 1A). The modular design facilitated the precise substitution and testing of one or more functional elements in otherwise identical plasmids. Luciferase (Lux) expression plasmids containing either the murine or human versions of the CMV enhancer (mC and hC, respectively) were constructed (pmCEFI-soLux-BGH and phCEFI-soLux-BGH) (Fig. 1B, Table 1). To measure the level and duration of transgene expression *in vivo*, plasmids were complexed with the cationic polymer PEI and delivered to the lungs of mice as an aerosol (Fig. 1C). At day 1 post-aerosol Lux activity from the pmCEFI-soLux-BGH containing the murine version of the enhancer was 3.5-fold higher than phCEFI-soLux-BGH ($p = 0.006$, MWU) but fell 25-fold by day 7. Lux activity from phCEFI-soLux-BGH increased such that by day 28 it was 2.2-fold higher than day 1 ($p = 0.006$, MWU). Crucially, Lux activity from phCEFI-soLux-BGH was higher than pmCEFI-soLux-BGH from day 7 until the end of the study ($p < 0.01$; MWU). These mouse lung gene expression profiles are similar to those observed previously an alternative gene transfer agent, cationic lipid (GL67A) [10]. Together these observations suggest that the inclusion of the mC enhancer in a plasmid may be associated with transient, high levels of gene expression in the mouse lung, whereas the hC enhancer element is associated with more persistent gene expression.

3.2. Comparison of plasmids containing dual CMV enhancers

New plasmids were constructed containing both mC and hC versions of the enhancer in order to explore the possibility that the properties of initial high-level transgene expression achieved with mC and persistent expression achieved with hC could be combined. Plasmids containing both mC and hC enhancers in head-to-tail arrangements were constructed (Fig. 1B and Table 1) and compared with the parent plasmid containing the respective single enhancers. Following transient transfection of HEK293T cells, Lux activity was 18-fold higher from pmCEFI-soLux-BGH than from phCEFI-soLux-BGH (Fig. 2A, $p = 0.004$, MWU) and performed similarly to the plasmid containing the dual enhancer (phCmCEFI-soLux-BGH) (hCmC) ($p > 0.05$, MWU). When the dual enhancer orientation was reversed (mC-hC) in plasmid pmChCEFI-soLux-BGH, there was a modest 3-fold increase in Lux activity relative to plasmid phCEFI-soLux-BGH containing the single hC enhancer ($p = 0.025$, MWU), but the expression level was still 5-fold lower than pmCEFI-soLux-BGH ($p = 0.004$, MWU). Together these data suggest that, at least in cell culture, the enhancer immediately upstream of the promoter has the dominant effect on the initial level of transgene expression.

The effect of the dual enhancers on the duration of expression is difficult to assess in cell culture because cell division rapidly leads to plasmid loss in the absence of selection. Therefore, persistence of expression from the dual enhancer plasmids was assessed in a mouse lung aerosol delivery model. Plasmids containing the dual enhancers in both orientations (hCmC and mChC) were delivered to mice as an aerosol of complexes of pDNA/PEI and compared with the single enhancer parent plasmids (Fig. 2B). There was no difference in Lux activity from pmCEFI-soLux-BGH and phCmCEFI-soLux-BGH at any time-point, nor was there any difference between phCEFI-soLux and pmChCEFI-soLux ($p > 0.05$, MWU). At later time-points (days 7, 21 and 63 post-dosing) there was a significant difference between the groups with higher activity levels from the two plasmids with the hCMV enhancer immediately upstream of the promoter ($p = 0.001$ kW). At the end of the study (day 63) Lux activity from these plasmids was ~20–40-fold higher than the equivalent plasmids containing the mC enhancer in this position. These results indicate that combining the two enhancers in one plasmid had no effect on the Lux expression profile relative to the respective parent plasmid. Moreover, plasmids containing a single hC enhancer appear to be superior both in terms of persistence and final levels of expression observed in the mouse lung.

3.3. Effect of the 3′UTR on transgene expression

To evaluate the effect of the 3′ UTR on transgene expression, plasmids were constructed containing three different 3′UTR regions: SV40, BGH and a 3′UTR element termed BGH2, which, in addition to the BGH 3′UTR also contains a tandem array of the BGH polyA signal and GRE (Fig. 3A). In cell culture, Lux activity from plasmids containing the SV40 and BGH2 elements was approximately 20-fold higher than phCEFI-soLux-BGH ($p = 0.008$, MWU) (Fig. 3B). To evaluate the effect in the context of an alternative promoter, plasmids were also constructed with the CpG-free human CMV enhancer/promoter (GZB promoter) [23] replacing the hCEFI promoter. A similar trend was observed, with activity from pGZB-soLux-SV40 and pGZB-soLux-BGH2 6- and 5-fold higher, respectively, than pGZB-soLux-BGH ($p = 0.008$, MWU). We also evaluated the hCEFI plasmids with the three different 3′UTRs in the mouse lung following delivery via a pDNA/PEI aerosol. Fig. 3C shows that there was no difference between the three plasmids at days 1 and 7 post-dose, however, by day 14 Lux activity from phCEFI-soLux-SV40 was significantly lower than the other two variants ($p = 0.012$ MWU). By day 28 post-dose Lux activity from phCEFI-soLux-BGH2 had also fallen, but activity from phCEFI-soLux-BGH was still 3-fold higher than the two other plasmids ($p < 0.05$) (Fig. 3C). These data indicate that Lux activity in the mouse lung from plasmids containing the BGH 3′UTR can lead to higher levels of reporter expression at later time-points, contributing to superior duration of transgene expression.

3.4. Duration of CFTR expression from plasmids containing the BGH and BGH2 3′UTR

Although reporter transgenes are useful for comparing plasmid performance, we wished to confirm that the findings obtained using the luciferase transgene in the mouse lung could be extrapolated to expression of CFTR, the gene of interest for the treatment of CF. Versions of the modular plasmids expressing CpG-free, codon-optimised CFTR were constructed to compare the duration of CFTR expression from plasmids containing either the BGH or BGH2 3′UTR (phCEFI-soCFTR-BGH and phCEFI-soCFTR-BGH2; Table 1). Using a cell culture iodide efflux assay [10], we have previously shown that different hCEFI-soCFTR transgene cassettes express similar levels of functional CFTR protein. The plasmids were used to transfect fully differentiated, human monolayer cultures grown at the airway liquid interface (hALI), and used as a model of the pseudostratified human airway epithelium. At days 2, 4 and 7 post-transfection total RNA was extracted from the cells and transgene expression determined by TaqMan RT-PCR (Fig. 4A). At day 2 and day 4 post-transfection there was no significant difference in the levels of expression from the two plasmids. However, by day 7, 7/8 of the phCEFI-CFTR-BGH replicates had fallen to below the level of quantification, while phCEFI-CFTR-BGH2 continued to exhibit robust expression at levels similar to day 2 and day 4

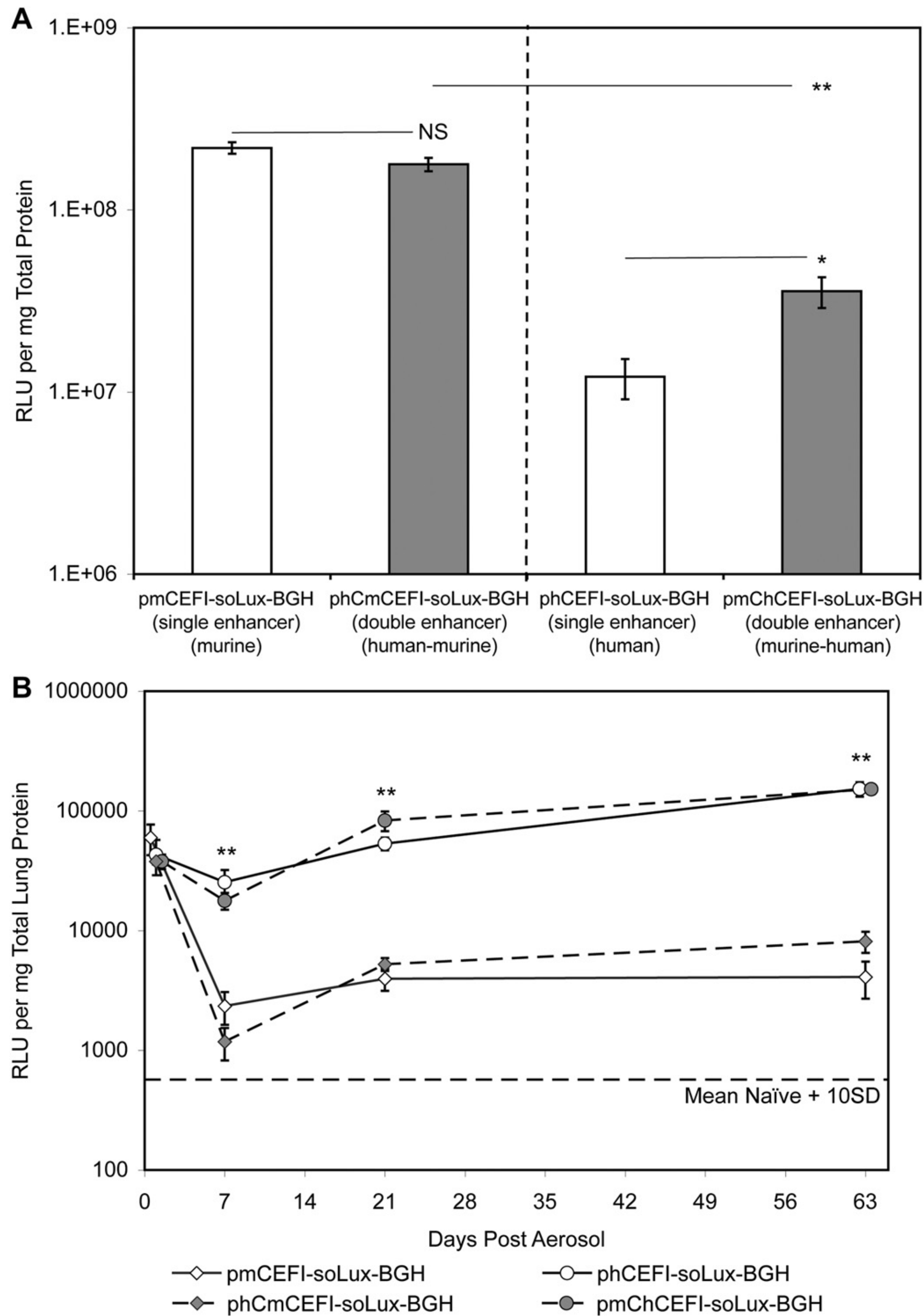

Fig. 2. Effect of dual CMV enhancers on reporter gene expression. (A) Luciferase activity from plasmids following transient transfection. HEK293T cells were transiently transfected with plasmids containing single (mC or hC) and dual enhancers (mChC) ($n = 6$ per group). At 24 h post-transfection Lux activity (RLU per mg total protein) was measured. Representative results from one of multiple repeat studies are shown. (B) Duration of Lux activity from plasmids containing the single (mC or hC) and dual enhancers (mChC or hCmC) following pDNA/25 kDa PEI aerosol delivery to the mouse lung ($N = 6$ per group). Lux activity (RLU per mg total lung protein) was determined at the time-points indicated. Error = 1 SEM. ★ indicates $p < 0.05$, ★★ indicates $p < 0.01$ by Mann–Whitney U-test (A) or Kruskal–Wallis analysis (B).

($p = 0.01$, MWU). The duration of expression from the two plasmids was also investigated in the aerosol lung delivery model lung model after complexing with GL67A cationic liposomes (Fig. 4B). At days 1 and 7 post-aerosol, expression was higher (approx 2-fold) from the BGH plasmid ($p = 0.044$ and 0.025, MWU) although there was no significant difference in expression by day 21.

3.5. *Effect of lung inflammation on plasmid-mediated transgene expression*

The mouse aerosol lung delivery model has been used extensively to investigate lung gene transfer, however, the lungs of CF patients are prone to chronic inflammation. Therefore we wished to

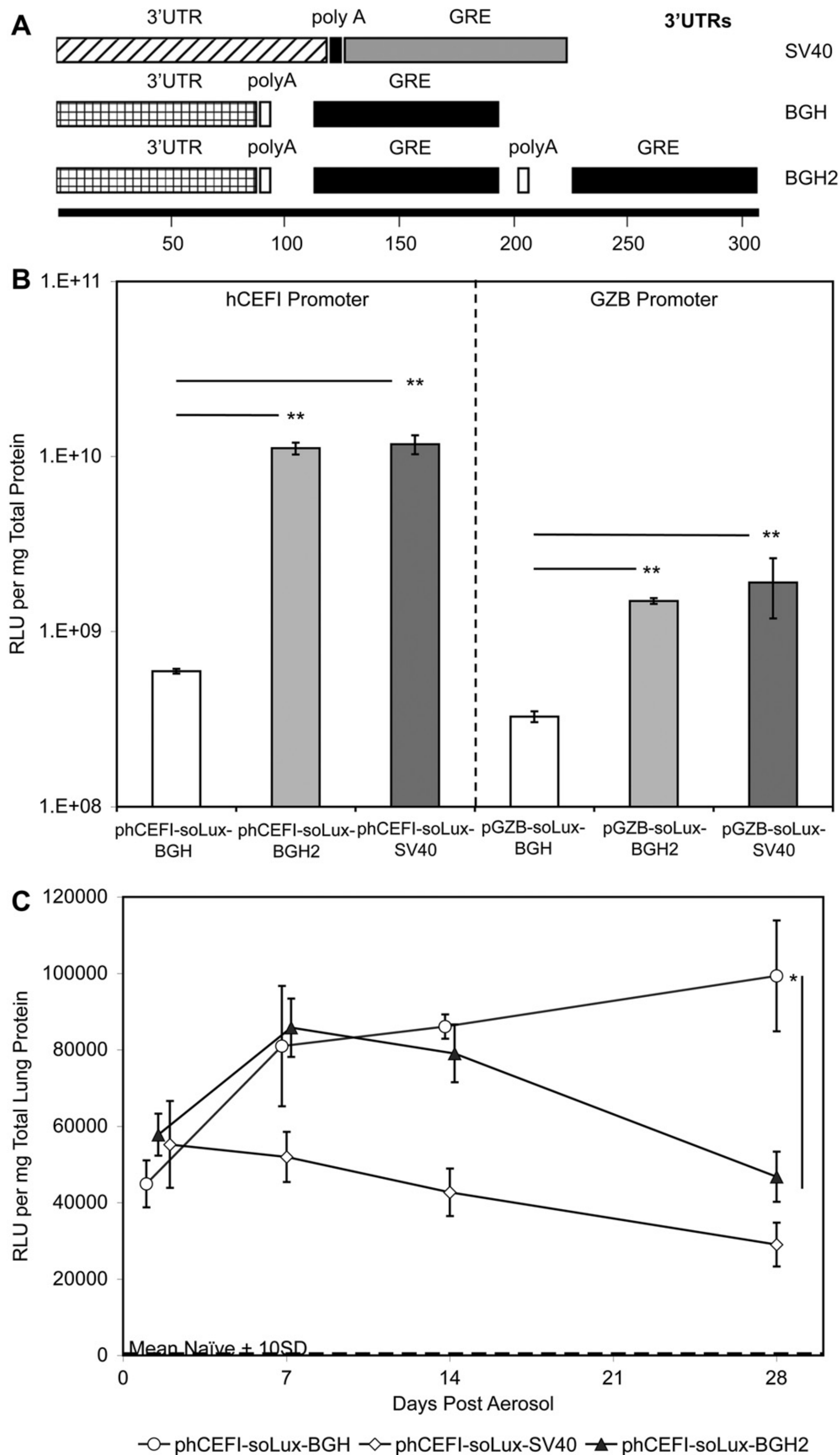

Fig. 3. Effect of 3′UTR sequence on reporter gene expression. Schematic representation of (A) 3′ un-translated regions (3′UTRs) with the constituent UTR, polyA and gene regulatory element (GRE) components of the SV40, BGH and BGH2 3′UTRs. (B) Lux activity from plasmids in cell culture. HEK293T cells were transiently transfected with 3′UTR modified plasmids containing either the hCEFI or GZB promoter ($n = 6$ per group). At 24 h post-transfection Lux activity was determined (RLU per mg total protein). Representative results from one of multiple studies are shown. (C) Duration of Lux activity from modified plasmids following aerosol delivery to the mouse lung. PEI complexes were formed with 3′UTR modified plasmids containing the hCEFI promoter and aerosolised to mice ($n = 6$ per group). Lux activity (RLU per mg total lung protein) was determined at the time-points indicated. Day 1 symbols are staggered for clarity. Error = 1 SEM. NS indicates no significant difference: ★ indicates $p < 0.05$, ★★ indicates $p < 0.01$ by Mann–Whitney U-test.

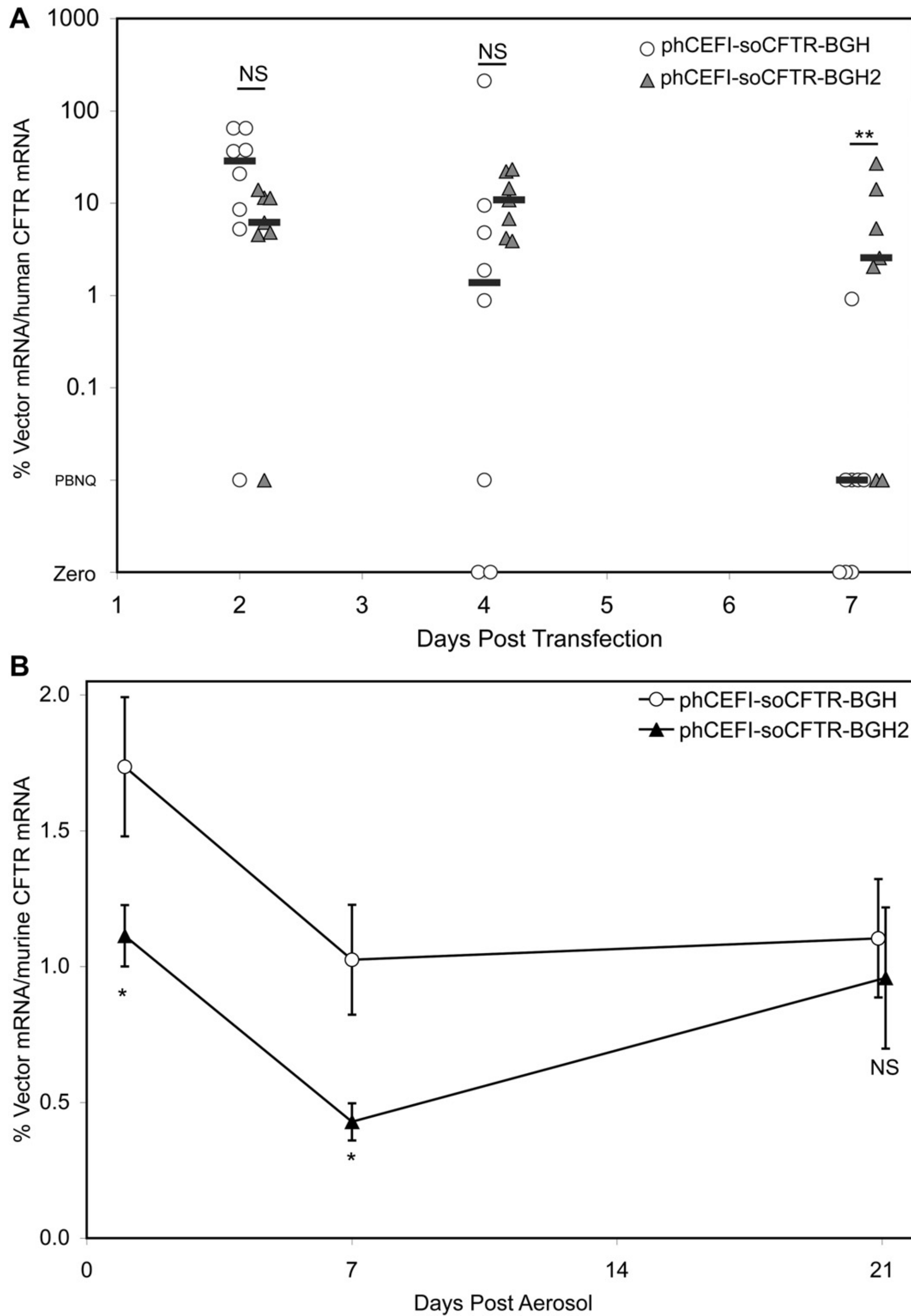

Fig. 4. Effect of 3′UTR sequence on CFTR transgene expression. (A) Human ALI cultures were transfected with plasmids phCEFI-soCFTR-BGH or phCEFI-soCFTR-BGH2 complexed with Lipofectamine. Total RNA was purified at the time-points indicated ($n = 7–8$ inserts per time-point). Quantitative RT-PCR was used to determine the absolute copy number of vector-derived mRNA normalised to the absolute copy number of human CFTR mRNA (% vector mRNA/human CFTR mRNA). (B) Plasmids phCEFI-soCFTR-BGH and phCEFI-soCFTR-BGH2 were complexed with GL67A and aerosolised to groups of mice ($n = 6$ per time-point). Total mouse lung RNA was purified at the time-points indicated and quantitative RT-PCR used to determine the absolute copy number of vector-derived mRNA normalised to the absolute copy number of mouse CFTR mRNA (% vector mRNA/murine CFTR mRNA). NS indicates no significant difference: ★ indicates $p < 0.05$, ★★ indicates $p < 0.001$ by Mann–Whitney *U*-test.

assess transgene expression in a simple model of lung inflammation. Lung instillation of GL67A complexes was used to initiate an acute inflammatory response characterised by neutrophil infiltration and elevation of pro-inflammatory cytokines [10,29]. On day 13 following aerosol delivery of PEI complexes of phCEFI-soLux-BGH ($n = 60$), groups of mice ($n = 12$) were instilled with either cationic lipid GL67A (GL67A), GL67A complexed with a CpG-free plasmid (GL67A/pCpG-free), or GL67A complexed with a CpG-rich plasmid (GL67A/pCpG-rich). Control animals were untreated (Fig. 5). The plasmids used for instillation did not express luciferase

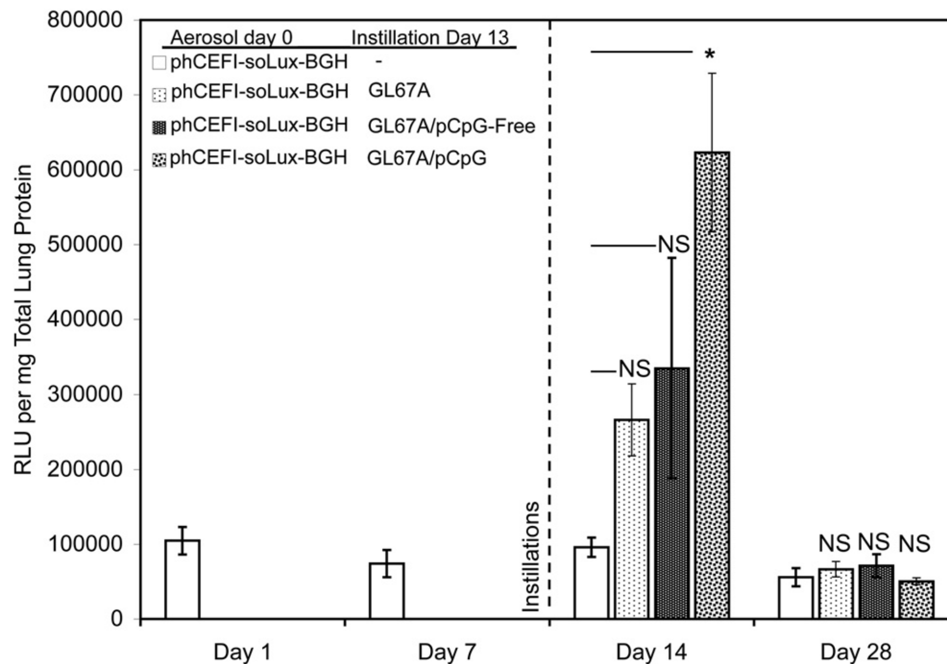

Fig. 5. Duration of luciferase activity following induction of an inflammatory response in the mouse lung. An aerosol of 25 kDa PEI/phCEFI-soLux-BGH was delivered to mice ($n = 60$). To stimulate an inflammatory response, on day 13 post-aerosol groups of mice ($n = 12$) were instilled with either cationic lipid GL67A (GL67A), GL67A complexed with a CpG-free plasmid (GL67A/pCpG-free), or GL67A complexed with a CpG-rich plasmid (GL67A/pCpG-rich). Control animals were untreated. Mouse lungs were harvested at the time-points indicated and Lux activity was determined (RLU per mg total lung protein). $n = 6$ mice per group per time-point, error = 1 SEM. NS indicates no significant difference: ★ indicates $p > 0.05$ by Mann–Whitney U-test.

so that Lux activity from the original PEI/phCEFI-soLux-BGH aerosol was not compromised and could continue to be measured in all groups at 14 and 28 days post-dose (1 and 14 days post-instillation, respectively). At day 1 post-instillation, the mice that received the GL67A/pCpG-rich instillation exhibited reduced mobility and piloerection consistent with the inflammatory responses that have previously been observed [10]. As expected, the mice that received GL67A alone, or GL67A/pCpG-free complexes, had normal behaviour and appearance (data not shown). At day 1 post-instillation, the induction of inflammation following the delivery of GL67A/pCpG-rich resulted in a 7-fold increase in Lux activity from phCEFI-soLux-BGH ($p = 0.018$, MWU), but no statistically significant changes were observed following the instillation of GL67A or GL67A/pCpG-free complexes (Fig. 5). However, by day 28 post-aerosol (day 14 post-instillation) the level of Lux activity observed in all three groups of mice was no different from the untreated control. This indicates that the creation of an acute inflammatory response in the model did not adversely affect the duration of expression from the phCEFI-soLux-BGH plasmid.

4. Discussion

The efficacy of non-viral gene therapy is highly dependent on the performance of the plasmid vector, yet relatively few attempts have been made to optimise plasmids before their use in the clinic. In the case of CF gene therapy, previous non-viral clinical trials have utilised plasmids with a limited range of viral promoters such as CMV [6,7,30] or RSV [31,32]. These studies reported evidence of transient CFTR expression, lasting for only a few days after dosing, which is perhaps unsurprising as viral promoters often lead to short lived gene expression, at least in the lung [11]. Prior to conducting further CF gene therapy clinical trials, we systematically optimised every element of the plasmid vector prior to the selection of the final clinical plasmid. The initial development of a CpG-free plasmid backbone and the selection of a codon-optimised CFTR cDNA sequence have been described previously [10], but here, the impact of other plasmid elements on gene expression performance has been assessed.

Studies have established that the EFI promoter directs only very low level expression in the mouse lung, with the addition of either the murine or human versions of the CMV enhancer greatly boosting its activity [10]. Although the two selected sequences share similar viral functions, they share only modest sequence similarity (38% identity; data not shown). It appears that the mC enhancer generally results in the highest absolute levels of expression but the addition of the hC enhancer results in far greater duration of expression (Fig. 1C). Since both of these characteristics may ultimately be desirable for effective transgene expression, the impact of combining both the mouse and human enhancers together in one plasmid was assessed, with the aim of combining their respective properties. This effect was not achieved in the mouse lung aerosol model; the expression profiles of each of the dual enhancer plasmids simply reflected the expression characteristics of the single enhancer located immediately 5′ of the promoter (Fig. 2B). This suggests that both versions of the enhancer most likely function by directly interacting with the promoter and that no synergistic benefit can be gained by including an additional enhancer further 5′. As no benefit was observed from dual enhancer plasmids, the hC enhancer alone was selected for the clinical plasmid design in order to favour persistent transgene expression.

The potential effect of the 3′UTR on plasmid expression has not been thoroughly investigated for lung gene transfer, despite its fundamental role in mRNA stability [22]. In this study, the effect of BGH2 and two commonly used 3′UTRs (BGH and SV40) (Fig. 3A) on the levels and duration of expression from our CpG-free plasmids was investigated. In cell culture models, reporter expression was greatly enhanced when the SV40 or BGH2 3′UTRs were used (Fig. 3B). However, in the more clinically relevant mouse lung aerosol model the BGH 3′UTR showed improved persistence of

expression compared with the other two 3′UTRs (Fig. 3C). To confirm this observation with the gene of interest for CF gene therapy, CFTR-expressing versions of the plasmids were created containing the BGH or BGH2 3′UTR, which were then assessed in the mouse lung model complexed with GL67A, our preferred non-viral GTA for clinical use. Expression from the BGH plasmid was higher at days 1 and 7 ($p < 0.05$) but there was no difference in activity at day 21 post-aerosol (Fig. 4B). Interestingly, in human ALI cultures the plasmid containing the BGH2 3′UTR was superior to the BGH 3′UTR plasmid, still strongly expressing in the majority of samples at 7 days (Fig. 4A). These results highlight a major difficulty in performing optimisation studies. Logic suggests that one should utilise several model systems, yet this is problematic when different models provide contradictory results. Numerous differences exist between the models here and together with normal experimental variables combine to make the optimisation of plasmid elements all the more challenging. Ultimately we anticipate that an *in vivo* model is a better predictor of lung gene transfer than cell culture models and that the high reproducibility of data from the mouse aerosol model is a more reliable guide to vector performance; and therefore we chose to incorporate the BGH 3′UTR in the clinical plasmid design.

Together, these studies have defined a clinical trial plasmid with the hCEFI promoter driving the expression of the soCFTR gene, together with the BGH 3′UTR. However, one remaining concern was that these studies were conducted in normal mice with healthy lungs whereas the lungs of individuals with CF are prone to chronic inflammation. The bronchoalveolar lavage fluid (BALF) from patients with CF contains elevated levels of the pro-inflammatory cytokines IL-1β, TNF-α, IL-6 and IL-8 compared with non-CF controls [33], which are mediators of cachexia and neutrophil-induced airway inflammation. Although neutrophils are intended to control infection, in CF the large numbers that infiltrate the airways cause tissue damage due to the secretion of excessive amounts of neutrophil elastase and other proteinases [34]. There are no simple mouse models available that readily allow the assessment of our gene transfer vectors in a diseased CF lung. However, it has previously been demonstrated that lung instillation of pDNA/GL67A complexes containing CpGs can initiate a broad-ranging inflammatory response in the mouse lung, including neutrophil influx and elevation of pro-inflammatory cytokines [10]. Therefore this method was used to induce an inflammatory environment in the mouse lung, hypothesising that the inflammation could have an adverse effect on expression either acutely or in the long-term. However, no detrimental effects on persistence of transgene expression were observed. Instead, at day 1 post-instillation a 7-fold increase in Lux activity from phCEFI-soLux-BGH was observed (Fig. 5). The reasons for this are unclear but could be due to the activation of transcription factors by the inflammatory response, which in turn increases expression from phCEFI-soLux-BGH. The results are encouraging and demonstrate that the selected plasmid design has some resilience in an inflammatory environment, which may be helpful for successful gene transfer and transgene expression in the human CF lung. Clinical evaluation of plasmid phCEFI-soCFTR2-BGH (also known as pGM169 [10]) has recently been undertaken. A single aerosol dose of pGM169 complexed with cationic lipid GL67A was delivered to the nose and lungs of CF patients, to assess the safety of this formulation. Clinical data show changes in nasal potential difference (PD) consistent with the correction of the CF chloride secretion defect [35]. Nasal PD changes were observed in 11/19 subjects with, in some cases, changes being sustained for up to 6 weeks post-dosing. These observations suggest that the optimised CFTR expression plasmid is capable of persistent transgene expression in the human airways, a substantial improvement over the plasmids used in early CF gene therapy clinical trials, where CFTR expression lasted only a few days. These observations also support the use of the mouse in gene transfer studies as a model for gene transfer to the human airways.

5. Conclusion

The results presented here demonstrate that the systematic evaluation of plasmid design can result in improved transgene expression *in vivo*. We have undertaken the systematic testing of the key functional elements of a plasmid construct in order to develop a potent expression plasmid for CF gene therapy. Together with the selection of an appropriate promoter sequence these studies have resulted in a plasmid capable of high-level and persistent transgene expression and confirm the benefits of plasmid optimisation in improving non-viral gene transfer. Observations suggest that the optimised CFTR expression plasmid is capable of persistent transgene expression in the human airways and also support the use of the mouse lung gene transfer studies as a model for gene transfer to the human airways.

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