An *araC* controlled bacterial *cre* expression system to produce DNA minicircle vectors for nuclear and mitochondrial gene therapy

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Running Title:

A bacterial *cre* expression system for minicircle production
Summary

The presence of CpG motifs and their associated sequences in bacterial DNA causes an immunotoxic response following the delivery of these plasmid vectors into mammalian hosts.

We describe a biotechnological approach to the elimination of this problem by the creation of a bacterial cre recombinase expression system, tightly controlled by the arabinose regulon. This permits the Cre mediated directed excision of the entire bacterial vector sequences from plasmid constructs to create supercoiled gene expression minicircles for gene therapy.

Minicircle yields using standard culture volumes are sufficient for most in vitro and in vivo applications whilst minicircle expression in vitro is significantly increased over standard plasmid transfection.

By the simple expedient of removing the bacterial DNA complement, we significantly reduce the size and CpG content of these expression vectors, which should also reduce DNA induced inflammatory responses in a dose dependent manner.

We further describe the generation of minicircle expression vectors for mammalian mitochondrial gene therapy, for which no other vector systems currently exist. The removal of bacterial vector sequences should permit appropriate transcription and correct transcriptional cleavage from the mitochondrial minicircle constructs in a mitochondrial environment and brings the realisation of mitochondrial gene therapy a step closer.
Introduction

There is increasing evidence to suggest that plasmid DNA used for non-viral gene delivery can cause unacceptable inflammatory responses in eukaryotes (1-5). These immunotoxic responses are largely due to the presence of unmethylated CpG motifs and their associated stimulatory sequences on plasmids, following bacterial propagation of plasmid DNA. Simple methylation of DNA in vitro may be enough to reduce an inflammatory response but is likely to result in severely depressed gene expression (6). The removal of CpG islands by cloning out, or elimination of non-essential sequences is more successful in reducing inflammatory responses but is time consuming and tedious (7).

Since bacterial DNA contains on average 4 times more CpG islands than mammalian DNA (8), a good solution is to entirely eliminate the bacterial control regions from gene delivery vectors during the process of plasmid production.

Removal of bacterial sequences needs to be efficient, using the smallest possible excision site, whilst creating supercoiled DNA minicircles, consisting solely of gene expression elements under appropriate mammalian control regions.

This can be achieved by the use of Cre recombinase, a bacteriophage P1 derived integrase (9-11), catalysing site-specific recombination between direct repeats of 34 base pairs (loxP sites).

In the case of a supercoiled plasmid containing DNA flanked by two loxP sites in the same orientation, Cre recombination produces two DNA molecules that are topologically unlinked, circular, and mainly supercoiled (10), each containing a single 34 bp loxP site. Efficient minicircle production requires the use of a stable bacterial based cre expression system for efficient production of supercoiled DNA. However, currently available bacterial strains do not have sufficient control of cre recombinase expression to avoid leakage during the bacterial and concomitant plasmid growth phases (12).
This leads to premature Cre-recombination, resulting in loss of the replication deficient minicircle due to out-competition by the replication competent and antibiotic resistant bacterial vector (Bigger unpublished).

We therefore utilised the tightly controlled arabinose expression system (13-15), for review see (16), to create a cre expressing bacterial strain, which is both stable and easily controllable by altering the carbon source available for metabolism by these bacteria.

In order to increase minicircle yield we have improved the kinetics of the cre/loxP reaction by modification of the loxP sites (17,18) to induce a shift in reaction equilibrium towards increased production of minicircle. This will also serve to reduce concatamer formation from multiple copies of minicircle DNA.

This approach to eliminating bacterial DNA from delivery vectors is also stimulated by our work on the development of expression vectors for use in mitochondrial gene therapy. Our aims is to express an ornithine transcarbamylase gene sequence, modified for mitochondrial translation (sOTC), within mitochondria (19). Since no vectors exist for mammalian mitochondrial gene expression, we have inserted the sOTC gene between two tRNA genes within the entire mouse mitochondrial genome, cloned into a bacterial plasmid vector for propagation (19,20). Due to the rarity of non-coding sequences within mammalian mtDNA the presence of a bacterial vector is likely to be deleterious to either or all of the processes of mitochondrial RNA splicing, replication and transcription. Elimination of the bacterial vector sequences should both overcome this problem and reduce the size of these vectors, increasing the ease of their introduction into mitochondria.

In this paper we describe the construction and testing of a bacterial strain exhibiting tightly controlled and efficient expression of cre recombinase. We have developed this system for DNA minicircle generation using a wide range of producer plasmids designed for both nuclear and mitochondrial gene expression with size ranges of 6 – 20 kb. We
also demonstrate the use of mutant \textit{loxP} sites to direct the Cre reaction resulting in improved yields of supercoiled luciferase minicircle, as well as showing significantly increased gene expression \textit{in vitro} of this construct over standard plasmid vectors.

\section*{Experimental Procedures}

\textit{Plasmids, strains and oligonucleotides}

Plasmid pBC SK (+) was purchased from Stratagene, Plasmid pDSRed1-N1 was purchased from Clontech. Plasmids p705Cre, pBAD33Cre and pSVpAX1, as well as bacterial strain MM294, were kind gifts from Dr. F. Buchholz and Dr. A.F. Stewart (EMBL). Plasmid pCIKluc was a gift from Dr. D. Gill and Dr. S. Hyde (Oxford University). Mitochondrial plasmids pRSmtOTCAP, and pRSmtJMC, were made as previously described (21). Oligonucleotides (Genosys) DLOX 5'-GGAATTCATA ACTTCGTATA ATGTATGCTA TACGAAGTTA TTAATCTCGA GTAATAACTT CGTATAATGT ATGCTATACG AAGTTATGGT ACCGCGCCCG-3' and REVDL 5'-CGGGCGCGGT ACCATAACT-3' were used to synthesise a DNA fragment with two \textit{loxP} sites to ultimately create plasmid pDlox3, as well as to reconstruct the ND5/ND6 junction to create pDlox1. Oligonucleotides LINK1 5'-TCGAGTCGAC TCTAGAGAT CGAGCTCCC CGGGAAGCTT CTGCAGT-3' and LINK2 5'-TCGAACTGCA GAAGCTTCCC GGGGAGCTCG GATCCTCTAG AGTCGAC-3' were used to create a polylinker sequence for the plasmid pDlox3. Oligonucleotides LoxF 5'-CTCGAATTCA TAACTTCGTA TAGCATACT TATACGAACG GTACTCGAGT ACCGTTCGTA TAGCATACT TATACGAAGT TATGGTACCA AAAA-3' and LoxR5'-TTTTTGTAC CATAACT-3' were used to create LE and RE mutant \textit{loxP} sites to ultimately create construct pFIX. Primers NsiICre 5'-GTGAATGATG TAGCCGTCGA G-3' (homologous to a sequence in the \textit{cre} gene) and
CreIntFwd 5’-CCATGATTAC GGATTCAC-3’ (homologous to nucleotides 2-18 of the chromosomal lacZ gene) were used to amplify a 1.9 kb region, demonstrating insertion of the cre-araC cassette into the bacterial genome.

All constructs were sequenced over the insertion regions and gene expression regions including loxP sites using the Big Dye kit (Perkin Elmer), on a Perkin Elmer 377 sequencing apparatus.

**Construction of the pBAD75Cre targeting plasmid**

Plasmid p705Cre was adapted by the excision of part of the cre gene, the promoter and most of the CI 857 temperature sensitive repressor, at NsiI/RsrII sites. This 583 bp fragment was then replaced with the 1624 bp control regions from pBAD33Cre, including the same part of the cre gene, the BAD promoter, and the araC regulator, also using NsiI/RsrII sites to create pBAD75Cre.

**Construction of the MM219Cre strain**

The recombination competent (recA+) bacterial strain MM294 was transformed with pBAD75Cre, and the cre/araC cassette inserted into the bacterial lacZ gene using the targeting method of Hamilton *et al* (22) (Figure 1), to produce strain MM219Cre.

**Construction of pDlox1 and pDlox3 dual loxP plasmids**

The SacI site was removed from pBC SK(+) by SacI digestion, filling-in with Klenow (Gibco BRL) and religation. Two loxP sites were inserted into the resulting pBC SK-Sacl<sup>+</sup> plasmid by annealing DLOX and REVDL oligonucleotides, endfilling with Klenow, digestion of both the fragment and the plasmid by EcoRI/KpnI and subsequent ligation to create pDlox1. The polylinker was removed by XbaI/PstI digestion, endfilled
with Klenow, and ligated to form pDloxB. Then a new polylinker formed by the annealing of LINK1 and LINK2 was introduced between the **loxP** sites of pDloxB at XhoI to create pDlox3.

**Construction of pNIXluc and mutant loxP containing pFIXluc nuclear plasmids**

Plasmid pNIXluc was created by the insertion of the BamHI/BglIII luciferase cassette from pCIKluc, into the BamHI site of pDlox3.

Dual mutant **loxP** sites (LE and RE) were introduced into pBCSK+ by annealing LoxF and LoxR oligos, filled-in with *Pfx* polymerase (Gibco BRL), further digestion with EcoRI/KpnI, and ligation to create pMlox1. The unwanted polylinker was removed from pMlox1 by PstI/XbaI digestion, Klenow treatment and self ligation to produce pMlox2. A replacement polylinker was added within the **loxP** sites by the insertion of the entire pDSRed1-N1 plasmid at XhoI (pMlox3), before removal of the remainder of pDSRed1-N1, excluding the polylinker, by BamHI/NheI digestion, endfilling using Klenow and subsequent ligation to create pFIX. Plasmid pFIXluc was created by the replacement of the pDSRed1-N1 BamHI/BglIII fragment from pMlox3 with the BamHI/BglIII luciferase cassette from pCIKluc.

**Construction of pMEV8, pMEV46, pMEV88 mitochondrial plasmids**

Construct pMEV8 was made by the insertion of pDloxB into the unique XhoI site of pRSmtOTCAP△XhoI (21). The ampicillin resistant vector pRS316 was removed from this construct by digestion with SacI and religation to form pMEV8.

Construct pMEV46 was formed by exchange of pRS406 with pDloxB at the BamHI site of pRSmtJMC (21). Construct pMEV88 was constructed by the deletion of the 16S and most of the 12S rRNA genes at the Klenow filled BlpI/SnaBI sites of pMEV46.
Minicircle production and purification

Electrocompetent MM219Cre cells (25µl) were electro-transformed (BioRad Gene pulser) according to manufacturers instructions, with the appropriate minicircle producer plasmids. Transformed cells were allowed to recover for 1 hour in Luria Bertani media (LB) containing 1% glucose, before plating on LB 1% glucose containing 30µg/µl chloramphenicol (Cm). Selected colonies were amplified in LB 1% glucose, Cm and frozen in 20% v/v glycerol. Transformed cells containing a minicircle producer plasmid were grown as a 5 ml starter culture overnight at 37°C in LB 1% glucose with Cm, before inoculation of 500 ml flasks. The most successful growth and cre induction conditions were as follows:

Technique 1
Cells were grown overnight in a shaking incubator at 37°C in modified M9 minimal media (with the addition of 0.2% yeast extract) (Difco) supplemented with 0.2% glucose, and 30 µg/µl Cm (Sigma Aldrich). Cells were pelleted at 5000 rpm for 10 minutes before resuspension in 1 volume of modified M9 minimal media. After washing, cells were re-pelleted at 5000 rpm and resuspended in the same volume of cre induction media (modified M9 minimal media supplemented with 0.5% L-arabinose (Sigma Aldrich)), and further grown in a shaking incubator at 37°C for 2 - 4 hours.

Technique 2
Cells were grown overnight at 37°C in LB supplemented with 0.5% glucose, and 30 µg/µl of Cm. Cells were pelleted at 5000 rpm for 10 minutes before resuspension in 1 volume of M9 minimal media. After washing, cells were re-pelleted at 5000 rpm and resuspended in the same volume of cre induction media (M9 minimal media supplemented with 0.5% L-arabinose) and further grown in a shaking incubator at 37°C for 4 - 6 hours.
1 litre of cells were treated in 5 mg/ml lysosyme in 40 ml Solution I (50mM glucose, 25mM Tris.Cl pH 8.0, 10mM EDTA), followed by lysis in 80 ml (0.2N NaOH, 1% SDS) and finally neutralised in 60 ml 3M potassium acetate (pH 4.8). The cleared supernatant was isopropanol precipitated and the resulting DNA solution further purified by RNA precipitation in 6M lithium chloride, RNAse treatment and phenol/chloroform extraction (23). This technique provides very high yields of DNA per litre of culture (~10 mg).

The resulting pool of DNA products, producer plasmid and excised bacterial vector were cut with the triple cutting PvuII for luciferase plasmids and with NotI for mitochondrial plasmids. Undigested supercoiled minicircle could then be density separated from linear producer plasmid and excised bacterial vector on a cesium chloride gradient using the intercalating agent etidium bromide (24), or more effectively, propidium iodide.

Removal of cesium chloride was achieved by dilution in 3 volumes of water, ethanol precipitation and two washes in 70% ethanol (25). Minicircle DNA was run through cation exchange columns AG50W-X8 (BioRad) to remove etidium bromide or propidium iodide according to manufacturers instructions in order to achieve maximal DNA yield from columns.

**Transfection of mammalian cells with minicircles and control plasmids**

2 x 10⁵ cells were seeded into a 24 well tissue culture plate in 1 ml of growth medium (DMEM (Life Technologies) + 10 % (v/v) fetal calf serum (FCS)) and incubated at 37°C until 50-80 % confluent (approximately 16 hours). 0.24 - 0.5 μg DNA in 100 μl OPTIMEM media (Life Technologies) was complexed to Lipofectamine (Gibco BRL) in 100 μl OPTIMEM media (2mg/ml) in the ratio of 10 μl lipofectamine/μg DNA, according to manufacturers instructions. In order to obtain 6 replicates per treatment, this reaction was appropriately scaled-up and the DNA-liposome complex allowed to
form at 37°C for 20 minutes. Cells were washed once in OPTIMEM and a 200 µl
reaction volume of complexed DNA in OPTIMEM was then overlaid onto the cells in
each well. 4 hours later 1 ml DMEM containing 10 % (v/v) FCS was added and the
incubation continued at 37°C. 24 hours after the start of transfection, the media was
exchanged (DMEM + FCS) and 24 hours following this, cells were harvested and
transgene activity measured.

Measurement of relative luciferase activity and statistical analysis

Luciferase activity was measured using the Luciferase Reporter Gene Assay kit (Roche
pharmaceuticals) on a Lucy1 luminometer (Anthos, Gibco Life Technologies, UK)
according to manufacturers instructions. The total protein per measurement was
determined in a colorimetric assay using the Micro BCA Protein Assay Reagent kit
(Pierce, Rockford, ILL, USA) according to manufacturers instructions. Relative light
units of luciferase activity per minute per measurement were then adjusted to that
obtained for 1 mg of total protein per measurement.

Significance tests were based on the mean from 6 replicates for each assay. In order to
satisfy requirements for analysis of variance (ANOVA), raw data was transformed by
taking the Log₁₀ of each figure. This results in data which are relatively normally
distributed (Shapiro-Wilk test) within treatments, with more equal treatment variances.

We have used the analysis of variance to determine the pooled variance for the 9
treatments and subsequently used a method for multiple comparisons based on the
studentised range (Q) between means, which is considerably more stringent than either
95% confidence intervals based on 1.96(standard error), or the least significant
difference test. Given that all sample sizes are equal between compared treatments (6
replicates each), this determines a critical value (ω) for the difference between the largest
and the smallest sample means and applies this to the whole experimental set to obtain a
95% confidence interval between any pair of means. The value of the Q method is such that when comparing all of the differences between means in this manner over a large number of treatments, the probability that no erroneous claims of significance are made is ≥95%.
**Results**

**Creation of a bacterial strain expressing cre recombinase under the control of the arabinose regulon**

We modified the vector pBAD33Cre, a direct derivative of the pBAD33 expression vector (26) containing the arabinose control regulon (araC), to create a new cre recombinase expressing bacterial strain (Figure 1).

The plasmid, p705Cre, which also expresses cre recombinase, has a leaky $\lambda P^\beta$ based expression cassette flanked by regions of homology to the bacterial lacZ gene, permitting targeted insertion into the bacterial genome by homologous recombination.

Replacement of the cre expression cassette in p705Cre with the cre/araC expression cassette from pBAD33Cre resulted in the creation of a targeting plasmid pBAD75Cre.

Controlled cre expression from this new plasmid was tested by co-transforming bacteria with pBAD75Cre and the Cre reporter construct pSVpaX1, which uses a convenient lacZ based assay for Cre activity (27). Growth on LB media containing arabinose led to Cre mediated excision of a 1.1 kb segment from this plasmid and lacZ inactivation giving white colonies. Growth on media containing glucose led to no Cre mediated excision, thus leaving the lacZ gene intact and resulting solely in blue colonies (not shown). This provides good evidence that plasmid based cre expression from the arabinose regulon is absent on growth in glucose containing media, whilst growth in arabinose containing media (in the absence of glucose) results in successful cre expression.

Targeted cre/araC insertion into the recA+ bacterial strain MM294 using pBAD75Cre was achieved by successive rounds of targeted recombination and excision at the lacZ
chromosomal locus and the use of the temperature sensitive plasmid replicon \textit{pSC101} \textsuperscript{ts} (22) (Figure 1).

A PCR based assay was used to determine successful targeted \textit{cre/araC} insertion into the \textit{lacZ} gene (Figure 1 inset) thus creating strain MM219Cre (\textit{F \lambda supE44 endA1 thi-1 hsdR17 lacZ::araC-Cre}).

\textbf{Construction of minicircle producer constructs}

To expedite the process of construct manufacture for both nuclear and mitochondrial expression, a multi-cloning plasmid containing dual \textit{loxP} sites flanking a polylinker (pDlox3) was created from the basic vector pBCSK(\textsuperscript{+}). This plasmid permits easy insertion of expression cassettes or mitochondrial sequences into the polylinker region, to create minicircle producer plasmids.

The initial construct for nuclear expression was generated by cloning of the luciferase reporter gene and CMV promoter from the high expression plasmid pCIKluc, into the \textit{loxP} flanked polylinker of pDlox3. The resulting plasmid pNIXluc contains a minimal sized luciferase expression cassette flanked by \textit{loxP} sites to permit removal of bacterial sequences by Cre recombination to create mNIXluc minicircle (Figure 2a).

We have previously created a 22 kb construct designed for mitochondrial expression based on the insertion of a modified OTC gene between two tRNA sites within the entire mouse mtDNA (19-21). This expression construct is difficult to modify due to its instability (21) and presents problems for introduction into mitochondria by electroporation, due to its large size (28) [Collombet unpublished]. In addition, the bacterial vector falls within the mitochondrial gene \textit{COXIII}, is not easily removable, and is likely to abolish mitochondrial gene function.
To ameliorate this situation, the \textit{loxP} flanked pDloX1 vector was inserted into pRSmtOTCAP at XhoI and the pRS316 vector removed to create the mitochondrial minicircle producer plasmid pMEV8. This XhoI site in mouse mtDNA is situated in a 14 bp area where the \textit{ND5} gene coded on the heavy strand overlaps the terminal coding region of the \textit{ND6} gene, oriented in the opposite direction on the light strand. The terminal regions of the \textit{ND5} and \textit{ND6} genes were reconstructed between the \textit{loxP} sites of the insertion vector pDloX1 to ensure complete transcription from these genes within pMEV8 (Figure 2b).

The mitochondrial minicircle resulting from Cre mediated excision of pDloX1\textDelta from pMEV8 (mMEV8), contains a single 34 bp \textit{loxP} site flanked by the reconstructed \textit{ND5} and \textit{ND6} genes. This should minimise the impact of incorrect splicing resulting from the presence of a foreign sequence on transcribed mitochondrial minicircle DNA.

Smaller mitochondrial constructs were also made to permit more efficient DNA transfer into mitochondria, by PCR amplification of key regions of the mitochondrial genome and the sOTC gene (21). Construct pMEV46 consists of the mitochondrial D loop, 12S, 16S rRNA, the origin of light chain replication and several tRNAs, with the \textit{loxP} flanked pDloX3 inserted at the already artificial Thr/Ser tRNA gene junction (Figure 2b). An even smaller 6.8 kb derivative, pMEV88 (not shown), lacks most of the 12S and 16S rRNA regions of pMEV46.

As tRNAs are believed to act as cleavage signals within polycistronic mtRNA transcripts (29,30) we anticipate that the 34 bp \textit{loxP} site will have minimal impact on mitochondrial transcription in these constructs.

All of these minicircle producer constructs are designed to permit excision of the bacterial vector (pDloX1\textDelta or pDloX3\textDelta) by Cre recombination to leave solely a 34 bp \textit{loxP} site within the resulting minicircle constructs (Figure 2).
Cre recombinase activity and minicircle production in MM bacterial strains

Our novel *E. coli* strain, MM219Cre expresses *cre recombinase* under tight control of the *araC* regulon. The AraC protein acts as both a positive and negative regulator of Cre activity. In the presence of arabinose in growth media, transcription from the *BAD* promoter is turned on; in its absence, transcription proceeds at a very low level. The addition of glucose to growth media, which lowers levels of 3′,5′ cyclic AMP, further down-regulates the catabolite-repressed *BAD* promoter (13-15).

MM219Cre cells transformed with different minicircle producer plasmids showed effective repression of *cre recombinase* over a range of media types using varying levels of glucose. We used minicircle production and the presence of excised bacterial vector as indicators of leaky *cre recombinase* expression. The three Media types used for bacterial growth in decreasing order of richness were; LB, modified M9 minimal media (containing 0.2% yeast extract) and M9 minimal media, incorporating a range of glucose concentrations from 0.2% to 2%. Rich media (LB) leads to the most rapid growth of both bacteria and plasmid but also results in the exhaustion of glucose. Bacterial growth in M9 minimal media gives comparatively poor bacterial and hence plasmid yields. Initial glucose concentrations higher than about 1% also lead to significant inhibition of bacterial growth, as a result of the Crabtree effect (16,31,32), although *cre* induction is still effectively repressed.

The best growth conditions were obtained using levels of 0.2 – 0.5% glucose with any of the media types, striking a balance between bacterial and thus plasmid replication and down-regulated *cre* expression.

However, growth of MM219Cre cells containing the largest plasmid, pMEV8 (20.7 kb), in LB 0.2%-0.5% glucose leads to a slight induction of *cre*, minicircle production and subsequent loss of minicircle during growth. Assuming that there is slight *cre* expression during bacterial growth using low glucose levels, the potential toxicity of the
largest mitochondrial construct may help to induce loss of replication deficient minicircle during plasmid replication under chloramphenicol selection.

We do not observe significant minicircle production (and subsequent loss) using the same low glucose media growth conditions in the case of any other minicircle producer constructs. This is in accordance with data on pBAD expression plasmids for which no significant gene induction effects have been observed under similar low glucose conditions (26). By changing media type to modified M9 minimal media, glucose levels could be kept low (0.2%) and still effectively down-regulate cre expression using pMEV8, whilst this richer media type permitted increased plasmid yields over that of minimal media alone.

Following bacterial and plasmid growth, induction of cre recombinase and thus minicircle production used either LB, modified M9 minimal media or M9 minimal media, containing levels of arabinose from 0.2% - 2%. Arabinose levels had little effect on overall minicircle yields, whilst incubation times of 4-6 hours produced the greatest yields of minicircle from smaller plasmids (Figure 3a), and shorter incubation times of 2-4 hours for the largest mitochondrial minicircle mMEV8 (Figure 3b).

The two best techniques for minicircle production were as follows.

Technique 1: Growth in modified minimal media, 0.2% glucose overnight, washing in modified minimal media and induction for 2-6 hours in modified minimal medium containing 0.5% arabinose.

Technique 2: Growth in LB, 0.5% glucose overnight, washing in minimal media and induction for 4-6 hours in minimal media containing 0.5% arabinose.

Following cre recombinase induction, supercoiled minicircle could be purified away from producer plasmid and excised bacterial vector by restriction enzyme digestion of the latter two forms and purification of supercoiled minicircle using a cesium chloride gradient.
Technique 1 was effective for minicircle production from smaller plasmids, with a purified minicircle yield of up to 200 µg/L culture, as well as being the only effective method for producing yields of 40 µg/L culture of minicircle from the large mitochondrial construct pMEV8. Interestingly, technique 2 produced slightly higher yields of minicircle using smaller plasmids, but was very ineffective for minicircle production from the larger pMEV8 construct, presumably due to minicircle loss during bacterial growth. Media step down from rich to minimal medium as observed in technique 2 did not seem to reduce cre expression as might be expected, but contrastingly led to a small increase in yields of supercoiled minicircle.

**Creation and testing of a mutant loxP containing construct**

Cre recombination may occur between and within minicircle constructs, producer plasmids and bacterial vectors resulting in double, triple etc concatamers as a result of the equilibrium kinetics exhibited by the reaction. Although a significant proportion of the minicircle produced is in the monomeric supercoiled form, reduction of the formation of minicircle concatamers as well as the ability to drive the Cre reaction towards minicircle production, should permit increased yields of minicircle.

Modification of the terminal 5 nucleotides on one side of the loxP site to create left element (LE) loxP sites, or vice versa to create right element (RE) loxP sites, results in a slightly reduced Cre interaction at these sites (17). Modification of both sides of the loxP site to produce LE/RE double mutant loxP sites results in a severely reduced Cre interaction (17,18). Recombination between two partially mutant loxP sites, one LE and one RE, leads to the production of a double mutant loxP site (LE/RE) and an unmutated wild type loxP site (WT) in the two products (Figure 4).
Reverse kinetics in this reaction are extremely poor, due to the reduced affinity of Cre for the LE/RE double mutant \textit{loxP} site. Thus there is a directed drive towards production of an LE/RE site (17,18).

Following this concept we created a producer plasmid to contain a mutant LE \textit{loxP} site and a mutant RE \textit{loxP} site flanking the polylinker region (pFIX). The CMV/luciferase cassette from pCIKluc was inserted between the LE and RE \textit{loxP} sites to create a new minicircle producer vector pFIXluc. Growth and induction of this producer plasmid pFIXluc using technique 2 resulted in increased levels of monomeric minicircle compared to excised bacterial vector (Figure 5). Since the construct has been designed such that the minicircle mFIXluc always contains the LE/RE double mutant \textit{loxP} site, this is probably a result of reduced minicircle concatamerisation and a shift in equilibrium towards minicircle production. This results in a significant increase in overall yield of mFIXluc minicircle over pFIXluc to 300 $\mu$g per litre of bacterial culture.

Although maximal obtainable yields of luciferase minicircle measured by spectrophotometry with 260/280 ratios approaching 1.8 were in the region of 5-600 $\mu$g/litre of bacterial culture, gel quantification of DNA did not support this data, giving levels approximately 30\% lower. Further RNase treatment and phenol/chloroform purification was performed in these cases to obtain agreement between spectrophotometry and gel data. This may have been the result of residual ethidium bromide/propidium iodide skewing spectrophotometry readings, thus emphasising the importance of cross-checking measurement data within batches using gel quantification methods.

The MM219Cre strain is \textit{recA}+, which probably explains the continued occurrence of supercoiled concatamers of mFIXluc minicircle (Figure 5), despite the severely compromised Cre interaction at the double mutant \textit{loxP} sites. Despite this, all mFIXluc concatamer forms could be resolved to the same size (3.1 kb) by enzymatic digestion (not shown), suggesting simple concatamerisation rather than rearrangements. The
possibility of large-scale rearrangements and plasmid deletions using MM219Cre seems unlikely, since the large mitochondrial clones pMEV8, and pRSmtOTCAP can be stably maintained with no observable rearrangements. In further support of this, it has been possible to clone and stably maintain a 150 kb BAC in MM219Cre cells (Howe, personal communication). A recA+ strain may actually encourage stable maintenance of some large constructs, by permitting repair of damaged constructs.

**Gene expression in vitro using luciferase minicircle constructs**

In order to test the versatility of luciferase expression from our latest nuclear minicircle within mammalian cells, we chose to perform three comparative tests using lipofectamine complexed to DNA to obtain cellular transfection. In each test we compared luciferase minicircle mFIXluc with its parent plasmid pFIXluc, as well as with the original plasmid from which pFIXluc was derived (pCIKluc), all of which contain a luciferase cassette driven by a CMV promoter. Treatment regimes over 6 replicates for each construct are summarised in Table 1 and Figure 6.
Table 1: Summary of the 3 treatment regimes used to transfecrt HeLa cells with DNA constructs using the same ratio of lipofectamine to DNA in each case (20:1 µg)

<table>
<thead>
<tr>
<th>Treatment per well</th>
<th>pFIXluc 6456bp</th>
<th>mFIXluc 3089 bp</th>
<th>pCIKluc 5632 bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mole:mole with stuffer DNA (pDlox2 stuffer 3409 bp)</td>
<td>0.5 µg</td>
<td>0.24 µg</td>
<td>0.44 µg</td>
</tr>
<tr>
<td>0 µg stuffer</td>
<td>0.26 µg stuffer</td>
<td>0.06 µg stuffer</td>
<td></td>
</tr>
<tr>
<td>Weight:weight</td>
<td>0.5 µg</td>
<td>0.5 µg</td>
<td>0.5 µg</td>
</tr>
<tr>
<td>Mole:mole without stuffer</td>
<td>0.5 µg</td>
<td>0.24 µg</td>
<td>0.44 µg</td>
</tr>
</tbody>
</table>

The initial treatment of mole:mole with stuffer compares equal molar ratios of each construct, with the total weight of DNA adjusted to 0.5µg per well using pDlox2 plasmid. This permits equal levels of lipofectamine to be used for transfection in each case, thus minimising differences resulting from the cytotoxicity of lipofectamine. It should therefore result in equal numbers of transcriptional luciferase units being delivered to cells in each case and is thus the most unbiased comparison of minicircle function. The weight:weight treatment compares equal weights of DNA from each construct. Lipofectamine levels are again equal throughout the treatment but 2.1 times the amount of minicircle luciferase cassettes should be transfected over pFIXluc. Finally the mole:mole without stuffer treatment allows comparison of molar ratios of constructs with variable lipofectamine quantities, whilst keeping the same ratio of lipofectamine to DNA (20:1µg). Whilst this permits the transfection of equal numbers of transcriptional luciferase units, the variable lipofectamine will give varying results depending on the cytotoxicity of lipofectamine.

Figure 6 demonstrates the results of these 3 treatments using 3 plasmids over six replicates in two different graphical representations. Firstly a) the means of raw data are presented for each plasmid on a semi-log scale, and secondly b) the means of log transformed data with 95% confidence limits between any pair of means are presented. The studentised Q test for multiple comparisons, as shown in this case, gives a single bar
representing the minimum distance required between any two means to provide 95% confidence in a significant difference. This is in contrast to a 95% confidence interval calculated for an individual mean (1.96x standard error), given by two opposite bars flanking the mean.

Basic luciferase expression from pFIXluc was roughly comparable to that of pCIKluc (its precursor) in the mole:mole + stuffer comparison, suggesting that gene expression and transfection efficiency from the adapted construct pFIXluc is undiminished. In the weight:weight comparison there was a slight but insignificant increase in luciferase activity by pCIKluc over pFIXluc as expected given the increased number of luciferase cassettes theoretically delivered (1.1 fold). Finally there was a significant increase of pCIKluc luciferase activity over pFIXluc in the mole:mole without stuffer treatment. Despite equal molar quantities of luciferase cassettes transfected per construct the difference is probably due to reduced lipofectamine in the case of pCIKluc producing less cytotoxicity.

Comparisons between the luciferase expression from pFIXluc and mFIXluc were quite conclusive in demonstrating increased minicircle luciferase expression over pFIXluc in all treatments.

Surprisingly, the mole;mole with stuffer treatment produced a 4.5 fold increase in luciferase activity for minicircle over pFIXluc, that was statistically significant (p≤0.05) within the treatment. Theoretically these transfection conditions represent those most likely to give equal levels of transfection in the case of each construct. It should be noted however that although all constructs were produced in the same way, minicircle production involved cre recombination, which produces multimeric concatamers of minicircle, as well as the predominant monomeric form. Multimeric plasmid forms have previously been shown to increase marker gene activity following transfection in vitro (33), perhaps because they provide a more efficient template for nuclear transcription.
Not surprisingly, weight:weight comparisons showed an 8.8 fold increase of minicircle transgene activity over parent plasmid (pFIXluc) (Significant at p<0.05), as expected given that 2.1 times more luciferase cassettes were transfected over the mFIXluc mole:mole with stuffer treatment.

Finally, minicircle luciferase activity over pFIXluc for mole:mole comparisons with no stuffer DNA is vastly increased (152 fold) (Significant at p<0.05). This increase should be treated with caution as it serves to highlight the limitations of lipofectamine as a transfection reagent, where reduced lipofectamine quantities in the case of minicircle transfection cause a huge increase in transgene activity despite equimolar transfection. Indeed transfection of 0.5 µg of DNA into HeLa cells using this reagent at the applied ratio 20:1 is already becoming toxic to these cells. This is also supported by the transfection of pCIKluc using the same treatment and only slightly less lipofectamine, giving a 4.5 fold increase over pFIXluc. Interestingly, transfection comparisons on HeLa cells using either mole:mole with stuffer or weight:weight ratios of 0.25 µg DNA (at lipofectamine levels not toxic to HeLa cells) still show increased minicircle luciferase activity over parental plasmid (not shown).

**Discussion**

We describe the creation of a bacterial strain expressing *cre recombinase* under the tight control of the *araC* regulon, which can be used to produce large quantities of DNA minicircle *in vivo*. We have also developed a range of minicircle constructs for both mitochondrial expression of *sOTC* and for nuclear *luciferase* expression. In addition we demonstrate both effective and substantially increased luciferase expression from nuclear minicircle constructs over both parental plasmids.
Previous techniques for minicircle production (34-36), have used bacterial phage λ integrase mediated recombination to produce minicircle DNA. This system results in attL or attR excision sites of 100 - 165 bp, following recombination (37). By contrast, the Cre mediated recombination system employed here results in a recognition site of only 34 bp (9-11), thus producing a minimal construct size.

Yields of over 300 µg of purified minicircle per litre of culture are sufficient for most in vitro and in vivo applications, whilst further scale up and optimisation of the process seems likely to be relatively straightforward.

The mitochondrial minicircles eliminate bacterial sequences which may be able to act specifically as potential mitochondrial origins of replication (38), or break-points for transcription. However, we cannot be sure that even a 34 bp loxP site insertion into gene junction sites will not disrupt transcription and maintenance of these constructs in mitochondria.

Although the reduced size mitochondrial constructs pMEV46 and pMEV88, made by gene deletion present additional concerns for stability in organello, the minicircle constructs resulting from these producer plasmids (mMEV46, mMEV88) are now of a size which should enable their electroporation into mitochondria (28). We are currently investigating the internalisation and functionality of these mitochondrial constructs.

The nuclear minicircle vectors mNIXluc and mFIXluc clearly possess the advantage of being approximately half the size of their plasmid counterparts. As such, these small constructs demonstrate 4.5 fold increased luciferase activity over their parental plasmid counterparts when transfected on a mole:mole basis (with stuffer DNA) and 8.8 fold increase on a weight:weight basis. The huge increase seen in the mole:mole without stuffer comparison (152 fold) only serves to highlight the versatility of these vectors in reducing the cytotoxic load of DNA/liposome complexes to cells whilst maximising the number of transcriptional units transfected. Indeed by the simple expedient of removing the entire bacterial DNA complement, we have also reduced the CpG content of most of
these expression vectors by more than 60%. As such, minicircle expression vectors are likely to provide a useful tool for reducing inflammatory responses in non-viral vector delivery *in vivo* as well as the increased transgene activity already demonstrated *in vitro*.

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**References**


**Figure legends**

**Figure 1: Insertion of cre/araC into the chromosomal lacZ locus of MM294 bacteria**

a) The plasmid pBAD75Cre contains the cre/ara expression cassette flanked by areas of homology to the bacterial lacZ gene (ΔlacZ1 and ΔlacZ2). The chromosomal lacZ gene has been represented here by five regions for simplicity of reference: lacZ start region, ΔlacZ1 region, lacZ Mid region, ΔlacZ2 region and finally lacZ end region, all of which make up the complete lacZ gene. Use of the temperature sensitive plasmid replicon, pSC101<sup>T</sup>, permits selection for integration of the entire plasmid into the lacZ locus, by
using conditions non-permissive for plasmid growth (44°C) and selection for white chloramphenicol resistant (Cm') colonies (loss of function of \(pSC101\) as shown by X).

A second recombination (excision) event, removing the bacterial vector sequences, is selected for by propagation at 30°C permissive for plasmid replication, and selection of white Cm' colonies. The excised plasmid is not capable of \(lacZ\) expression because it still lacks the start and end of the \(lacZ\) gene. Cm' selection may be dropped for 3 days, resulting in loss of the Cm' plasmid, giving white chloramphenicol sensitive colonies containing the integrated \(cre/ara\) cassette.

b) Targeted insertion of the \(cre/ara\) cassette was tested by PCR amplification of a 1.9 kb fragment using one primer in the \(cre\) gene and another in the chromosomal part of the \(lacZ\) gene. Colony 44\(_2\) was the result of the first recombination event to insert the entire pBAD75Cre plasmid into the \(lacZ\) gene, and serves here as a positive control. Colonies 218 and 219 are the result of a second recombination (excision) event leaving solely the \(cre/ara\) cassette in the chromosome at \(lacZ\). Colony 252blue has resulted in the excision of the entire plasmid and serves as a negative control.

Figure 2: Minicircle producer constructs

a) Plasmid pNIXluc for constitutive mammalian luciferase expression was constructed by insertion of the \(CMV/luc\) cassette from pCIKluc into pDlox3. This construct will form minicircles by the Cre directed excision of bacterial vector sequences at \(loxP\) sites. Differential digestion of the resulting products with an enzyme that cuts only in the bacterial vector and not in the expression minicircle, permits purification of supercoiled minicircle from unwanted linearised producer plasmid and excised bacterial vector using cesium chloride density separation gradients. In the case of mitochondrial constructs, NotI was used to digest the bacterial vector, whilst PvuII was used to digest bacterial vector from luciferase constructs for nuclear gene delivery.
b) Plasmid pMEV8 was constructed as described in experimental procedures. In order to further reduce the size of mitochondrial constructs, regions of mitochondrial DNA were PCR amplified and cloned (3 regions arrowed in blue), to create pMEV46 (8.7 kb), including the D loop, the 12S and 16S rRNA regions, the sOTC gene and the origin of light chain replication.

**Figure 3: Time courses of minicircle production from nuclear and mitochondrial constructs**

a) MM219Cre cells containing construct pNIXluc (6.5 kb) were grown overnight in LB + 0.5% glucose before induction of cre recombinase expression by media exchange to M9 minimal media + 0.5% arabinose for 2 – 24 hours. This results in the appearance of two new supercoiled excision products; bacterial vector pDlox3Δ (3.4 kb) and luciferase minicircle mNIXluc (3.1 kb). The additional bands above 6.5 kb supercoiled probably represent various alternate concatenations (linear, open circular) of the original plasmid pNIXluc, as well as supercoiled concatamers of both pDlox3Δ and mNIXluc (induced lanes only). The best induction times for effective production of minicircle were between 4 – 6 hours.

b) MM219Cre cells containing mitochondrial producer construct pMEV8 were grown overnight in LB + 2% glucose, prior to cre recombinase induction in M9 minimal media + 0.5% arabinose for 10 – 150 minutes. All products were digested with EcoRI. Induction of cre was evident from the appearance of bands corresponding to mitochondrial minicircle mMEV8 (15 kb, 2 kb 0.2 kb) in addition to those of pMEV8 (13.9 kb, 4.5 kb, 2 kb, 0.2 kb) as well as a linear excised vector (pDlox1Δ) band at 3.4 kb. Cre induction appears to initiate as soon as 10 minutes after initial media change, is obvious after 60 minutes, and reaches equilibrium at 120-150 minutes.
Figure 4: Driving the Cre recombinase reaction to completion by the use of mutant 
loxP sites

We constructed a new luciferase expression construct, identical to pNIXluc but 
containing mutant loxP sites; with respectively, a left element (LE) (bracketed text) and 
right element (RE) (mutation in lower case text) mutation in the last 5 base pairs of each 
site – (pFIXluc). Recombination between a LE loxP and a RE loxP site results in an 
excised bacterial vector product containing a wild type loxP site (pMlo3Δ) and a 
minicircle product (pFIXluc) containing a double mutant LE/RE loxP site. Cre 
recombinase has a slightly reduced affinity for either a LE site or a RE site; however it 
has a severely compromised recognition of a LE/RE site, which results in a shift in the 
equilibrium towards minicircle production. In addition, since LE/RE double mutant sites 
do not easily recombine with each other, the formation of minicircle concatamers should 
be reduced

Figure 5: Comparison of the dynamics of the Cre/loxP interaction for normal or 
mutant loxP sites

MM219 cells were transformed with either pNIXluc (normal loxP sites) or pFIXluc 
(mutant loxP sites) and grown overnight in LB + 0.5% glucose. Cre induction was 
carried out in M9 minimal media + 0.5% arabinose for 4 hours. All plasmids are 
undigested. Cre recombination of either producer plasmid (pNIXluc or pFIXluc – each 
6.4 kb) produces the respective supercoiled minicircle (mNIXluc or mFIXluc - 3.1 kb) as 
shown, including excised bacterial vector (pDlo3Δ/pMlo3Δ - 3.4 kb). Cre 
recombination of pNIXluc resulted in roughly equal quantities of the three major 
reaction components - producer plasmid, minicircle and excised vector – (6.5 kb, 3.1 kb, 
3.4 kb respectively). However, recombination of pFIXluc (6.4 kb) although not 
complete, produces a greater quantity of minicircle mFIXluc (3.1 kb) compared to 
excised bacterial vector (3.4 kb). This is probably due to a reduced ability of Cre to
recombine minicircle mFIXluc products with either themselves or the producer plasmid because of the double mutant loxP site in the minicircle. Cesium chloride purified minicircle mFIXluc does show some concatamerisation (6.2 kb - minicircle X2, 9.3 kb - minicircle X3, etc), probably as a result of general recombination from the MM219Cre recA+ strain but most of the minicircle DNA was in the single 3.1 kb supercoiled concatamer form. Supercoiled mFIXluc minicircle yields from 1 litre of bacterial culture of 0.75 mg were however considerably higher than those of pNIXluc (0.25 mg) from the same culture volume.

Figure 6: Comparisons of luciferase activity from HeLa cells transfected with liposome/DNA complexes using different minicircle and plasmid constructs

a) Means of six replicates of luciferase activity following transfection with DNA/lipofectamine complexes (ratio at 20ug lipofectamine/µg DNA). Treatment regimes of mole:mole ratios with stuffer DNA, weight:weight and mole:mole without stuffer comparisons are given in table 1. Plasmids pFIXluc and pCIKluc gave roughly similar levels of luciferase activity in mole:mole ratios with stuffer, demonstrating similar gene expression and transfection abilities. Minicircle luciferase activity was increased over pFIXluc by 4.5 fold in mole:mole ratios with stuffer DNA, 8.8 fold in weight:weight ratios and 152 fold in mole:mole ratios without stuffer. The first increase demonstrates an intrinsic increase in minicircle transfection ability or gene expression, probably as a result of multimeric concatamers of minicircle. The second shows that the increased (2.1 fold) number of transcriptional units gives a concomitant increase in transgene activity without changing lipofectamine quantities. The final figure demonstrates the cytotoxicity of lipofectamine, as reduced quantities of this reagent with minicircle result in vastly increased transfection efficiency. Although these figures are adjusted for total protein quantities per measurement, cell cytotoxicity will still result in reduced gene expression from the surviving cells.
b) Log_{10} transforming data from luciferase activity provides a method for satisfying the conditions required to perform analysis of variance (normality of data and equal variances). In this case F is extremely significant at \(p \leq 1.7 \times 10^{-18}\). We have then used the studentised values of Q to perform a multiple comparisons test between any two pairs of means from these values. The resulting bar shows the minimum distance required between any two means for at least 95% confidence in a significant difference. We can see that comparative increases in luciferase activity from minicircle over either pFIXluc or pCIKluc within each treatment are significant at this level (\(p \leq 0.05\)) in all cases.
Figure 1: Insertion of cre/araC into the chromosomal lacZ locus of MM294 bacteria

Transform plasmid into cells containing functional lacZ (blue)

pSC101ts = Temperature sensitive origin
Permissive at 30°C
Non permissive at 44°C

Select at 44°C with chloramphenicol for recombination

Bacterial Genome

Single lacZ recombination gives white colonies

Select at 30°C with chloramphenicol for excision

White Cm resistant colonies demonstrate excision at second lacZ site

Drop selection for 3 days Plasmid is lost

Check cre by PCR and activity of cre protein

Check PCR

1.9 kb product

1.9 kb

1 kb ladder

442 white (+Ve)

218

219

252 blue (−Ve)

1 kb ladder
Figure 2: Minicircle producer constructs

a) Nuclear luciferase construct and minicircle

b) Mitochondrial constructs for minicircle production
Figure 3: Time courses of minicircle production from nuclear and mitochondrial constructs

a) Cre induction time course for pNIXluc (undigested)

b) Cre induction time course for pMEV8 (EcoRI digested)
Figure 4: Driving the Cre recombinase reaction to completion by the use of mutant loxP sites

Digestion with PvuII destroys plasmid and bacterial vector
Purification of supercoiled minicircle on CsCl gradient
Figure 5: Comparison of the dynamics of the Cre/loxP interaction for normal or mutant loxP sites
Figure 6: Comparisons of luciferase activity from HeLa cells transfected with liposome/DNA complexes using different minicircle and plasmid constructs.
An araC controlled bacterial cre expression system to produce DNA minicircle vectors for nuclear and mitochondrial gene therapy
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