

pFARs, Plasmids free of antibiotic resistance markers, display high-level transgene expression in muscle, skin and tumour cells

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Abstract

Background Nonviral gene therapy requires a high yield and a low cost production of eukaryotic expression vectors that meet defined criteria such as biosafety and quality of pharmaceutical grade. To fulfil these objectives, we designed a novel antibiotic-free selection system.

Methods The proposed strategy relies on the suppression of a chromosomal amber mutation by a plasmid-borne function. We first introduced a nonsense mutation into the essential *Escherichia coli thyA* gene, resulting in thymidine auxotrophy. The bacterial strain was optimized for the production of small and novel plasmids free of antibiotic resistance markers (pFARs) and encoding an amber suppressor t-RNA. Finally, the potentiality of pFARs as eukaryotic expression vectors was assessed by monitoring luciferase activities after electrotransfer of LUC-encoding plasmids into various tissues.

Results The introduction of pFARs into the optimized bacterial strain restored normal growth to the auxotrophic mutant and allowed an efficient production of monomeric supercoiled plasmids. The electrotransfer of LUC-encoding pFAR into muscle led to high luciferase activities, demonstrating an efficient gene delivery. In transplanted tumours, transgene expression levels were superior after electrotransfer of the pFAR derivative compared to a plasmid carrying a kanamycin resistance gene. Finally, in skin, whereas luciferase activities decreased within 3 weeks after intradermal electrotransfer of a conventional expression vector, sustained luciferase expression was observed with the pFAR plasmid.

Conclusions Thus, we have designed a novel strategy for the efficient production of biosafe plasmids and demonstrated their potentiality for nonviral gene delivery and high-level transgene expression in several tissues. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords biosafety; DNA delivery; electrotransfer; gene therapy; nonviral; plasmid vector

Introduction

High expression levels of therapeutic proteins in transfected mammalian cells require an optimal intracellular gene delivery. Although viral vectors are considered to be efficient vehicles for gene transfer, their use is restricted by several major limiting factors, such as the occurrence of immune responses

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to viral components, possible insertional mutagenesis into the host genome and high production costs [1–4]. These drawbacks can be avoided by using easily produced nonviral expression vectors that remain in a non-integrative state inside eukaryotic cells [5,6]. Furthermore, the development of physical methods for *in vivo* plasmid administration (e.g. hydrodynamic delivery or electrotransfer) allows a significant increase in protein expression levels [7,8] and the reconsideration of nonviral expression vectors as therapeutic gene vehicles.

Nonviral expression vectors consist of three main classes: plasmidic DNA, MIDGE vectors [9] and minicircles [10–12]. The latter two are produced from a parental plasmid, which contains a eukaryotic expression cassette flanked by either restriction enzyme- or recombinase-recognized specific sequences, respectively. After plasmid propagation, the expression cassette is released by either enzymatic digestion or intramolecular recombination and further purified [9–12]. MIDGE vectors and minicircles present the main advantage of being totally free of bacterial sequences that appear to promote heterochromatin formation, leading to transgene silencing in certain organs such as liver [13]. In muscle, however, prokaryotic sequences do not appear to impair prolonged transgene expression [5], thus making possible the use of alternative nonviral vectors for gene delivery in this organ. The third group of nonviral vectors is represented by plasmidic DNA that is produced from dividing bacterial cells grown in the presence of antibiotics to ensure plasmid maintenance and propagation. Although plasmids encoding proteins that inactivate antibiotics of the aminoglycoside class (such as kanamycin which is not extensively used in human health) have been approved for some clinical applications, several alternatives have been envisioned to replace them. Thus, the risk of dissemination of antibiotic resistance markers to mammalian-colonizing endogenous flora and/or putative anaphylactic shocks as a result of the presence of antibiotics in plasmid preparation is eliminated. Antibiotic-free selection systems are based on the principle of the growth of bacterial conditional mutants relying on the presence of plasmids. Plasmidic expression vectors can harbour either: (i) *lac* operator sequences that titrate a repressor bound to an essential chromosomal gene promoter/operator region [14,15]; (ii) a suppressor t-RNA coding sequence enabling the complete translation of a protein playing a role in the arginine biosynthetic pathway [16,17]; and (iii) a replication origin encoding an RNA molecule involved in the regulation of an essential [18,19] or a lethal [20] chromosomal *Escherichia coli* gene via an RNA/RNA interference mechanism.

In the present study, we describe a novel combination of *E. coli* producer strain/plasmids free of antibiotic resistance markers (pFARs). First, a nonsense mutation (CAT>TAG) was introduced into the essential *E. coli thyA* gene that encodes a thymidylate synthase, an enzyme required for DNA precursor synthesis. The *thyA* mutant was further optimized for the production of a family of small pFAR vectors that encode an amber suppressor t-RNA. Finally, pFAR efficiency as eukaryotic expression

vectors was assessed using the luciferase reporter gene expressed from the cytomegalovirus (CMV) promoter, after plasmid injection into muscle, skin and tumour cells, followed by electrotransfer. This physical method consists of employing electric field-mediated intracellular delivery via a mechanism mostly comprising cell permeabilization and DNA uptake through electrophoresis, allowing an increase in intracellular gene delivery and protein expression levels [8,21–23]. We showed that combining electrotransfer with optimized nonviral gene vectors results in a further increase in plasmid delivery into various tissues.

Materials and methods

Microbiological and molecular biology manipulations

Escherichia coli strains were grown in either complex (Luria-Bertani: LB [24] or Mueller Hinton: MH [25]; Sigma-Aldrich, Lyon, France) or minimal (M9) [24] media supplemented with either antibiotics [24] or thymidine (30 µg/ml). The DH5 α strain [26] was used for routine cloning procedures that were carried out as described by Sambrook *et al.* [24].

Escherichia coli mutants were isolated from strain MG1655 obtained from 'The Coli Genetic Stock Center' (USA) (<http://cgsc.biology.yale.edu>) because, unlike most laboratory strains, it does not contain suppressor t-RNA alleles (Accession number #U00 096) [27].

Construction of *E. coli* mutants

Escherichia coli mutants were isolated essentially as described by Posfai *et al.* [28] using pST76-C to introduce mutated genes into the bacterial genome, and pST76-AsceP to select those strains in which the second event of recombination had occurred. Both plasmids have a thermosensitive origin of replication and are therefore easily cured from bacteria by growing them at 43 °C in the absence of antibiotics.

To construct the *thyA* mutant, a 2-kb region covering the *thyA* gene was amplified by the polymerase chain reaction (PCR) using the ExTaq polymerase (Takara, Lonza, Verviers, Belgium), MG1655 genomic DNA and primers (ThyA-F: 5'-CATGCGGTATTGCGCAGGC-3' and ThyA-R: 5'-CGCTGTATCTGTTCCGTGTCT-3'). The primers were designed to place the amber mutation in the centre of the 2-kb fragment. The PCR product was ligated to the cloning vector PCR2.1 (Invitrogen, Illkirch, France) and sequenced. An amber mutation (His 147: CAT>TAG) was introduced into the *thyA* gene by site directed mutagenesis using the QuikChange® II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) and ThyA-His147-F (5'-CGCTGGCACCGTGCTAGGCATTCTTCCAGTTC-3') and ThyA-His147-R (5'-GAACTGGAAGAATGCCTAGCAGGTGCCAGCG-3') as primers. The mutated *thyA* region

was then entirely sequenced to confirm that the insert only contained the amber mutation. Plasmids PCR-thyA* and pST76-C were ligated after digestion by *EcoRV* and *SmaI*, respectively. Finally, the pUC replication origin from pCR2.1 was deleted by digesting the fusion plasmid by *BamHI*. The mutated *thyA* region was then introduced into the MG1655 genome as described above, selecting for thymidine auxotrophic strains. The amplification, by PCR, of a 2.6-kb fragment covering the mutated region and sequence analysis confirmed that the mutated strain only contained an amber mutation in *thyA* and that the adjacent genes have not been affected by the crossing-over events.

To construct the double *thyA endA* mutant, two 1-kb fragments, covering regions upstream (*yggI*) and downstream (*rsmE*) from *endA*, were amplified by PCR using MG1655 genomic DNA as a template, the ExTaq polymerase (Takara) and two pairs of primers: 5'-GTCCGTTTTGCCATGAGTGC-3' and 5'-taccgggTAGACAAATAACGGTACATCAC-3', and 5'-ttccgggAGAGCTAACCTACTAGCG-3' and 5'-CCA CTCTTCGTAGTTCTGCT-3', respectively. The PCR fragments were entirely sequenced after cloning into PCR2.1 (Invitrogen). The 1-kb *SmaI-XbaI* fragment from PCR2.1-*yggI* was ligated to PCR2.1-*rsmE* digested with *SmaI-SpeI*, to give PCR2.1 delta *endA*. This latter plasmid (digested with *ApaI* and Klenow-treated) was fused to pST76-C (digested with *SmaI*). A *BamHI* deletion gave a plasmid only carrying the thermosensitive replication origin. The deleted *endA* region was introduced into the *thyA* mutant genome as described above.

To construct the triple *thyA endA recA* mutant, two 1-kb fragments located upstream (*ygaD*) and downstream (*recX*) from *recA* were amplified by PCR using the MG1655 genomic DNA as a template, the ExTaq polymerase (Takara) and two pairs of primers: 5'-GTAATGGCAAACGGTCAGGC-3' and gatccgggGTCGATAGCCATTTTTACTCC-3', and 5'-catccgggGAAGGGTAGCAGAACTAAC-3' and 5'-GT CGCCGAAGCTGAAGTTG-3', respectively. *ygaD* and *recX* were fused using the same protocol as previously described. PCR2.1.delta.*recA* was ligated to pST76-C after digesting the plasmids with *XbaI* (Klenow-treated) and *SmaI*, respectively. The deleted *recA* region was introduced into the *thyA endA* mutant as described above.

Construction and preparation of pFAR plasmids

pFAR1 is a derivative of pVAX2 [29] that was obtained by replacing the cytomegalovirus promoter of pVAX1 (Invitrogen) by that of pCMV- β (Clontech, Ozyme, Saint-Quentin-en-Yvelines, France). The histidine suppressor t-RNA gene was amplified from pGFIB-1.His(AS) [30] by PCR using primers: 5'-gtccatggCTGGCGCCGCTTCTTTGAGC-3' and 5'-ccccatgg ACGACGGCCAGTGCCAAG-3'. The PCR fragment was digested with *NcoI* and inserted into pVAX2 digested with

BspHI. The digestion of the resulting plasmid by *HindIII* (Klenow-treated) and *PvuII*, followed by a self-ligation, allowed the deletion of the kanamycin resistance gene, to give pFAR1.

To construct pFAR1-LUC, the 1.7-kb *BamHI-XhoI* fragment containing the firefly cytoplasmic luciferase-encoding gene was isolated from pVAX2-LUC [29] and introduced into pFAR1 digested with the same enzymes. To optimize the expression vector and eliminate intergenic sequences, a new plasmid, called pFAR4, was entirely synthesized (Geneart, Regensburg, Germany). The approximately 1.7 kb *MluI-PvuII* fragment containing the expression cassette CMV.LUC.BGH cleaved from pVAX2-LUC was introduced into pFAR4 digested with *MluI* and *EcoRV*, to give pFAR4-LUC.

For *in vivo* studies, plasmids were purified using Endofree preparation kits (Qiagen, Hilden, Germany). Endotoxin levels were determined by Lonza, using the LAL (Limulus Amoebocyte Lysate) procedure. They were all below the detection limits (<0.05 EU/ml). The quality of plasmids was assessed by calculating the ratio of light absorption (260 nm/280 nm) and by visualization on ethidium bromide-stained 1% agarose gels. Light absorption at 260 nm was used to determine DNA concentration.

In vivo studies

Six- to 8-week-old female BALB/c mice (Charles River, L'Arbresle, France), 6-week-old female NMRI mice (Université catholique de Louvain, Brussels, Belgium) and 8-week-old male C57BL/6 mice (Janvier, Le Genest Saint Isle, France) were used for plasmid intramuscular, intradermal and intratumoural injection, respectively. Prior to all procedures, the animals were anaesthetized by intraperitoneal injection of ketamine and xylazine as described by Bloquel *et al.* [31] and Vandermeulen *et al.* [21]. Studies were conducted in accordance with the recommendations of the European Convention for the Protection of Vertebrates Animals used for Experimentation. All experimental protocols were approved by the Local Ethic Committees for animal care and use.

Plasmids (1.64 pmol) diluted in 30 μ l of physiological serum were injected into BALB/c mouse tibial cranial muscles and electrotransferred, as described by Bloquel *et al.* [31] using two stainless steel plate electrodes placed on each side of the shaved leg and a G250 electropulsator (Sphergen, Evry, France) to deliver eight square-wave electric pulses (200 V/cm, 20 ms, 2 Hz). For intradermal electrotransfer, the abdomen skin was shaved 1 day prior to the experiments with a depilatory cream (Veet for Sensitive Skin, Belgium). Plasmids (16.4 pmol in two volumes of 15 μ l PBS) were injected into the dermis at two 5-mm apart different sites, using a Hamilton syringe and a 30-gauge needle. Then, a cutaneous fold was performed and injection sites were placed between 2 mm spaced-plate electrodes (IGEA,

Carpi, Italy) [21]. A short high voltage pulse (700 V/cm, 100 μ s) immediately followed by a low voltage pulse (200 V/cm, 400 ms) were applied approximately 1 min after plasmid injection [32], using a Cliniporator system (Cliniporator; IGEA). For all experiments, conductive gel was used to ensure electrical contact with the skin (EKO-GEL, Egna, Italy).

For the assessment of pFAR4 efficiency in tumours, B16F10 cells were suspended and cultured at 37°C in DMEM complete medium (Invitrogen) supplemented with fetal bovine serum, penicillin and streptomycin. 10^6 cells in 50 μ l of phosphate-buffered saline (PBS) were injected into the flank of mice using an insulin syringe. The size of the tumours was calculated, based on the volume of a spheroid ($4/3 \cdot \pi b^2 a$), after measurement of their transverse (b) and anteroposterior (a) diameters using an electronic caliper. Twenty picomoles of plasmids (in a volume of 50 μ l PBS) were injected into 10–25-mm³ tumours, using an insulin syringe. One minute after DNA injection, the plasmids were electrotransferred using 4-mm spaced plate electrodes and a combination of a high voltage pulse (1250 V/cm, 100 μ s) followed by a low voltage pulse (140 V/cm, 400 ms) separated by a 1-s time interval [33].

Luciferase activities were recorded using charged-coupled-device (CCD) cameras that allow *in vivo* studies in living animals. Following intramuscular plasmid electrotransfer, 150 μ l of Luciferin (LUX Biotechnology, Edinburgh, UK) diluted in phosphate buffer (pH 9.0) (2.5 mg/ml) were injected intraperitoneally. Twenty minutes later, luciferase activities were recorded for two minutes using a Photon-Imager camera (Biospace, Paris, France). To measure luciferase activities in transfected skin or tumours, luciferin diluted in PBS without Mg²⁺ and Ca²⁺ (Xenogen corporation, Alameda, CA, USA) was injected intraperitoneally (i.p., 3 mg/100 μ l). Optical imaging was acquired using an IVIS50 system (Xenogen Corp., Alameda, CA, USA). The duration of luminescence acquisition was between 10 s and 60 s and was initiated 10 min after injection of the substrate. In all cases, luminescence levels were integrated in region of interest (ROI) drawn by hand around luminescence zones. Background luminescence was subtracted according to values obtained in ROI drawn on a nontransfected zone of the mice [31].

Titration of anti-LUC antibodies

Antibodies against luciferase in mouse serum were titrated by enzyme-linked immunosorbent assay, essentially as described by Bloquel *et al.* [31] using luciferase (Promega, Madison WI, USA) to coat the microplates and a horseradish peroxidase-conjugated goat anti-mouse secondary antibody (GE Healthcare, Uppsala, Sweden). To establish a standard curve, a serial dilution of a monoclonal antibody anti-luciferase (Sigma-Aldrich, Lyon, France) was used.

Results

Description of a novel combination of *E. coli* producer strain/plasmids devoid of antibiotic resistance markers

To propagate plasmids free of antibiotic resistance markers, a nonsense mutation (TAG) was introduced into the *E. coli thyA* gene that encodes a thymidylate synthase (EC 2.1.1.45). This enzyme is required for *de novo* synthesis of thymidine monophosphate (dTMP) from deoxyuridine monophosphate. Mutation in *thyA* leads to thymidine auxotrophy [34] that can be overcome by adding exogenous thymidine in the growth medium. The supplement penetrates into bacterial cells, via a nucleoside transporter, and is transformed into dTMP by a thymidine kinase (EC 2.7.1.75). In the absence of thymidine, only the strains that contain a plasmid encoding a suppressor t-RNA enabling the full translation of ThyA can grow (Figure 1).

An amber mutation, which substitutes the CAT encoding His147 to TAG, was introduced into the cloned *thyA* region and subsequently inserted into the nonpathogenic *E. coli* strain MG1655 genome by homologous recombination. The selected mutant did not grow on minimal medium unless thymidine was added. To facilitate cloning procedures and plasmid preparations for routine laboratory assays, we identified another selective complex medium: the Mueller Hinton (MH) broth known to contain only traces of thymidine [35] and in which the *thyA* mutant did not grow.

In parallel, a plasmid devoid of antibiotic resistance markers was constructed. First, the histidine suppressor t-RNA gene was introduced into pVAX2, an expression vector that carries a kanamycin resistance gene [29]. The suppressor t-RNA gene is an allelic form of *hisR*, which codes a histidine t-RNA with a modified anticodon (AUG>CUA) followed by two AA to yield 5'-CUAAA. This additional modification was shown to improve the suppression efficiency of the nonsense mutation [36]. The expression of the t-RNA suppressor is under the control of the *E. coli* lipoprotein promoter region (*lpp*) and the transcription terminator sequence from the *rrnC* operon [37], which comprise two cis-regulatory elements only active in prokaryotic cells. The kanamycin resistance gene was subsequently deleted from pVAX2.sup.t-RNA and the resulting plasmid was named pFAR1. To validate the proposed strategy, pFAR1 was introduced into the *thyA* mutant by transformation. Whereas bacteria electroporated with water did not form colonies on selective medium (MH), pFAR1-transformed cells grew well even in the absence of thymidine, indicating that the suppression of the nonsense mutation could sustain bacterial growth. All tested transformants grown overnight in selective medium appeared to contain pFAR1, with the expected size and enzymatic restriction profiles. All these data show that this novel combination of *E. coli thyA* mutant/pFAR plasmid can be used to produce

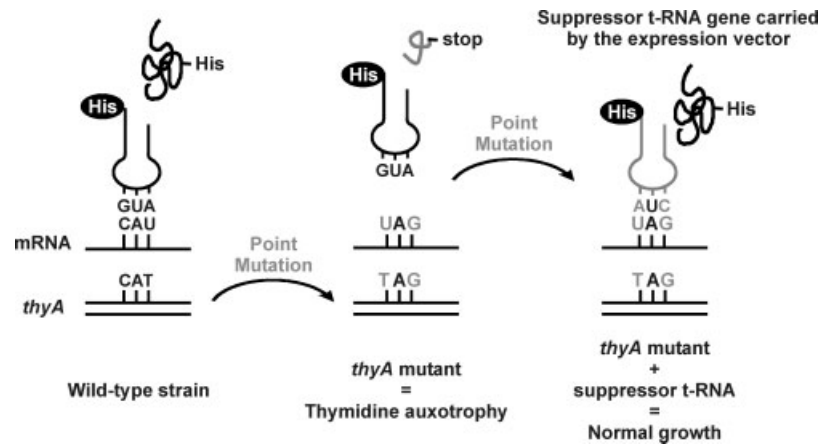


Figure 1. Description of the strategy used to propagate pFAR plasmids. A nonsense mutation TAG was introduced into the *E. coli thyA* gene, resulting in thymidine auxotrophy. The mutant strain was isolated in the presence of thymidine that can penetrate into bacterial cells where it is transformed into dTMP, a precursor required for DNA polymerization. In selective medium, only the strains containing a plasmid encoding a suppressor t-RNA (i.e. inserting an amino acid in response to UAG) can grow

plasmids devoid of antibiotic resistance markers and was therefore further optimized.

Optimization of the bacterial producer strain and of the eukaryotic expression vector

The use of plasmid DNA for clinical trials requires that they meet some defined criteria such as purity and topology [38,39]. Because most laboratory strains contain suppressor t-RNA genes, the amber mutation had to be introduced into the wild-type reference strain MG1655. To reach optimal conditions for plasmid production, the *thyA* mutant needed to be further optimized. DNA quality produced by bacterial strains can be improved by mutating the *endA* gene that encodes the endonuclease 1. Although the function of this enzyme is not fully understood, the quality of plasmid DNA purified from *endA* + strains appears inconsistent, degrading upon long-term storage [40]. To overcome this, an internal region of *endA* (coding amino acids 7–234 of the 234 amino acids EndA protein) was deleted from the chromosome of the *thyA* mutant, to give a double *thyA endA* mutant.

During plasmid propagation and replication, intermolecular recombination between homologous sequences can occur, leading to multimers formation and an increase in plasmid size. With plasmid copy number being controlled by the replication origin concentration, recombination events will influence the monomers/multimers ratio, resulting in inconsistency from one plasmid batch to another, which is not suitable for pharmaceutical applications [38,39]. To avoid multimers formation, the internal region of *recA* that encodes a recombinase (353 amino acids) was deleted (amino acids 5–343) from the double *thyA endA* mutant genome. As shown Figure 2a, plasmids prepared from the triple mutant predominantly formed monomeric closed circular DNA, whereas those purified from the simple *thyA* mutant also appeared as dimers or multimers.

Having optimized the bacterial producer strain, the next objective was to further develop the plasmid vector. To minimize bacterial sequences and plasmid size, a new DNA vector was entirely synthesized and named pFAR4 (Figure 3). pFAR4 has a small size (1.1 kb), it contains a pUC-type origin of replication, the same histidine suppressor t-RNA gene as pFAR1 and a multiple cloning site to allow easy cloning procedures. When introduced into the triple *thyA endA recA* mutant, pFAR4 showed the same properties as pFAR1, such as the ability to restore normal growth to the thymidine auxotrophic strain. In selective MH medium, the growth curve of the optimized *thyA* mutant containing a pFAR4 derivative was similar to that of the strain DH5 α harbouring pVAX2-LUC propagated in the presence of kanamycin (Figure 2b). Furthermore, at a similar optical density ($OD_{600} \sim 0.9$) or after overnight growth, the amount of pFAR4 plasmids purified from the triple *thyA endA recA* mutant was equivalent to that of a pVAX2 derivative prepared from DH5 α grown in Luria–Bertani (LB) medium supplemented with kanamycin (Figure 2c). Thus, bacterial and plasmid optimization led to the production of plasmids devoid of antibiotic resistance markers with a good yield and appropriate criteria for *in vivo* studies.

Assessment of pFAR plasmids as eukaryotic expression vectors

To assess whether pFAR plasmids could be used for *in vivo* studies, the cytoplasmic firefly luciferase-encoding gene was cloned into pFAR1 and into the *de novo* synthesized pFAR4 plasmid (Figure 3). The reporter gene is under the control of the ubiquitous CMV promoter and the bovine growth hormone (BGH) polyadenylation signal. Similar amount (1.64 pmol) of pVAX2-LUC, pFAR1-LUC and pFAR4-LUC, which contain the same expression cassette, were injected into mouse tibial cranial muscles and subsequently electrotransferred. Luciferase activities were determined after intraperitoneal injection of the substrate

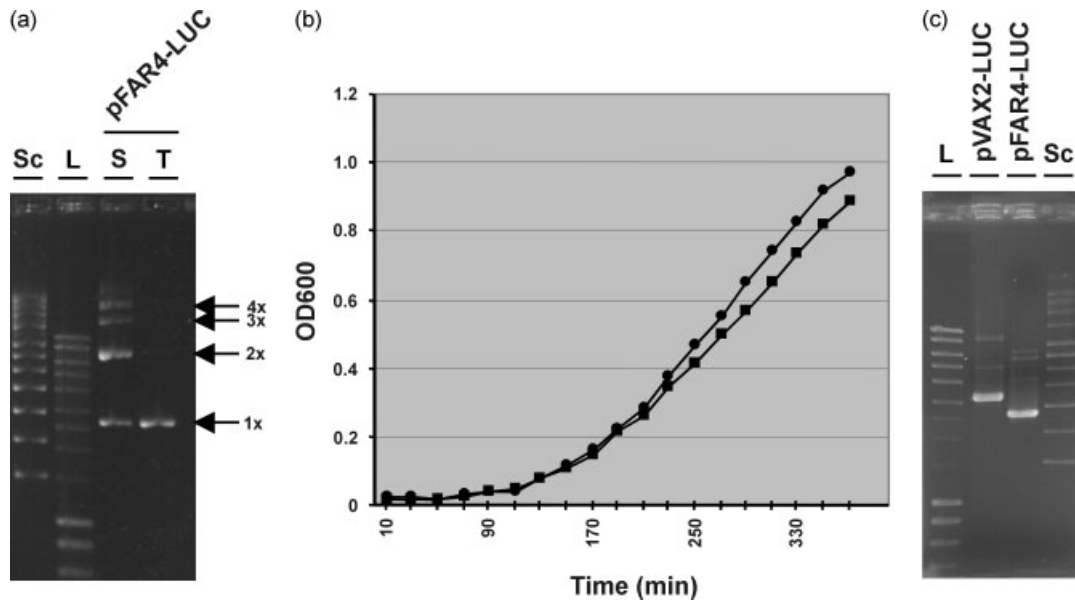


Figure 2. Properties of pFAR plasmids. (a) Topology of pFAR plasmids prepared from the *thyA* mutants. Native plasmid DNA (pFAR4-LUC: 3.7 kb) prepared from distinct bacterial strains was loaded onto a 1.0% agarose gel and stained with ethidium bromide after electrophoresis. The pFAR4 derivative produced from the single *thyA* mutant (S) formed multimers (1x: mono, 2x: di . . .) whereas plasmids prepared from the triple *thyA recA endA* mutant (T) were mainly present as monomeric (noted 1x) closed circular DNA. Plasmid multimerization was determined by comparison with the markers: Sc, Supercoiled DNA ladder (Invitrogen; bands correspond to supercoiled DNA with the sizes: 2, 3, 4, 5, 6, 7, 8, 10, 12, 14 and 16 kb); L, Smart Ladder (Eurogentec, Seraing, Belgium; bands correspond to linearized DNA fragments of 0.6, 0.8, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8 and 10 kb). (b) Introduction of pFAR plasmids into the optimized *thyA* mutant restored bacterial growth. Overnight cultures grown in MH broth were used to inoculate fresh medium to an initial optical density of OD₆₀₀: 0.015. Cultures were incubated at 37°C under constant agitation and absorbance was measured every 20 min. The introduction of pFAR4 derivatives into the *thyA endA recA* mutant restored normal growth to the thymidine auxotroph strain, leading to high-density cultures (squares). For comparison, a growth curve obtained with the strain DH5 α -pVAX2-LUC propagated in MH supplemented with kanamycin is also indicated (circles). Data points are the means of duplicate experiments. (c) Production of pFAR plasmids. pFAR4 derivatives prepared from the optimized *thyA* mutant grown in MH medium are produced with a yield similar to that of pVAX2-LUC propagated in DH5 α cultivated in LB medium supplemented with kanamycin. Both plasmids were prepared from strains having a similar OD₆₀₀ (~0.9) using plasmid Nucleospin® columns (Macherey Nagel, Hoerd, France). Ten microlitres of undigested DNA were loaded onto a 1% agarose gel, which was stained with ethidium bromide

(luciferin), using a CCD camera that allows monitoring gene expression in living animals (Figure 4). Until day 15, the luciferase activity recorded after injection of either plasmid was similar, indicating that both pFAR1 and the newly synthesized pFAR4 vector display efficient *in vivo* gene delivery. At later time points, however, luciferase activities started to decrease in two pFAR1-LUC-transfected muscles of the same mouse (Figure 4). When sera were collected by retro-orbital punctures, they appeared to contain antibodies directed against the exogenous luciferase protein. The amount of antibody was 14.5 $\mu\text{g/ml}$ 30 days after plasmid injection, and reached 70 $\mu\text{g/ml}$ at day 60 (data not shown).

To assess luciferase expression in another tissue, 16.4 pmol of pVAX2-LUC and pFAR4-LUC were injected into mouse skin and electrotransferred. Figure 5 shows that luciferase activities in pVAX2-LUC-transfected cells decreased within a few weeks. By contrast, the injection of the pFAR4 derivative led to sustained luciferase expression levels. In another independent experiment, a prolonged expression was observed for more than 3 months (data not shown). Furthermore, at all time points, a higher luciferase activity was monitored in skin after pFAR4-LUC electrotransfer as compared to

the value obtained with pVAX2-LUC ($p = 0.039$, two-way analysis of variance), suggesting an increased transfection efficiency. Nevertheless, expression at the background level was detected when either pVAX2-LUC or pFAR4-LUC were injected intradermally without electroporation, confirming the requirement of this physical method for an efficient gene delivery in skin.

Finally, to determine whether pFAR4 could efficiently drive eukaryotic transgene expression in tumours, cultured murine melanoma B16F10 cells were transplanted into the flank of mice. When the tumours reached a volume of 10–25 mm³, 20 pmol of either pVAX2-LUC or pFAR4-LUC were injected and electrotransferred. Luciferase activities were monitored two days later (Figure 6). Interestingly, the tumours transfected with the pFAR4 derivative displayed, on average, almost a one-log higher reporter gene activity than those expressing luciferase from pVAX2-LUC.

Discussion

The production of recombinant therapeutic proteins or of plasmids most often requires growing bacteria in the

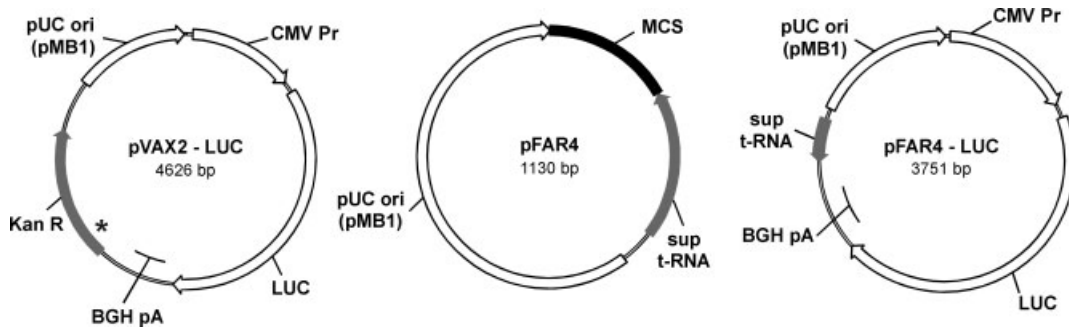


Figure 3. Maps and plasmid sizes of pVAX2-LUC and pFAR derivatives. pFAR4 is a new and small plasmid vector. It has a size of 1.1 kb, it contains an origin of replication of pUC-type, a histidine suppressor t-RNA gene expressed from prokaryotic regulatory elements and a multiple cloning site (MCS) to facilitate cloning procedures. The luciferase-expressing pFAR4 derivative is approximately 0.9 kb smaller than pVAX2-LUC, has a reduced CpG content and lacks the motif GAGGTT (*) considered to mediate inflammatory responses in mice [45,46]

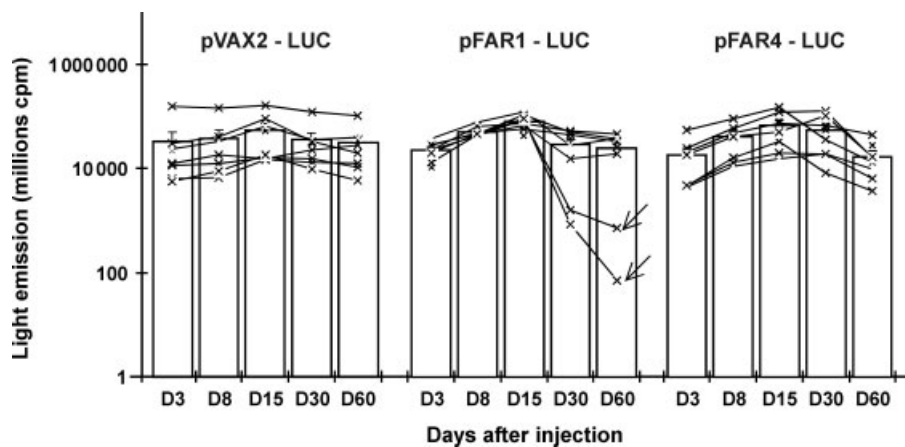


Figure 4. Luciferase activities in mouse tibial cranial muscles. Some 1.64 pmol of plasmids (pVAX2-LUC, pFAR1-LUC or pFAR4-LUC) diluted in 30 μ l of physiological serum were injected into each tibial cranial muscle of four BALB/c mice and subsequently electrotransferred. Luciferin was injected intraperitoneally and luciferase activities were recorded in living animals using a CDD camera 3, 8, 15, 30 and 60 days later. Bars represent the mean \pm SEM. Crosses indicate individual values measured in each muscle. A dramatic decrease in luciferase activity (arrows) was observed in both pFAR1-LUC-transfected muscles from the same mouse, which appeared to produce high levels of anti-luciferase antibodies

presence of antibiotics to ascertain vector maintenance and propagation in prokaryotic cells. To produce biosafe plasmids, we designed a new antibiotic-free selection system, which is based on the suppression of a nonsense amber mutation, introduced into the essential *E. coli thyA* gene, by a plasmid encoding a suppressor t-RNA.

Nonsense mutations are of three types: amber, ochre or opal, resulting in UAG, UAA or UGA chain-terminating codons, respectively. We chose to introduce an amber nonsense codon into the *thyA* gene for two main reasons: (i) several studies have shown that amber mutations can be efficiently suppressed [30,36,37] and (ii) the distribution of translation termination codons is: 7.6% for UAG, 63% for UAA and 29.4% for UGA [27]. Thus, the introduction of UAG suppressor t-RNA into *E. coli* was expected to have a moderate impact on host metabolism. This is also supported by the fact that most laboratory strains and a significant number of wild-type *E. coli* isolates naturally contain amber suppressor t-RNA genes, with no apparent selective disadvantage [41,42]. Furthermore, all nonsense mutations are not suppressed

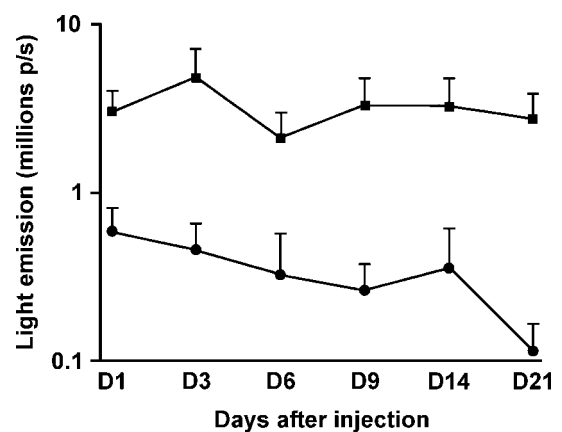


Figure 5. Luciferase expression after intradermal injection and electrotransfer of pVAX2-LUC and pFAR4-LUC. Similar molarity (16.4 pmol) of either pVAX2-LUC (●) or pFAR4-LUC (■) were injected and electrotransferred into the skin of 7 and 8 mice, respectively. One, 3, 6, 9, 14 or 21 days later, luciferin was injected intraperitoneally and luciferase activities were monitored using a CDD camera. The symbols (● and ■) represent the mean \pm SEM values

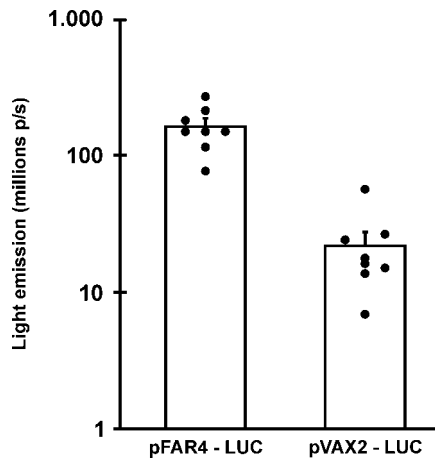


Figure 6. Luciferase expression after intratumoural injection and electrotransfer of pVAX2-LUC and pFAR4-LUC. B16F10 tumour cells were transplanted into the flank of eight mice and transfected with 20 pmol (in 50 μ l) of either pVAX2-LUC or pFAR4-LUC. Two days after plasmid injection, luciferin was injected intraperitoneally and luminescence was acquired for 60 s using a CCD camera. Luciferase activities appeared to be approximately one-log higher in the pFAR4 derivative-transfected tumour cells than after pVAX2-LUC delivery (statistical analysis: *t*-test: $p = 0.0002$). Bars represent the mean \pm SEM values

with an equal efficiency. Several factors appear to play a role, such as the nucleotides surrounding the nonsense codon being suppressed [36], as well as the selected suppressor t-RNA [30,36,37]. Taking into account all of these parameters and a previous study by Michiaels *et al.* [43] on the insertion of nonsense codon in the thymidylate synthase coding region, we introduced the amber mutation in the *thyA* nucleotide sequence encoding histidine 147, which lies in the substrates binding pocket of the enzyme [43]. This mutation resulted in thymidine auxotrophy that could be efficiently suppressed upon introduction of pFAR4.

pFAR4 is a new plasmidic DNA vector that was entirely synthesized. It carries an allelic form of *hisR* which encodes a histidine suppressor t-RNA which inserts the expected amino acid with a high efficiency (up to 100%) [36,43]. Indeed, the introduction of pFAR plasmids into the optimized *thyA* mutant allowed the selection of plasmid-containing bacteria, the restoration of a normal growth to thymidine auxotrophic strains and a high yield production of monomeric supercoiled expression vectors, as required for pharmaceutical products.

In comparison with other antibiotic-free plasmid selection systems, the strategy that we have developed presents several advantages. First, the *E. coli* producer strain was created by generating a point mutation into the *thyA* gene, precluding the occurrence of a leaky phenotype as observed when the expression of the essential gene is under the control of a not tightly regulated promoter [14,19]. Second, in contrast to pCOR plasmids, which are produced from an *E. coli argE* mutant grown in minimal medium lacking arginine or any source of proteins, pFARs can be easily and efficiently propagated

using a commercially available thymidine-free medium. Having demonstrated that pFARs are efficient eukaryotic expression vectors (see below), our next objective is to determine and optimize bacterial growth conditions for plasmid production in large-scale fermentation processes. Finally, the *de-novo* synthesis of pFAR4 allowed: (i) the removal of dispensable and potentially inflammatory prokaryotic DNA sequences; (ii) the reduction in plasmid size, promoting higher transfection efficiency [10]; and (iii) the insertion of a multiple cloning site for easy insertion of any eukaryotic expression cassette (Figure 3).

The efficiency of pFAR plasmids as both nonviral gene vehicles and *in vivo* eukaryotic expression vectors was assessed after electrotransfer of a luciferase-encoding pFAR4 derivative into muscle, skin and tumours. Luciferase activities measured in pFAR derivatives- or pVAX2-LUC-transfected muscles were similar until the production of antibodies directed against the exogenous firefly luciferase protein. The specific humoral immune response against the reporter protein and probable cytotoxicity against luciferase-expressing muscular fibres may account for the decrease in enzymatic activities. Similar observations have already been reported after injection and electrotransfer of other nonviral vectors [31].

Sustained luciferase expression observed in pFAR4-LUC-transfected skin cells was somehow unexpected. There exist a few differences between pVAX2-LUC and pFAR4-LUC: (i) pVAX2-LUC is approximately 0.9 kb larger (Figure 3) and (ii) contains 12 additional CpG motifs that are potential inflammatory hexanucleotides made of CG flanked by two purines and two pyrimidines [44,45]. Furthermore, whereas pVAX2-LUC harbours one GACGTT motif (Figure 3), which was reported to have the most immunostimulatory effect in mice [45,46]; the pFAR4 derivative carries none. The co-injection of pFAR4 with an immunostimulant CpG oligonucleotide (ODN 1668) [45,46] did not influence temporal luciferase gene expression in skin (G. Vandermeulen and V. Pr at, unpublished data). Attempts made aiming to identify the type of transfected skin cells using pFAR4 or pVAX2 derivatives expressing betagalactosidase did not provide an explanation for this observation. Nevertheless, this interesting property of pFAR vectors may allow the use of skin cells to secrete therapeutic proteins at several defined sites but at a low dose, which might be beneficial for some applications. For high expression levels, skeletal muscle might be a better target because it represents a long life span organ with a large volume of accessible tissue capable of producing local and systemic factors [47].

For the eradication of established tumours, the intratumoural electrotransfer of plasmids encoding cytokines such as interleukin (IL)-12 has been reported to be more efficient than intramuscular electrotransfer, probably as a result of a local increase in IL-12 and in interferon- γ -dependent antitumour factors and the infiltration of immune cells to the tumour site [48,49]. Furthermore, intratumoural plasmid injection eliminates the risk of

adverse side-effects associated with systemic IL-12 recombinant protein therapy. The data obtained in the present study, which show an increased transgene expression level after the electrotransfer of a pFAR4 derivative within established subcutaneous B16.F10 melanoma, suggest that the improvement of both physical methods and gene vectors could lead towards a more efficient cancer therapy.

Thus, pFAR plasmids appear to be efficient biosafe eukaryotic expression vectors that are suitable for the treatment of various diseases. Our next objectives are to assess this feasibility, to pursue the evaluation of transgene expression in other organs or tissues (e.g. liver) and to further develop pFAR4 plasmids for genetic immunization.

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