pFAR plasmids: New Eukaryotic Expression Vectors for Gene Therapy, devoid of Antibiotic Resistance Markers

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Running title: Development of biosafe plasmid vectors

Final Word count: 4,162

Summary

Efficient production of eukaryotic expression vectors requires the selection of plasmid-containing bacteria. To avoid the risk of dissemination of antibiotic resistance markers, we developed a new system to produce a family of **p**lasmids **F**ree of **A**ntibiotic **R**esistance genes, called pFARs. The strategy is based on the suppression of a chromosomal nonsense mutation by a plasmid-borne function. The amber mutation was introduced into the *Escherichia coli thyA* gene that encodes a thymidylate synthase required for dTMP synthesis, resulting in thymidine auxotrophy. In parallel, a small plasmid vector that carries an amber suppressor t-RNA gene was entirely synthesised. The introduction of pFAR plasmids into an optimised *thyA* mutant restored normal growth to the auxotrophic strain, and led to an efficient production of monomeric supercoiled plasmids, as required for clinical trials. Luciferase activities measured after intramuscular injection and electrotransfer of LUC-encoding pFAR vector were similar to those obtained with a commercial vector containing the same expression cassette. Interestingly, whereas luciferase activities decreased within three weeks after intradermal electrotransfer of conventional expression vectors, sustained levels were observed with the pFAR derivative. Thus, pFAR plasmids represent a novel family of biosafe eukaryotic expression vectors, suitable for gene therapy.

Keywords: Biosafety, plasmid vector, DNA delivery, Gene Therapy, Electrotransfer

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 to viral components, possible insertional mutagenesis into host genome and high production costs.[1-4] These drawbacks can be avoided by using easily produced non-viral expression vectors that remain in episomal state inside eukaryotic cells.[5,6] Furthermore, the development of physical methods (e.g: hydrodynamic delivery or electrotranfer) used to administer plasmids *in vivo*, allowed a significant increase in protein expression levels[7,8] and reconsidering non-viral expression vectors as therapeutic gene vehicles.

Non-viral expression vectors consist of three main classes: plasmidic DNA, MIDGE vectors,[9] and minicircles.[10-12] The two last ones 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 seem to promote heterochromatin formation, leading to gene silencing in certain organs such as liver.[13] In muscles, however, prokaryotic sequences do not appear to impair prolonged gene expression,[5] thus making dispensable laborious preparations of MIDGE or minicircle vectors. The third group of non-viral vectors is represented by plasmidic DNA that are 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 aminoside class (such as kanamycin which is not normally used in human health) have been approved for some clinical applications, several

alternatives have been envisioned to avoid them for safety concerns. All strategies are based on the same following principle: growth of bacterial conditional mutants relies upon the presence of plasmids. Plasmidic expression vectors can harbour either (i) several copies of the *lacZ* 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 complete traduction of a protein playing a role in the arginin biosynthetic pathway[16,17] (iii) a replication origin encoding an RNA molecule involved in the regulation of an essential *E. coli* gene via an RNA/RNA interference mechanism.[18,19]

In this paper, we describe a novel combination *E. coli* producer strain / plasmids **F**ree of **A**ntibiotic **R**esistance markers, referred as pFARs. On one side, 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 optimised for the production of a family of new and 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 and skin, followed by electrotransfer. This physical method consists of applying electric field-mediated intracellular delivery via a mechanism mostly composed of cell permeabilisation and DNA uptake through electrophoresis, allowing an increase in protein expression level by a two-log factor in both organs.[8,20-22]

Results

Description of a novel combination of an 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 (dUMP). Mutation in *thyA* leads to thymidine auxotrophy[23] 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 thimidine kinase (EC 27.1.75). In the absence of thymidine, only the strains that contain a plasmid encoding a suppressor t-RNA enabling full traduction of ThyA can grow (Fig. 1).

An amber mutation, which substitutes the CAT encoding His147 to TAG, was introduced into the cloned *thyA* region and subsequently inserted into the non-pathogenic *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[24] 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.[25] 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.[26] 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,[27] 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, **F**ree of **A**ntibiotic **R**esistance marker, was called pFAR1. To validate the proposed strategy, pFAR1 was introduced into the *thyA* mutant by transformation. Whereas no bacteria electroporated with water appeared on selective medium (MH), pFAR-transformed cells grew well even in the absence of thymidine, indicating that the suppression of the nonsense mutation could sustain bacterial growth. Plasmid DNA were prepared from several transformants grown overnight in selective medium. They all appeared to contain pFAR1, with the expected size and restriction profiles. All these data showed that this novel combination of *E. coli thyA* mutant / pFAR plasmid can be used to produce plasmids devoid of antibiotic resistance markers and was therefore further optimised.

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

The use of plasmid DNA for clinical trials requires that they meet some defined criteria such as purity and topology.[28,29] As 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 therefore to be further optimised. 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.[30] To overcome this, an internal region of *endA* (coding amino acids 7-234 of the 234 AA-long EndA protein) was deleted from the *thyA* mutant chromosome, to give a double *thyA endA* mutant.

During plasmid propagation and replication, intermolecular recombination between homologous sequences can occur, leading to multimers formation and increase in plasmid

size. Plasmid copy number being controlled by the replication origin concentration, recombination events will have an influence on the monomers/multimers ratio, resulting in inconsistency from one plasmid batch to another, which is not suitable for pharmaceutical applications.[28,29] To avoid multimers formation, the internal region of *recA* that encodes a recombinase (353 amino-acids) was deleted (AA: 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 optimised the bacterial producer strain, our next objective was to further develop the plasmid vector. To minimize bacterial sequences and plasmid size, a new DNA vector was entirely synthesised and called pFAR4 (Fig. 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 optimised *thyA* mutant containing a pFAR4 derivative was similar to that of the strain DH5α harbouring pVAX2-LUC propagated in the presence of kanamycin (Fig. 2b). Furthermore, at a similar optical density (OD600~0.9) 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 DH5α grown in LB medium supplemented with kanamycin (Fig. 2c). Thus, bacterial and plasmid optimisation 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 pFAR4 (Fig. 3). The reporter gene is under the control of the ubiquitous cytomegalovirus (CMV) promoter and the bovine growth hormone (BGH) polyadenylation signal. Plasmids pVAX2-LUC, pFAR1-LUC and pFAR4-CMV LUC BGH, 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, luciferin, using a charged-coupled device (CCD) camera that allows monitoring gene expression in living animals (Fig. 4). Until day 15, luciferase activities recorded after injection of either plasmids were similar, indicating that pFAR1 or the newly synthesised vector pFAR4 do not exert adverse effects after muscle transfection. At later time points, however, luciferase activities started decreasing in some mice. When sera were collected by retro orbital puncture, they appeared to contain antibodies directed against the exogenous luciferase protein. In the mouse showing a great decrease in luciferase activity, the amount of anti-LUC antibodies was of 14.5 µg/ml 30 days after plasmid injection, and reached 70 µg/ml at day 60.

To assess luciferase expression in another tissue, pVAX2-LUC and pFAR4-CMV LUC BGH were then injected into mouse skin and electrotransferred. Figure 5 shows that luciferase activities in pVAX2.LUC-transfected cells decreased within a few weeks time. In marked contrast, the injection of the pFAR derivative led to sustained luciferase expression levels. In another experiment, a prolonged expression in skin cells was observed for more than three months (data not shown).

Discussion

Production of recombinant therapeutic proteins or of plasmids most often implies growing bacteria in the presence of antibiotics to ascertain vector maintenance and propagation in prokaryotic cells. To avoid the risk of dissemination of antibiotic resistance markers to mammalian-colonizing endogenous flora and/or putative anaphylactic chocks due to the presence of these compounds in plasmid preparations, we developed a new system to produce **p**lasmids **F**ree of **A**ntibiotic **R**esistance genes referred as pFARs. The strategy 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[26,27,31] and (ii) the distribution of translation termination codons is as follows: 7.6% for UAG, 63% for UAA and 29.4% for UGA.[32] 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 (~30%) of wild-type *E. coli* isolates naturally contain amber suppressor t-RNA genes, with no apparent selective disadvantage.[33,34] Furthermore, all amber mutations are not suppressed with an equal efficiency. Several factors seem to play a role, such as the nucleotides surrounding the nonsense codon being suppressed[26] as well as the selected suppressor t-RNA.[26,27,31] Taking into account all of these parameters and work reported by Michiaels et al[35] on the insertion of nonsense codon in the thymidylate synthase coding region, we chose to introduce the amber mutation in the *thyA* nucleotide sequence encoding histidine 147, which lies in the substrates binding pocket of the enzyme.[35] 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 synthesised. It carries an allelic form of *hisR*, the expression of which is under the control of prokaryotic regulatory elements, and encoding a histidine suppressor t-RNA that allows the insertion of the expected amino acid with a high efficiency (up to 100%).[26,35] Indeed, the introduction of pFAR plasmids into the optimized *thyA* mutant allowed the selection of plasmid-containing bacteria, restoration of a normal growth to thymidine auxotrophic strains and high yield production of monomeric supercoiled expression vectors, as required for clinical trials.

To assess the efficiency of pFAR plasmids as both non-viral gene vehicles and *in vivo* eukaryotic expression vectors, a luciferase-encoding pFAR derivative was injected and electrotransferred into mouse muscle and skin. 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 activity. Similar observations have already been reported after injection and electrotransfer of other non-viral vectors.[36]

Sustained luciferase expression observed in pFAR derivative-transfected skin cells was somehow unexpected as, it had, to our knowledge, never been observed before.[37] There exist a few differences between pVAX2-LUC and pFAR4-CMV-LUC-Bgh: (i) pVAX2-LUC is ~0.9-kb bigger (Fig. 3) and (ii) it contains 12 additional CpG motifs that are potential inflammatory hexanucleotides made of CG flanked by two purines and two pyrimidines.[38,39] Furthermore, whereas pVAX2-LUC harbours one GACGTT motif (Fig. 3), which was reported to have the most immunostimulatory effect in mice,[39,40] the pFAR4 derivative carries none. Co-injection of pFAR4 with an immunostimulant CpG oligonucleotide (ODN 1668)[39,40] did not influence temporal luciferase gene expression in skin (our unpublished data). Further

analyse will be performed to determine the factors playing a role in the sustained gene expression level as well as the type of transfected skin cells. Anyhow, this interesting property of pFAR vectors may allow using 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 as it represents a long life span organ with a large volume of accessible tissue capable of producing local and systemic factors.[41]

Thus, pFAR plasmids appear to be efficient eukaryotic expression vectors suitable for the delivery of therapeutic genes and the treatment of a large range of human diseases. Our next objective is to assess this feasibility.

Materials and methods

Microbiological and Molecular Biology manipulations

Escherichia coli strains were grown in either complex (Luria-Bertani[42] or Mueller Hinton[43] (Sigma-Aldrich, Lyon, France)) or minimal (M9)[42] media supplemented with either antibiotics[42] or thymidine (30 µg/ml). The DH5α strain[44] was used for routine cloning procedures that were carried out as described in Sambrook *et al.*[42]

E. coli mutants were isolated from strain MG1655 obtained from "The Coli Genetic Stock Center" (USA) (http://cgsc.biology.yale.edu) since, unlike most laboratory strains, it does contain suppressor t-RNA alleles (Accession number #U00096).[32]

Construction of E. coli mutants

E. coli mutants were isolated essentially as described by Posfai *et al.*[45] 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 PCR using the ExTaq polymerase (Takara, Lonza, Verviers, Belgium), MG1655 genomic DNA and primers (ThyA-F: CATGCGGTATTGCGCAGGC and ThyA-R: CGCTGTATCTGTTCCGTGTCT). 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

(CGCTGGCACCGTGCTAGGCATTCTTCCAGTTC) and ThyA-His147-R (GAACTGGAAGAATGCCTAGCACGGTGCCAGCG) as primers. The mutated *thyA* region was then entirely sequenced to ascertain that the insert only contained the amber mutation. Plasmids PCR-thyA* and pST76-C were ligated after digestion by *Eco*RV and *Sma*I, respectively. Finally, the pUC replication origin from pCR2.1 was deleted by digesting the fusion plasmid by *Bam*HI. The mutated *thyA* region was then introduced into the MG1655 genome as described above, selecting for thymidine auxotrophic strains. 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, Lonza, Verviers, Belgium) and two pairs of primers (GTCGGTTTTGCCATGAGTGC and ta*cccggg*TAGACAAATAACGGTACATCAC) and (tt*cccggg*AGAGCTAACCTACACTAGCG and CCACTCTTCGTAGTTCTGCT), respectively. The PCR fragments were entirely sequenced after cloning into PCR2.1 (Invitrogen, Illkirch, France). The 1-kb *Sma*I-*Xba*I fragment from PCR2.1-yggI was ligated to PCR2.1-rsmE digested with *Sma*I-*Spe*I, to give PCR2.1 delta endA. This latter plasmid (digested with *Apa*I and Klenow-treated) was fused to pST76-C (digested with *Sma*I). A *Bam*HI 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, Lonza, Verviers, Belgium) and two pairs of

primers (GTAATGGCAAACGGTCAGGC and gat*cccggg*GTCGATAGCCATTTTTACTCC) and (cat*cccggg*GAAGGCGTAGCAGAAACTAAC and GTCGCCGAAGCTGAAGTTG), 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 X*ba*I (Klenow-treated) and *Sma*I, respectively. The deleted *recA* region was introduced into the *thyA endA* mutant as described above.

Construction and preparation of pFAR plasmids

Plasmids that are Free of Antibiotic Resistance markers are called pFARs. pFAR1 is a derivative of pVAX2[25] that was obtained by replacing the cytomegalovirus promoter of pVAX1 (Invitrogen, Illkirch, France) by that of pCMV-β (Clontech, Ozyme, Saint-Quentin-en-Yvelines, France). The Histidine t-RNA suppressor gene was amplified from pGFIB-1.His(AS)[31] by PCR using primers (gt*ccatgg*CTGGCGCCGCTTCTTTGAGC and cc*ccatgg*ACGACGGCCAGTGCCAAG). The PCR fragment was digested with *Nco*I and inserted into pVAX2 digested with *Bsp*HI. The digestion of the resulting plasmid by *Hin*dIII (Klenow-treated) and *Pvu*II, followed by a self-ligation, allowed the deletion of the kanamycin resistance gene, to give pFAR1.

To construct pFAR1-LUC, the 1.7-kb *Bam*HI-*Xho*I fragment containing the firefly cytoplasmic luciferase-encoding gene was isolated from pVAX2-LUC[25] and introduced into pFAR1 digested with the same enzymes. To optimise the expression vector, a new plasmid, called pFAR4, was entirely synthesised (Geneart, Regensburg, Germany). The ~1.7 kb *Mlu*I-*Pvu*II fragment containing the expression cassette CMV-LUC-BGH cleaved from pVAX2-LUC was introduced into pFAR4 digested with *Mlu*I and *Eco*RV, to give pFAR4-CMV LUC BGH.

For *in vivo* studies, plasmids were purified using Endofree preparation kits (Qiagen, Hilden, Germany). Endotoxin levels were determined by Lonza (Verviers, Belgium), using the LAL 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 visualisation on Ethidium Bromide-stained 1% agarose gels. Light absorption at 260 nm was used to determine DNA concentration.

in vivo studies

6-8 weeks old female BALB/c mice (Charles River, L'Arbresle, France) and 6 weeks old female NMRI mice (Université catholique de Louvain, Brussels, Belgium) were used for plasmid intramuscular and intradermal injection, respectively. Prior to all procedures, the animals were anaesthetised by intraperitoneal injection of ketamine and xylazine as described by Bloquel *et al.*[36] and Vandermeulen *et al.*[20]. Studies were conducted following 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 diluted in 30 µl of physiological serum were injected into BALB/c mouse tibial cranial muscles and electrotransferred, as described in Bloquel *et al.*,[36] 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, 2Hz). For intradermal electrotransfer, the abdomen skin was shaved 1 day prior to the experiments with a depilatory cream (Veet for sensitive skin, Belgium). Plasmid DNA (in a volume of 15 µl) was 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).[20] A short High

Voltage pulse (700 V/cm 100 µs) immediately followed by a Low Voltage pulse (200 V/cm 400 ms) were applied approximately one minute after plasmid injection[37], using a Cliniporator system (Cliniporator, IGEA, Carpi, Italy). For all experiments, conductive gel was used to ensure electrical contact with the skin (EKO-GEL, ultrasound transmission gel, Egna, Italy).

Luciferase activities were recorded by using charged-coupled device (CCD) cameras that allow *in vivo* studies in living animals. Following intramuscular plasmid electrotransfer, 150 µl 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, luciferin diluted in PBS without Mg^{2+} and Ca^{2+} (Xenogen corporation, Alameda, CA, USA) was injected intraperitoneally (i.p., 3 mg/100 µl). Optical imaging was acquired using an IVIS50 system (Xenogen corporation, Alameda, CA, USA). The duration of luminescence acquisition was between 10 s and 60 s and was initiated 10 minutes after injection of the substrate. In both 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 non-transfected zone of the mice.[36]

Titration of anti-LUC antibodies

Antibodies against luciferase in mouse serum were titrated by ELISA, essentially as described by Bloquel *et al,*[36] using luciferase (E1702, Promega, Madison WI, USA) to coat the microplates and a horseradish peroxidase-conjugated goat anti-mouse secondary antibody (NXA931, GE Healthcare, Uppsala, Sweden). To establish a standard curve, a serial dilution of a monoclonal antibody anti-luciferase (L2164, Sigma-Aldrich, Lyon, France) was used.

Acknowledgements

This work was supported by the European Commision under the MOLEDA STREP grant of the Sixth Framework Programme [Grant number 512034] and the "Fonds de la Recherche Scientifique Médicale" (Belgium). GV is a "Fonds National de la Recherche Scientifique" (Belgium) research fellow.

Dr. György Pósfai (Biological Research Center, Szeged, Hungary) is deeply acknowledged for providing us with pST76-C and pST76-AsceP plasmids. We also thank Magdalena Ibañez-Riuz who suggested to us to mutagenise the *thyA* gene. C.M. is grateful to Michel Francis Bureau (CNRS-UMR8151, Paris, France) for stimulating discussions.

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Titles and legends to figures

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 polymerisation. 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.

Figure 2: Properties of pFAR plasmids. Fig. 2a. **Topology of pFAR plasmids prepared from the *thyA* mutants.** Native plasmid DNA (pFAR4-CMV LUC BGH: 3.7 kb) was loaded onto a 1.0% agarose gel and stained with ethidium bromide after electrophoresis. The pFAR 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 multimerisation was determined by comparison with the following markers: Sc: Supercoiled DNA ladder (Invitrogen, Illkirch, France ; bands correspond to supercoiled DNA with the following size: 2, 3, 4, 5, 6, 7, 8, 10, 12, 14 and 16 kb) and L: Smart Ladder (Eurogentec, Seraing, Belgium ; bands correspond to linearised DNA fragments of 0.6, 0.8, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8 and 10 kb). **Fig. 2b. Introduction of pFAR plasmids into the optimised *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 minutes. The introduction of pFAR 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 DH5α-pVAX2-LUC propagated in MH supplemented with kanamycin is also indicated (circles). Data points are the means of duplicate experiments. **Fig. 2c. Production of pFAR plasmids.** pFAR derivatives prepared from the optimised *thyA* mutant grown in MH medium are produced with a yield similar to that of pVAX2-LUC propagated in DH5α cultivated in LB medium supplemented with kanamycin. Both plasmids were prepared from strains having a similar OD_{600} (~ 0.9) using plasmid Nucleospin® columns (Macherey Nagel, Hoerd, France). Ten µl of undigested eluted DNA were loaded onto a 1% agarose gel, which was stained with ethidium bromide.

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 ~0.9 kb smaller than pVAX2-LUC, has a reduced CpG content and lacks the motif GACGTT (*) thought to mediate inflammatory responses in mice[39,40].

Figure 4. Luciferase activities in mouse tibial cranial muscles. 5 µg of pVAX2-LUC and an equimolar amount of luciferase expression cassette harboured by pFAR1-LUC and pFAR4-CMV LUC BGH diluted in 30 µl of physiological serum were injected into each tibial cranial muscle of 4 Balbc mice and subsequently electrotransferred. 3, 8, 15, 30 and 60 days later, luciferin was injected intraperitoneally and luciferase activities were recorded in living animals using a CDD camera. Bars represent the mean values (± SEM). Crosses indicate individual values measured in each muscle. A dramatic decrease in luciferase activity (arrows) was observed in both muscles of a mouse, which appeared to produce high levels of anti-luciferase antibodies.

Figure 5. Luciferase expression after intradermal injection and electrotransfer of pVAX2-LUC and pFAR4 – CMV LUC BGH. 50 µg of pVAX2-LUC and an equimolar amount of luciferase expression cassette carried by pFAR4 CMV LUC BGH were injected and electrotransferred into the skin of 7 or 8 mice. Luciferin was injected intraperitoneally, 1, 3, 6, 9, 14 or 21 days later and luciferase activities were monitored using a CDD camera. Bars represent the mean values (± SEM).

Figure 1.

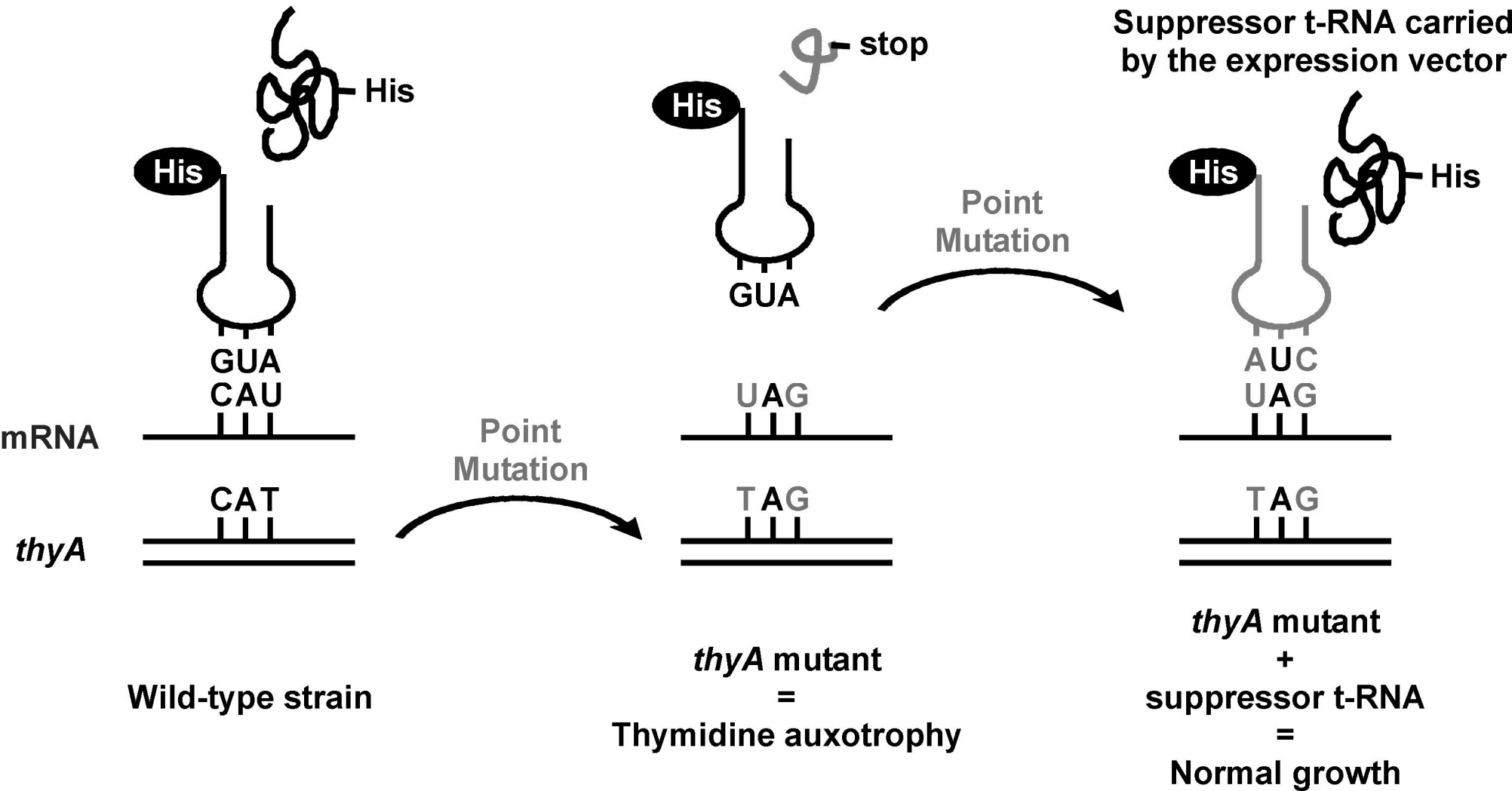
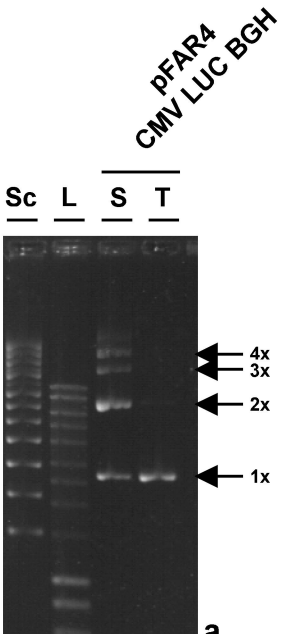
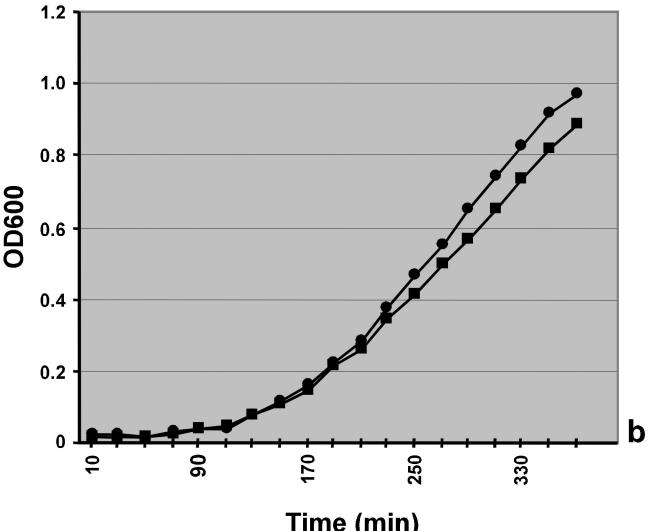

His
His
GUA
CAU
mRNA
CAT
thyA
Point
Mutation
stop
His
GUA
UAG
TAG
Point
Mutation
Suppressor t-RNA carried
by the expression vector
His
His
AUC
UAG
TAG
Wild-type strain
thyA mutant
=
Thymidine auxotrophy
thyA mutant
+
suppressor t-RNA
=
Normal growth

Figure 2.


pFAR4
CMV LUC BGH
Sc
L
S
T
4x
3x
2x
1x
a


1.2
1.0
0.8
0.6
0.4
0.2
0
OD600
10
90
170
250
330
Time (min)
b

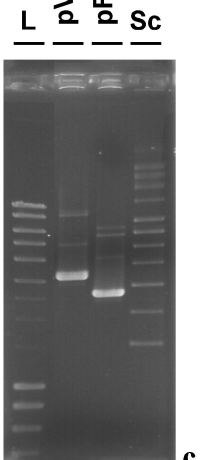
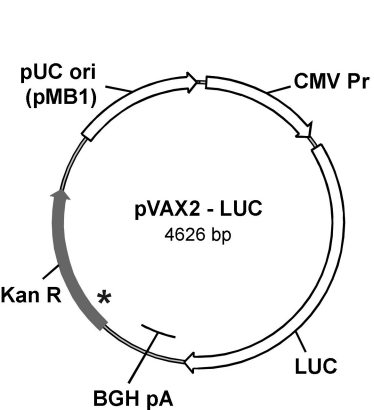
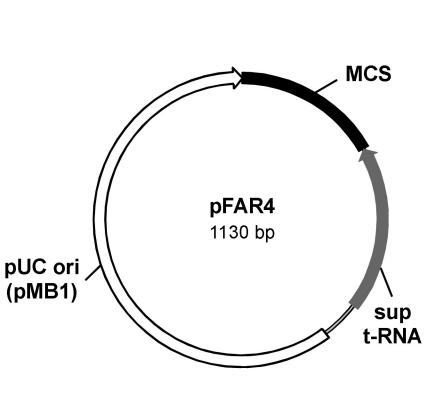

L
pVAX2-LUC
pFAR4 – CMV LUC BGH
Sc
c

Figure 3.


pUC ori
(pMB1)
CMV Pr
pVAX2 - LUC
4626 bp
Kan R
*
LUC
BGH pA


MCS
pFAR4
1130 bp
pUC ori
(pMB1)
sup
t-RNA

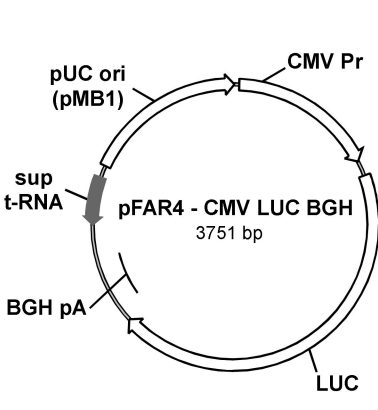

pUC ori
(pMB1)
CMV Pr
sup
t-RNA
pFAR4 - CMV LUC BGH
3751 bp
BGH pA
LUC

Figure 4.

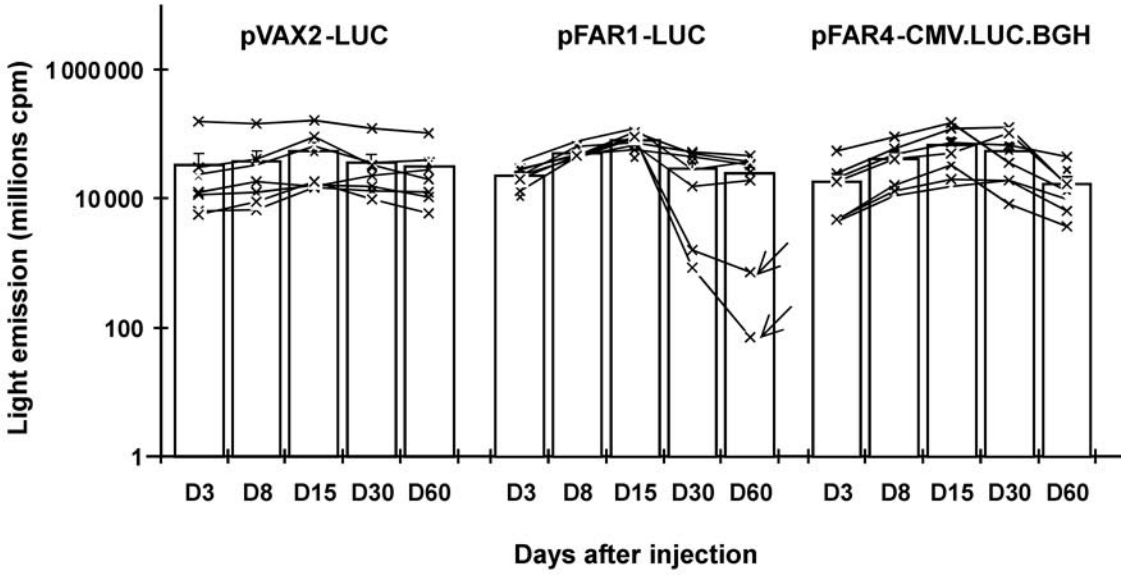
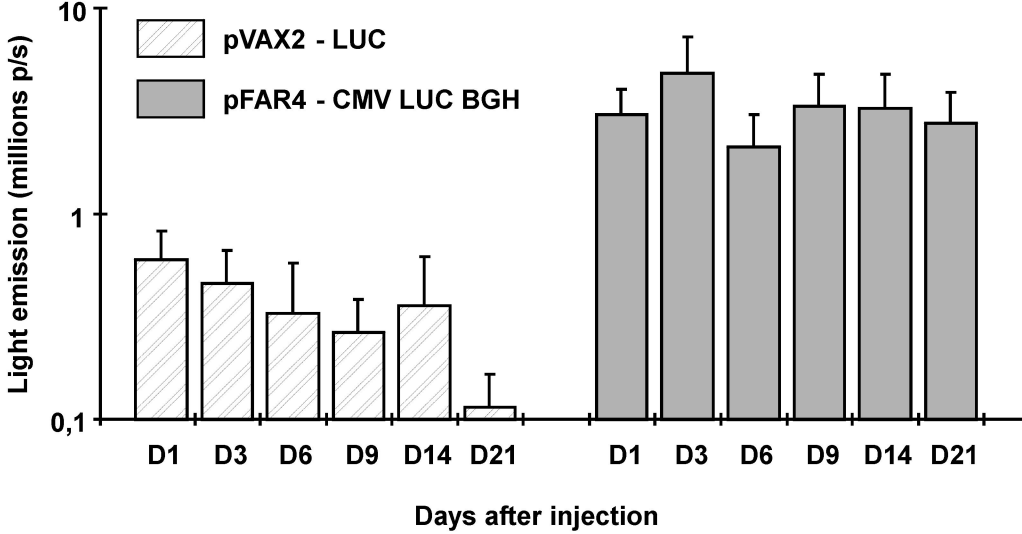

pVAX2-LUC
pFAR1-LUC
pFAR4-CMV.LUC.BGH
Light emission (millions cpm)
1 000 000
10 000
100
1
D3 D8 D15 D30 D60
D3 D8 D15 D30 D60
D3 D8 D15 D30 D60
Days after injection

Figure 5.


10
1
0,1
Light emission (millions p/s)
pVAX2 - LUC
pFAR4 - CMV LUC BGH
D1
D3
D6
D9
D14
D21
D1
D3
D6
D9
D14
D21
Days after injection