Sustained Gene Expression in the Retina by Improved Episomal Vectors

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Gene and cellular therapies are nowadays part of therapeutic strategies for the treatment of diverse pathologies. The drawbacks associated with gene therapy—low levels of transgene expression, vector loss during mitosis, and gene silencing—need to be addressed. The pEPI-1 and pEPito family of vectors was developed to overcome these limitations. It contains a scaffold/matrix attachment region, which anchors its replication to cell division in eukaryotic cells while in an extrachromosomal state and is less prone to silencing, due to a lower number of CpG motifs. Recent success showed that ocular gene therapy is an important tool for the treatment of several diseases, pending the overcome of the aforementioned limitations. To achieve sustained gene delivery in the retina, we evaluated several vectors based on pEPito and pEPI-1 for their ability to sustain transgene expression in retinal cells. These vectors stably transfected and replicated in retinal pigment epithelial (RPE) cells. Expression levels were promoter dependent with constitutive promoters cytomegalovirus immediate early promoter (CMV) and human CMV enhancer/human elongation factor 1 alpha promoter yielding the highest levels of transgene expression compared with the retina-specific RPE65 promoter. When injected in C57Bl6 mice, transgene expression was sustained for at least 32 days. Furthermore, the retina-specific RPE65 promoter showed higher efficiency *in vivo* compared to *in vitro*. In this study, we demonstrate that by combining tissue-specific promoters with a mitotic stable system, less susceptible to epigenetic silencing such as pEPito-based plasmids, we can achieve prolonged gene expression and a sustained therapeutic effect.

Introduction

OVER THE LAST DECADES, gene therapy has been a driving force for the development of strategies to treat genetic and acquired diseases. The major disadvantages of DNA-based gene therapy are the epigenetic silencing due to unmethylated CpG motifs present in the bacterial backbone of plasmids grown in bacteria[1] and vector loss during mitosis, due to their incapacity to integrate the genome and replicate in the host cell.[2–4] The use of plasmids with the capacity to replicate in mammalian cells, less frequent silencing events, and species/tissue-specific promoters could lead to sustained gene expression.[4] To address these issues, Piechaczek *et al.* developed a new vector, named pEPI-1, which contains a scaffold/matrix attachment region (S/MAR), derived from the human interferon β-gene.[5] Other authors have shown the involvement of the S/MARs in DNA duplex destabilization and strand opening, suggesting these sequences to be involved in DNA replication and gene

expression.[6,7] Additionally, it was shown that the S/MAR-containing vectors prevent epigenetic silencing of gene expression by shielding the transgene sequence from adjacent regulatory sequences and heterochromatinization,[8] maintaining the vector in a transcriptionally active state.[9] The original pEPI-1 vector contains two mammalian transcription units and a total of 305 CpG motifs, most of them located in the elements of the vector required for bacterial propagation. To reduce the CpG content in the bacterial backbone of pEPI-1 and obtain increased transgene expression *in vitro* and *in vivo*, a new nonviral vector, pEPito, was created by Haase *et al.*[10] pEPito was constructed by cloning the pEPI-1 plasmid replicon in a plasmid backbone containing 60% less CpG motifs and excluding the second transcription unit. A comparative study between pEPito and pEPI-1 constructs showed pEPito-based vectors to be more efficient, both *in vitro* and *in vivo*,[10] explained by CpG depletion of the bacterial backbone, which exhibits a strong influence on epigenetic silencing events.[1,10–12]

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The eye is an attractive target for gene therapy because it is an accessible and immune-privileged organ.[13,14] Moreover, due to its small size, only a small amount of a therapeutic agent is necessary to observe a therapeutic effect.[14] Diseases like glaucoma, retinitis pigmentosa, and age-related macular degeneration are blinding disorders that contribute to more than 25% of blindness cases, worldwide.[15] The ineffectiveness of current treatments makes these diseases ideal targets for gene therapy.[14,15] Moreover, in most cases, its genetic etiology is well known and there is easy access to the photoreceptors or retinal pigment epithelial/epithelium (RPE) by localized injection.[16] Studies with viral vectors, based on adeno-associated viruses (AAVs), have as a major limitation, the small size of the therapeutic gene that can be incorporated into the viral vector.[17] Therefore, our goal is to combine a nonviral vector with an efficient gene expression system both in duration and expression levels, to overcome the need of repeated injections, thus increasing patient compliance.

In this study, pEPI-1- and pEPito-based vectors were evaluated for their ability of sustained transgene expression both *in vitro*, in RPE cells and *in vivo*, in the mouse retina. We found pEPito to be an excellent gene expression system, adequate for sustained gene expression.

Materials and Methods

pEPI-1- and pEPito-based vectors

Table 1 illustrates the five different plasmids tested in this study, which are based on pEPI-1 and pEPito vectors.

The pEPI-1 vector contains an S/MAR sequence, derived from the human interferon β-gene, two mammalian transcription units, and a cytomegalovirus immediate early promoter (CMV) constitutive promoter. The derivative pEPito vectors contain an S/MAR sequence and four different promoters: CMV promoter, human CMV enhancer/human elongation factor 1 alpha promoter (hCMV), human CMV enhancer/RPE65 (hCMV/RPE65), and RPE65, an RPE tissue-specific promoter.[18] pEPito vectors also contain an eGFP-BSD cassette, in which an enhanced green fluorescent protein (eGFP) and blasticidin (BSD) are expressed through an internal ribosomal entry site. In addition to the promoter, the backbones of the vectors vary in the CpG content and size (between 5.2 and 7.8 kb).

Chemically competent *Escherichia coli* GT115 bacteria (Invivogen) were transformed using 30 ng of each plasmid. Transformed bacteria were selected on LB plates containing either ampicillin (Sigma) or kanamycin (Sigma). After bacterial propagation, pDNA was isolated using the QIAGEN® Plasmid Maxi Kit (Qiagen), according to the manufacturer's instruc-

tions. The restriction map of the plasmids was confirmed using endonuclease digestion and subsequent gel electrophoresis.

Transfection efficiency assay

In this study, the transfection efficiency of the plasmids was evaluated in a human RPE cell line, D407. This cell line was derived from an eyeball of a 12-year-old child.[19] These cells have been extensively used as a model of the retina and retinal pigmented epithelium.[20,21]

D407 cells were cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 5% fetal bovine serum (PAA), 1% of L-glutamine (Sigma), and 1% penicillin/streptomycin (Sigma).

For the transfection assay, 2×10^5 cells were seeded in a six-well tissue culture plate (Orange Scientific). Twenty-four hours after plating, the cells were transfected with 1 μg of DNA and FuGENE® HD (Promega) as the transfection reagent using a 3:1 (μL of FuGENE HD:μg of DNA) ratio, according to the manufacturer's instructions.

Forty-eight hours after transfection, cells were suspended, washed three times with phosphate-buffered saline (PBS), and GFP expression analyzed using a FACSCalibur (Becton Dickinson) device and JDS Uniphase® laser, with 100,000 events recorded.

Controls included nontransfected cells cultured in the same conditions.

Colony-forming assay

Forty-eight hours post-transfection, D407 cells were transferred from the six-well tissue culture plate to 10-cm Petri dishes (BD Biosciences) and selected in the presence of blasticidin (Sigma). Due to conflicting reports in the literature regarding the blasticidin concentration for selection of stably transfected cells,[22,23] we have determined the adequate blasticidin concentration to select D407 transformed cells by an MTT assay, where six different concentrations of blasticidin were tested—0.01, 0.1, 1, 3, 5, and 10 μg/mL. The selected concentration of blasticidin was 1 μg/mL (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/tea).

After 32 days of selection with blasticidin, three colonies of each plasmid were extracted and maintained in culture for 2 additional months under selection (up to 3 months in total), with eGFP-positive cells quantified by flow cytometry as described in the previous section.

The remaining colonies were fixed with 4% paraformaldehyde (PFA; Sigma) in PBS, stained with 2% methylene blue in methanol (VWR), and counted.

TABLE 1. pEPI AND pEPito VECTORS USED IN THIS STUDY

Plasmid	Size (kb)	Backbone	Promoter	Transgene	CpG motifs (backbone)
pEPI-1	6.7	pEPI	CMV	eGFP	206
pEPito-CMV-eGFP	5.3	pEPito	CMV	BSD-eGFP	37
pEPito-hCMV-eGFP	5.2	pEPito	hCMV	BSD-eGFP	37
pEPito-RPE65-eGFP	7.5	pEPito	RPE65	BSD-eGFP	37
pEPito-hCMV/RPE65-eGFP	7.8	pEPito	hCMV/RPE65	BSD-eGFP	37

BSD, blasticidin; CMV, cytomegalovirus immediate early promoter; hCMV, human CMV enhancer/human elongation factor 1 alpha promoter; eGFP, enhanced green fluorescent protein; RPE, retinal pigment epithelial.

Injection of pEPito vectors in C57Bl6 mice and eGFP expression

To test the *in vivo* efficiency of the pEPito-derived vectors, FuGENE HD-plasmid complexes were injected into the eye of C57Bl6 mice pups. All methods involving animals were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Litters and nursing dams were housed in plastic cages in a pathogen-free environment, with continuous access to food and water on a 12-h light–12-h dark schedule.

The DNA-FuGENE complex used *in vivo* was prepared by mixing 2 μg of plasmid DNA and 1 μL of FuGENE HD in a total volume of 20 μL of PBS. Pups at postnatal age of 5 days (P5) were anesthetized on ice. Using a needle, the sclera was punched in the temporal side and 1 μL of the DNA-FuGENE complex was injected into the vitreous cavity. After the injection, a topical ointment consisting of gentamicin and prednisolone acetate was applied to reduce the pain and the risk of infection. The contralateral noninjected eye was used as control.

At 3, 7, 14, 21, and 32 days postinjection (dpi), eight mice per plasmid DNA were humanely sacrificed, the eyes enucleated, and fixed in ice-cold 4% PFA in PBS, for 24 h. This was followed by an overnight immersion in 30% sucrose (Sigma) in a PBS solution and embedded in OCT (Tissue-Tek) for cryosection. Ten-micrometer-thick serial sections were performed, counterstained with DAPI and mounted with Fluoromount G (Electron Microscopy Sciences).

In vivo transfection was evaluated by eGFP expression using an AxioImager Z2 Fluorescence microscope (Zeiss), with AxioCam HRm and magnifications of $50 \times$ and $200 \times$. The transfected area was quantified using ImageJ software, by quantifying both the GFP-positive and the ganglion cell layer (GCL) areas, and calculating the percentage of GFP-positive area relative to the GCL area.

Statistical analyses

Statistical analyses were performed using the SPSS Software, version 19.0.

One-way ANOVA was used to compare the *in vitro* transfection efficiency of the different plasmids and the *in vitro* colony-forming efficiency of stably selected D407 cells. In the event of a significant ANOVA, the Tukey test was used for *post hoc* analysis.

Data are expressed as mean ± standard deviation and $*p < 0.05$.

Results

Transfection efficiency of pEPI-1 and pEPito in RPE cells is promoter dependent

In vitro experiments were performed in D407 human RPE cells, and transfection efficiency was analyzed by flow cytometry for eGFP-positive cells, 48 h post-transfection.

Our results showed that plasmids pEPito-CMV, pEPito-hCMV, and pEPI-1, containing either the CMV or hCMV constitutive promoters, presented transfection efficiencies in the range of 30% (Fig. 1). The plasmid containing the hCMV enhancer element and the RPE65 promoter (pEPito-hCMV/RPE65) had the lowest transfection efficiency (6%), compared with the plasmids containing either the CMV or hCMV promoters (Fig. 1). No eGFP-positive cells were detected in
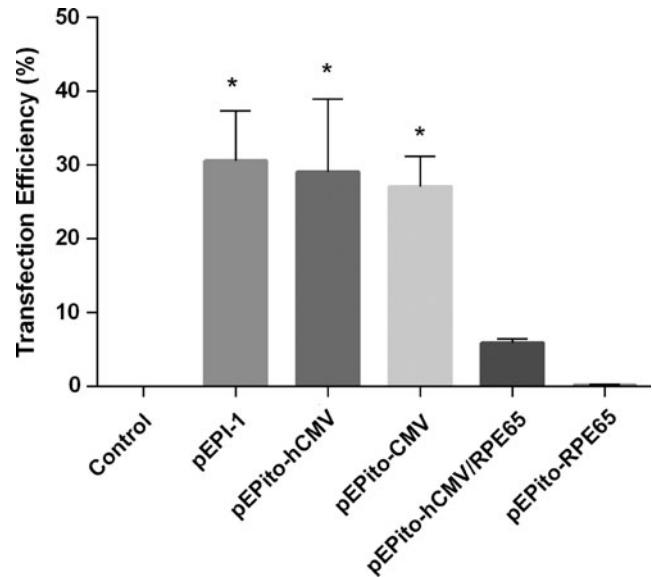

FIG. 1. Transfection efficiencies for transiently transfected D407 cells with the five different plasmids. Mean values are derived from four independent experiments and statistical significance was determined with one-way ANOVA followed by a *post hoc* Tukey Test. The statistical difference is indicated by a star (*) symbol ($*p < 0.05$).

conditions transfected with pEPito-RPE65 (Fig. 1), and for this reason, this vector was not further tested *in vitro*.

pEPito plasmids express eGFP for 3 months in mitotically active RPE cells

As previously stated, one of the major advantages of the S/MAR-containing plasmids is their ability to be maintained in an episomal state and to be replicated during mitosis.[9] This eliminates one of the major drawbacks of plasmid gene therapy—the vector dilution effect—occurring during cell division. To test the capacity of these plasmids to replicate during cell division and to form a stable transfected cell colony, D407 cells were transfected with pEPito-CMV, pEPito-hCMV, and pEPito-hCMV/RPE containing the blasticidin resistance gene (BSD), for mammalian cell selection. Our assay to determine the lowest blasticidin concentration to select pEPito-modified cells showed this value to be 1 μg/mL (Supplementary Fig. S1). This concentration was used in all the selection experiments.

All tested plasmids were able to originate stably transfected colonies of D407 cells (Fig. 2).

We found that the plasmids containing either hCMV or CMV promoter were more efficient establishing stable colonies than the plasmid containing the cell-specific promoter (Fig. 3). The plasmid that originated a higher number of colonies after 32 days of selection was pEPito-hCMV, with an average of 510 colonies (Fig. 3). pEPito-CMV originated 360 colonies (Fig. 3) and for pEPito-hCMV/RPE65, the number of colonies formed was markedly lower, resulting in about 200 colonies (Fig. 3). This result was not surprising because pEPito-hCMV/RPE65 had a lower transfection efficiency (Fig. 1) compared with the plasmids containing either CMV or hCMV promoter. This result indicates a direct correlation between the transfection efficiency and the number of formed colonies.
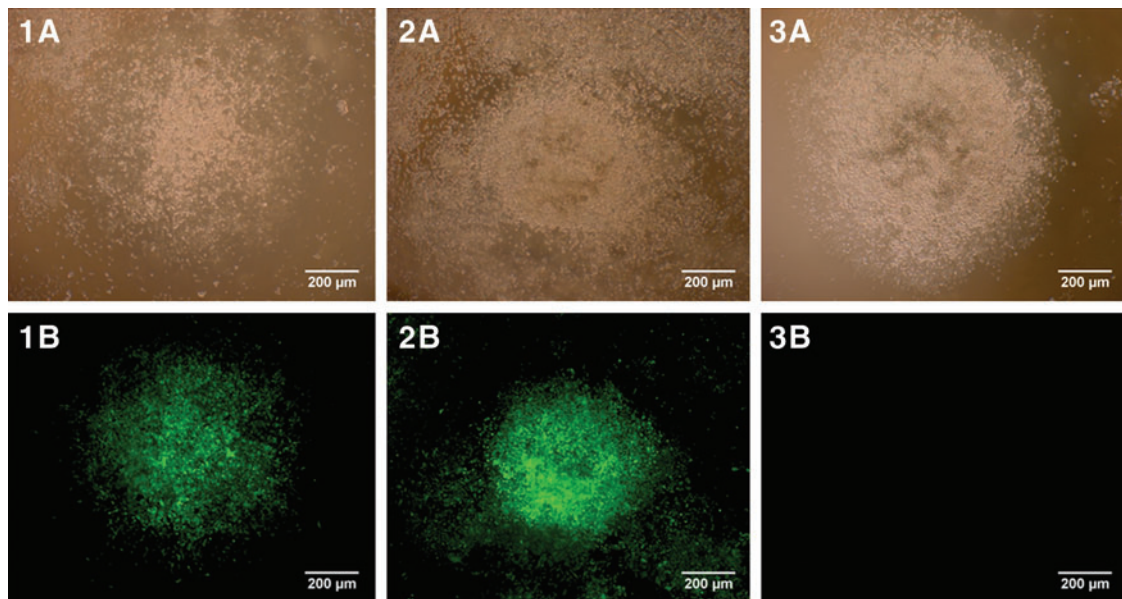
FIG. 2. Brightfield **(A)** and fluorescence **(B)** microscopy of a stably transfected D407 cell line colony with pEPito-CMV-eGFP-BSD **(1)**, pEPito-hCMV-eGFP-BSD **(2)**, and pEPito-hCMV/RPE65-eGFP-BDS **(3)** 32 days post-transfection. BSD, blasticidin; CMV, cytomegalovirus immediate early promoter; hCMV, human CMV enhancer/human elongation factor 1 alpha promoter; eGFP, enhanced green fluorescent protein; RPE, retinal pigment epithelial. Color images available online at www.liebertpub.com/tea

After 1 month of selection, the intensity of the fluorescence decreased over time in the colonies transfected with pEPito-CMV. On the other hand, in the colonies transfected with pEPito-hCMV, the intensity of fluorescence remained h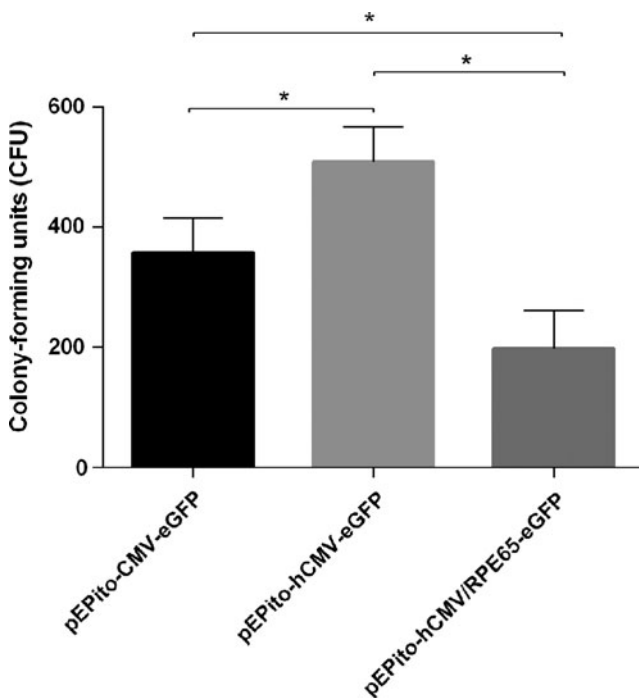igh. These results were further confirmed by flow cytometry: after 2 months of selection, eGFP expression was detected and about 19% of the cells transfected with pEPito-hCMV presented fluorescence (Fig. 4B). However, only 3% of the cells transfected with pEPito-CMV presented fluorescence (Fig. 4C) and 1% of cells with pEPito-hCMV/RPE65 (Fig. 4D). After 3 months of selection, almost no eGFP-positive cells were detected in cells transfected with either pEPito-CMV or pEPito-hCMV/RPE65 (Fig. 4G, H). In contrast, 2% of the cells transfected with pEPito-hCMV remained fluorescent (Fig. 4F).

pEPI and pEPito gene transfer to mouse retinas

An intravitreal injection of 1 μL solution of each plasmid DNA and FuGENE (containing 100 ng of plasmid DNA) was performed in 5-day postnatal (P5) C57Bl6 mice. After the injection procedure, the eyes had a normal appearance and an absence of inflammation similar to the noninjected, contralateral eye.

At 3, 7, 14, 21, and 32 dpi, the eGFP expression was analyzed in 10 μm eye sections. Since the administration method was by intravitreal injection, we expected the retinal GCL to be transfected, because it was in direct contact with the vitreous cavity and with the injection site. Indeed, we have observed that the cells expressing the GFP were clustered along the border of the GCL (Fig. 5).

The overall analysis of the retinal sections showed that the eGFP was expressed by all plasmids, up to 32 days after injection. Table 2 shows the number of retinas, per plasmid and per time point, where eGFP expression was detected. Since it was not possible to follow the same animal over time, the results are shown as number of retinas (animals) with eGFP expression.

As expected, 3 dpi was the time point in which less retinas exhibited GFP expression, as this is too short a time period for the cellular machinery to start the transcription of the genetic material within the plasmid. The number of retinas
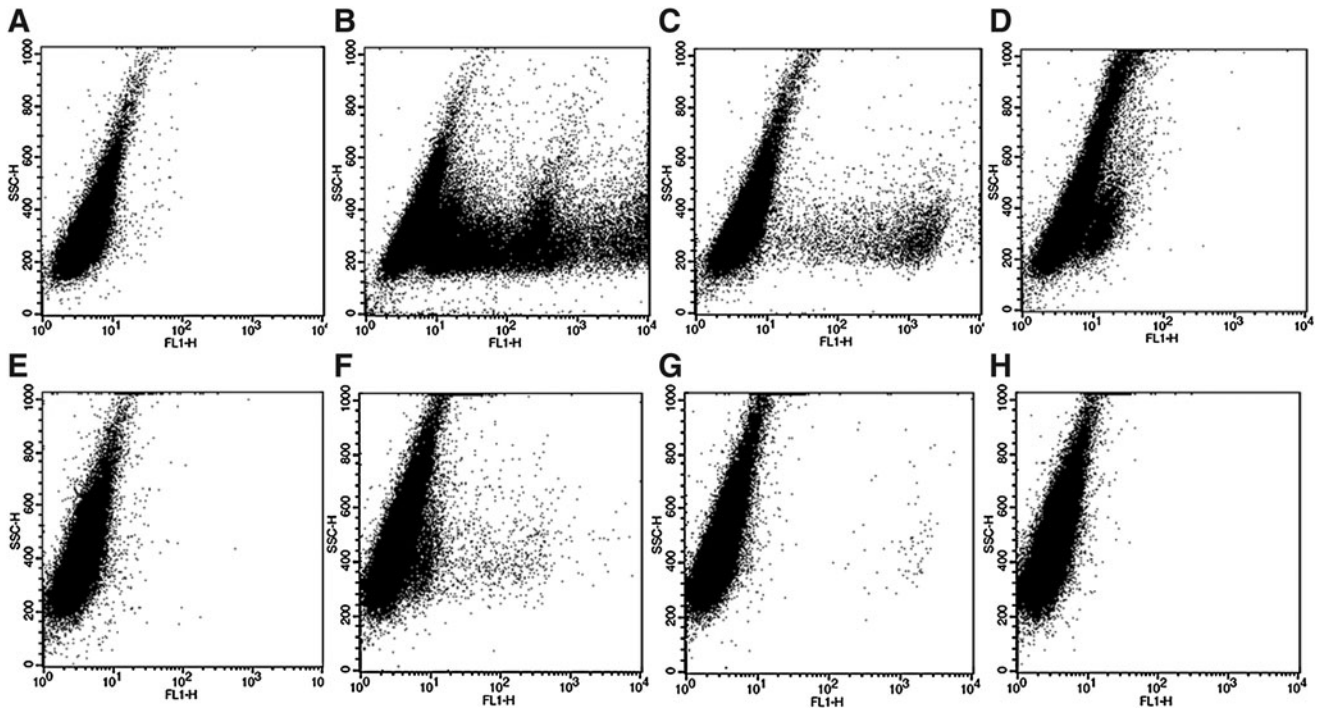

FIG. 3. Colony-forming efficiency of D407 cells selected with blasticidin, for each of the pEPito plasmids. Mean values are derived from three independent experiments and statistical significance was determined with one-way ANOVA followed by a *post hoc* Tukey Test. Bars labeled with * indicate statistical difference ($p < 0.05$).

FIG. 4. Flow cytometry of D407 colonies stably transfected with pEPito-hCMV (**B, F**), pEPito-CMV (**C, G**), and pEPito-hCMV/RPE65 (**D, H**). Upper panels represent cells after 2 months of selection with BSD, and lower panels correspond to cells after 3 months of selection. (**A**) and (**E**) are the control nontransfected cells.

eGFP-positive was lower for both pEPI-1 and pEPito-CMV than for pEPito-hCMV.

Even though the *in vitro* experiments using pEPito-hCMV/RPE65 had lower transfection efficiencies than those containing either the CMV or hCMV promoter, this result was reverted *in vivo,* since the number of retinas expressing the eGFP was comparable to the one observed for pEPito-hCMV. As expected, no fluorescence was detected in the retinas of the contralateral noninjected eye (Fig. 5A, B).

After quantification, we conclude that the transfected area was about 1.5–2% of the GCL area. This area was not affected by the plasmid used.

Discussion

In this study, the potential of plasmids containing S/MARs, pEPI-1, and its derivative pEPito, as expression systems for gene transfer to the retina, was tested *in vitro* and *in vivo*. The aim was to assess whether these expression systems could sustain prolonged expression in the retina, as observed previously for the liver.[8–10,24]

Our transfection assay showed that in D407 retinal cells, the constructs with the RPE-specific promoter were less efficient than the constructs with either the CMV or hCMV promoter. One possible explanation lies in the fact that plasmids containing the RPE65 promoter are larger (7.8 kb, Table 1) than the ones with the constitutive promoter (5.2–6.7 kb, Table 1), and since the *in vitro* experiments were performed by transfecting the same amount (in mass) of plasmid DNA and not equimolar ratios of vector molecules that may account for differences regarding the higher transfection efficiencies observed for the smaller plasmids.

Our colony-forming assay, aimed at testing the ability of these vectors to be replicated during mitosis, showed that all plasmids were able to originate stably transfected colonies for at least 32 days post-transfection. pEPito-hCMV originated the highest number of colonies and pEPito-hCMV/RPE65 the lowest. The latter result was expected since pEPito-hCMV/RPE65 had a lower transfection efficiency compared with the plasmids with either CMV or hCMV promoter. Moreover, cells from the colonies transfected with pEPito-hCMV maintained GFP expression for 2 further months, whereas in colonies transfected with pEPito-CMV or pEPito-hCMV/RPE65, no GFP-positive cells were detected after this period. This result emphasizes the importance of the promoter in the expression profile of a gene, with the CMV promoter being more susceptible to silencing by epigenetic events than the hCMV promoter. Moreover, the plasmids were able to stably replicate for over 100 cell divisions *in vitro*, indicating that *in vivo*, in mostly postmitotic retinal cells, gene expression will be maintained for a prolonged time.

In vivo, our results evidence that all plasmids were able to express the eGFP in mouse retinas for at least 32 dpi. The number of retinas expressing eGFP was lower for pEPI-1 and for pEPito-CMV than for pEPito-hCMV, which indicates that in the plasmids containing the CMV promoter, the eGFP expression was silenced. This result is supported by the literature, where it is described that plasmids with a higher CpG content either in the backbone or in the promoter are more susceptible to epigenetic silencing events,[1,11,12] and the pEPI-1 and pEPito-CMV have more CpG motifs than the pEPito-hCMV.[10] This result supports the importance of a CpG-depleted vector/promoter for a sustained gene expression profile.

On the other hand, we observed a higher number of retinas displaying fluorescence in the eyes injected with
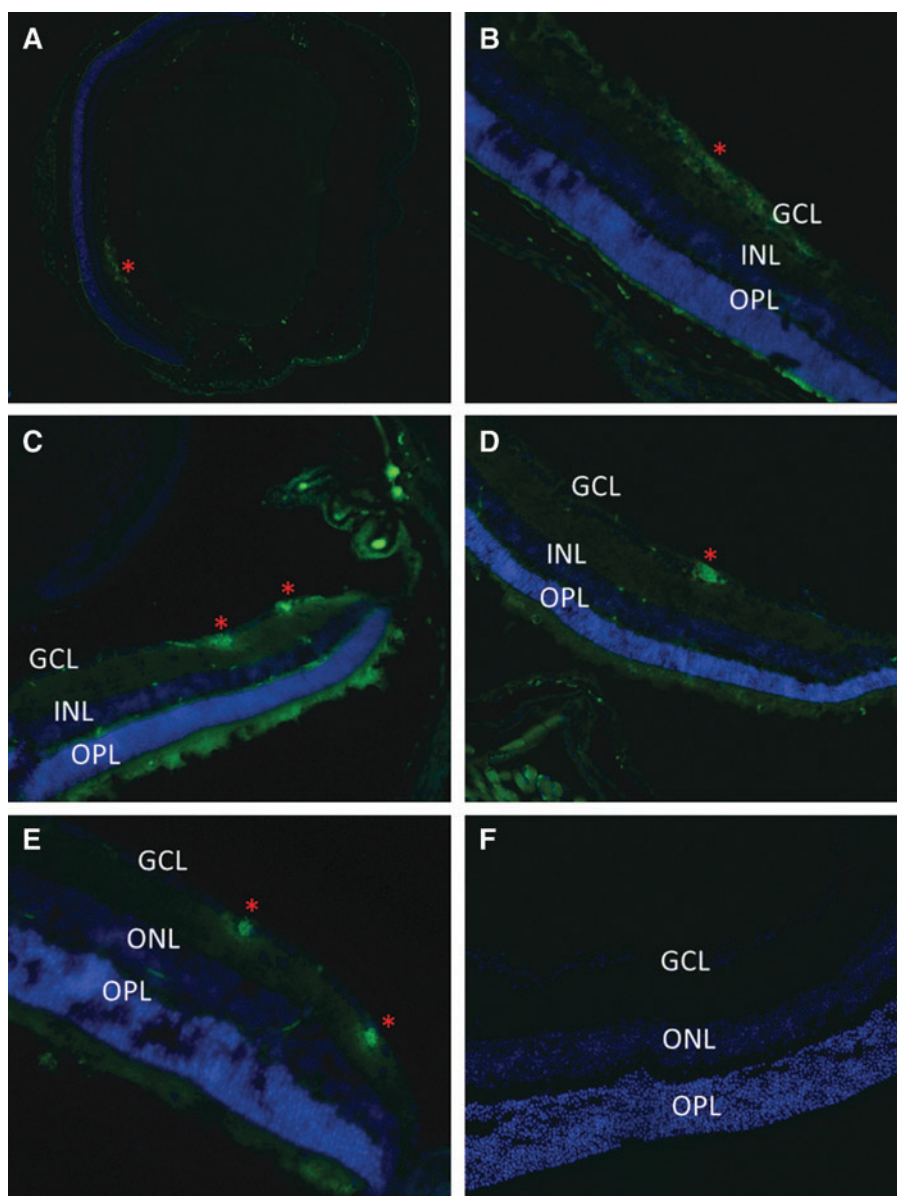
FIG. 5. Transversal sections of a mouse retina injected with pEPI-1- and pEPito-based vectors, sacrificed 32 days postinjection. * Indicates clusters of eGFP-expressing ganglion cells transfected with pEPito-hCMV **(A)** (magnification: 50×); **(B)** corresponds to an amplification of image **(A)** (magnification: 200×), pEPito-CMV **(C)** (magnification: 200×), pEPI-1 **(D)**, and pEPito-hCMV/RPE65 **(E)**. **(F)** A noninjected retina, without GFP expression. DAPI (blue) stains nuclei. GCL, ganglion cell layer; INL, inner nuclear layer; OPL, outer plexiform layer. Color images available online at www.liebertpub.com/tea

pEPito-hCMV than with pEPito-CMV. This is in accordance to what is described by Haase *et al.*,[10] which has shown these plasmids to originate the strongest luciferase expression *in vivo*, as the hCMV promoter is less affected by epigenetic silencing events than CMV.[12]

Although our *in vivo* study has shown a low number of transfected cells, upon comparison with other studies, where

TABLE 2. SUMMARY OF THE NUMBER
OF GFP-POSITIVE RETINAS (N = 8)

dpi	pEPi-1	pEPito-CMV	pEPito-hCMV	pEPito-hCMV/RPE65
3	1	0	4	3
7	3	3	6	4
14	4	4	5	5
21	4	4	5	5
32	3	3	5	4

dpi, days postinjection.

these systems were combined with chitosan nanoparticles for corneal gene delivery[25] or AAVs used for retinal transduction,[26] the amount of DNA we used was significantly lower than in other studies (100 ng compared with 1.5 μg[25]). However, our results show that by using only 100 ng of DNA, we could achieve a transfection efficiency of 1.5–2% of the ganglion cells.

Finally, pEPito-hCMV/RPE65 showed a lower transfection efficiency than other plasmids in our *in vitro* experiments, but a greater efficiency *in vivo,* which highlights the relevance of a tissue-specific promoter. Moreover, the expression of the pEPito-hCMV/RPE65 containing an RPE-specific promoter observed in ganglion cells can be explained by the presence of the hCMV enhancer, which has been described as a strongly potentiating gene expression.

With this study, we prove that these improved episomal vectors containing S/MARs and less CpGs—the pEPitos—can be used for efficient gene transfer to the retina. The combination of an enhancer that is less affected by epigenetic silencing effects with a tissue-specific promoter is

crucial to sustain a prolonged gene expression *in vivo* and contribute for an efficient gene therapy strategy.

Disclosure Statement

No financial interests exist for any of the authors.

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