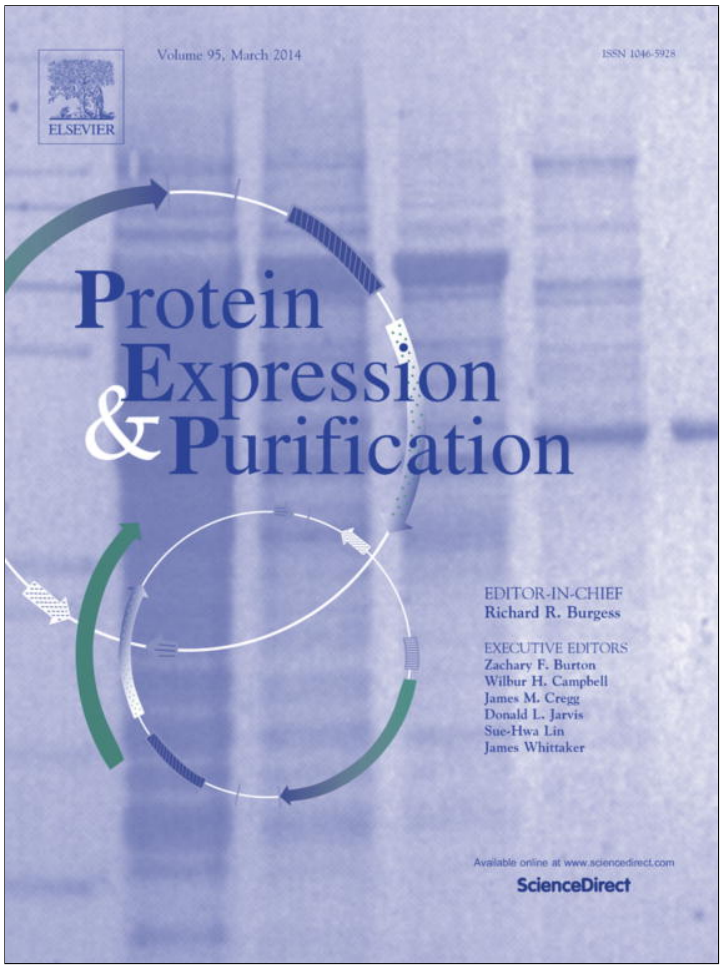

Protein Expression and Purification 95 (2014) 50–56

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

Development of lentiviral vectors for transient and stable protein overexpression in mammalian cells. A new strategy for recombinant human FVIII (rhFVIII) production

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ARTICLE INFO

Article history:
Received 19 August 2013
and in revised form 17 October 2013
Available online 22 November 2013

Keywords:
Lentiviral vector
rhFVIII production
Protein overexpression
CMV enhancer/promoter
EF-1α promoter

ABSTRACT

Background: Recombinant protein overexpression in mammalian cells constitutes a real challenge in therapeutic protein production. Following the discovery of intron functionality in gene expression, various expression vectors that include them in their sequences have been developed. In this study, the main goal was to develop new lentiviral vectors (LVs) carrying different promoter and intron-containing 5′UTR (5′ untranslated region) combinations and the design of LVs for rhFVIII production in Chinese hamster ovary (CHO) cells.

Results: By combining the human cytomegalovirus (CMV) or the elongation factor 1α (EF-1α) promoters along with different 5′UTRs that included leader introns, between 2 and 12-fold increases were reached, when transient and stable expression of the enhanced green fluorescent protein (EGFP) and rhFVIII were analyzed. Also, new LVs provided with promoters and 5′UTRs from high expression genes, according to a gene database, were designed. Three of them were shown to be superior to the EF-1α promoter in three widely used cell lines.

Conclusion: In the present work, LVs containing different promoters and 5′UTRs were designed. In transient and stable assays some of these constructs have shown higher activity compared with commercial promoters and, therefore, constitute promising candidates for therapeutic protein production.

Introduction

The need for high levels of protein expression has prompted the search for new strategies, including technologies to obtain cells with a high number of copies of a given gene [1] or mechanisms that enhance its transcriptional activity obtaining a greater number of transcripts from a single DNA molecule. The latter is possible through strong activity promoter sequences that include binding sites for transcriptional factors that ultimately allow a more efficient RNA polymerase II enzyme binding. Another way to improve gene expression is through an optimized mechanism at the post-transcriptional level [2–4]. This is achieved through different ways that include an increased mRNA stability, a more efficient transport across the nuclear pore complex as well as using strategies to achieve better transcript recognition by the ribosomal complex, once it reached cell cytoplasm [5–7].

Increased gene expression mediated by introns is a process known in literature as IME (Intron Mediated Enhancement) [8–10] and may occur at different levels. There are introns that contain enhancer elements capable of increasing transcript amount [11,12], and there have been cases in which the regulation occurs at post-transcriptional level [13], through mechanisms dependent on splicing process. Such mechanisms lead to greater efficiency in polyadenylation, mRNA stability or exporting transcript through the nuclear pore complex to the cytoplasm [14,15]. This remarkable phenomenon has been described in certain genes belonging to different organisms, suggesting the presence of a conserved gene expression mechanism across species [16–19]. Moreover, an algorithm that allows predicting the intron functionality in a certain gene in rice and *Arabidopsis* was designed by Rose [20]. The importance of these sequences prompted several researchers to obtain recombinant vectors that included strong promoters accompanied by introns. Those introns were generally located between the promoter region and the gene of interest, i.e. in the 5′UTRs and are known as *leader introns*.

Taking into account the role of these sequences in the regulation of gene expression, it is not surprising to see an increasing number of attempts to achieve "the best combination promoter sequence-5′UTR" [21–23]. For instance, experiments with the human cytomegalovirus major immediate early gene virus

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http://dx.doi.org/10.1016/j.pep.2013.11.005

promoter (pCMV)[1] and the elongation factor 1 alpha promoter (pEF-1α) were performed [24]. Moreover, these sequences are part of expression vectors widely used for recombinant gene expression.

An expression mechanism derived from lentiviral vectors (LVs) was developed some years ago [25,34]. Such vectors are viral based gene delivery systems that are able to stably transfer genes into several cell lines with an elevated efficiency. LVs bind to target cell protein using an envelope which allows the release of the LV RNA gene sequence contained in the cell. The LV's RNA is then converted into DNA by a reverse transcription process. Then, the DNA pre-integration complex enters the nucleus and efficiently integrates into the target cell chromosomal DNA [26,27].

In the present study, the first goal was to develop new LVs containing different promoter and 5′UTR combinations in order to reach improved versions. These sequences were fused to either the gene encoding the enhanced green fluorescent protein (EGFP) or the B-domain deleted recombinant human Factor VIII (rhFVIII). The latter is involved in blood coagulation cascade and congenital or acquired deficiencies can produce a disease known as Hemophilia A. Also, it was shown that removal of the FVIII B-domain increased the manufacturing yield without compromising the biological activity of the molecule [28,29]. Moreover, comprehensive structural and functional analyses have established that this version retains the necessary structural integrity required for full bioactivity in vivo [30,31].

On the other hand, by means of a gene database search where expression levels and patterns are reported based on microarrays data, seven genes with elevated expression levels were chosen, two of them belonging to the *Mus musculus* species and the remaining of human origin. Thereby, their putative promoters and their respective 5′UTRs were amplified and cloned into a LV, upstream from the *egfp* reporter gene. The genes whose sequences were tested include: *Mus musculus* heterogeneous nuclear ribonucleoprotein K (*MmHNRNPK*) and glyceraldehyde-3-phosphate dehydrogenase (*MmGAPDH*); *Homo sapiens* glyceraldehyde-3-phosphatedehydrogenase (*HsGAPD*); heterogeneous nuclear ribonucleoprotein K (*HsHNRNPK*); RNA binding motif protein 4 (*HsRBM4*); serine/arginine-rich splicing factor 5 (*HsSRSF5*) and growth hormone 1 (*HsGH1*) gene. Different cell lines expressing EGFP under the command of those sequences, in transient or stable conditions, were analyzed by flow cytometry using the cellular promoter pEF-1α as control. This analysis revealed that some of these constructs constitute suitable candidates for therapeutic protein production.

Materials and methods

Vector construction

For transient and stable expression a LV (Fig. 1A) was used.

Recombinant LVs (containing EGFP or rhFVIII coding sequences) carrying different combinations of pCMV or pEF-1α with the following sequences: Chimeric Intron derived from pCINeo® (Promega), constituted by the 5′-donor site from the first intron of the human β-globin and the branch and 3′-acceptor site from the intron located between the leader and body of an immunoglobulin gene heavy chain variable region [32]; the human elongation factor 1 alpha (HsEF-1α) 5′UTR and the *Cricetulus griseus* elongation factor 1 alpha (CgEF-1α) 5′UTR, were constructed. Such sequences were obtained from PCR reactions using appropriate templates and primers. All constructs (Fig. 1B) were checked by DNA sequencing.

A search for genes with high expression levels in human and mouse cells in the AceView database (http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/) was carried out. Promoter regions from genes with AceView scores higher than eight were selected. Then, by searching the NCBI database, putative promoter and 5′UTR sequences were identified (Supplementary Data), which were amplified by PCR using appropriate oligonucleotide primers. DNA fragments were digested with restriction enzymes and cloned into a LV upstream from the *egfp* gene (Fig. 1C).

Oligonucleotide primers, templates, restriction enzymes and recombinant vectors are described in Table 1 (Supplementary Data).

Cell lines

For CHO cell line growth and maintenance, Dulbecco's Modified Eagle's Medium (DMEM); DMEM/Ham's Nutrient Mixture F-12 (50:50); 5% (v/v) Fetal Calf Serum (FBS) and 2 mM glutamine were used. For HEK and HeLa cell lines, DMEM and 2 mM glutamine supplemented with 10% FBS were used. In all cases the pH was adjusted to 7.0. Media, Fetal Calf Serum (FCS) and consumables for cell culture were obtained from Gibco (USA), PAA (Argentina) and Greiner (Germany).

Transient expression (lipofection)

Adherent HEK293T, CHO-K1 and HeLa cells were seeded in a 12-well plate (2×10^5 cells per well) one day before transfection. Experiments were performed with the LipofectAMINE Reagent (Invitrogen) according to the supplier's instructions by using a mix of 3 μg of each vector and 3 μl of the LipofectAMINE Reagent. Intracellular fluorescence activity was measured on a Guava® EasyCyteTM cytometer (Guava Technology, USA) at 48 h after transfection.

LV particles production and titration

Research grade HIV-based LVs particles containing EGFP or rhFVIII transgenes were manufactured following the protocol suggested by Naldini [33] and Dull [34]. Following the lipofection protocol mentioned above, adherent HEK293T cells (1.2×10^7 cells per well) were simultaneously co-transfected with four plasmids: the packaging construct (pMDLg/pRRE) [34], the VSV-G-expressing construct (pMD.G) [33], the Rev-expressing construct (pRSV-Rev) [34], and the self-inactivating (SIN) promoter-containing LV. Lentiviral particles (LVPs) containing supernatant were collected 72 h after transfection.

LVPs were tittered by performing the QuickTiter™ Lentivirus Titer Kit (Lentivirus-Associated HIV p24) following instructions suggested by the manufacturer (Cell Biolabs Inc., USA) and the titer (TU ml^{-1}) was determined. The titer was used to calculate the necessary supernatant dilutions to transduce cells at the same multiplicity of infection (MOI).

Stable expression (lentiviral transduction)

Transductions were carried out by incubating 6.0×10^4 cells per well seeded onto 6-well plates (Greiner) with LVPs in a final MOI of sixty. Twenty-four hours post-transduction, media were replaced with fresh media. In order to eliminate the remaining wild type

[1] *Abbreviations used:* EGFP, enhanced green fluorescent protein; TU ml^{-1}, Transduction Unit per ml; IRES, internal ribosome entry sites; 5′UTR, 5′ untranslated region; pCMV, human cytomegalovirus major immediate-early gene promoter; pEF-1α, elongation factor 1 alpha promoter and 5′UTR; rhFVIII, recombinant human Factor VIII; *MmHNRNPK, Mus musculus* heterogeneous nuclear ribonucleoprotein K; *MmGAPDH,* glyceraldehyde-3-phosphate dehydrogenase; *HsGAPD, Homo sapiens* glyceraldehyde-3-phosphatedehydrogenase; *HsHNRNPK,* heterogeneous nuclear ribonucleoprotein K; *HsRBM4,* RNA binding motif protein 4; *HsSRSF5,* serine/arginine-rich splicing factor 5 and *HsGH1,* growth hormone 1 gene; CI, Chimeric Intron; CgEF-1α, *Cricetulus griseus* elongation factor 1 alpha; DMEM, Dulbecco's Modified Eagle's Medium; FBS, Fetal Bovine Serum; FCS, Fetal Calf Serum; h, hour; CHO, Chinese hamster ovary; HEK, Human Embryonic Kidney.

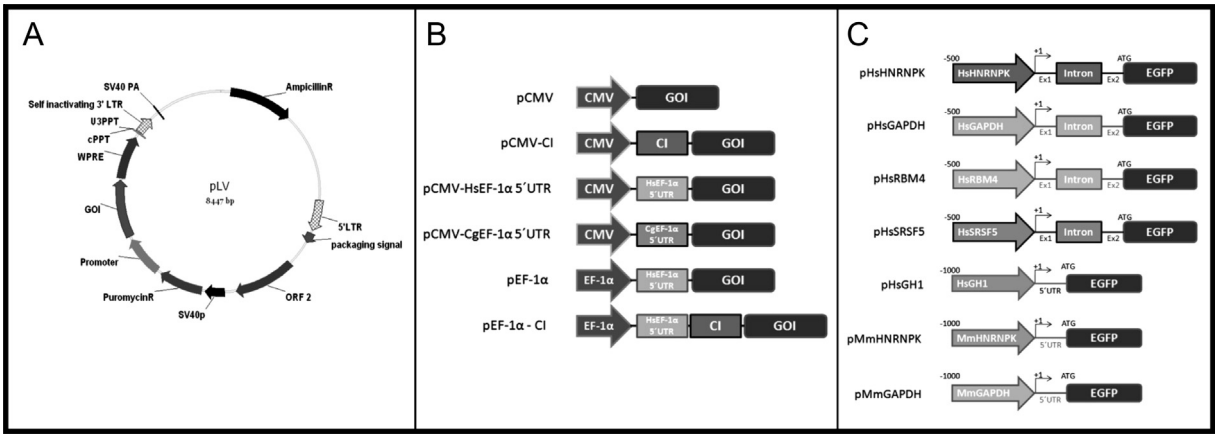

Fig. 1. Schematic representation of expression vectors. (A) Lentiviral vector used for protein overexpression. (B) Different combinations of promoter regions and 5′UTRs fused to GOI, gene of interest (*egfp* or *rhfviii*). (C) Promoter and 5′UTR sequences from predicted elevated expression genes fused to EGFP.

cells, 96 h post-transduction supernatants were replaced by fresh growth medium containing 10 μg ml^{-1} puromycine (Sigma Aldrich, USA). Selective medium was changed every 3–4 days until control cell death. Transduced cells were expanded for EGFP analysis or FVIII quantitation.

Fluorescence measurements

Flow cytometry was performed on a Guava® EasyCyteTM cytometer (Guava Technology, USA). Data acquisitions and analysis were performed using Guava CytoSoftTM 3.6.1 software. For each sample 5000 events were collected gating on the forward scatter (FSC) vs side scatter (SSC) dot plot. Flow calibration and optical alignment was performed with the aid of Flow-Check Fluorospheres (Guava® Check kit, Hayward, CA, USA) before each determination. Cells were analyzed for the percentage of cells expressing EGFP above the untransfected control levels and the fluorescence intensity.

EGFP expression was determined by multiplying the percentage of EGFP positive cells by the fluorescence intensity as obtained by flow cytometry 48 h post-transfection [1,46]. Data are representative of three (transduction) or five (transfection) independent experiments. Error bars represent ± SEM.

rhFVIII quantitation by sandwich ELISA

Sandwich enzyme-linked immunosorbent assays (ELISA) were performed in 96-well plates (Greiner), coated with a capture monoclonal antibody developed in our laboratory (internal code P2E5). Coating was achieved by incubating the plates with 100 ng per well of an antibody solution in carbonate buffer (Na_2CO_3 50 mM, pH 9.4) for 1 h at 37 °C and then ON at 4 °C. Plates were then washed 6 times with phosphate buffer saline (PBS: 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 14 mM NaCl, 2.7 mM KCl, pH 7.40) containing 0.05% (v/v) Tween 20 (PBS-T). Plates were incubated with 200 μl per well of 2% (v/v) Bovine Serum Albumin (BSA) in PBS for 1 h at 37 °C in order to eliminate nonspecific binding. After a washing step using PBS-T, 200 μl of each collected sample were added and seven serial dilutions were made in diluent solution (0.2% (v/v) BSA in PBS) and incubated for 1 h at 37 °C. After washing with PBS-T, plates were incubated with 100 ng per well of rabbit anti-human FVIII polyclonal antibody for 1 h at 37 °C. After washing with PBS-T, plates were incubated for 1 h at 37 °C with peroxidase-labelled rabbit anti-mouse immunoglobulins (DAKO, Denmark) appropriately diluted with PBS-T-FCS 0.5% (v/v). After washing and incubating in the dark with 50 mM citric acid-phosphate buffer, pH 5.3, containing 3 mg ml^{-1} o-phenylenediamine and 0.12% (v/v) H_2O_2 (substrate solution), the absorbance was measured at 492 nm with a microtiter plate reader (Labsystems Multiskan MCC/340, Finland). The assay was reproduced by triplicate.

Results

Comparison of promoter – 5′UTR combinations for EGFP transient expression

In this study the first aim was to compare promoter strengths considering the pCMV and the pEF1-α as initial constructions. Fluorescence levels from CHO, HEK293T and HeLa cell lines transfected with LVs described in Fig. 1B were determined. Results are relative to the pCMV or pEF1-α containing vectors and are summarized in Fig. 2. Along the three cell lines the pCMV–HsEF-1α5′UTR containing vector was allowed to reach the maximum EGFP expression, since fluorescence levels yielded between 3 and 4 times higher than those obtained with pCMV. In this context, the CI incorporation to the pEF1-α showed an increase between 8 and 12-fold depending on the cell line. This assay was reproducible from five independent transfections.

Stable EGFP expression

Considering the wide applicability of protein production for therapeutic purposes under a stable expression system, promoter-containing LVs were analyzed in such expression conditions.

To achieve this objective, promoter-containing LVPs were assembled. Those particles were used to transduce (at the same MOI) CHO, HEK293T and HeLa cell lines.

After puromycin selection, fluorescence levels from the different transgenic cell lines were determined. Results are shown in Fig. 3. The best expression vector was the one that included the pCMV–CgEF-1α5′UTR sequence, which yielded expression levels 50% higher than the pCMV in CHO and HEK293T cells, and 300% higher than the same control in HeLa cells. In case of pEF1-α, the CI incorporation allowed to reach between 7 and 13-fold increase.

rhFVIII stable production in CHO cells

Based on the results deriving from previous experiments, the promoter-containing constructs were used to produce a protein of interest in the pharmaceutical industry as in the case of rhFVIII.

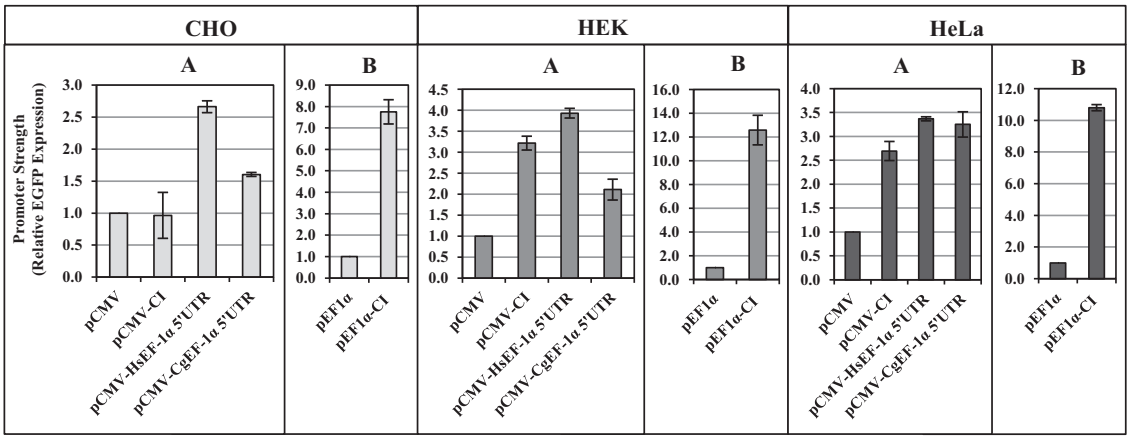

Fig. 2. Effect of addition of different 5′UTRs downstream of the pCMV or pEF1-α from a transient expression assay in CHO, HEK293T and HeLa cells, expressed as the fold-increase in EGFP expression relative to the pCMV (A) or pEF1-α (B). Results were obtained from five separate transfections ± standard error.

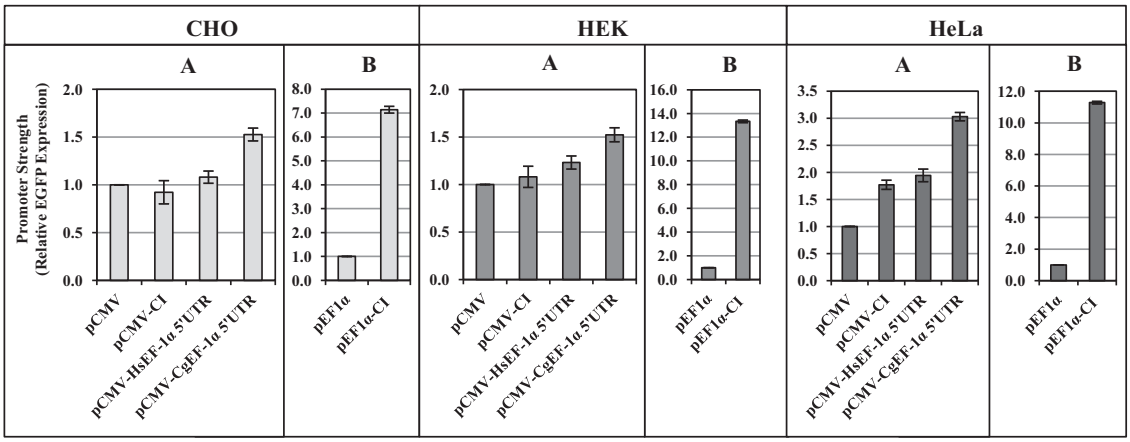

Fig. 3. Stable expression assay to evidence the effect of different 5′UTR incorporations downstream of the pCMV or pEF1-α in different mammalian cells, expressed as the fold-increase in EGFP expression relative to the original promoters. Cell lines were transduced with the same MOI in each case. Results were obtained from three separate transductions ± standard error.

To achieve this, *egfp* gene was replaced by the cDNA encoding for rhFVIII. Then, rhFVIII containing LVs were used for assembling LVPs. CHO cells were transduced with those LVPs at the same MOI. Transductions were performed in triplicate in each case. After 96 h culture medium was replaced with fresh puromycin containing medium at a final concentration of 10 μg ml^{-1}. Once complete wild type cell death was verified, rhFVIII production was determined by sandwich ELISA. Results were plotted in Fig. 4. Once again, the incorporation of different 5′UTRs to the original promoters (pCMV and pEF-1α) led to significant increases in expression levels. In this case the best combination was pEF-1α-CI that yielded productions in the range of 800 ng ml^{-1} (a 4-fold increase compared with pEF-1α). Also, these levels were barely higher than those produced by the pCMV-CI sequence-containing construct.

Search for putative promoters for the development of new expression vectors

A second part of the present work consisted of a search for genes with high expression levels in human and mouse cells, assuming that their enhanced expressions are consequence of an increased transcriptional/post-transcriptional activity which is provided by their respective promoter and 5′UTR sequences. To carry out this, a survey in the AceView database was conducted

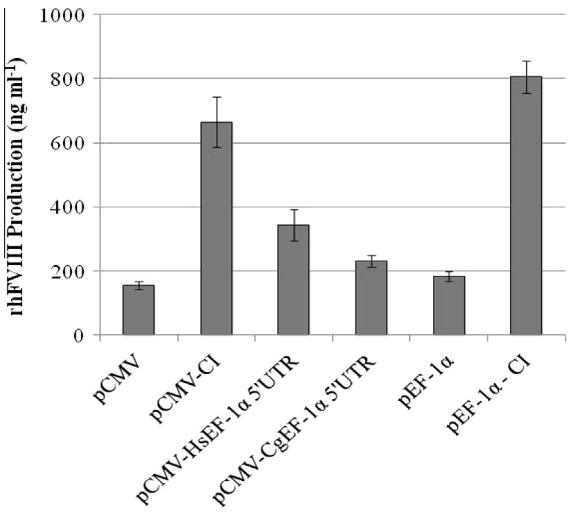

Fig. 4. rhFVIII productivities in CHO cells transduced with different promoter/5′UTR combinations and selected with 10 μg ml^{-1} puromycin. Determinations were made by sandwich ELISA from culture supernatants and using an appropriate standard. Results were obtained from three separate transductions events ± standard error.

Table 1
Putative promoters and 5′UTRs from genes with high expression levels according to the AceView database.

Gene	Promoter + 5′UTR[a] Size (bp[b])	Intron Size (bp)	AceView (gene expression times over the average[c])
Growth Hormone (GH1)	1062	–	12.8
Human Heterogeneous Nuclear Ribonucleoprotein K (HsHNRPK)	705	93	8.5
Human Glyceraldehyde 3-phosphate dehydrogenase (HsGAPDH)	811	240	10.2
Human RNA-binding protein 4 (HsRBM4)	1595	947	10.5
Human Splicing factor, arginine/serine-rich 5 (HsSFRS5)	1542	757	9.9
Mouse Heterogeneous Nuclear Ribonucleoprotein K (MmHNRPK)	1000	–	12.8
Mouse Glyceraldehyde 3-phosphate dehydrogenase (MmGAPD)	1000	–	29.1

[a] Untranslated Region.
[b] Base pair.
[c] Promoter regions from genes with AceView scores higher than eight were selected as they are considered genes with very high expression levels.

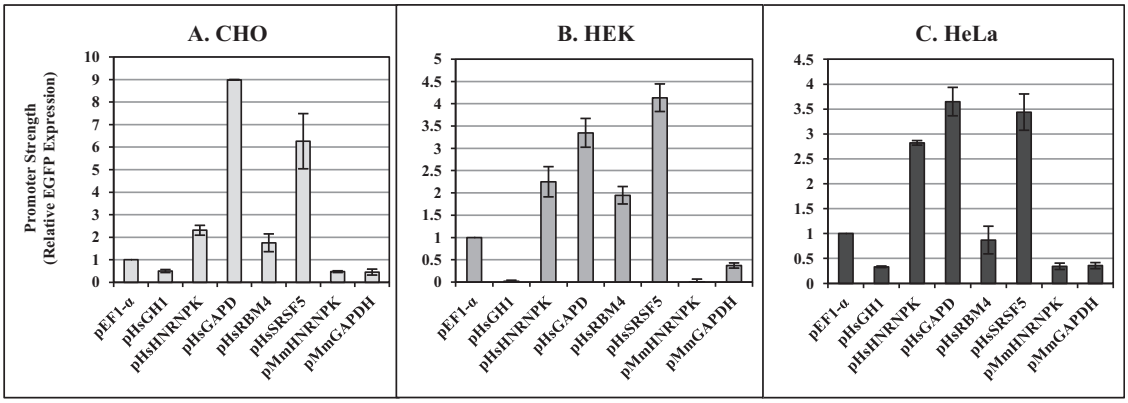

Fig. 5. Comparison of promoter strength from different putative promoter sequences and their respective 5′UTRs using the pEF1-α as control in a transient expression system. Transfections were performed in quintuplicate and the results represent the mean value and its standard deviation (error bar).

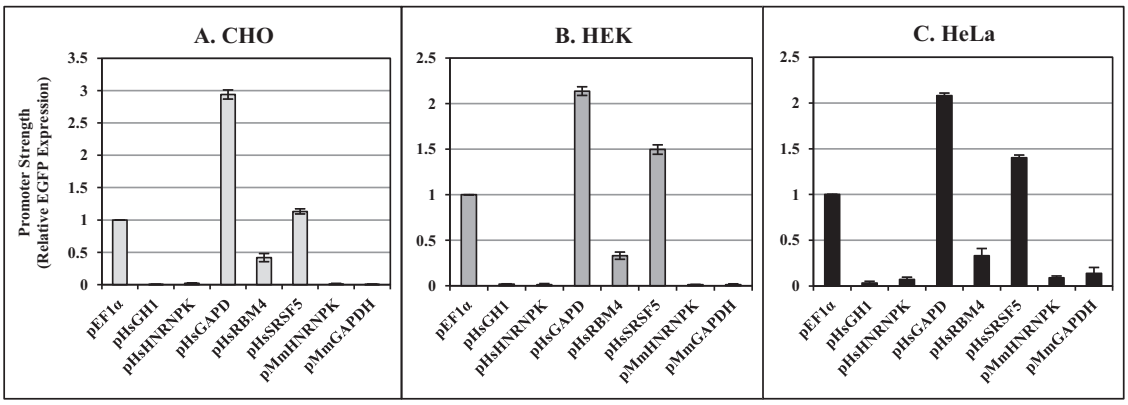

Fig. 6. Evaluation in a stable expression system of promoter strength from different putative promoter sequences and their respective 5′UTRs compared with the pEF1-α. Determinations were made from cell lines transduced in triplicate.

in order to identify those genes having this feature. A total of seven genes (five of human origin and the remaining two of murine source) were identified and fused to the *egfp* gene. A brief description of these sequences is shown in Table 1.

Transient expression

Putative promoter-5′UTR sequences-containing LVs were used to transfect CHO, HEK293T and HeLa cells. EGFP expression from those cells was determined. Results are relative to the pEF1-α-containing vector and are shown in Fig. 5. In all three cell lines the pHsGAPDH and pHsSRSF5 allowed to reach the highest fluorescence levels, between 3 and 9 times higher compared to the pEF-1α. The experiment was reproducible from five independent transfections.

Stable expression

In order to assess the behavior of putative promoter-5′UTR sequences once integrated into the cellular genome, LVPs with

such sequences were generated. These particles were used to transduce CHO, HEK293T and HeLa cell lines, carrying out three separate transduction events in each case. Fluorescence levels were determined and results are shown in Fig. 6. pHsGAPDH and pHsSRSF5 sequences emerged again as the best of the seven analyzed in this work, showing a 1.5 to 3-fold increase compared to the pEF-1α.

Discussion and conclusion

There is vast evidence about the importance of intron sequences located at 5′UTRs in gene expression [35–37], and in this case, they are known as *leader introns*.

In this study, the first aim was to attempt to optimize pCMV and pEF-1α-containing vectors, widely used for recombinant protein overexpression [1,36,38,39]. To carried out this, pCMV was combined with different *leader intron*-containing 5′UTR and the pEF-1α was fused to the Chimeric Intron from the pCINeo® vector. Transient and stable expression assays were used to compare the promoter strength. In the first case, the best combination was found to be the one containing the pCMV accompanied by the (HsEF-1α) 5′UTR (Fig. 2), whereas in the second case the incorporation of the (CgEF-1α) 5′UTR produced the highest performance (Fig. 3). It should be stressed that in all three cell lines these combinations yielded the best results, revealing the possible presence of common transcriptional/post-transcriptional cues among them. However, a discrepancy in yields between transient and stable expression systems was observed. This behavior reflects the different factors that govern gene expression in both cases [34,40]. The lower increases observed in stable conditions are living proof of impact of gene integration into a genome region and, thus, the consequent exposure to regulatory mechanisms that govern gene expression in this context. Also, since that the addition of splice signals downstream of the internal promoter might increase the risk for illegitimate splicing reactions and premature splicing of the introduced intronic sequences in packaging cells, the transferred DNA integrity was confirmed by PCR (Fig. 1 in Supplementary Data) and this possibility was discarded.

Another aspect that should be considered is that even though most of the combination increased gene expression, some of them exhibited a cell-specific effect. This phenomenom is consistent with other publications [44,45] and remains to be elucidated.

Since the human EF-1α 5′UTR insertion behind the EF-1α promoter in which already included the EF-1α first intron did not produce an increase in gene expression [22], those combinations were not considered in this study. However, the CI incorporation downstream the pEF-1α has produced an enhancement in EGFP expression in all three cell lines and in both production platforms (Figs. 3 and 4). Moreover, this addition showed an increase greater than those achieved with other combinations that included the pCMV.

In order to apply these results to the pharmaceutical industry, *egfp* gene from LVs was replaced by the coding sequence for the B-domain deleted recombinant human Factor VIII (rhFVIII) [41–43]. The production assay in CHO cells from a stable expression system revealed that the CI incorporation in both promoter regions (pCMV and pEF-1α) produced the best results (Fig. 4). Although pCMV-CI combination is part of the widely used pCINeo® vector [32], there is no evidence describing the CI incorporation effect to the pEF-1α. Another aspect that should be highlighted is the different performance between pCMV – HsEF-1α 5′UTR and pCMV – CgEF-1α 5′UTR-containing vectors, fused to both EGFP and rhFVIII. A plausible explanation may lie in the differences between both proteins, such as molecular size and cellular localization. This reflects the importance of extrapolating reporter protein production systems to the therapeutic protein of interest regardless the results achieved with the former.

A second part of this work focus on attempting to design new cellular promoter-containing LVs. From the query in the AceView gene database seven genes with high expression levels were identified. This feature was regarded as an evidence of high promoter activity. Therefore, such putative sequences accompanied by their respective 5′UTRs were isolated and cloned into an EGFP containing-LV. By using the pEF-1α as control, transient expression assays showed that pHsGAPDH and pHsSRSF5 reached the highest EGFP levels in CHO, HEK293 and HeLa cell lines (Fig. 5). For this reason these particular sequences could be suitable candidates considering the importance of transient expression systems in therapy.

By testing the behavior of these sequences in a stable production platform, the ones mentioned above showed again the best performance (Fig. 6). However, relative fluorescence levels were lower compared with the transient expression condition. It should also be noted that it is possible for the promoter regions isolated in this study that relevant transcriptional sequences and/or enhancer elements have been excluded. Therefore, it will be meaningful to investigate larger promoter regions as well as the evaluation of the sequential shortening effect to identify those elements involved and the gene expression level on which they act (see Table 2 in Supplementary Data). Also, the use of these sequences for biotechnological protein production will be considered.

In conclusion, in this study new pCMV and pEF-1α-containing LVs along with different 5′UTRs were evaluated. Some of these constructs allowed to reach higher EGFP and rhFVIII levels than those shown by the original promoters. Moreover, in an effort to identify new cellular promoter sequences for protein overexpression, better alternatives to a widely used at present (pEF1-α) have been found. Such sequences revealed a great performance in three different cell lines both in a transient and a stable platform, constituting promising candidates for pharmacological protein production.

Acknowledgements

This work was supported by Universidad Nacional del Litoral (SAT No. 356,698) and Zelltek SA. RK, ME and EFM are members of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.pep.2013.11.005.

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