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Original Article

Comparing the Yield of Recombinant Human Factor VII Protein Expressed by the rDNA-Promoter with the CMV-Promoter in Iranian Lizard *Leishmania*

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Abstract

Background: Iranian Lizard *Leishmania* (I.L.L) is a nonpathogenic *Leishmania* strain. Due to its advantages, several recombinant proteins have been produced in this host. However, I.L.L shows a lower yield of recombinant protein expression compared to other commercial hosts. Considering the role of protease enzymes in protein digestion, we selected cysteine protease B (CPB) to investigate its impact on recombinant protein yield in I.L.L.

Methods: we generated gene knockouts by utilizing homologous recombination (HR) and CRISPR methods. To assess the efficacy of the designed construct, we compared the yield of recombinant human factor VII (rhFVII) production between cells transfected with the pLEXSY-hyg2-FVII vector and the CMV-promoter-based construct (pF7cmvneo).

Results: The knockout of a single *CPB* gene allele through the HR method or the complete knockout of all alleles through the CRISPR method led to cell death. This outcome suggests that even the deletion of a single *CPB* gene allele diminishes the protein to a level insufficient for the survival of I.L.L, indicating a critical dependency on the presence of this protein for the organism's viability. rhFVII exhibited a greater expression yield with the pLEXSY construct compared to the pF7cmvneo construct in I.L.L. The lower expression rate of pF7cmvneo may be influenced by epigenetic factors related to the *CPC* gene or the RNA polymerase used for the expression of that promoter.

Conclusion: Therefore, considering alternative integration targets for CMV-promoter-based constructs and incorporating UTR sequences of I.L.L high-expression proteins in the vector may enhance recombinant protein expression rates.

Introduction

Leishmania is a unicellular eukaryotic parasite employed as an expression host for the production of various recombinant proteins (1). Among the diverse *Leishmania* species, most strains of *L. tarentolae* stand out for their non-pathogenicity to humans (2). In addition, *L. tarentolae* has mammalian-like post-translational modifications (PTMs), the ability to produce multi-subunit proteins, ease of culturing, and cost-effective protein production (3).

The *Leishmania P10* strain is commercially available through the Jena Bioscience, serving as a host for the expression of recombinant proteins (2). While this system has demonstrated protein yields of up to 500 mg/L in some studies, the typical recombinant protein expression yield in this strain ranges from 0.1 to 30 mg/L (3, 4). This company also has introduced pLEXSY vectors designed for both constitutive and inducible protein expression in *Leishmania*. The genome integrative vectors utilize the chromosomal ssu-locus as the integration site (5).

Kazemi et al. (6) isolated a strain of *Leishmania*, is known as Iranian Lizard *Leishmania* (I.L.L). This strain is non-pathogenic to mammals and offers a cost-effective option for laboratory cultivation (7). The transcriptome in the promastigote of I.L.L was identified by the NGS system (8). Using pLEXSY plasmids, I.L.L was utilized to produce recombinant proteins, yielding between 75 µg/L and 4 mg/L (9, 10). Additionally, with a CMV-promoter-based plasmid, the recombinant protein yield was 0.2 ng/mL (11).

Protease activity within host cells can lead to the degradation of unnecessary proteins, including recombinant proteins (12). Among these proteases, cysteine proteases have been identified for their negative impact on recombinant proteins (13). Silencing these proteases in a transgenic host has the potential to prevent protein degradation (14). Notably, cysteine protease B (CPB), which express at high levels in several *Leishmania* strains (15), was successfully knocked out (16) or inhibited (17) in *L. mexicana*.

Promoters play a crucial role in influencing protein expression yields. Among popular promoters, the simian cytomegalovirus immediate early promoter (sCMV) is well-known for its use in achieving high expression rates in mammalian and non-mammalian systems (18). Kazemi et al. further extended its application to non-mammalian research, developing an expression vector based on the CMV-promoter (11).

We aimed to knockout the CPB gene in I.L.L. We hypothesized that this gene knockout would enhance the recombinant protein yield. Additionally, the expression rates of recombinant human Factor VII (rhFVII) were compared using the pLEXSY vector and a modified vector containing the CMV-promoter.

Materials & Methods

I.L.L culture medium

The I.L.L strain was cultured routinely at the Cellular and Molecular Biology Research Center, Shahid Beheshti University of Medical Sciences, Iran between 2022-2023 at 26 °C in $RPMI_{1640}$ medium supplemented with 15% heat-inactivated FBS and 100 µg/mL of Pen-Strep. To maintain the cultures, they were passaged to fresh medium with 10-fold dilution every three days.

Construction, vectors, and donor

CPB13 and CPBR primers were designed by referencing the *CPB* gene sequence registered in tritrypdb.org under the accession number LtaPh_0808201. These primers were employed to amplify a specific segment of the *CPB* gene from the genomic DNA of I.L.L. The donor cassette was synthesized with DoBleCPF and DoBleCPR primers using the pSPble vector (Addgene ID: 63561) as the template. For the knockout of the *CPB* gene

with the CRISPR method, the pLdCN plasmid (Addgene ID: 84290) was employed. For the constitutive expression of hFVII, the pLEXSY-hyg2-FVII plasmid (19) was utilized. Additionally, the pKDB-cpc vector (11) served as the foundation for the pF7cmvneo vector**.**

Generation of CPB-bleomycin-fragment

The CPB-bleomycin targeting fragment was employed for *CPB* knockout. This fragment was generated through overlapping PCR in two steps: (i) Three separate PCR amplifications were performed: one for the 302-bp CPB 5'-flanking sequence using primers CPB13 and R1CPBle, another for the 584-bp bleomycin-expression-cassette with primers donor249F and donor249R, and a third for the 472-bp CPB 3'-flanking sequence with primers F2CPNNble and CPNNR. (ii) The PCR fragments obtained in step (i) were pooled and employed as templates for a conclusive SOEing PCR reaction utilizing CPB13 and CPNNR primers. This yielded a specific 1278-bp CPB-bleomycin-fragment (Fig. 1).

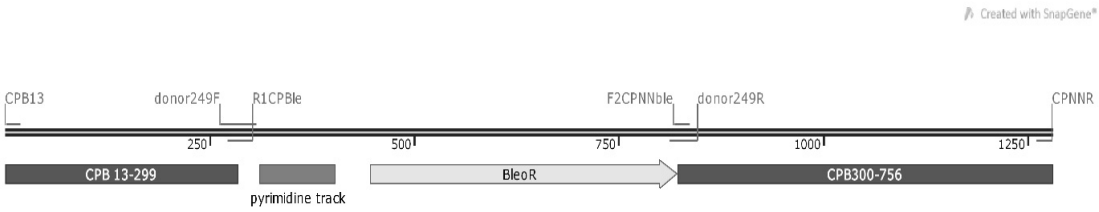

Fig. 1: The construction of the CPB-bleomycin-targeting-fragment. Bleomycin-Expression-Cassette contains the bleomycin-resistance-gene along with a pyrimidine track sequence and two homology arms

gRNA and primer design and synthesis

We designed gRNA utilizing the CRISPOR online tool (http://crispor.tefor.net/). Then, the gRNA was cloned into the pLdCN vector. For designing PCR primers, Oligo 7 primer analysis software and SnapGene® software (snapgene.com) were utilized which are listed in Table 1.

Table 1: Sequences of all primers and gRNA.

Name	*Sequences (5'to3')*
CPB13	AGGGTCCTTCTTTGCGTTG
CPBR	CCAGCTCGCACCCCACGAA
R1CPBle	CGATCCGATGAAGATCTCAGGTAGCGCGCGG
donor249F	CGGAGTTCGCCGCGCGCTACCTGAGATCTTCATCGGATCGGGTAC
donor249R	CGCCTCTGTGAAGTACGCGGCGCCGTCAGTCCTGCTCCTCGG
F2CPNNble	CTGACGGCGCCGCGTACTTC
CPNNR	AGCCATCTCGTCTTCGTTG
gRNA249F	TTGTGTTCGCCGCGCGCTACCTGAG
gRNA249R	AAACCTCAGGTAGCGCGCGGCGAAC
F7cmvF	AAAAAAGAATTCATGGTCTCCCAGGCCCTCAG
F7cmvR	TTTTTTCTCGAGGATAGAAATTTTGCATTTATAGG
LtaF1	GTCTTTTACCAGGTCTGTGCTGTGCC
DoBleCPF	AGGCGGAGTTCGCCGCGCGCTACCTATCTTCATCGGATCGGGTAC
DoBleCPR	CTTCGCCTCTGTGAAGTACGCGGCGTCAGTCCTGCTCCTCGG

pF7cmvneo plasmid construction

The pF7cmvneo plasmid was generated through the following steps: (i) A 1400-bp PCR fragment containing the hFVII expression sequences was obtained from the pLEXSY-hyg2-FVII plasmid using F7cmvF and F7cmvR primers, which incorporated *EcoRI* and *XhoI* restriction sites at the 5'-end. (ii) The PCR fragment from step (i) was digested with the *EcoRI* and *XhoI*, and the resulting fragment was purified. (iii) The purified fragment was subsequently ligated into the *EcoRI* and *XhoI* sites of the pKDB-cpc. The pKDB-cpc vector had been pre-digested with the same enzymes. This process resulted in the creation of the pF7cmvneo vector (Fig. 2).

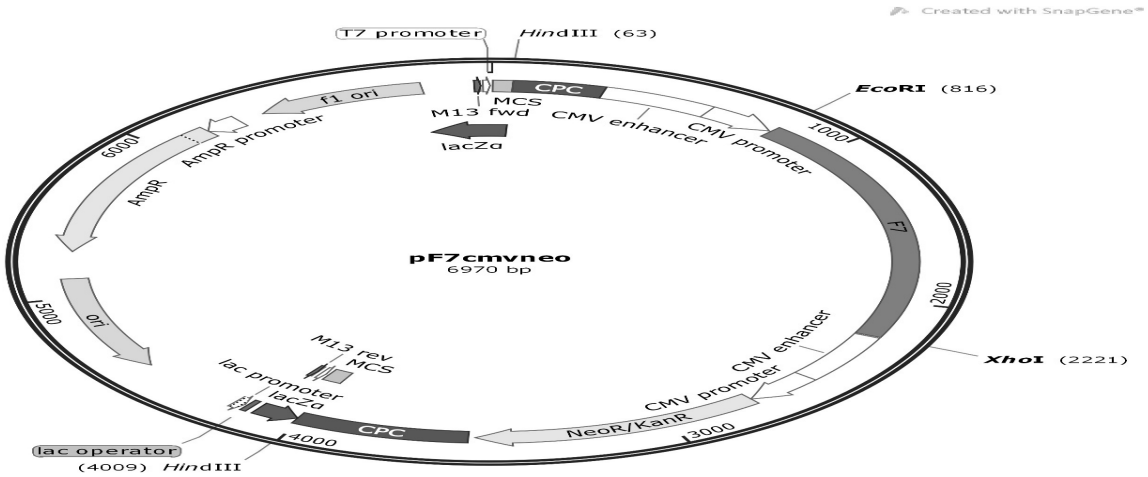

Fig. 2: The pF7cmvneo plasmid. The pF7cmvneo construct provides a platform for the expression of hFVII under the control of CMV-promoter

I.L.L transfection and selection

2×10^7 *Leishmania* promastigotes from the I.L.L strain in the middle log phase to the early stationary phase were used for each transfection. These cells were suspended in an electroporation buffer (20). For the CRISPR method, I.L.L was transfected with 10 µg of gRNA-cloned-pLdCN plasmid and 4 µg of purified donor cassette. Cell selection followed the previously described protocol (21). In the *CPB* gene replacement with the HR method, 4 µg of purified CPB-bleomycin PCR product was used for transfection. Additionally, we transfected the pLEXSY-hyg2-FVII construct following established method (19). The pF7cmvneo plasmid was digested with *HindIII*, and the pure expression cassette was used for transfection.

Quantifying antibiotic-resistance-rates in I.L.L

To quantify the G418 and bleomycin resistance-rates in I.L.L, the parasites were transfected with a gRNA-free-pLdCN vector and a pSPble vector separately. Initially, the cells were incubated in an antibiotic-free medium. Subsequently, the medium was changed, and the selection process commenced with 25 µg/mL of antibiotics. To impose stringent-selection-conditions, the antibiotic-concentration was incrementally increased over the course of a week. The parasite cells demonstrated viability until reaching a concentration of 100 µg/mL of G418 in pLdCN-transfected cells and 125 µg/mL of bleomycin in pSPble-transfected cells.

Expression and purification of rFVII

After selecting positive transfected cells, 1.8×10^8 cells were cultured in serum-free medium for 24 hours. The pellet from a 1 mL shake flask was obtained and then resuspended in lysis buffer (Tris-HCl 50mM, SDS 1%, NaCl 50mM, EDTA 50mM, pH 8) and sonicated twice for 20 seconds. Cell debris was separated from proteins by centrifuging. The proteins were subsequently precipitated with acetone and stored at -20°C overnight, followed by centrifugation.

Determination of rFVII concentration

After extracting whole proteins, we assessed the retained coagulation activity of the FVII light chain peptide and its quantity using the Factor VII Human Chromogenic Activity Kit (Abcam, ab108830). We followed the manufacturer's instructions for the test, measuring the absorbance at 405 nm. A standard curve was constructed with standard concentrations on the x-axis and the change in absorbance per minute (ΔA/min) on the y-axis. The best-fit line was determined through regression analysis and employed to determine the concentration of the unknown sample (Fig. 3).

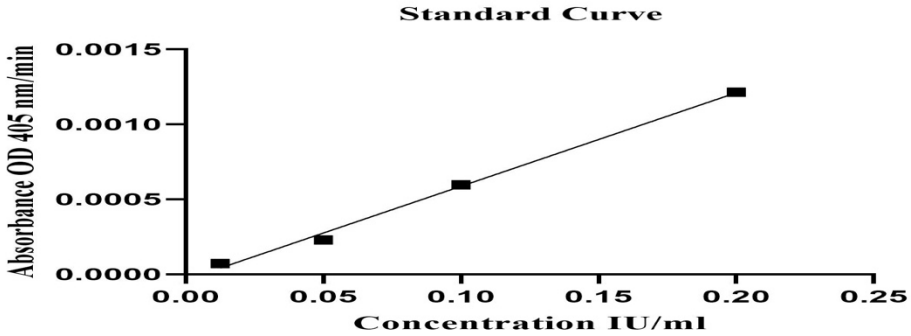

Fig. 3: rhFVII standard curve. GraphPad Prism 9.0.0 Software was used to generate the standard curve of rFVIIa. The R^2 value was determined 0.9959

Protein purification and expression analysis

Whole proteins were diluted in a wash buffer (Tris 20mM, NaCl 100mM, CaCl2 10mM) and applied to an affinity column containing specific factor VII polyclonal antibodies coupled to CNBr-activated sepharose 4B resin. rhFVII bound to the column antibody in the presence of calcium ions and unbound by omitting calcium ions. rhFVII was eluted using an elution buffer (Tris 20mM, NaCl 100mM, EDTA 20mM). Acetone precipitation was performed, and the precipitated product was resuspended and denatured by adding NuPAGE LDS Sample Buffer (Thermo Fisher Scientific) and boiling for 5 minutes at 95°C. The samples were then run-on gel electrophoresis using an ExpressPlus™ 4-20% Bis/Tris SDS-polyacrylamide gel (GenScript) and MES/SDS-running buffer (Invitrogen). After gel electrophoresis, a wet transfer to a polyvinylidene difluoride membrane was performed. rhFVII was visualized by incubating in PonceauS staining solution. The membrane was scanned with an Amersham ImageQuant™ 800, and the densitometric measurement of rhFVII yield was determined using ImageJ.1.8.0_172 software.

Results

The CPB gene sequence

Sequencing of this partial *CPB* gene fragment was performed and aligned to the reference sequence (LtaPh_0808201). The results substantiated the existence of six mutations in the I.L.L *CPB* gene (C492T, C508T, T534G,

G805T, G816A, G823A), among which only two induced amino acid changes (A269S, G275S). This finding was subsequently validated through the sequencing of five additional amplified fragments. The sequence of the I.L.L *CPB* gene was deposited in the Gene Bank database under the accession number MZ018026.

Generation of CPB knockout cells

For generating *CPB* knockout cells using the HR method, the I.L.L was transfected with the CPB-bleomycin-fragment. Cells were selected by increasing the bleomycin concentration to 125 µg/mL for a week. No cells survived at this dose.

To generate *CPB* knockout cells with the CRISPR method, I.L.L were transfected with the gRNA-cloned-pLdCN. Cells were selected by increasing the G418 concentration to 100 µg/mL for a week. No cells survived at this dose. Then, I.L.L cells were transfected with the gRNA-cloned-pLdCN and the donor fragment at the same time, and Cells were selected by increasing the bleomycin concentration to 125 µg/mL and the G418 concentration to 100 µg/mL for a week. No cells survived at these doses in this experiment too.

In different experiments, electroporated parasites with the CPB-bleomycin targeting fragment and the gRNA-cloned-pLdCN, either alone or with the donor fragment, were allowed to grow in an antibiotic-free medium. After 2 weeks, genome amplification using CPB13 and CPBR primers showed the same pattern in PCR products as untransfected parasites.

Gene expression potency of pLEXSY and pF7cmvneo vectors

The concentration of rhFVII expressed by pLEXSY-hyg2-FVII-transfected cells was 126 ng/mL, while pF7cmvneo-transfected cells expressed rhFVII at a concentration of 60 ng/mL. Purified rhFVII from transfected I.L.L was detected on a gel (Fig. 4). The protein expression yield in pLEXSY-hyg2-FVII-transfected cells was compared to pF7cmvneo-transfected cells using ImageJ.1.8.0_172 software, revealing that I.L.L transfected with pLEXSY-hyg2-FVII expressed rhFVII 1.5 times more than pF7cmvneo-transfected cells.

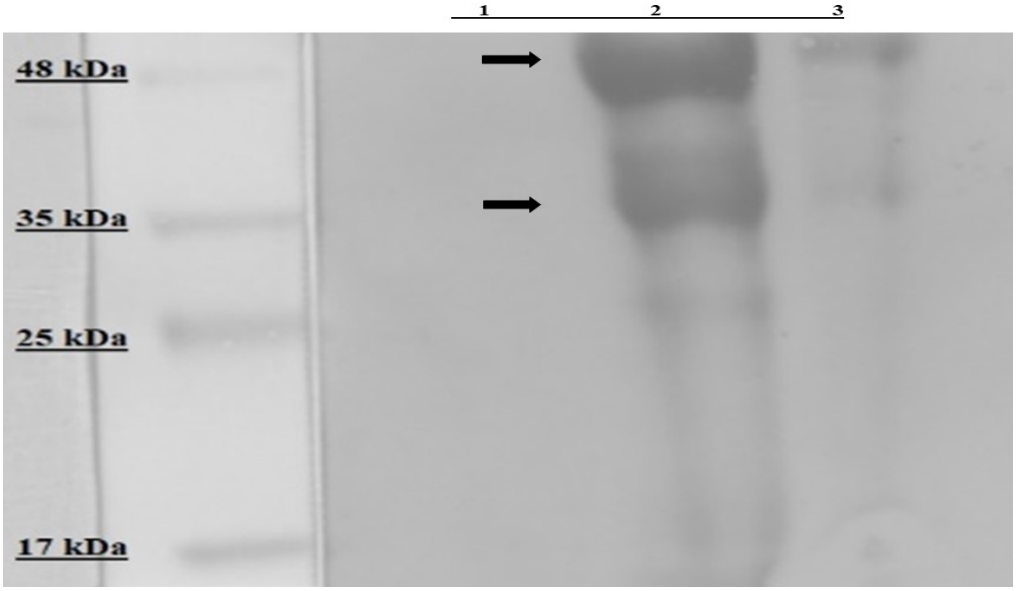

Fig. 4: ~50 kDa rhFVII electrophoresis gel. Lane 1, the purified sample from the non-transfected I.L.L; Lane 2, the purified rhFVII from the pLEXSY-hyg2-FVII-transfected I.L.L. One of the activated forms of rhFVII is detectable; Lane 3, the purified rhFVII from the pF7cmvneo-transfected I.L.L

Discussion

Generating CPB gene knockout

Cysteine proteases play diverse roles in parasites, encompassing functions in differentiation, nutrition, and the host cell infection process (22). Pathogenic *Leishmania* manifests a multicopy presence of the *CPB* gene, in contrast to the single-copy status of *CPA* and *CPC* genes (23). The I.L.L *CPB* gene was amplified and sequenced. Six variations in the sequence were identified when compared to the reference sequence. Preliminary analysis, based on the similarity between the original and modified amino acids, suggested that these changes may not alter protein function. Nevertheless, additional experiments are warranted to conclusively determine the impact of these variations on protein functionality.

The HR method has been employed for gene deletion in *Leishmania* since the 1990s (24). In the HR method, the success rate of recombination is directly proportional to the length of homology arms (25). In a previous study, Donyavi et al. used homology arms of 229 and 417 bp to facilitate HR in I.L.L (11). In our research, two homology arms with lengths of 287 and 457 bp were designed to enhance HR within the *CPB* gene. To identify successfully recombined cells, we positioned the bleomycin sequence between these homology arms. The *bleomycin*-resistance-gene expression cassette included a pyrimidine-track upstream of the *bleomycin* gene, which is essential for the trans-splicing of the downstream gene (26).

Given that *Leishmania* is naturally considered diploid (27), confirming genome editing in all alleles typically requires at least two selection markers (28). In this study, the utilization of a single selection marker suggests that both alleles of the *CPB* gene were not completely deleted, resulting in the demise of I.L.L due to the deletion of just one allele and the subsequent reduction in the amount of CPB enzyme below the necessary threshold for cell survival. Previous studies have demonstrated that a reduction in the quantity of certain proteins, even without complete gene knockout, can lead to cell death. Examples include TbRRM1 (29) γ-Glutamylcysteine synthetase (γ-GCS) (30) in *T. brucei*, highlighting instances where cell death occurred due to gene knockdown.

Due to various challenges encountered during the utilization of the HR method, in recent years, the CRISPR method has emerged as a valuable alternative for targeting gene knockouts (31). In this investigation, the pLdCN vector was applied for generation gene knockout via the CRISPR method. Following the use of this method, no cells survived in a medium with the antibiotic.

The presence of CPB within the intracellular environment of *Leishmania* was documented (32), and its involvement in the regulation of other proteins was reported (33). Despite successful gene deletion in other strains (16), we encountered challenges in generating I.L.L cells with a *CPB* gene knockout. It's important to note that all those strains are pathogenic for mammalian cells, whereas I.L.L is nonpathogenic. Previous study indicated the absence of many virulence factors in *L. tarantolae* (34), and the presence of the *CPB* gene in the I.L.L strain might be due to its essential role in this particular strain. This suggests that the I.L.L strain may have lower copy numbers of the *CPB* gene than pathogenic *Leishmania*, and/or the amount of protein expressed by them is essential for the viability of the I.L.L strain. Therefore, unlike other strains, *I.L.L* cannot tolerate not only the complete deletion of CPB but also the removal of just one allele of CPB. However, confirming the essentiality of CPB in I.L.L may require the use of techniques such as Cre-LoxP or episomal vectors for expressing CPB.

rhFVII expression rate

In a previous study (19) rhFVII had been expressed in I.L.L cells using the pLEXSY-hyg2-FVII vector, resulting in concentrations of up to 10 µg/10 mL. In this experiment, we achieved a rhFVII yield of 126 ng/mL. The variation in rhFVII levels between this study and the previous one may be attributed to differences in the methods used to quantify recombinant protein production.

We also employed the CMV-promoter for rhFVII expression in the I.L.L and observed recombinant protein accumulation at concentrations of up to 60 ng/mL. Previously, rhIL-12 had been produced by this contract with a concentration of 246 pg/mL. These variations in protein expression can be attributed to differences in protein characteristics and the strategies used for quantification in each study. IL-12 is a two-subunit protein, and a signal peptide had been utilized for its secretion into extracellular. In contrast, rhFVII is a single subunit protein produced intracellularly.

The findings of this study suggest that the vector designed based on the CMV-promoter is less effective compared to the pLEXSY vector. The diminished expression observed with the CMV-promoter may be attributed to its regulation by highly controlled RNA Polymerase II (Pol II). The feedback loop between protein overexpression and RNA Pol II activity leads to the down-regulation of Pol II (35). Additionally, the lower expression rate of the CMV-promoter could be a consequence of epigenetic modifications, which might lead to reduce expression of the *CPC* gene and the target gene inserted into that specific genomic area. The epigenome serves as a major regulatory interface in the genomes and its role in the transcriptional regulation of *Trypanosoma* was observed (36). It is important to note that the pLEXSY vector integrates multiple recombinant genes and is mediated by non-feedback response RNA Polymerase I (35).

Consequently, we hypothesize that optimizing recombinant protein expression using the CMV promoter-based vector may involve implementing various strategies include utilizing different homology arms for integrating constructs into other genes in the genome such as *glycoprotein GP63*, which has multiple copies in *Leishmania* (37). Furthermore, numerous studies have emphasized the importance of translation efficiency and protein half-life in achieving higher expression rates (38, 39). Hence, incorporating the 3'-UTR and 5'-UTR sequences (40) of I.L.L genes with high expression levels on both sides of recombinant genes can enhance the stability of recombinant genes and promote their translation.

Conclusion

In this study, our primary objective was to perform knockouts of *CPB* to modify this strain and enhance recombinant protein production. Despite utilizing both the HR and CRISPR methods, generating a *CPB* knockout proved to be challenging, it suggests that the CPB plays an essential role in the I.L.L strain. Additionally, the pLEXSY vector outperformed the CMV-promoter-based vector in terms of expression yield.

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Conflicts of Interest

The authors declare no conflict of interest.

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