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Effect of human β-Globin second intron on transient gene expression in mammalian cell lines

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Gene therapy

Intron-Mediated Enhancement (IME)

Nonsense-Mediated Decay (NMD)

pVAX

ABSTRACT

Exogenous protein expression in mammalian cells is necessary to produce therapeutic proteins and modern medical applications like developing DNA vaccines and gene therapy. This study examines the human-Globin (hBG) second intron's capacity for intron-mediated enhancement (IME) in various mammalian cell lines. Our study's main aim is to investigate the effect of the incorporation and arrangement of the second intron of the human Beta-globin gene into the pVAX-1 expression cassette on improving the expression of foreign genes. Two plasmids were constructed, one with the hBG second intron positioned upstream and the other downstream in the expression cassette. EGFP expression was evaluated at the mRNA and protein levels after transfection using Lipofectamine 2000 using One-way ANOVA analysis. Results showed that the pVAX-1 harbouring the hBG second intron did not lead to enhanced transient EGFP expression and did not exhibit Intron Mediated Enhancement (IME) in tested mammalian cell lines. Further investigations are necessary to understand factors contributing to the lack of enhancement and explore alternative intron options for optimizing foreign gene expression in cell lines.

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1 Introduction

Mammalian cell lines are crucial for the manufacture of complicated biopharmaceuticals. Production in mammalian cells improves the quality and effectiveness of biologics, including antibodies, growth factors, and many more, by facilitating complex post-translational changes (Tan et al. 2021). Similarly, foreign gene expression in mammalian cells is critical in gene therapy and DNA vaccine development. An effective expression vector with appropriate regulatory elements is necessary to create a functional protein. Polyadenylation (Poly-A) signals, promoters, upstream enhancers, and terminators are common components of the expression cassette found in various expression systems to increase or optimize gene expression in mammalian cells (Barrett et al. 2012). The promoter sequence in the expression vector determines the expression efficiency of the protein of interest. Thus, to effectively express foreign genes at high levels, it is necessary first to develop an optimal promoter (Wang et al. 2017; Dou et al. 2021; Grose et al. 2021). In addition to an optimal promotor, noncoding enhancer sequences such as introns can boost transcript levels by influencing transcription rates, nuclear mRNA export, transcript stability, translational efficiency, and mRNA degradation, a phenomenon called Intron-Mediated Enhancement (IME)(Carron et al. 2021). Many commercial vectors include heterologous introns, which are known to dramatically increase exogenous gene expression depending on the gene type and cell line (Samadder et al. 2008; Bartlett et al. 2009; Gallegos and Rose 2015; Laxa 2017). Furthermore, studies have demonstrated that promoter-proximal introns can improve the initiation of transcription and the processivity of RNA polymerase II, thereby increasing pre-mRNA synthesis. Studies have also shown that for effective 3'-end cleavage and polyadenylation, the 3'-splice acceptor intronic region is necessary (Furger et al. 2002; Noe Gonzalez et al. 2021).

Previous research has shown that the hBG introns enhance foreign gene expression in various cell types (Kang et al., 2005; Haddad-Mashadrizeh et al., 2009; Pereverzev et al., 2014). In this study, we investigated whether the presence of the second intron of hBG in the expression cassette and its position within the cassette impacted its IME potential. Accordingly, we constructed mammalian expression plasmids based on pVAX-1 with hBG second intron and evaluated the transient expression in five cell lines viz., CHO-K1, HEK-293, HELA, NRK-52E, and NIH-3T3.

2 Materials and Method

2.1 Plasmid construction for transient expression

The hBG gene containing the second intron was amplified and sequenced using human genomic DNA as a template. The sequence-verified DNA was used as the template for cloning the hBG second

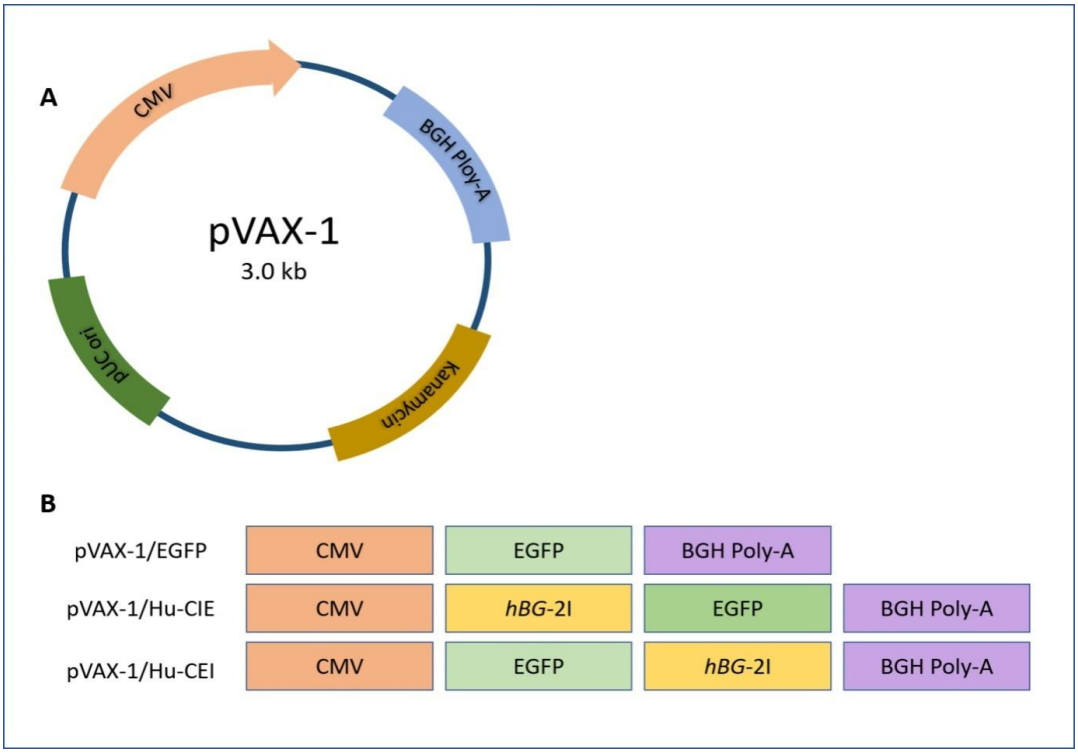

Figure 1 Plasmid pVAX-1/Hu-CIE and pVAX-1/Hu-CEI construction (A) A vector map of the plasmid pVAX-1; (B) A schematic illustration of an intron-cloned and intron-less plasmid; CMV - human cytomegalovirus immediate-early promoter; BGH Poly-A - bovine growth hormone polyadenylation signal; EGFP - enhanced green fluorescent protein; hBG-2I - hBG second intron

Table 1 Primer sets used for hBG second intron sequencing and plasmid construction

Gene	Primer	Sequence	Restriction site
hBG second intron	Forward	5'- TGAGTGAGCTGCACTGTG -3'	-
	Reverse	5'- TCTTTGCCAAAGTGATGG -3'	-
	Forward-1	5' -ATATGCTAGCTGAGTGAGCTG -3'	NheI
	Reverse-1	5'- ATATCTTAAGTCTTTGCCAAAGTG -3'	AflII
	Forward-2	5'- TATATGAATTCTGAGTGAGCTGC -3'	EcoRI
	Reverse-2	5'- TATATTCTAGATCTTTGCCAAAGTG -3'	XbaI
EGFP	Forward	5'- TATGAAGCTTATGGTGAGCAAGGG -3'	HindIII
	Reverse	5'- ATGCGGATCCTTACTTGTACAGCT -3'	BamHI

intron into the mammalian expression vector. The intron and intron-less pVAX-1-based expression plasmids were constructed based on our previously reported methodology (Vijayakumar et al. 2023). Briefly, the EGFP sequence with HindIII and BamHI restriction sites was PCR amplified and cloned into pVAX-1 to generate a control plasmid with no intron (pVAX-1/EGFP). Two plasmids with intron viz., pVAX-1/Hu-CIE and pVAX-1/Hu-CEI, were prepared by PCR amplifying and cloning hBG gene containing second intron with NheI and AflII restriction sites and EcoRI and XbaI restriction site respectively into pVAX-1/EGFP. A schematic diagram of the intron-less plasmid and hBG gene containing second intron positioning in the expression cassette of the expression vectors is shown in the Figure 1, and the primers used for intron sequencing and plasmid construction are given in Table 1.

2.2 Cell culture, Transfection

The cell lines utilized throughout this study were procured from NCCS Pune and were carefully maintained and cultured in a humid incubation environment at 37°C with 5% CO_2. The panel of cell lines encompassed HEK-293, HELA, NRK-52E, NIH-3T3, and CHO-K1 cells. Specifically, HEK-293, HELA, NRK-52E, and NIH-3T3 cells were cultured and maintained in Dulbecco's Modified Eagle Medium (DMEM), while CHO-K1 cells were cultured and maintained in the RPMI medium. These culture media were supplemented with 10% foetal bovine serum (FBS) and 1X Penicillin-Streptomycin (Pen-Step) antibiotics to support optimal cell growth and viability. A day before transfection, the appropriate amount of exponentially growing cells were seeded in the complete media in 6-well plates. During the transfection process, the entire media was removed, and the cells were subsequently rinsed with 1X phosphate-buffered saline (PBS). The transient transfection of cell lines was conducted using Lipofectamine 2000 (ThermoFisher). The transfection involved using pVAX-1/EGFP (control plasmid) or pVAX-1/Hu-CIE or pVAX-1/Hu-CEI plasmids at a ratio of 1:2 (plasmid to Lipofectamine), following the instructions provided by the manufacturer. The transfection experiments were conducted in triplicate to ensure the accuracy of the results.

2.3 Fluorescent Microscopy and RT-PCR

For qualitative analysis, the Olympus CKX53 inverted fluorescent microscope was used to observe green fluorescence, and RTq-PCR was used for quantitative analysis to determine the presence of EGFP in cells transfected with the plasmid. TRIZOL was used to

Table 2 Real-time PCR primers used for relative quantification of EGFP mRNA transcript

Host system	Gene	Primer	Sequence	NCBI Accession number
-	EGFP	Sense	5'- AAGCTGACCCTGAAGTTCATCTGC -3'	-
		Antisense	5'- CTTGTAGTTGCCGTCGTCCTTGAA -3'	
Human	GAPDH	Sense	5'- GTCTCCTCTGACTTCAACAGCG -3'	NM_001289745.3
		Antisense	5'- ACCACCCTGTTGCTGTAGCCAA -3'	
Mouse	GAPDH	Sense	5'- TTCACCACCATGGAGAAGGC -3'	BC023196.2
		Antisense	5'- GGCATGGACTGTGGTCATGA -3'	
Rat	GAPDH	Sense	5'- TGGGGCTGGCATTGCTCTTA -3'	XM_032885257
		Antisense	5'- CTGGGTGGTCCAGGGTTTCT -3'	
Chinese Hamster	GAPDH	Sense	5'- GAAAGCTGTGGCGTGATGG -3'	NM_001244854.2
		Antisense	5'- CATACTTGGCAGGTTTCTCCAG -3'	

extract the total RNA, and Takara's PrimeScript™ RT Master Mix was then used to quantify and convert the extracted total RNA to cDNA. Utilizing Takara's TB Green® Premix Ex TaqTM II (Tli RNase H Plus) following the manufacturer's instructions, RTq-PCR was performed on a Himedia InstaQ48m real-time PCR instrument. The relative mRNA expression level of EGFP was quantified using the GAPDH gene as an internal control (Table 2), and the calculation of the fold change was performed using the $2^{-\Delta\Delta Ct}$ method. Briefly, 2µl of 50ng/µl cDNA, 10µl of 2X Master mix, 0.8µl of 10mM primers (0.4µM each), and 6.4µl of PCR grade water was added to a final volume of 20µl. PCR conditions for EGFP expression were as follows: an initial denaturation step at 95°C for 30 seconds, followed by denaturation at 95°C for 5 seconds, and subsequent annealing/extension at 60°C for 35 seconds. This cycle was repeated for a total of 40 times. Subsequently, a melt curve analysis was performed to assess the PCR amplification's efficacy.

2.4 Western Blot

To gain further insights into the EGFP expression at the protein level, western blot analysis was performed on HEK-293 cells. The following western blot protocol was used for this study (Subbarayan et al. 2018). Briefly, the protocol entailed trypsinizing the cells and then centrifuging them for 10 minutes at 500 x g at 4 °C. The resulting cell pellet was re-suspended in 400 µl of ice-cold lysis buffer. 4 µl of a mixture of protease inhibitors were added. The remaining proteins were carefully isolated after further incubation and centrifugation, and the total protein content was assessed using a bicinchoninate test. A total of 30 g of protein extract was resolved using sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) gel electrophoresis, followed by subsequent transfer onto nitrocellulose membranes. Each membrane was subjected to incubation with primary anti-EGFP antibody [F56-6A1.2.3] (Abcam, #ab184601) and anti-GAPDH antibody [6C5] with the Loading Control (Abcam, #ab8245) at a 1:1000 dilution, followed by goat anti-mouse IgG H+L secondary antibody (ThermoFisher #31430) at a 1:5000 dilution.

2.5 Statistical analysis

All experimental data of biological triplicates were represented as the mean and standard deviation from the three experiments. Statistical significance between groups was calculated using One-way ANOVA analysis using GraphPad Prism (9.0) (**** $p < 0.001$).

3 Results and Discussions

3.1 Influence of human β-Globin second intron in transgene expression

Two different sets of plasmids were created to examine the effects of the hBG second intron on transgene expression in mammalian cell lines. The intron was positioned upstream in the expression cassette of the first plasmid, pVAX-1/Hu-CIE, between the CMV promoter and the EGFP sequence and downstream in the expression cassette of the second plasmid, pVAX-1/Hu-CEI, between the EGFP sequence and the BHG Poly A-tail. The pVAX-1/EGFP plasmid without an intron was used as the control plasmid for comparison. Comparing cells transfected with pVAX-1/Hu-CIE to those transfected with pVAX-1/EGFP, there was no discernible difference in the levels of EGFP transcripts. Concerning the location of the intron inside the expression cassette, CHO-K1, HEK-293, and NRK-52E cells transfected with pVAX-1/Hu-CEI as opposed to pVAX-1/EGFP showed a significant reduction in EGFP transcript levels. In a similar vein, CHO-K1 and NRK-52E cells transfected with pVAX-1/Hu-CEI showed noticeably lower levels of EGFP transcripts than pVAX-1/Hu-CIE. Between pVAX-1/Hu-CEI and the intron-less control plasmid pVAX-1/EGFP, however, there was no appreciable difference in the expression of EGFP between HELA and NIH-3T3 cells (Figures 2 and 3).

Furthermore, Western blot analysis extended the validation of the observed expression pattern to the proteome level. Remarkably, the Western blot findings for all HEK-293 cells subjected to transfection exhibited concordance with their corresponding RTq-PCR outcomes, affirming the direct correspondence between protein expression and mRNA levels. In the pVAX-1/Hu-CIE group, EGFP expression was not significantly altered compared to the control group with the intron-less plasmid pVAX-1/EGFP. However, the EGFP expression was substantially lower in the pVAX-1/Hu-CEI group compared to the control intron-less group and the pVAX-1/Hu-CIE group (Figure 4).

It is well known that introns can affect heterologous gene expression in many ways, including transcription rate, mRNA export, polyadenylation, and mRNA decay (Bonnet and Palancade 2015; Misra and Green 2016; Schlautmann and Gehring 2020; Watts et al. 2021). Kang et al. (2005) have shown enhanced murine cytomegalovirus (MCMV) promotor activity with hBG second intron and stronger expression when compared to the human cytomegalovirus (HCMV) promoter (Kang et al. 2005). Similarly, Haddad-Mashadrizeh et al. (2009) have reported a synergistic effect of hBG second intron and Kozak sequence on the expression level of human factor IX (hFIX) in CHO cells (Haddad-Mashadrizeh et al. 2009). However, we discovered no substantial enhancement in exogenous EGFP expression by the hBG second intron in all cells in our current investigation (Figure 2-4).

Regarding intron positioning in the expression cassette, multiple studies have reported increased gene expression by inserting an intron between the gene's promoter and the gene of interest, and no enhancement in gene expression or even decreased gene expression at the 3' UTR (Furger et al. 2002; Agarwal and Ansari

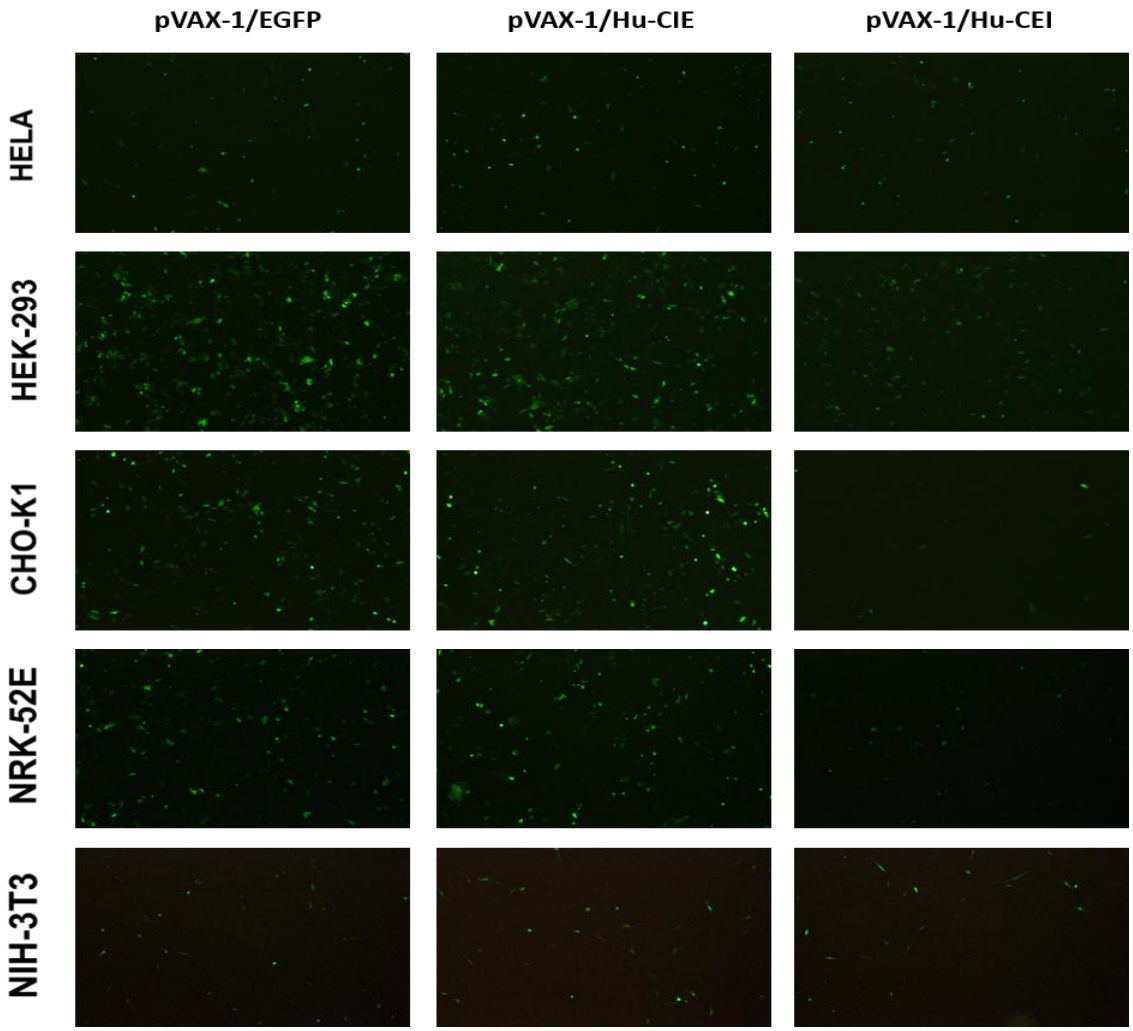

Figure 2 Expression of EGFP in CHO-K1, HEK-293, HELA, NRK-52E, and NIH-3T3 cells transfected with pVAX-1/E or pVAX-1/Hu-CIE or pVAX-1/Hu-CEI plasmid.

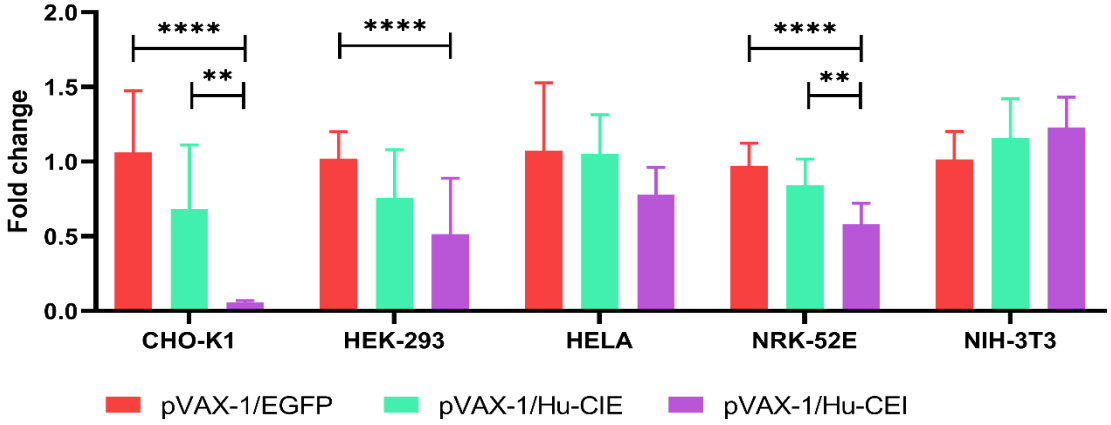

Figure 3 One-Way ANOVA analysis of transient EGFP expression in CHO-K1, HEK-293, HELA, NRK-52E, and NIH-3T3 cells transfected with pVAX-1/E or pVAX-1/Hu-CIE or pVAX-1/Hu-CEI using GraphPad Prism (9.0)(**** $p < 0.001$).

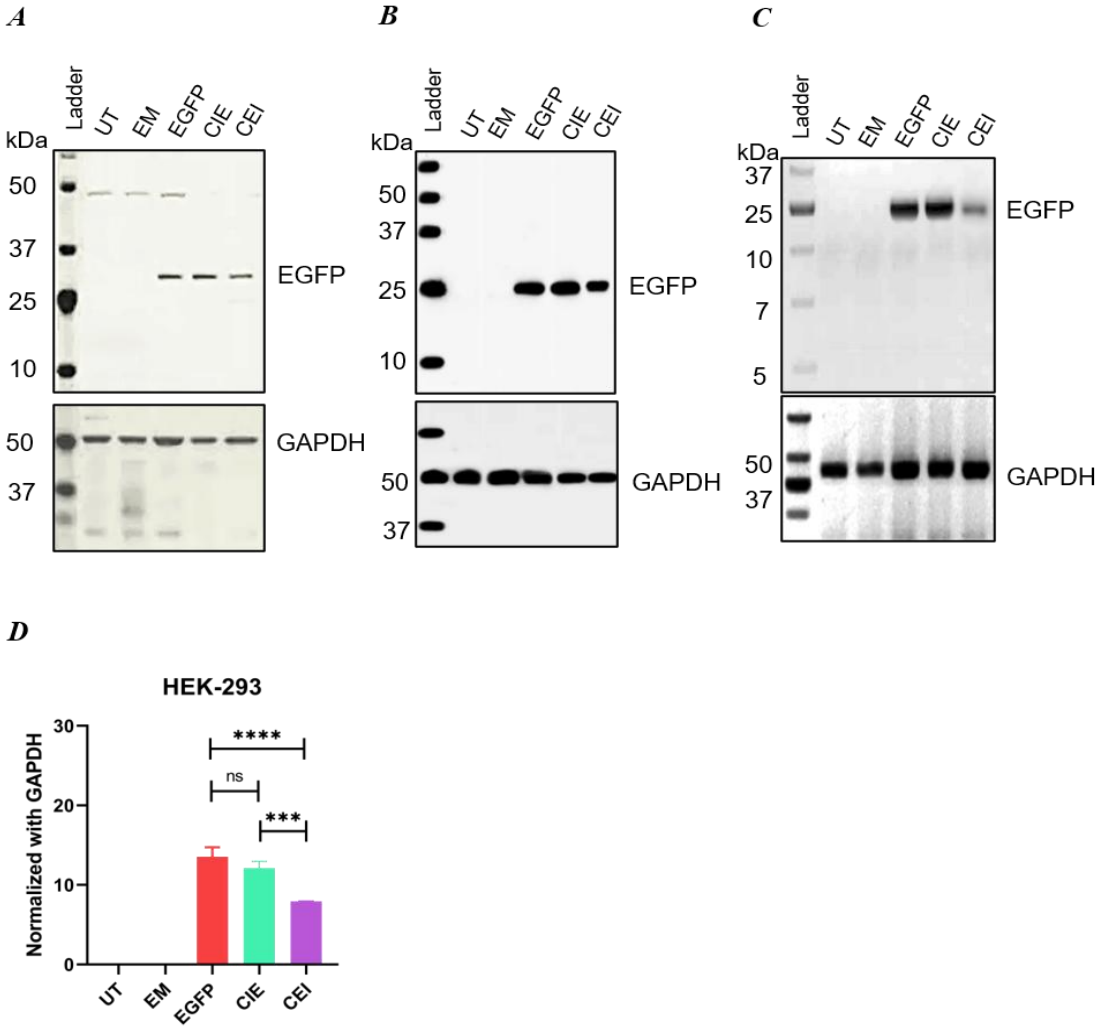

Figure 4 Western Blot confirmation of transient expression of EGFP in HEK-293 (A) First set; (B) Second set; (C) Third set; UT - Untreated; EM - Empty vector; EGFP, pVAX-1/EGFP; CIE - pVAX-1/Hu-CIE; CEI - pVAX-1/Hu-CEI, and (D) One-Way ANOVA analysis of transient EGFP expression in HEK-293 followed by the Bonferroni multiple comparison test using GraphPad Prism (**** $p < 0.001$).

2016). Consistent with previous studies, our results demonstrated similar patterns in EGFP expression in cells transfected with the plasmid carrying the hBG second intron located downstream within the expression cassette (Figures 2 and 3). A plausible rationale behind this expression discrepancy might be the increased distance between the transcription start site and the recognition sites of U1 snRNA within the intron. When the intron is positioned distantly from the transcription start site, the association between the human Transcription Factor II (TFIIH), U1 snRNA, and RNA Polymerase II (Pol-II) may not occur efficiently, leading to a compromised transcription re-initiation efficiency (Bieberstein et al. 2012; Almada et al. 2013; Engreitz et al. 2014).

Another possible explanation could be the rapid degradation of mRNA by Nonsense-Mediated Decay (NMD). The impact of introns on nuclear export and nonsense-mediated mRNA decay (NMD) has been ascribed to the interaction between ribosomes and the Exon Junction Protein Complex (EJC). If a premature termination codon is detected, the EJC binds to mRNA upstream at the exon-exon junction and may enter the nucleus and change cytoplasmic mRNA metabolism by NMD (Le Hir et al. 2000; Schlautmann and Gehring 2020; Lejeune 2022). However, Pereverzev et al. (2014) showed that the hBG second intron in 3' UTR of the expression cassette can improve exogenous GFP expression by 1.8-fold, contradicting our findings and earlier studies. According to the research, the enhanced expression was due to a shorter distance between termination codons and exon junctions of less than 50 nucleotides, which reduced the risk of mRNA decay via NMD (Pereverzev et al. 2014).

Conclusion

Including introns in gene expression constructs could affect the expression of the target gene, depending on the specific intron sequence and placement, particularly when located near the 5' UTR in the expression cassette between the promotor and the gene. We believe that selecting the optimal promoter, intron, gene, and intron position, along with other factors, including chromosomal position, physiological conditions, and cell type, can enable introns to enhance gene expression. When introns are utilized, cautious vector design is required because cryptic splicing signals have the potential to result in abnormal processing of the mRNA transcript, which can lead to decreased translation levels and inadequate or defective protein products.

Author's contribution

Shakila Harshavardhan and Kevin Kumar Vijayakumar conceived and planned the study. Kevin Kumar Vijayakumar and Humera Khathun Abdul Hameed did the experiments and data analysis. The paper was written and proofread by Shakila Harshavardhan and Kevin Kumar Vijayakumar.

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Conflict of interest

The authors did not declare any possible conflict of interest.

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