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Research article

Synthetic enhancers including TFREs improve transgene expression in CHO cells

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ABSTRACT

The human cytomegalovirus major immediate early gene (CMV) promoter is currently the most preferred promoter for recombinant therapeutic proteins (RTPs) production in CHO cells. To enhance the production of RTPs, five synthetic enhancers including multiple transcription factor regulatory elements (TFREs) were evaluated to enhance recombinant protein level in transient and stably transfected CHO cells. Compared with the control, four elements can enhance the report genes expression under both two transfected states. Further, the function of these four enhancers on human serum albumin (HSA) were investigated. We found that the transient expression can increase by up to 1.5 times, and the stably expression can maximum increase by up to 2.14 times. The enhancement of transgene expression was caused by the boost of their corresponding mRNA levels. Transcriptomics analysis was performed and found that transcriptional activation and cell cycle regulation genes were involved. In conclusion, optimization of enhancers in the CMV promoter could increase the production yield of transgene in transfected CHO cells, which has significance for developing high-yield CHO cell expression system.

1. Introduction

In the last 30 years, the good efficacy of recombinant therapeutic proteins (RTPs) in clinical trials has driven the sustained and rapid growth of recombinant monoclonal antibodies (mAbs) worldwide [1,2]. RTPs have been produced in different expression systems. Since post-translational modifications (PTMs) are directly related to the efficacy and immunogenicity of RTPs, the expression system capable of performing PTMs are the suitable hosts for biopharmaceutical production. Chinese hamster ovary (CHO) cells are currently the most common used system for the yield of RTPs due to their ability for executing appropriate protein folding and assembly, human-like PTMs, stable integration of transgenes, and high-density suspension cultures [3–8].

To obtain high-yield production systems, innovative approaches are needed to overcome some of the disadvantages of CHO, such as

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low cell productivity, restricted growth, unstable expression, and high production costs [8,9]. To satisfy the growing market requirement of biopharmaceuticals, the approaches such as gene modification, process optimization, expression vector and cell line modification are necessary to enable higher production capacity, higher product quality, and lower production costs [10–12]. Among of them, the modification of expression vector is a positive method to improve the yield of recombinant proteins, and expression vector cassette engineering and using chromatin-modifying elements can enhance the production level and stability of genes of interest (GOI) [13–17].

As the first procedure during gene expression, transcription is particularly important to improve transcription efficiency by constructing efficient expression vectors [18]. The strong human cytomegalovirus major immediate early gene (CMV) promoter is widely applied to express the GOI in CHO cell system. However, CMV promoter is inherently prone to silencing, leading to drop in yield during long-time expression [19–24]. The CMV promoter mainly consists of an upstream regulatory sequence (URS), CMV enhancer, core polymerase II promoter, first exon, and intron A [25,26]. However, cytosine methylation within CpG islands makes them susceptible to epigenetic silencing, leading to reduce productivity during long-term culture [27–39]. In addition, viral promoters exhibit cell cycle dependence and induce cellular stress responses [30]. To fundamentally overcome the shortcomings of present applied CMV promoters, synthetic promoters are an attractive alternative because they allow the design of predictable recombinant gene transcripts, removing unclear and uncontrollable elements from expression vectors [31,32]. CMV enhancer elements consist of many transcription factor regulatory elements (TFREs). Previous studies have shown that robust transcription can be driven by repetitive arrays of multiple TFREs [33]. Here, we combined different TFREs to synthesize five different enhancer elements, and replaced the naturally existing enhancer of the CMV promoter, further evaluate their effects on **GOI** expression in CHO cells.

2. Material and methods

2.1. Vector construction

According to the original pEGFP and pSEAP (Fig. 1A), the unique region sequence at the 5′ end of the CMV promoter was first deleted to form the truncated promoter vectors pEGFP-S1 and pSEAP-S1 (Fig. 1B). Five synthetic enhancers E1, E2, E3, E4, and E5, consisting of different TFREs (Fig. S1), replaced the CMV enhancers in pEGFP-S1 and pSEAP-S1 vectors, to produce pEGFP-E1, pEGFP-E2, pEGFP-E3, pEGFP-E4, pEGFP-E5 and pSEAP-E1, pSEAP-E2, pSEAP-E3, pSEAP-E4, pSEAP-E5 expression vector, respectively (Fig. 1C). According to the results of the reporter gene, the more effective synthetic enhancers were selected and further used to express human serum albumin (HSA) to form the new expression vectors pHSA-E1, pHSA-E2 pHSA-E4, pHSA-E5, respectively (Fig. 1D). The vector constructs were completed by General Biosystems Biological Co., Ltd.

2.2. Cell culture and transfection

CHO–S cells from Life Technologies (#A11557-01; Carls-bad, CA, USA) were cultured in DMEM/12 (ProteinEasy Biological Co., Ltd., Xinxiang, China) medium supplemented with 10% FBS and 1% penicillin and streptomycin at 37 °C, 5% CO_2. Cells were seeded at 4×10^5 cells/mL in 12-well plates the day pre-transfection, and vectors were transfected using Lipo 2000 Transfection Reagent (Biosharp, China) according to the manufacturer's instructions. At 48 h after transfection, the cells were suspension cultured at 5×10^5 cells/mL using CHO Pro Exp® serum-free medium (SFM) (ProteinEasy Biological Co., Ltd., Xinxiang, China). The viable cell density (VCD) and viability were detected every day using cell counting instrument (Countstar, China). When the cell viability decreased below 50%, the same volume of supernatant was harvested to assay the expression of GOI. For EGFP production, the same number cells were harvested for test.

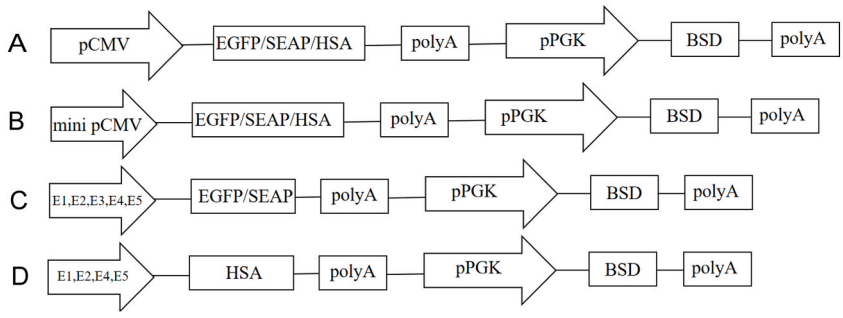

Fig. 1. Schematic representation of the different expression vectors. A: The original pEGFP, pSEAP, pHSA vector. B: Truncated promoter vectors. C: Expression vectors containing different synthetic enhancers for expression of reporter genes: D: Expression vectors containing different synthetic enhancers for expression of human serum albumin.
pCMV: CMV promoter; mini CMV: Truncated CMV promoter; E1, E2, E3, E4, E5 are different synthetic enhancers.

2.3. Analysis of EGFP expression

At 48 h after transfection, the fluorescence intensity was evaluated. The mean fluorescence intensity (MFI) of EGFP was assessed by flow cytometry when the cell viability decreased below 50% under suspension culture state.

2.4. Analysis of SEAP expression

The samples were collected and the cell supernatant was harvested after centrifugation at 1000 rpm for 5 min. Following, to discard cell debris and impurities, the cells supernatant was centrifuged at 13,000 rpm for 10 min at 4 °C. Samples were assayed according to the steps of the Alkaline Phosphatase Assay Kit (Beyotime, Shanghai, China). The absorbance value of each sample was detected at 450 nm and the data was processed and analyzed according to the instructions.

2.5. Construction of stable transfected cell pools

After 48 h transfection, CHO cells were screened with 10 μg/ml blasticidin (BSD) for 5–7 days, un-transfected cells were eliminated. Then the cells incubated continuously for 10 days in a medium containing 8 μg/ml BSD. During this time, the medium was changed or passaged every 2–3 days until stable cell pools were produced. Then, the cells were cultured in suspension at 5×10^5 cells/mL using CHO Pro Exp® serum-free medium (ProteinEasy Biological Co., Ltd., Xinxiang, China). The VCD and viability were measured every day. When the cell viability decreased below 50%, the same volume of supernatant was collected for protein expression analysis.

2.6. Western blot

The EGFP transfected cell were collected in the same number, and supernatants of HSA were collected in the same volume. EGFP and HSA were electrophoresed on 12% SDS–polyacrylamide gel on 8% SDS–polyacrylamide gels. The protein gel was transferred to a polyvinylidene fluoride membrane, blocked with 5% skimmed milk for 1.5 h at room temperature and washed 3 times with TBST. EGFP was incubated with EGFP antibody (BGI Genomics Co., Ltd., Shenzhen, China) at a dilution of 1:2000 and human serum albumin was incubated with His antibody (BGI Genomics Co., Ltd., Shenzhen, China) at a dilution of 1:3000. TBST washed 3 times and then incubated with a 1:2000 dilution of goat anti-mouse secondary antibody (Affinity Biosciences, Cincinnati, Ohio, USA) for 1.5 h at room temperature. Densitometric analysis was performed using ImageJ software.

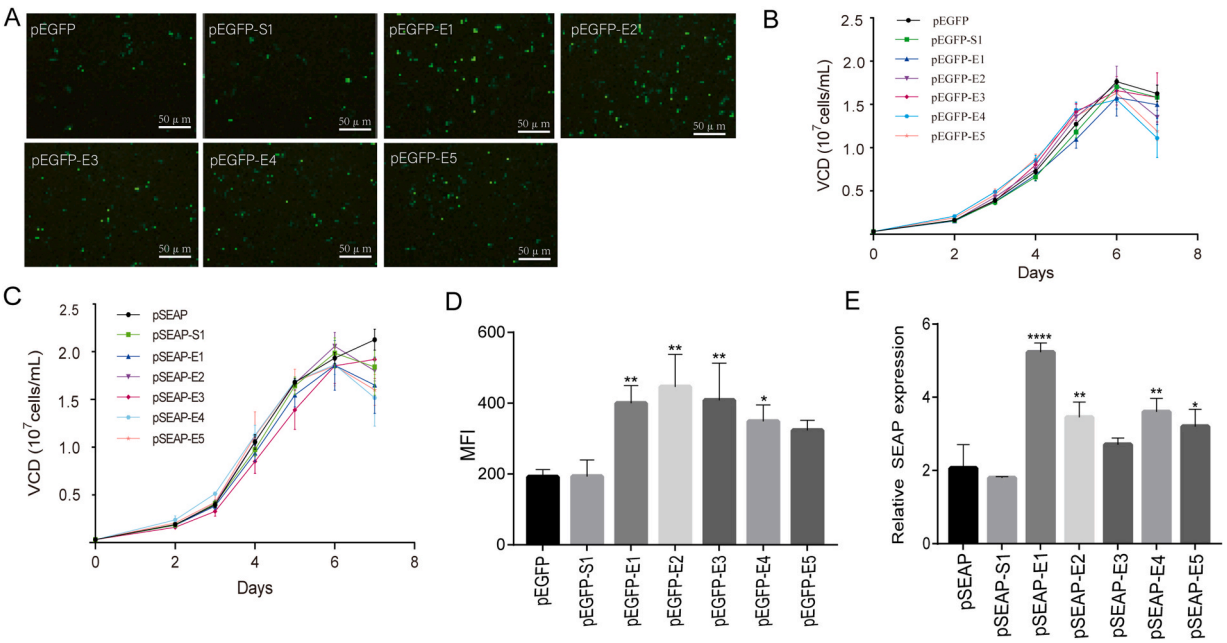

Fig. 2. Effect of different synthetic enhancers on the transient expression of reporter genes. A: The fluorescence microscopy images at 48 h post-transfection. B: Viable cell density of EGFP in CHO cells. C: Viable cell density of SEAP in CHO cells. D: Median fluorescence intensity of EGFP. E: Transient expression of SEAP.
Compared to control, $^*P < 0.05$, $^{**}P < 0.005$. $^{***}P < 0.0001$.

2.7. Analysis of relative mRNA levels

The relative mRNA levels were determined using real-time quantitative PCR (qRT-PCR) as described previously [24]. Total RNAs were extracted from 5×10^6 cells using the Gentra Puregene Cell Kit (Qiagen, Hilden, Germany) and the RNAqueous-4PCR kit (Ambion, Austin, TX), respectively. β-Actin were used to normalize the variation in input amount and quality of RNA, respectively.

2.8. Transcriptomics analysis

Three samples from pHSA-E4 high expression cell line and the control cell line were taken, and all samples were subjected to transcriptome sequencing by Shanghai biotechnology corporation. Raw read from sequencing was performed quality control and filtered reads containing adapters, reads containing N with a ratio greater than 10%, and reads with low quality (Qphred $\leq$ 20 and base numbers accounting for more than 50% of the entire read length) to obtain high-quality clean data. The transcript level FPKM expression matrix was obtained through the HISAT2 analysis process, and edgeR differential analysis was carried out using web tools (https://www.omicshare.com/tools/Home/Soft/Getsoft/search/edgR). The different expressed genes were screened [false discovery rate less than or equal to 0.05, absolute value of log 2 (fold-change) greater than or equal to 1, differences set to 2, and dispersion coefficient set to 0.01]. GO differential genes were performed GO (https://www.omicshare.com/tools/Home/Soft/Gogseasoner) and KEGG.

(https://www.omicshare.com/Tools/Home/Soft/pathwaygsesensor)enrichment analysis, selecting *P*-value for plotting.

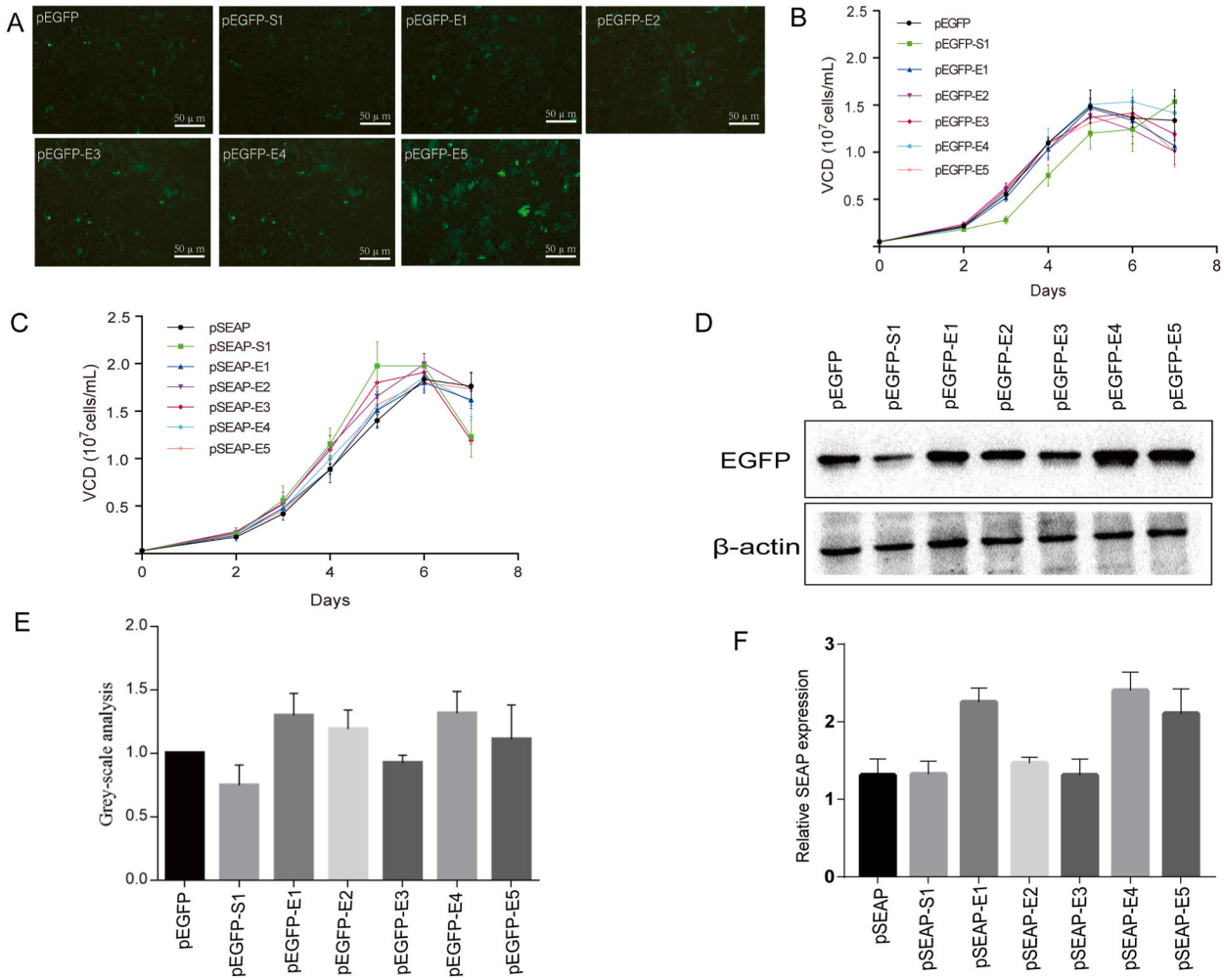

Fig. 3. Effect of different synthetic enhancers on the stable expression of reporter genes. A: Fluorescence microscope of a stable cell pool. B: Viable cell density of EGFP stable expression. C:Viable cell density of SEAP stable expression. D: Western blot results. E: Grey-scale analysis of EGFP Western blot results. F: Stable expression of SEAP.

2.9. Statistical analysis

The study was graphically and analyzed using GraphPad Prism 7 software and a one-way ANOVA, with statistically significant when $P < 0.05$.

3. Results

3.1. Transient expression of reporter genes

The vectors containing different synthetic enhancers were transfected into CHO–S cells, and EGFP and SEAP transient expression were analyzed. At 48 h after transfection, the fluorescence intensity of EGFP was observed under fluorescence microscopy. As shown in Fig. 2A, the fluorescence intensity of five synthetic enhancers was stronger than the original and the truncated vector. The VCD of cells transfected with different vectors showed the same growth trend (Fig. 2B and C). When the cell viability decreased below 50% under suspension, the same number of cells were collected and the MFI of EGFP transient expression was measured by flow cytometry, the results showed that MFI was increased by 2.09, 2.33, 2.1, 1.8, 1.71 times by E1, E2, E3, E4 and E5 compared with the control (Fig. 2D). For SEAP transient expression, the E1 had the highest expression, with a 2.5-fold increase compared to the control vector pSEAP, pSEAP-E2, pSEAP-E3, pSEAP-E4, and pSEAP-E5 were 1.67, 1.32, 1.75, and 1.55-fold, respectively (Fig. 2F).

3.2. Stable expression of reporter genes

CHO cells were transfected with the different vectors and subjected to the BSD selection to establish stable pool and reporter genes expression levels were analyzed. The fluorescence microscopy showed the similar results with transient expression, five synthetic enhancers can increase the fluorescence intensity (Fig. 3A). Moreover, the cells of different stable cell pools exhibited the similar growth trends (Fig. 3 B, C). Compared to the control vector, EGFP of E1, E2, E3, E4, and E5, were increased by 1.32, 1.19, 0.85, 1.31, and 1.1-fold respectively (Fig. 3 D, E). SEAP expression of E1, E4 and E5 were 1.72, 1.83 and 1.61-fold higher compared to the original vector pSEAP, respectively (Fig. 3F).

3.3. HSA transient expression

To further evaluate the effect of synthetic promoters on the expression of recombinant proteins. Four synthetic enhancers, including E1, E2, E4, E5, which are effective in enhancing the expression of reporter genes, were further used for expressing human serum albumin. The control vector and the vector containing E1, E2, E4, E5, four different synthetic enhancers were transfected into CHO–S cells. The results showed that there was no significant difference in VCD during the culture process (Fig. 4 A). Compared with the control, the E1 containing vector had higher HSA expression levels by 1.55-fold (Fig. 4B and C).

3.4. HSA stable expression

CHO cells were transfected with the HSA vectors containing different and subjected to the selection to establish stable cell pools, and the expression of HSA were analyzed. The cell growth trend was similar in the and control groups during the culture process (Fig. 5A). The stable results showed E1 can increased HSA expression 1.79-fold compared to control. E2, E4 and E5 were increased by 2.07, 2.16 and 2.14-fold respectively (Fig. 5B and C).

3.5. Analysis of relative mRNA levels

The HSA and GAPDH gene were used the target and internal reference, respectively. The relative mRNA levels of the HAS stable cell pool were detected using qPCR. Compared with the control pHSA, the mRNA of pHSA-E1, pHSA-E2, pHSA-E4, and pHSA-E5 were all

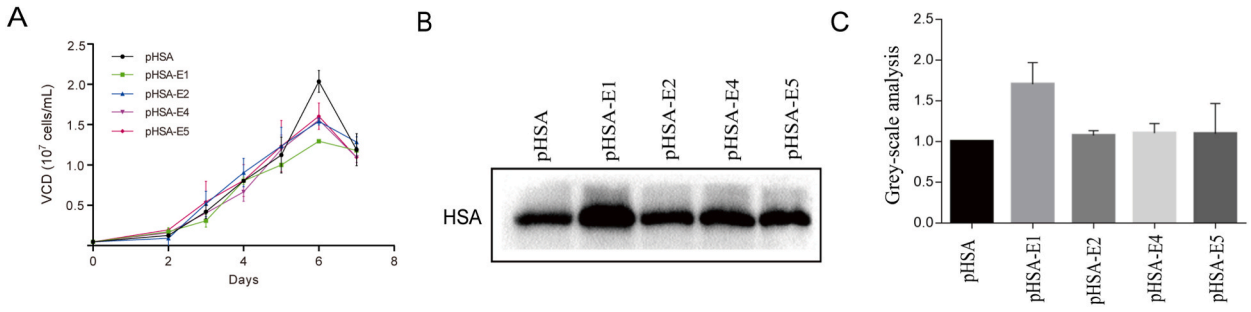

Fig. 4. Effect of different synthetic enhancers on the transient expression of human serum albumin A:Viable cell density; B:Western blot results; C: Grey-scale analysis of human serum albumin Western blot results;

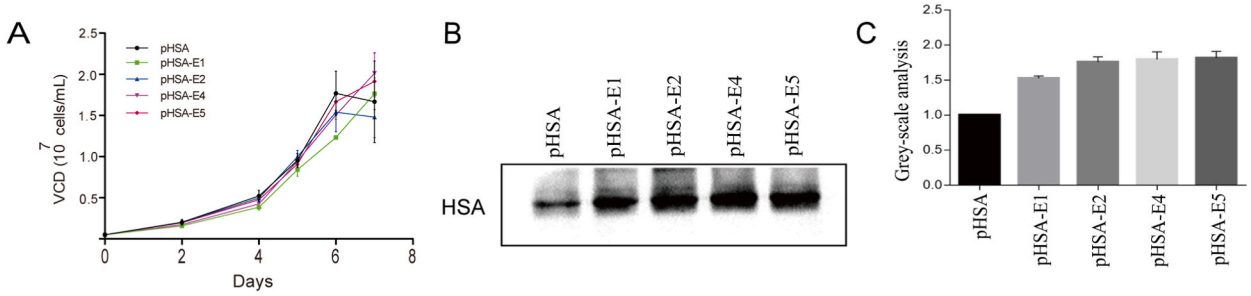

Fig. 5. Effect of different synthetic enhancers on the stable expression of human serum albumin. A: Viable cell density; B: Western blot results; C: Grey-scale analysis of human serum albumin Western blot;

increased (Fig. 6A). Then, a correlation analysis was conducted between HSA expression level and mRNA level, with a correlation coefficient of R2 = 0.6066, indicating a correlation between increased protein expression level and mRNA level (Fig. 6B).

3.6. Transcriptomics analysis

Transcriptomics analysis was performed on enhancer stable cell lines and control, the results showed that a total of 51 different expressed genes were found (q-value < 0.05, Fold change > 2). Among them, 23 genes were upregulated and 28 genes were down-regulated (Fig. 7), including genes related to transcriptional regulation and cell cycle processes, such as transcriptional activation or inhibition, and cell cycle regulation. These genes perform different functions, such as E74 like factor 3 (Elf3) and forkhead box H1 (Foxh1) participating in the positive regulation of RNA polymerase II transcription, cyclin dependent kinase inhibitor 1C (Cdkn1c) participating in the regulation of cell cycle division, and zinc finger protein 541 (Zfp541) related to histone deacetylase complexes.

4. Discussion

CHO cells are the most common host cells for the commercial production of RTPs. Different strategies have been used to increase the productivity and quality of protein in CHO expression systems [7,10,13,16]. Expression vectors are one of key elements that influence recombinant protein yield and quality; thus, optimization of expression vectors can greatly increase transgene expression levels [13,16,17,21]. The promoter is the main component of the vector expression cassette, and optimization of promoter can improve targeting specificity and gene expression [34,35]. In this study, we obtained an efficient expression vector by replacing the enhancer in the CMV promoter with enhancer containing different TFTEs, which have significance in promoter design and recombinant protein production.

Five synthesis enhancers elements containing different TFREs were designed to replace the natural enhancers in the CMV promoter to drive EGFP and SEAP reporter genes expression. The transient and stable results showed that synthetic enhancers E1, E2, E4, and E5 all significantly increased the expression level of the reporter genes, and the maximum transient expression results was obtained by E1 enhancer, which can increase SEAP by 2.5-fold. Johari et al. showed a 2.5-fold higher activity saturation of the synthetic promoter compared to CMV. It is speculated that this may be the highest level of CHO cell-specific recombinant gene transcription [32]. The use of synthetic promoter led to the change of TFREs, which acts by binding to transcription factors in cells. Several transcription factor families have been identified and their activity is regulated by various mechanisms, including interactions with other transcription

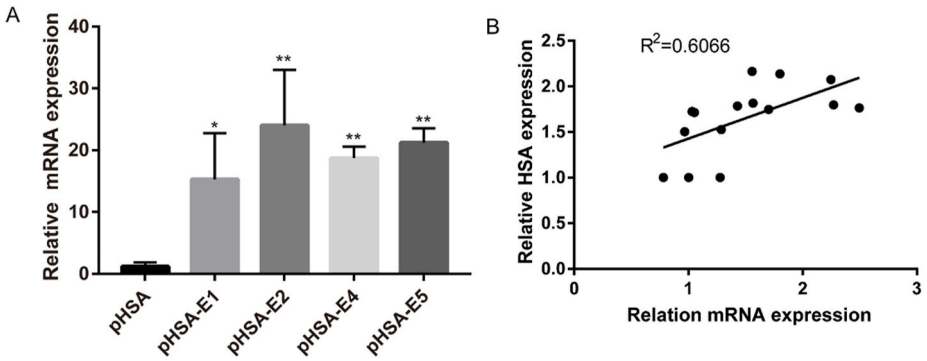

Fig. 6. Analysis of mRNA relative expression levels in HSA stably cell pools. A: mRNA expression levels in HSA stable cell pools; B: The correlation between HSA expression level and mRNA level was evaluated using a correlation coefficient (R2). Compared to control (pHSA), ***P* < 0.05**. ****P* < 0.005.**

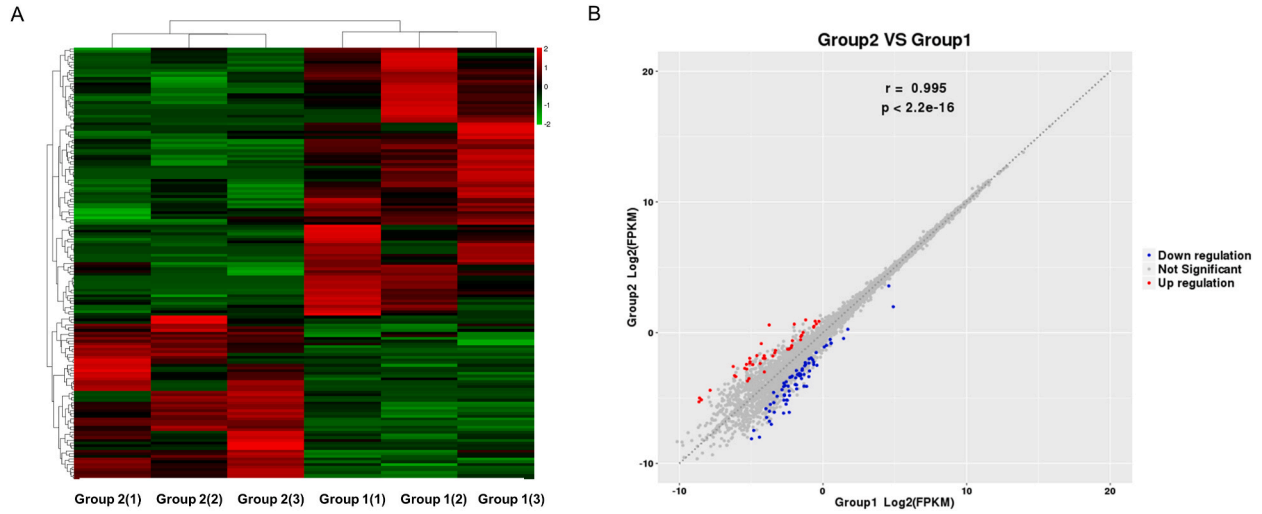

Fig. 7. Analysis of gene expression differences between high expression and control cell lines. A: Differential gene heatmap; B: Expression correlation scatter plot: Group 2 and Group 1 represents highly expressed strain and control cell line, respectively.

factor proteins (TF-TF) or DNA binding elements (TF-TFRE) [36,37]. It has been shown that the activity of synthetic promoters is largely dependent on the relative composition of the promoters. Brown et al. first described the creation of a library of 140 synthetic promoters specifically designed to regulate the expression of recombinant genes in CHO cells, with the most active promoters containing relatively high levels of nuclear factor kB (NF-kB) and E-box TFREs [38]. The efficient enhancers E1, E2, E4 and E5 in this study also contained NF-kB and removed the negatively regulated cAMP RE (CRE).

The stable results were as similar with the transient expression, E1, E2, E4 and E5 can all increase the gene of interest (GOI) expression, but the improvement effect on secreted proteins is more significant. E4 can increase EGFP, SEAP 1.31 and 1.83-fold respectively, suggesting the function of enhancers is related to the type of protein. Intrinsic regulation of the promoter is determined by the chromatin structure on the promoter. The formation of a repressed chromatin conformation on the promoter prevents TF from binding to the target site, thus altering the active state of the promoter [39,40]. Promoter silencing is a common cause of unstable production [41]. In CHO cells, promoters can be designed to enhance expression stability by removing or adding TFRE sequence features that promote or prevent silencing respectively. To reduce promoter silencing, TFREs that have been shown to have transcriptional repressive functions, such as CRE and D-box, can be eliminated when designing synthetic promoters. As overexpression of recombinant proteins in CHO cells may induce an unfolded protein response (UPR), the sequence of TFREs containing endoplasmic reticulum stress response elements (ERSE) may also be removed [42].

Efficient synthetic enhancers selected for the expression of HSA based on the results of reporter gene experiments. Results show that selected synthetic enhancers increase the expression of HSA, and E1 can increase HSA 1.55-fold. It has been proposed that synthetic promoters are able to precisely control the expression of recombinant genes in CHO host cells over a wide dynamic range. For readily expressed proteins, where transcription rates have been shown to exert a high level of control over production [43,44].

To further investigate the mechanism of the synthetic enhancer function, transcriptomics analysis was performed. The results showed that genes related to transcriptional regulation and cell cycle processes, such as transcriptional activation or inhibition, and cell cycle regulation were different, suggesting that they are related to the regulation of enhancers, and the underlying mechanisms need to be further explored.

The effect of synthetic promoters on the expression of recombinant proteins is currently being studied mainly in CHO–S cell lines. The same TFREs sequence plays different roles in different cell lines and the function of the synthetic promoter can be tested in different cell lines. Regulation of promoter activity is highly cell-type specific, as different cells contain different transcription factors and co-regulatory factor [19,32], In addition, synthetic promoters with reduced promoter interference effects can be designed for the expression of multiple genes on a single vector by constructing promoters containing different TFREs types.

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CRediT authorship contribution statement

Hui-Ning Liu: Investigation. **Xiao-Yin Wang:** Methodology. **Ying Zou:** Data curation. **Wen-Bao Wu:** Software. **Yan Lin:** Validation. **Bo-Yu Ji:** Validation. **Tian-Yun Wang:** Writing – review & editing, Writing – original draft, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Tian-yun Wang reports was provided by National Natural Science Foundation of China. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Abbreviations

BSD	blasticidin
Cdkn1c	cyclin dependent kinase inhibitor 1C
CHO	Chinese hamster ovary
CMV	cytomegalovirus
Elf3	E74 like factor 3
ERSE	endoplasmic reticulum stress response elements
FBS	fetal bovine serum
Foxh1	forkhead box H1
GOI	gene of interest
HSA	human serum albumin
mAbs	monoclonal antibodies
MFI	median fluorescence intensity
NF-kB	nuclear factor kB
PTM	post-translational modification
qRT-PCR	real-time quantitative PCR
RTP	recombinant therapeutic protein
TFREs	transcription factor regulatory elements
TF	transcription factor
URS	upstream regulatory sequence
UPR	unfolded protein response
VCD	viable cell density
Zfp541	zinc finger protein 541

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e26901.

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