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Full Length Research Paper

Enhancement of plasmid-mediated stable gene expression by woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) in human embryonic kidney (HEK293) cells

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Influence of random integration site on the expression of transgene in mammalian cells makes it a major challenge to achieve high productivity of recombinant proteins. Optimization of expression vector is one of the most popular strategies to resolve this problem. Among this, woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) is a possible enhancer of gene expression in mammalian cells that promotes efficient export of unspliced (RNA) into the cytoplasm, as has been proved in transient transfection. In this study, WPRE was evaluated for enhancing stable gene expression levels in two industrial cell lines, human embryonic kidney (HEK293) and CHO-S, using the enhanced green fluorescent protein (EGFP), prourokinase (pro-UK) and protein C (PC) as the reporter gene. Based on the mean fluorescence intensity (MFI), WPRE exerted a clear positive effect on gene expression in HEK293 cells with an increase of EGFP expression level by approximately 2.5- to 3-fold independent of the promoter used in plasmid vector. In contrast, in Chinese hamster ovary (CHO)-S cells, only a marginal effect on plasmid-mediated EGFP expression by WPRE was observed. The measurable increase of EGFP expression at the protein level was paralleled by an increase of EGFP RNA. Further test of the effect of WPRE on plasmid-mediated gene expression with two therapeutic proteins showed substantial increase of stable pro-UK and PC expression only in HEK293 by about 2.2-fold and 6.1-fold, respectively. The data of PC expression levels obtained from the random HEK293 cell clones transfected with WPRE-containing or lacking vector further demonstrated the enhancement of stable plasmid-mediated gene expression by WPRE in HEK293 cells. These results in stable transfectants show the positive effect of WPRE on transgene expression is cell-type dependent and promoter-independent, and provide valuable information to improve vectors for efficient and stable gene expression in HEK293 cells.

Key words: Mammalian cells, plasmid vector, stable gene expression, protein therapeutics, woodchuck hepatitis virus post-transcriptional regulatory element (WPRE).

INTRODUCTION

Over the past two decades, mammalian cell-derived protein therapeutics has changed the landscape of human healthcare. The importance of protein therapeutics has motivated the search for more cost-effective and efficient cell lines capable of producing protein products of high quality (Andersen and Krummen, 2002; Seth et al., 2006). The most widely used mammalian expression system in the production of protein therapeutics is Chinese hamster ovary (CHO) cell expression system, but a variety of alternative

mammalian expression systems, such as mouse myeloma (NS0), baby hamster kidney (BHK), and human embryonic kidney (HEK293) cell expression system, have gained regulatory approval for recombinant protein production (Bulter, 2005).

The HEK293 cell line has been extensively used as a mammalian host for transient expression of recombinant proteins. The principal attributes which make the HEK293 cell line a popular choice as a transient expression system includes its quick and easy reproduction and maintenance, high efficiency of transfection and protein production and faithful translation and processing of proteins (Baldi et al., 2007). The added advantages of HEK293 cell expression system are that it ensures that the produced recombinant proteins receive a human profile of glycosylation and a full propeptide cleavage and gamma carboxylation (Thomas and Smart, 2005; Durocher and Bulter, 2009).

It is known that the incorporation of the exogenous gene in the host genome is a random process and the transcription of the inserted exogenous gene in the genome of mammalian cells is often influenced by the site of integration (Gorman and Bullock, 2000). This may in turn affect the specific productivity and clonal stability of the resulting cell line, probably due to the influence of neighboring condensed chromatin (Moreira, 2008). Several strategies have been developed to overcome the negative position effects of random integration. Protective *cis*-regulatory elements include insulators (Pikaart et al., 1998), locus control regions (Li et al., 1999), scaffold or matrix attachment regions (S/MARs) (Zahn-Zahal et al., 2001), ubiquitous chromatin opening elements (UCOE) (Benton et al., 2002), conserved anti-repressor elements (Kwaks et al., 2003) and flanking transgenes with these elements reduces the negative effects of heterochromatin and allows stable expression of the transgene. Despite recent progress in fine-tuning mammalian expression systems for high-level recombinant gene expression, the practicality of achieving reliable, stable and high-level transgene expression in mammalian cells remains a major challenge.

Previous efforts at enhancing recombinant gene expression have mostly been directed at boosting transcription. However, the level of transgene expression also depends on other steps, including post-transcriptional events concerning the recombinant mRNA. The woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) has been shown to be a *cis*-acting element which promotes the efficient transport of unspliced RNA into the cytoplasm (Donello et al., 1998; Huang et al., 1999) also effect on nuclear processing and the transport receptor, CRM1-mediated export of (mRNA) into cytoplasm (Popa et al., 2002). WPRE has widely been used to enhance transgene expression or virus titers in a series of virus vectors (Paterna et al., 2000; Brun et al., 2003; Hlavaty et al., 2005; Lee et al., 2005; Mähönen et al., 2007). Recently, the effect of WPRE for enhancing transient gene expression levels in HEK293 cells has been evaluated in a few of research publications (Backliwal et al 2008; Kim et al., 2009; Mariati et al, 2010). However, studies on the use of WPRE in efficient production of therapeutic proteins are not fully substantiated. Therefore, it will be meaningful to investigate the effect of WPRE on the stable expression of exogenous gene in mammalian expression system.

In this study, we evaluated the use of WPRE in pcDNA3.1-based plasmid vectors carrying either the cytomegalovirus immediate early gene promoter (CMV promoter) or the human elongation factor 1alpha promoter (hEF-1α promoter) in mediating the expression of three representative proteins in HEK293 and CHO-S cells. In our results, WPRE shows significant enhancement on recombinant gene expression in HEK293 cells independently of the promoters used in pcDNA3.1-based plasmid vector, but only a marginal effect in CHO cells.

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Abbreviations: BGH, Bovine growth hormone; **BHK,** baby hamster kidney; **CHO,** Chinese hamster ovary; **CMV,** cytomegalovirus; **DMEM/F12,** a mixture of Dulbecco's modified Eagle's medium and Ham F12 medium; **EGFP,** enhanced green fluorescent protein; **ELISA,** enzyme-linked immunosorbent assay; **FACS,** fluorescence activated cell sorting; **FBS,** fetal bovine serum; **GAPDH,** glyceraldehyde 3-phosphate dehydrogenase; **HEK,** human embryonic kidney; **hEF-1α,** human elongation factor 1alpha promoter; **MMTV,** mouse mammary tumour virus; **PBS,** phosphate-buffered saline; **PC,** protein C; **pro-UK,** prourokinase; **MFI,** mean fluorescence intensity; **RT-PCR,** reverse transcription polymerase chain reaction; **S/MARs,** scaffold or matrix attachment regions; **UCOE,** ubiquitous chromatin opening elements; **UTR,** untranslated regions; **WAP,** murine whey acidic protein; **WHV,** woodchuck hepatitis virus; **WPRE,** woodchuck hepatitis virus post-transcriptional regulatory element.

MATERIALS AND METHODS

Plasmid construction

Vectors for evaluating WPRE were constructed based on pcDNA3.1 (+) vector (Invitrogen). The reporter gene, the enhanced green fluorescent protein (EGFP) was inserted into the multiple cloning sites of pcDNA3.1 (+) to generate the control vectors (pCE, Figure 1). Then, WPRE was synthesized according to the nucleotides 1094 to 1684 of woodchuck hepatitis virus (WHV) X protein (GenBank accession number J02442) and inserted 3′ end of the reporter gene (pCEW, Figure 1). To generate a pcDNA3.1 (+)-based plasmid vector carrying hEF-1α promoter, pcDNA3.1 (+) plasmid was digested with *Mlu*I/*Nhe*I and *Xba*I/*Bspe*I to remove the fragments harboring CMV promoter and bovine growth hormone (BGH) polyadenylation signal. And then, hEF-1α 5' and 3' untranslated regions sequences (EFU5 and EFU3) were amplified

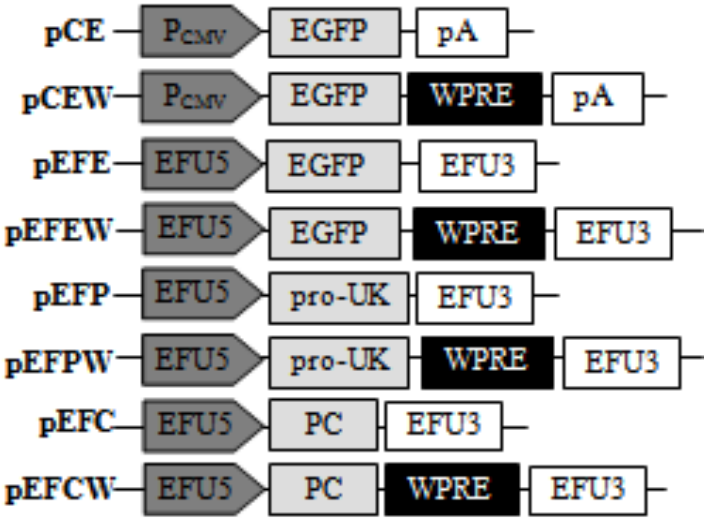

Figure 1. pcDNA3.1(+)-based plasmid vectors expressing EGFP, pro-UK, or PC gene: pCEW, pEFEW, pEFPW and pEFCW were WPRE-containing vectors expressing EGFP, pro-UK or PC under control of the CMV promoter or the hEF-1α promoter. Corresponding control vectors without WPRE were pCE, pEFE, pEFP and pEFC, respectively.

from human genome (GenBank accession number NT_007299.13) and inserted into *Mlu*I/*Nhe*I site and *Xba*I/*Bspe*I site, respectively. Again, reporter genes, EGFP, pro-UK and protein C (PC) were inserted into the pcDNA3.1-based vector carrying hEF-1α promoter to generate the control vectors (pEFE, pEFP and pEFC, Figure 1) and WPRE was placed 3′ end of the reporter gene (pEFEW, PEFPW and pEFCW, Figure 1).

Cell culture, transfection and selection

HEK293 cells and CHO-S cells (Invitrogen) were grown in a mixture of Dulbecco's modified Eagle's medium and Ham F12 medium (DMEM/F12) supplemented with 5% fetal bovine serum (FBS, HyClone) and 2 mM L-glutamine (Invitrogen) and maintained at 37°C in 5% CO_2. Transfection was performed using the Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For stable transfection, the transfected cells were selected in DMEM/F12 containing 0.4 mg/ml geneticin (G418; Invitrogen). The selective medium was changed every 3 to 4 days until the transfected colonies formed after 2 weeks. Then the positive cells were expanded to be subjected to the determination of exogenous gene expression, or single-cloned to quantitatively compare the expression of exogenous gene.

Analytic assays

EGFP was detected by fluorescence activated cell sorting (FACS). In brief, the selected cells were trypsinized, washed twice with phosphate-buffered saline (PBS) and then 10,000 cells per sample were analyzed using a fluorescence activated cell sorting (FACS) Calibur (BD Biosciences). The number and mean fluorescence intensity (MFI) of fluorescent cells were determined. The fibrinolytic activity of pro-UK was determined by an *in vitro* fibrin lysis assay as described previously (Jespersen and Astrup, 1983) using the national urokinase standard as the reference (National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China). PC was detected by a two-site enzyme-linked immunosorbent assay (ELISA) method using Human Protein C ELISA Kit (Aniara Corporation) according to the manufacturer's instructions.

Nucleic acid extraction and real time RT-PCR

Total RNA from the transfected cells were extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The relative amounts of cellular EGFP RNA were determined in duplicates by real-time quantitative polymerase chain reaction (RT-qPCR) using the QuantiTect Probe RT-PCR Kit (Qiagen) according to the instructions of the manufacturer and the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). In order to generate a specific probe for the detection of EGFP RNA by RT-PCR, a part of the EGFP gene was amplified by PCR using the oligonucleotide primers EGFP-f (bp289) (5′-CGCACCATCTTCTTCAAGGAC-3′) and EGFP-r (bp379C) (5′-TCAGCTCGATGCGGTTCAC-3′).

RESULTS

Effect of WPRE on plasmid-mediated EGFP expression

Firstly, the post-transcriptional element, WPRE, was evaluated for its ability to enhance plasmid-mediated expression level in HEK293 and CHO-S cells, with EGFP as the reporter gene and analyzed by FACS. Transfection of each EGFP vector lacking or containing WPRE was carried out twice in 6-well plate. Based on the mean fluorescence intensity (MFI), WPRE exerted a clear positive effect on gene expression in HEK293 cells with an increase of EGFP expression level of approximately 2.5- to 3-fold. In contrast, in CHO-S cells, only a marginal

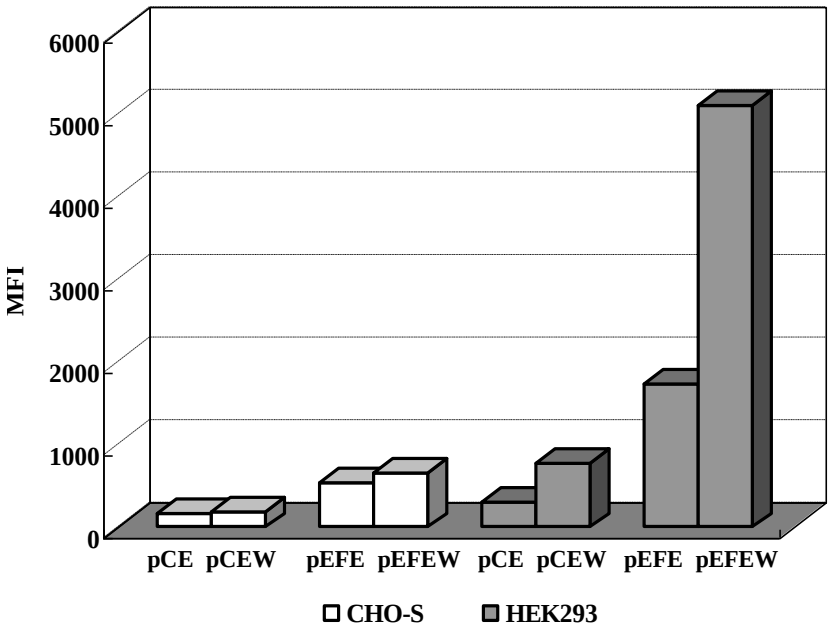

Figure 2. Effect of WPRE on EGFP expression levels in HEK293 (grey bars) and CHO-S (blank bars) cells: The effect of WPRE was evaluated in HEK293 and CHO-S cells, respectively with EGFP driven by the CMV promoter (pCE and pCEW) or the hEF1α promoter (pEFE and pEFEW) as the reporter gene. Results are expressed as the mean value of duplicate samples from different experiment.

effect on plasmid-mediated EGFP expression by WPRE was observed (Figure 2). In all cases, cells stably transfected with the hEF-1α promoter-containing vectors showed more efficient EGFP expression than the corresponding CMV promoter-containing vectors (Figure 2).

Difference in RNA level is reflected on EGFP expression level

Since WPRE has been known to enhance gene expression by its effect on nuclear mRNA processing, mRNA export and translation (Loeb et al., 1999), the observed increase in EGFP expression in WPRE-containing vector-transfected HEK293 cells is likely due to post-transcriptional regulation. The basal amounts of EGFP transcripts in HEK293 and CHO-S cells transfected with EGFP vectors lacking or containing WPRE were determined by qPCR (Figure 3). Consistent with the data obtained by FACS analysis, EGFP RNA levels significantly increased in HEK293 cells stably transfected with EGFP vector containing WPRE compared to the cells transfected with EGFP vector lacking WPRE. No significant differences in EGFP RNA levels were observed between CHO-S cells stably transfected with EGFP vector containing and lacking WPRE, both much lower than that in HEK293 cells. Again, cells stably transfected with the hEF-1α promoter-containing vectors showed much higher EGFP RNA levels than corresponding cells transfected with the CMV promoter-containing vectors in all cases (Figure 3).

Effect of WPRE on expression of therapeutic proteins

The hEF-1α promoter-containing vectors showed more efficient EGFP expression than the CMV promoter-containing vectors in both HEK293 and CHO-S cells. Hence, the effect of WPRE on plasmid-mediated gene expression was further tested with two therapeutic proteins, prourokinase (pro-UK) and PC in the hEF-1α promoter-containing vectors. In HEK293 cells, WPRE still exhibited strong positive effect on the expression of the two therapeutic proteins. The expression of pro-UK increased from 161 IU/10^6 cells/day to 359 IU/10^6 cells/day and PC from 81 ng/10^6 cells/day to 495 ng/10^6 cells/day, with enhancement of pro-UK and PC expression by about 2.2-fold and 6.1-fold, respectively (Figure 4). Consistent with the previous observations for EGFP expression, WPRE did not exert positive effect on the expression levels of both pro-UK and PC in CHO-S cells (Figure 4). Random cell clones were obtained from HEK293 cells stably transfected with PC-expressing vector containing or lacking WPRE. The PC expression levels of the cell clones transfected with WPRE-containing vector were about five-fold higher than those of the cell clones transfected with WPRE-lacking vector, further validating the enhancement of stable plasmid-mediated gene expression by WPRE in HEK293 cells (Figure 5).

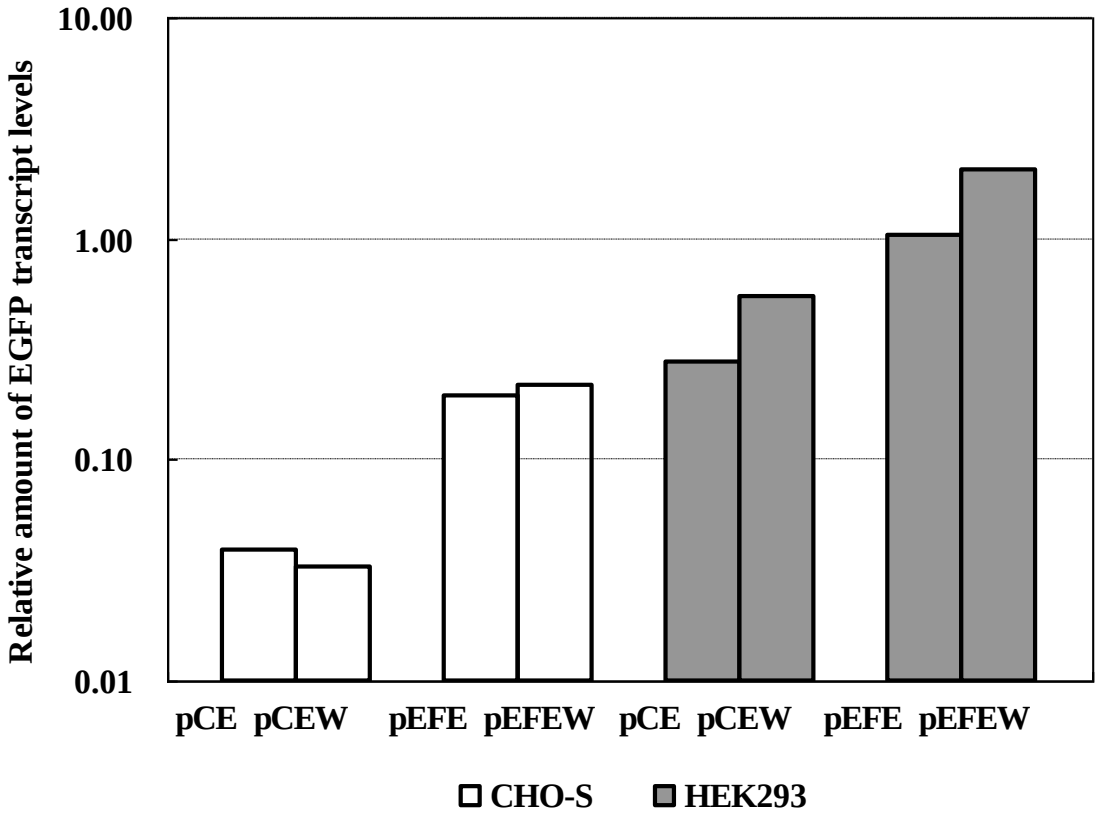

Figure 3. Relative RNA levels of EGFP in HEK293 (grey bars) and CHO-S (blank bars) cells transfected with EGFP vector containing or lacking WPRE. EGFP was expressed under the control of the CMV promoter (pCE and pCEW) or the hEF1α promoter (pEFE and pEFEW), respectively. Results are expressed as the mean value of duplicate samples from different experiment.

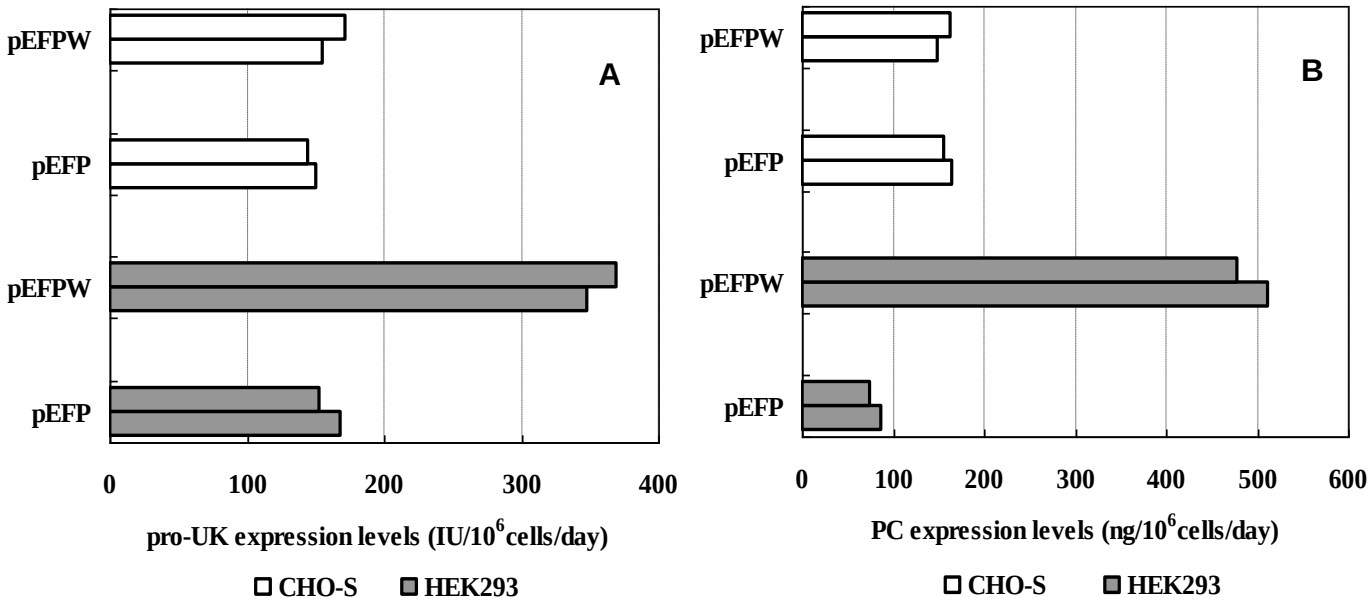

Figure 4. Effect of WPRE on prourokinase (A) and protein C (B) expression levels in HEK293 (grey bars) and CHO-S cells (blank bars). The expressions of these two interest proteins were driven by the same promoter of hEF1α. Each individual bar represents a protein expression level from one individual transfection.

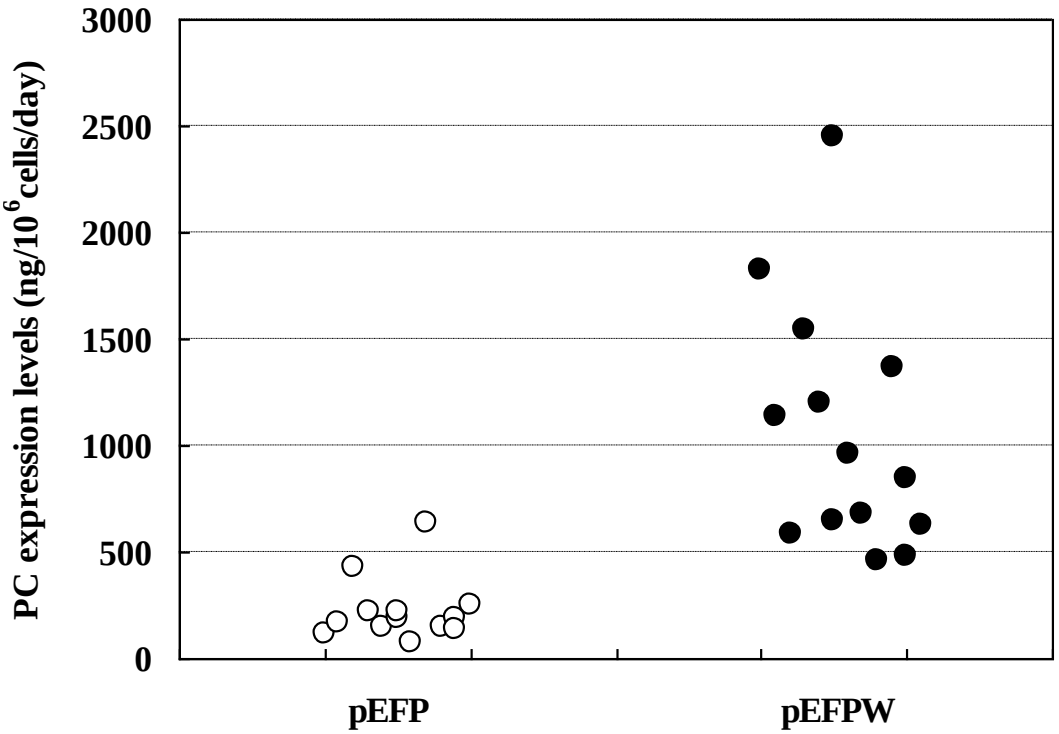

Figure 5. PC expression levels of the random cell clones derived from the HEK293 cells stably transfected with WPRE-containing (pEFPW) or lacking (pEFP) vector. Each individual point represents a protein expression level from one individual transfection. The two groups of data were significantly different by statistic student's *t*-test ($P < 0.05$).

DISCUSSION

In this study, we evaluated WPRE for enhancing plasmid-mediated gene expression in two commonly-used industrial cell lines, CHO-S and HEK293. The performances of WPRE were tested using three representative proteins, EGFP, pro-UK and PC. The genes of interest in the basic vector were under the control of the CMV promoter or the hEF-1α promoter. We observed a reproducible positive effect of WPRE on plasmid-mediated gene expression in HEK293 cells, but not in CHO-S cells independent of the genes of interest and the promoters used in the vectors. Furthermore, consistent with the data obtained by FACS analysis, the higher amount of EGFP in WPRE-harboring vector-transfected HEK293 cells obviously translated into the respective amounts of protein as shown in Figures 2 and 3.

In agreement with most publications which suggested that the effects of WPRE on expression is cell line-specific in lentiviral vectors, we observed that WPRE in plasmid vectors also exhibited cell line specificity with respect to the expression of three representative proteins in stably-transfected HEK293 and CHO-S cells (Hlavaty et al., 2005; Klein et al., 2006). The underlying mechanism of such phenomenon remains to be investigated. Emerging evidence indicates that the cell-specific effect of deoxyribonucleic acid (DNA) elements can be attributed to the different distribution of cellular factors in different cell lines (Lee et al., 2007). Klein et al. (2006) tested WPRE in combination with the mouse mammary tumour virus (MMTV) promoter or the murine whey acidic protein (WAP) promoter in retroviral vectors and noticed that WPRE-mediated enhancement of gene expression was promoter-specific and the differences in RNA levels were not reflected on protein level.

Recently, Mariati et al. (2010) evaluated WPRE for enhancing transient gene expression levels in CHO-K1 and HEK293 cells with respect to interferon γ and Trastuzamab monoclonal antibody expression, respectively. They showed that WPRE did not exert positive effect on the expression levels of both interferon γ and Trastuzamab. In contrast to previous report, no difference in the extent of the positive effect of WPRE on EGFP expression driven by the CMV promoter and the hEF-1α promoter was observed and the measurable increase of EGFP expression at the protein level was paralleled by an increase of EGFP RNA. Moreover, the positive effect of WPRE on stable transgene expression in HEK293 was revealed in all genes of interest tested without exception. These results were consistent with the previous reports which demonstrated that WPRE inside the cell plays a role in cooperation with several alternative pathways to facilitate nucleocytoplasmic transport of RNA and acts on additional post-

transcriptional mechanisms to stimulate expression of heterologous complementary deoxyribonucleic acid (cDNAs) (Popa et al., 2002; Zufferey et al., 1999).

This report demonstrates that the inclusion of WPRE can substantially increase stable gene expression levels in HEK293 cells independent of the promoter used in the plasmid vectors. Although WPRE action inside the cell is likely much more complex than assumed and design of vectors for high level gene expression still requires a deeper understanding of the interaction of cellular factors and regulatory DNA sequences to regulate the gene expression, it would be practical to use the benefit of this element for rational design of plasmid vectors for high-level gene expression in HEK293 cells.

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