

A transient expression vector for recombinant protein production in Chinese hamster ovary cells

Mimi ML Liao¹ and Noelle-Anne Sunstrom^{1,2*}

¹Department of Biotechnology and Biomolecular Sciences, University of New South Wales, Sydney NSW 2052, Australia

²ACYTE Biotech Pty Ltd, Sydney, NSW 2052, Australia

Abstract: An expression vector was specifically designed for use in Chinese hamster ovary (CHO) cells to enhance the level of protein production in a transient expression system. Two key components that can increase protein production transiently are the promoter used to drive recombinant gene expression and the template copy number. In this study the modified and metal-inducible metallothionein (M2.6) promoter was shown to be superior to the human cytomegalovirus (CMV) and to the simian virus SV40 promoters. Plasmid replication was achieved using the Polyoma (Py) virus origin of replication (PyOri) and the Py Large T antigen (PyLT). An expression vector containing Py elements was shown to replicate extensively in CHO cells. The combination of the metal-inducible M2.6 promoter and episomal replication of the expression vector, named pPyOriLT resulted in elevated levels of transgene expression following transient transfection of CHO cells

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Keywords: Chinese hamster ovary (CHO) cells; transient transfection; promoter; Polyoma virus; episomal replication

INTRODUCTION

High throughput screening in the drug discovery process has intensified the need to develop expression systems to rapidly produce milligram to gram quantities of recombinant protein. To accomplish this need, transient expression systems have attracted much interest over the traditional stable expression technology. As an alternative to stable cell line generation, large-scale transient expression of recombinant proteins in mammalian cells has been used to generate sufficient amounts of recombinant protein for early product analysis and high throughput screenings.^{1–3} Such large-scale transient expression employs the transformed human embryonic kidney (HEK293) cells engineered to express the Epstein–Barr Virus (EBV) nuclear antigen-1 (EBNA-1). The combination of *Orp* and EBNA-1 contributes to elevated levels of transgene expression following transient transfection.⁴ Chinese hamster ovary (CHO) cells are the most commonly used cell line in commercial production of biopharmaceutical products.^{5–7} Therefore, the availability of a high-level transient expression vector for CHO cells would be beneficial.

In this study, two key components of a transient expression vector were examined: the promoter driving transgene expression and the ability for the

plasmid DNA to replicate in transfected CHO cells. The use of a strong promoter is critical since the time-frame for product generation is relatively short (days). For this purpose three promoters were compared for transient transgene expression in CHO: the human cytomegalovirus immediate early enhancer/promoter (CMV promoter), the simian virus 40 early enhancer/promoter (SV40 promoter), and a modified and metal-inducible metallothionein (M2.6)^{8,9} promoter. The relative strengths of the CMV, SV40 and M2.6 promoters were compared using the reporter gene encoding the destabilised green fluorescence protein (dsGFP).¹⁰

The level of transient production can be increased when more plasmid copies persist in transfected cells. To establish plasmid episomal replication in CHO cells the Py virus-based replication system was chosen due to its simple requirements: only two exogenous elements, namely Py origin of replication (PyOri) and Py large T antigen (PyLT), are required for replication in CHO cells.¹¹

In combination, the metal-inducible M2.6 promoter and plasmid replication of the expression vector, pPyOriLT, were examined for transgene expression using two model proteins: (i) green fluorescent protein (GFP) to measure protein expression on a per cell

* Correspondence to: Noelle-Anne Sunstrom, Department of Biotechnology and Biomolecular Sciences, University of New South Wales, Australia

E-mail: n.sunstrom@unsw.edu.au

(Received 30 March 2005; revised version received 31 May 2005; accepted 1 June 2005)

Published online 18 October 2005

basis and (ii) secreted alkaline phosphatase (SEAP) to measure overall transient protein yield. This study demonstrates that pPyOriLT is an efficient expression vector for high-level transient expression in CHO cells.

MATERIALS AND METHODS

Cell culture and transfection

CHO-XL99 was adapted to grow in single cell suspension from the attached CHO-K1 cell line (ATCC, CCL-61) and cultured in EX-CELL™ 302 medium (JRH Biosciences, Lenexa, KS), in a humidified 37 °C incubator with 5% CO₂.

Transfection quality DNA was prepared using Nucleobond® AX columns (Macherey-Nagel, Duren, Germany). DNA concentration and purity were independently assayed using analytical gel electrophoresis, restriction enzyme digestions and spectrophotometry.

Transfections were performed in six-well plates (Iwaki, Tokyo, Japan). CHO-XL99 was washed once by centrifugation and resuspension in PBS (phosphate-buffered saline: 137 mmol L⁻¹ NaCl, 2.7 mmol L⁻¹ KCl, 4.3 mmol L⁻¹ Na₂HPO₄·7H₂O, 1.4 mmol L⁻¹ KH₂PO₄). Cell number was determined using the Trypan Blue exclusion method¹² to prepare 2.5 mL of 1.2 × 10⁶ cells per mL culture in OPTI-MEM® I medium (Invitrogen, Carlsbad, CA, USA) for each well. DNA and LIPOFECTAMINE™ 2000 (Invitrogen) complexes were prepared according to the manufacturer's instructions. The complexes were then added to cells and cultured on a shaker platform. Four hours post-transfection, one volume of fresh medium was added using EX-CELL™ 302. Combinations of metals were also added to the culture where indicated. Final concentrations of 100 μmol L⁻¹ ZnSO₄ and 2 μmol L⁻¹ CdCl₂ were used in experiments where indicated. These metal concentrations were chosen based on the optimal induction conditions reported for the M2.6 promoter.¹³ All chemicals used were purchased from Sigma (St Louis, MO, USA).

Vector construction

Three vectors were constructed to compare the promoter activities of CMV, M2.6 and SV40. To ensure an accurate comparison of promoter activities, each promoter was individually cloned into pd2EGFP-1 (Clontech, Palo Alto, CA, USA). This vector encodes the protein degradation domain of mouse ornithine decarboxylase, which is fused to the C-terminus of the GFP gene to direct rapid protein turnover.¹⁰ The pCMV-d2EGFP vector was constructed by inserting the BamHI and SalI digested 909 bp fragment of CMV immediate early enhancer/promoter fragment from pCEP4 (Invitrogen) into the multiple cloning site (MCS) of pd2EGFP-1 using BamHI and SalI restriction sites. The pM2.6-d2EGFP vector was constructed by inserting the M2.6 promoter from pNK vector⁸ into the MCS of pd2EGFP-1 using SalI and ApaI sites. The pSV-d2EGFP vector was constructed

by inserting the SV40 promoter fragment from pSV-Zeo (Invitrogen) into the MCS of pd2EGFP-1 using AgeI and BamHI sites.

To construct a vector that carries both a strong promoter and the Py-based replicating elements (PyOri and PyLT), plasmid cloning was performed in two stages. The first stage entailed the construction of a backbone vector that carries (i) the M2.6 promoter to direct strong protein expression and (ii) the ampicillin resistance gene for selection in bacteria. The M2.6 promoter was excised from the pNK plasmid⁸ using AccI and MluI. The excised 2023 bp fragment was then inserted into AccI and NruI digested pSVL vector (Amersham Pharmacia Biotech, Piscataway, NJ, USA) to form the pSVL-M2.6 plasmid. The second stage involved the insertion of the genomic PyOri and PyLT genes into the backbone. The genomic DNA that contained the PyOri and PyLT was isolated from pPy3A-1 (ATCC) by HindII and BamHI digestions. The 3629 bp fragment was then subcloned into EcoNI-digested pSVL-M2.6, resulting in pPyOriLT (Fig 1(A)).

The constructed vector was evaluated qualitatively and quantitatively using EGFP and SEAP respectively as reporters. The pPyOriLT-EGFP was constructed by inserting the EGFP gene into the MCS of pPyOriLT using NotI. For pPyOriLT-SEAP, the SEAP fragment was extracted from the pSEAP2-basic plasmid (Invitrogen) using XhoI and SalI.

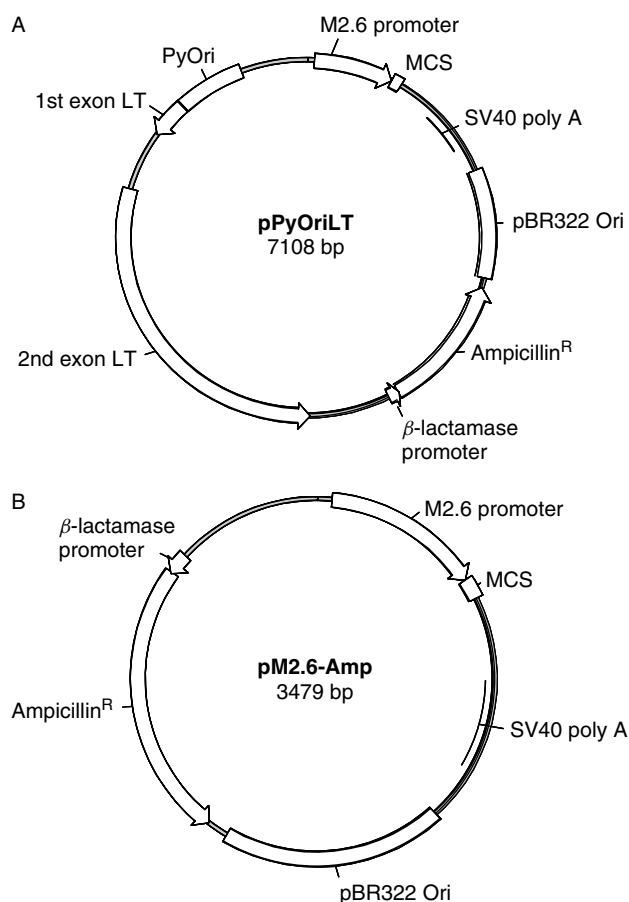


Figure 1. Vector maps for (A) pPyOriLT and (B) pM2.6-Amp.

Subsequently, this 1837 bp fragment was cloned into XhoI digested pPyOriLT. In both constructs, the M2.6 promoter was used to drive expression of GFP and SEAP.

A control plasmid was also constructed in parallel to assess the level of transgene expression in the absence of episomal replication. This plasmid, pM2.6-Amp, was obtained by self-relegating the EcoNI digested pSVL-M2.6 plasmid (2234 bp). A map of the pM2.6-Amp vector is shown in Fig 1(B). Using the same restriction sites and plasmid sources as described for pPyOriLT-EGFP and pPyOriLT-SEAP, the EGFP and SEAP genes were inserted into the MCS of pM2.6-Amp to form pM2.6-EGFP and pM2.6-SEAP.

Episomal replication assay

Low molecular weight plasmid DNA was extracted using the method described by Hirt.¹⁴ Extracted plasmid DNA was resuspended in TE buffer (10 mmol L⁻¹ Tris-HCl, 1 mmol L⁻¹ EDTA, pH 7.6) and subsequently digested with *SpeI* and *DpnI*. *SpeI* was used to linearise the plasmid, whereas *DpnI* was used to cleave any non-replicated DNA. The plasmid DNA used for transfection was prepared in DNA adenine methylase (*dam*) positive bacteria, where adenine nucleotides within GATC sites become methylated. In contrast, plasmids that replicate in mammalian cells are not methylated due to the absence of the *dam* enzyme. The endonuclease, *DpnI* cleaves a methylated recognition sequence and does not cleave plasmid that has undergone replication in mammalian cells.¹⁵ Digested plasmid DNA was electrophoresed through a 0.8% agarose gel and transferred to a nitrocellulose membrane. Southern hybridisation was performed on the membrane with ³²P-labelled PyOri DNA used as a probe. Following washing, the membrane was exposed to a Hyperfilm-ECL film (Amersham).

Fluorescence measurements

Flow cytometry was performed on a MoFlo Cytometer (Cytomation, Fort Collins, CO, USA) equipped with a multi-line 200 mW argon ion laser emitting light at 488 nm. A neutral density filter, ND 1.3, which absorbs light over the entire visual spectrum and limits the light reaching the photo-multiplier tube, was used where indicated. Analysis was performed on viable cells, gated by forward and side scatter and verified by propidium iodide staining. Cell emission spectra were detected on FL-1. Acquisition and data analysis were performed using the CyCLOPS Summit operating system (Cytomation). At least 10 000 events were acquired. Flow calibration and optical alignment was performed with the aid of Flow-Check Fluorospheres (Beckman Coulter Inc, Fullerton, CA, USA) before each analysis.

Cells were evaluated for the percentage of positive cell (percentage of cells expressing GFP above the levels of untransfected control) and the fluorescence

intensity as measured by mean relative fluorescence units (mean RFU).

SEAP assay

Culture medium (1 mL) was heated to 65 °C for 5 min to inactivate endogenous phosphatases. Cell debris was separated in a subsequent centrifugation step for 2 min at 14 000 rpm. A 100 µL aliquot of supernatant and 100 µL of 2× SEAP buffer (2 mol L⁻¹ diethanolamine, 1 mmol L⁻¹ magnesium chloride and 20 mmol L⁻¹ L-homoarginine) were transferred to a flat-bottom 96-well plate. The sample was pre-warmed at 37 °C for 10 min. The enzymatic reaction was initiated by adding 20 µL of 120 mmol L⁻¹ *p*-nitrophenol phosphate (Sigma). The increase in light absorbance at 405 nm was measured every 2 min for 1 h. The linear change in absorbance was calculated using SoftMax[®] Pro software (Molecular Devices, Sunnyvale, CA, USA), which is directly proportional to SEAP activity.¹⁶

RESULTS

Comparison of promoters

To select the optimal promoter for transient protein production in CHO cells, the promoter strengths of CMV, SV40 and M2.6 were compared. Promoter strength was determined from GFP expression of cells transfected with pCMV-d2EGFP, pSV40-d2EGFP or pM2.6-d2EGFP expression vectors encoding the destabilised EGFP protein. GFP expression was calculated by multiplying the percentage of GFP positive cells by the fluorescence intensity as determined by flow cytometry 24 and 48 h post-transfection.⁴ The total GFP expression (sum of GFP expression 24 and 48 h post-transfection) is shown in Fig 2 as the fold-increase in total GFP

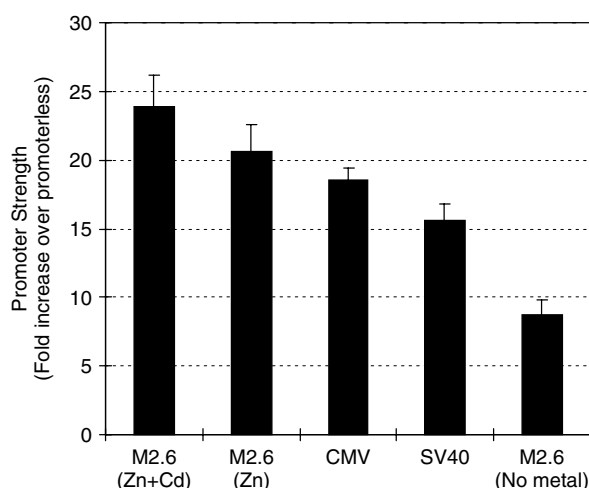


Figure 2. A comparison of promoter strengths in transient expression assay. Promoter strength is expressed as the fold-increase in total GFP expression (sum of GFP expression⁴ at 24 and 48 h post-transfection) from the promoter-containing construct relative to the promoterless construct (pd2EGFP-1) transfected in parallel. Results were obtained from five separate transfections repeated on separate days ± standard error.

expression from the promoter-containing construct relative to the promoterless construct (pd2EGFP-1) transfected in parallel.¹⁷ The rank order of expression is metal-induced M2.6 > CMV > SV40 > un-induced M2.6. This trend is reproducible from five separate transfections. These results indicate that the metal induced M2.6 promoter can be used to efficiently drive transgene expression in a transient expression system. The M2.6 promoter was thus selected and incorporated into the vector design for transient protein expression in CHO.

Episomal replication in CHO

The expression vector pPyOriLT (Fig 1(A)) was transfected into CHO cells to demonstrate episomal replication. Accordingly, pPyOriLT contains PyOri and encodes PyLT, the two viral elements necessary and sufficient to initiate plasmid DNA replication in CHO cells.¹⁸ Plasmid DNA replication was monitored over 3 days. Low molecular weight DNA was purified from transfected cells as described in Materials and Methods. Plasmid replication was detected by resistance to cleavage by *DpnI*, which cleaves only when its recognition site is methylated. Episomal replication is detected as early as 1 day post-transfection (Fig 3). Replicated DNA reaches its maximum levels 2 days post-transfection, increasing 4.6 times the initial levels of plasmid DNA. These results demonstrate the potential of Py-based episomal replication in CHO for elevated transient transgene expression by increasing template DNA through replication.

The effect on plasmid replication was examined following metal addition to the culture media following transfection. CHO cells were transfected with pPyOriLT-SEAP following addition of zinc alone or in combination with cadmium which has been previously shown to fully induce expression from the M2.6 promoter.^{8,9} Low molecular weight DNA was harvested 2 days post-transfection and the amount of replicated DNA was determined. The results shown in Fig 4 represent relative replicated plasmid copy number determined from cultures with

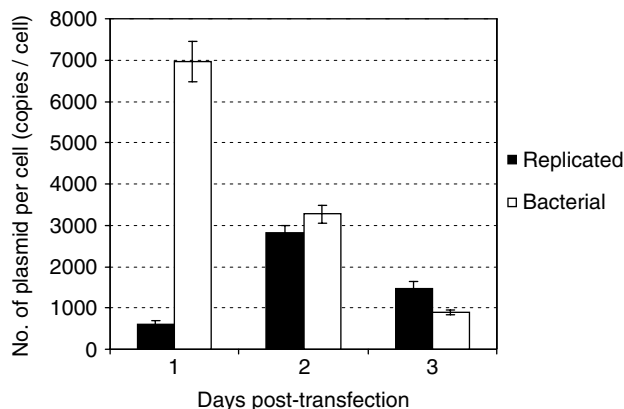


Figure 3. The strength of Py replication in CHO. The plasmid copy number \pm standard error was calculated from five Hirt extractions.

metal versus cultures without metal. The number of replicated plasmids extracted from the transfected culture without metal was set to 100%. The addition of 100 $\mu\text{mol L}^{-1}$ zinc, had no significant effect on plasmid replication compared with those cultures without metal (Fig 4). However, the addition of 100 $\mu\text{mol L}^{-1}$ zinc + 2 $\mu\text{mol L}^{-1}$ cadmium resulted in low plasmid copy number, about 10% of cultures without metal. This demonstrates that while the presence of zinc ions in the culture media has minimal effect on plasmid replication, the addition of both zinc and cadmium adversely affects plasmid replication.

Transient GFP expression

The replication competent expression vector pPyOriLT-EGFP encoding the enhanced green fluorescent protein (EGFP) driven by the M2.6 promoter was used to determine transgene expression in a transient assay. Flow cytometry was used to compare fluorescence in CHO cells transfected with either replication competent pPyOriLT-EGFP or replication incompetent pM2.6-EGFP vectors over 4 days. As shown in Fig 5(A), the pPyOriLT-EGFP transfected cultures show a significant increase in the percentage of GFP positive cells over time compared with pM2.6-EGFP transfected cultures. This is evident whether or not metal is present in the media. The percentage of GFP positive cells in a pPyOriLT-EGFP-transfected culture 4 days post-transfection is 80% in the absence of metal, 99% with zinc alone and 95% with zinc + cadmium. This is in contrast to cells transfected with pM2.6-EGFP where the percentage of GFP positive cells declines after 3 days post-transfection.

Fluorescence intensity was also determined in transfected cells using flow cytometry (Fig 5(B)). The mean RFU values were significantly higher in the pPyOriLT-EGFP transfected cultures compared with pM2.6-EGFP transfected cultures for 3 days following transfection. The mean RFU of pPyOriLT-EGFP transfected cultures decreased rapidly to that

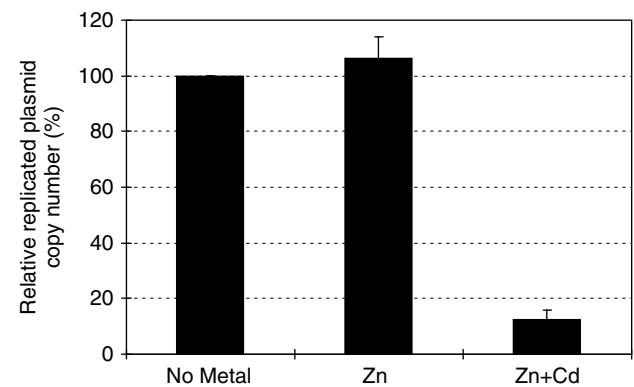


Figure 4. Effects of metal on Py replication. The data are expressed as the relative replicated plasmid copy number from culture treated with metal versus culture without metal addition. The amount of replicated plasmid DNA extracted from the transfected culture without metal was set to 100%. The data were obtained from three transfections \pm standard error.

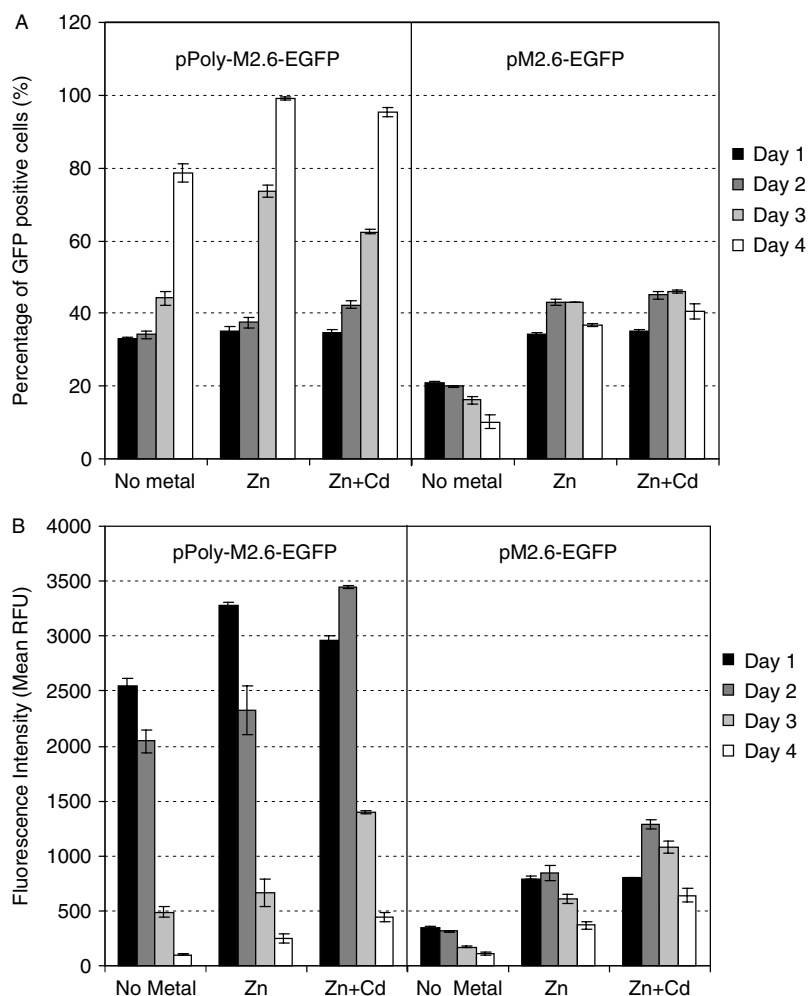


Figure 5. Transient GFP expression: (A) percentage of GFP-positive cells and (B) fluorescence intensity. Flow cytometry was used to measure (A) percentage of GFP-positive cells and (B) fluorescence intensity in CHO cells transfected with either replication competent pPyOriLT-EGFP or replication incompetent pM2.6-EGFP vectors over 4 days. Transfections were done in triplicate.

observed in M2.6-EGFP transfected cells. The decline in fluorescence intensity on the fourth day post-transfection may be a result of the corresponding increase in GFP-positive cells. To explain, the fluorescence intensity on an individual cell basis declines as more cells are capable of expressing GFP. Plasmid replication results in a greater number of cells acquiring the transgene through cell division even though fewer copies remain per cell.

Transient SEAP expression

In combination, Py-based plasmid replication and the metal-inducible M2.6 promoter were examined for efficient transient transgene expression of secreted alkaline phosphatase (SEAP). CHO cells transfected with the replication competent pPyOriLT-SEAP resulted in a substantially greater amount of measured activity than cells transfected with the replication incompetent pM2.6-SEAP (Fig 6). A comparison of SEAP activity as measured on the fourth day post-transfection, shows an increase of 1.5-fold for zinc-treated cultures, 2.6-fold increase in zinc + cadmium-treated cultures and 14-fold increase in the absence of any metals for the replication competent

vector containing cells. Interestingly, SEAP activity from zinc-induced cultures were only marginally greater than those without zinc addition in cells transfected with pPyOriLT-SEAP. These results suggest that an increase in plasmid copy number due to episomal replication can significantly improve the transient protein production in CHO.

DISCUSSION AND CONCLUSION

Large-scale transient expression technology is a promising approach for the rapid generation of recombinant protein. High level transient protein expression has been demonstrated successfully in HEK293 cells.^{4,19} This study focuses on two key factors that affect the level of protein expression: the utilisation of a strong promoter and the incorporation of elements that support episomal replication.

A transient gene expression assay was used to compare promoter-directed gene activity which eliminates the potential influence of chromosomal integration site on promoter activity.^{20,21} To minimise variations in transfection efficiencies due to plasmid size and vector sequence, DNA-containing promoter sequences were

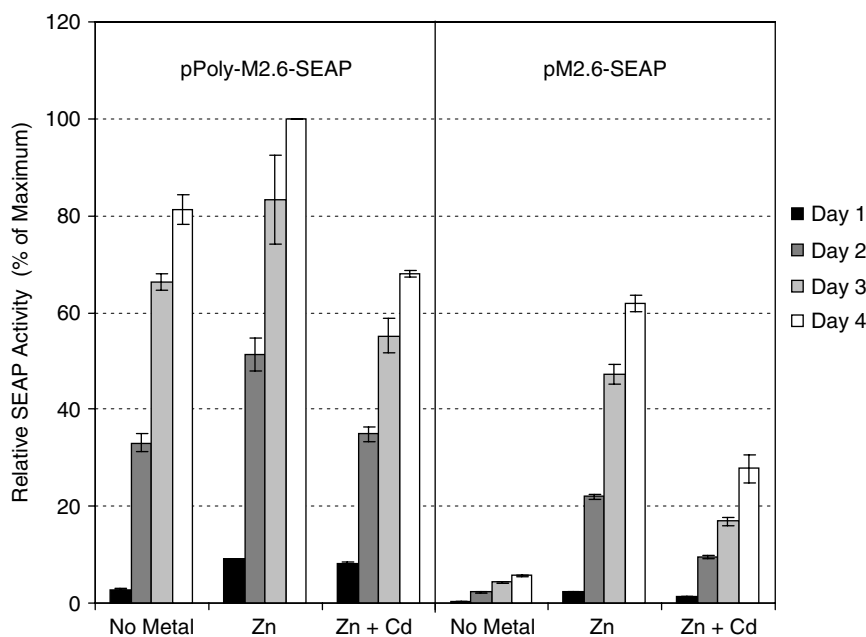


Figure 6. Transient SEAP expression. Relative SEAP activity in transfected cultures expressed. SEAP activity obtained from the zinc-induced pPyOriLT-SEAP transfected culture at 4 days post-transfection was set to 100%. Transfections were performed in triplicate.

individually cloned into the pd2EGFP-1 upstream of the coding sequence for destabilised GFP. The rapid turnover of destabilised GFP enables reporter activity to be tracked at finer intervals, providing a closer examination of promoter strength in a transient expression assay.¹⁰ For CHO, the rank order of expression levels in this study is: M2.6 (metal-induced) > CMV > SV40 > M2.6 (without metal).

Although it has been previously shown that conditions for the optimal induction of the metal-inducible M2.6 promoter is with the addition of $100 \mu\text{mol L}^{-1}$ zinc + $2 \mu\text{mol L}^{-1}$ cadmium,¹³ this combination of metal severely affected plasmid replication (Fig 4). For the purpose of transient expression, $100 \mu\text{mol L}^{-1}$ zinc is sufficient to induce expression of the M2.6 promoter (Figs 5 and 6). It has been previously demonstrated that the addition of zinc alone is sufficient for high level protein production employing the M2.6 expression system at various scales (spinner flask and bioreactor).²²

Plasmid copy number is a key element that can elevate the level of protein production in transient expression systems. The level of recombinant protein production can be elevated when more plasmid copies persist in transfected cells. One way to accomplish this is to enable episomal replication of transfecting DNA by incorporating a host cell specific viral origin of DNA replication into the expression vector. A Py-based vector was chosen to establish episomal replication in CHO, requiring only two exogenous elements, namely PyOri and PyLT.¹¹ The DNA encoding PyOri and PyLT was used to construct the expression vector, pPyOriLT. The plasmid was shown to replicate in CHO cells. Plasmid replication was maximal at 48 h post-transfection with approximately 3000 replicated plasmid copies per

cell (Fig 3), which exceeds other mouse cell lines transfected with Py-based plasmids.^{23,24} Although the amount of replicated plasmid DNA declined rapidly after 48 h in cells transfected with the replication competent vector, a significant increase in the percentage of cells expressing the transgene, GFP was observed. Up to 99% of the pool expressed measurable amounts of GFP protein 4 days post-transfection (Fig 5). This was not observed for the replication incompetent vector indicating that the Py-based episomal replication can significantly enrich the percentage of protein expressing cells in culture within 4 days following transfection. In this study, the replication-competent expression vector pPyOriLT-SEAP resulted in a 14-fold increase in SEAP expression compared with the replication-incompetent pM2.6-SEAP.

Further evidence that Py-based episomal replication can significantly improve recombinant protein production in CHO cells was demonstrated by Kunaparaju *et al.*²⁵ These authors demonstrated that suspension-growing CHO cells expressing PyLT transfected with PyOri containing plasmid resulted in a final concentration of 75 mg L^{-1} of recombinant protein in culture supernatants 11 days post-transfection.

In conclusion, plasmid replication and transgene expression driven by the zinc-induced M2.6 promoter resulted in elevated plasmid copy within transfected cells, a high percentage of transgene-expressing cells and high protein production. The expression vector pPyOriLT may prove to be beneficial for the scaled production of recombinant protein in transiently transfected CHO cells. The use of more cost-effective transfection reagents such as PEI^{1,26} instead of Lipofectamine 2000 used in this study may need to be explored.

REFERENCES

- 1 Durocher Y, Perret S and Kamen A, High-level and high-throughput recombinant protein production by transient transfection of suspension-growing human 293-EBNA1 cells. *Nucleic Acids Res* **30**:e9 (2002).
- 2 Girard P, Derouazi M, Baumgartner G, Bourgeois M, Jordan M, Jacko B and Wurm FM, 100-liter transient transfection. *Cytotechnology* **38**:15–21 (2002).
- 3 Meissner P, Pick H, Kulangara A, Chatellard P, Friedrich K and Wurm F, Transient gene expression: recombinant protein production with suspension-adapted HEK293-EBNA cells. *Biotechnol Bioeng* **75**:197–203 (2001).
- 4 Pham PL, Perret S, Doan HC, Cass B, St Laurent G, Kamen A and Durocher Y, Large-scale transient transfection of serum-free suspension-growing HEK293 EBNA1 cells: peptone additives improve cell growth and transfection efficiency. *Biotechnol Bioeng* **84**:332–342 (2003).
- 5 Werner RG, Noe W, Kopp K and Schluter M, Appropriate mammalian expression systems for biopharmaceuticals. *Arzneimittelforschung* **48**:870–880 (1998).
- 6 Chu L and Robinson DK, Industrial choices for protein production by large-scale cell culture. *Curr Opin Biotechnol* **12**:180–187 (2001).
- 7 Andersen DC and Krummen L, Recombinant protein expression for therapeutic applications. *Curr Opin Biotechnol* **13**:117–123 (2002).
- 8 Bailey CG, Baig M, Gray PP and Sunstrom NA, A rapid selection/amplification procedure for high-level expression of recombinant protein in a metal-amplifiable mammalian expression system. *Biotechnol Tech* **13**:615–619 (1999).
- 9 McNeill J, Sanchez A, Gray PP, Chesterman CN and Sleight MJ, Hyperinducible gene expression from a metallothionein promoter containing additional metal-responsive elements. *Gene* **76**:81–88 (1989).
- 10 Li X, Zhao X, Fang Y, Jiang X, Duong T, Fan C, Huang CC and Kain SR, Generation of destabilized green fluorescent protein as a transcription reporter. *J Biol Chem* **273**:34 970–34 975 (1998).
- 11 Heffernan M and Dennis JW, Polyoma and hamster papovavirus large T antigen mediated replication of expression shuttle vectors in Chinese hamster ovary cells. *Nucleic Acids Res* **19**:85–92 (1990).
- 12 Warburton S and James R, Hemocytometer cell counts and viability studies, in *Cell and tissue culture: Laboratory procedures*, ed by Doyle A, Griffiths JB and Newell DG. John Wiley & Sons Ltd, New York, 4B:1.1–1.5 (1993).
- 13 Bailey CG, Tait AS and Sunstrom NA, High-throughput clonal selection of recombinant CHO cells using a dominant selectable and amplifiable metallothionein-GFP fusion protein. *Biotechnol Bioeng* **80**:670–676 (2002).
- 14 Hirt B, Selective extraction of Polyoma DNA from infected mouse cell cultures. *Journal of Molecular Biology* **26**:365–369 (1967).
- 15 Nelson M and McClelland M, The effect of site-specific methylation on restriction-modification enzymes. *Nucleic Acids Res* **15**:219–230 (1987).
- 16 Berger J, Hauber J, Hauber R, Geiger R and Cullen BR, Secreted placental alkaline phosphatase: a powerful new quantitative indicator of gene expression in eukaryotic cells. *Gene* **66**:1–10 (1988).
- 17 Shillitoe EJ and Noonan S, Strength and specificity of different gene promoters in oral cancer cells. *Oral Oncol* **36**:214–220 (2000).
- 18 Cole CN and Conzen SD, Polyomavirinae: the viruses and their replication, in *Fields Virology*, ed by Knipe DM and Howley PM. Lippincott Williams & Wilkins, Philadelphia, pp 2141–2174 (2001).
- 19 Wurm F and Bernard A, Large-scale transient expression in mammalian cells for recombinant protein production. *Curr Opin Biotechnol* **10**:156–159 (1999).
- 20 Eszterhas SK, Bouhassira EE, Martin DI and Fiering S, Transcriptional interference by independently regulated genes occurs in any relative arrangement of the genes and is influenced by chromosomal integration position. *Mol Cell Biol* **22**:469–479 (2002).
- 21 Cranston A, Dong C, Howcroft J and Clark AJ, Chromosomal sequences flanking an efficiently expressed transgene dramatically enhance its expression. *Gene* **269**:217–225 (2001).
- 22 Huang EP, Marquis CP and Gray PP, Process development for a recombinant Chinese hamster ovary (CHO) cell line utilizing a metal induced and amplified metallothionein expression system. *Biotechnol Bioeng* **88**:437–450 (2004).
- 23 Kern FG and Basilico C, An inducible eukaryotic host-vector expression system: amplification of genes under the control of the polyoma late promoter in a cell line producing a thermolabile large T antigen. *Gene* **43**:237–245 (1986).
- 24 O'Hare K, Replication of polyoma plasmid recombinants in mouse cells. *J Mol Biol* **151**:203–210 (1980).
- 25 Kunaparaju R, Liao M and Sunstrom NA, *Epi-CHO*, an episomal expression system for recombinant protein production in CHO cells. *Biotechnol Bioeng* (2005). (in press).
- 26 Tait AS, Brown CJ, Galbraith DJ, Hines MJ, Hoare M, Birch JR and James DC, Transient production of recombinant proteins by Chinese hamster ovary cells using polyethyleneimine/DNA complexes in combination with microtubule disrupting anti-mitotic agents. *Biotechnol Bioeng* **88**:707–721 (2004).