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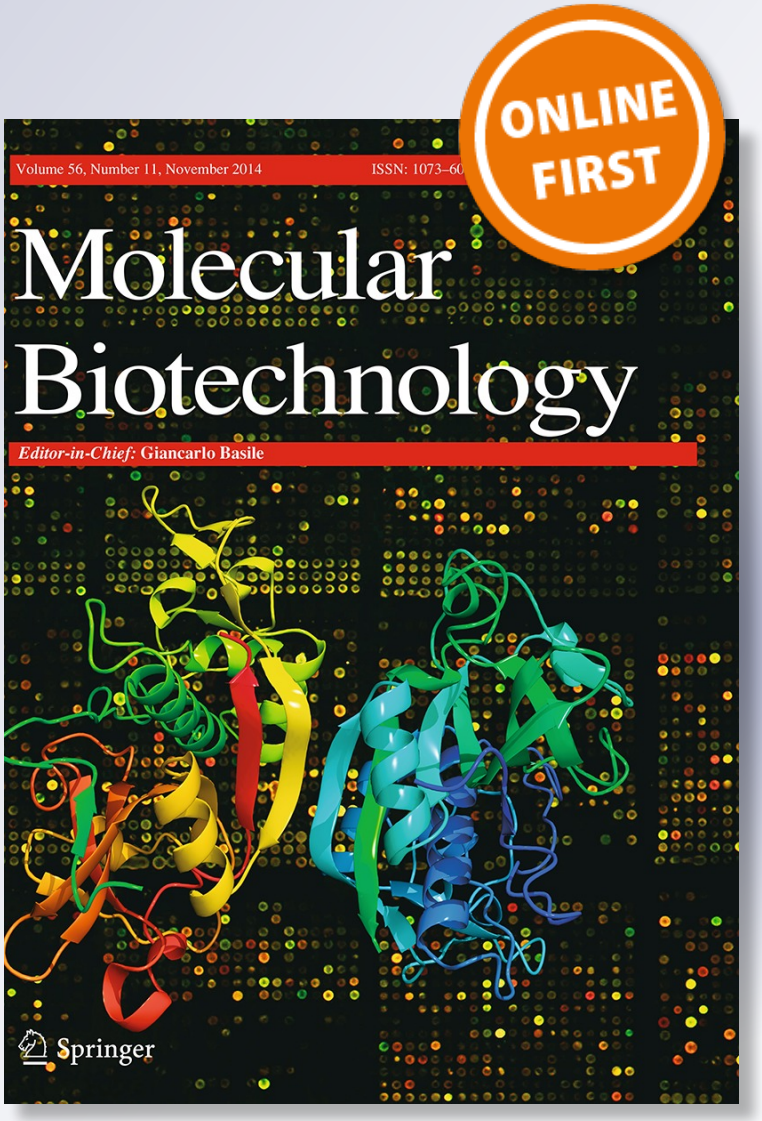

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RESEARCH

Impact of Using Different Promoters and Matrix Attachment Regions on Recombinant Protein Expression Level and Stability in Stably Transfected CHO Cells

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Abstract High expression level and long-term expression stability are required for therapeutic protein production in mammalian cells. Three commonly used promoters from the simian virus 40 (SV40), the CHO elongation factor 1α gene (EF1α), and the human cytomegalovirus major immediate early gene (CMV) and two matrix attachment regions from the chicken lysozyme gene (cMAR) and the human interferon β (iMAR) were evaluated for enhancing recombinant gene expression level and stability in stably transfected CHO cells. In the absence of MAR elements, the SV40 promoter gave lower expression level but higher stability than the EF1α promoter and the CMV promoter. The inclusion of MAR elements did not increase the integrated gene copies for all promoters but did enhance expression level for only the SV40 promoter. The enhanced gene expression was due to an increase in mRNA levels. Neither MAR elements enhance gene expression stability during long-term culture. The combinations of SV40 promoter and MAR elements are the best for obtaining both high expression level and stability. The information presented here would be valuable to those developing vectors for generation of CHO cell lines with stable and high productivity.

Keywords Matrix attachment region (MAR) · CMV promoter · SV40 promoter · CHO elongation factor 1α promoter · Stable recombinant protein expression · CHO cells

Introduction

Mammalian cells, such as Chinese hamster ovary cells (CHO), are predominant for commercial production of therapeutic proteins because of their capacity to perform proper protein folding, assembly, and post-translational modifications [1, 2]. Generating a therapeutic protein producing cell line starts with transfecting the mammalian host cells with a plasmid vector carrying the gene for the respective therapeutic protein. Subsequent selection is performed to isolate stably transfected clones with high productivity and long-term stable production. Expression stability also needs to be maintained without the aid of any selection pressure as these drugs add to production costs and can complicate downstream purification [3]. Recombinant protein productivity and stability in a clone are influenced by both the composition of the plasmid vector and the site of vector integration on the chromosome [4–6]. Apart from the gene of interest, components of the plasmid include promoters, polyadenylation signals, and other expression augmenting elements like matrix attachment regions (MAR).

A commonly used promoter for high level recombinant protein production in mammalian cells is the human cytomegalovirus major immediate early gene promoter (CMV) [7]. While CMV is a strong promoter, there are reports that the promoter is intrinsically susceptible to silencing, resulting in declined productivity during long-term culture [3, 8–16]. Promoters derived from the simian virus 40 (SV40) and

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S. C. L. Ho · Mariati · J. H. M. Yeo · S. G. Fang · Y. Yang (✉)
Agency for Science, Technology and Research (A*STAR), Bioprocessing Technology Institute, 20 Biopolis Way, #06-01 Centros, Singapore 138668, Singapore
e-mail: yang_yuansheng@bti.a-star.edu.sg

Y. Yang
School of Chemical and Biomedical Engineering, Nanyang Technological University, N1.2-B2-33, 62 Nanyang Avenue, Singapore 637459, Singapore

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CHO elongation factor 1α gene (EF1α) are also strong for therapeutic protein production in mammalian cells [7, 17]. EF1α promoter used in conjunction with flanking regions of the CHO EF1α gene was more active in CHO cells than using CMV and SV40 promoters alone [17]. Protein production instability in CHO cells when using the SV40 promoter has been reported [18, 19]. Some studies indicated that promoters of endogenous mammalian genes like the EF1α can be more resistant to silencing than viral promoters [20–25]. Although there are many separate reports of promoter studies, it can be difficult to make comparisons of the promoters when each report is performed in differing culture conditions and cell lines. A comparison of transgene expression level and stability in stably transfected CHO cell clones under similar conditions without selection pressure would aid in choosing between the CMV, SV40, and EF1α promoters for recombinant protein production in CHO cells.

MARs are DNA elements which may be involved in anchoring DNA/chromatin to the nuclear matrix to define the boundaries of independent chromatin domains [26, 27]. MARs were reported to shield transgenes from chromosomal position effects and increase transgene expression level in stably transfected cell lines [28–37]. There are conflicting reports on whether MAR elements can prevent transgenes from silencing in stably transfected cell lines. Inclusion of MARs into viral vectors increased their resistance to gene silencing [38, 39]. When MARs were included into plasmid vectors, the preferred vectors for safe production of therapeutic proteins in mammalian cells, enhanced resistance to silencing was observed for the SV40 promoter but not for the CMV promoter [19, 37], suggesting that the effect of MARs on gene silencing may be dependent on the promoters.

Among many MAR elements which have been identified to date, chicken lysozyme (cMAR) and human interferon β (iMAR) MAR elements have shown to be very effective at enhancing transgene expression in stably transfected cells [29, 37]. In this work, we evaluated the SV40, EF1α, and CMV promoters for recombinant protein expression level and stability in stably transfected CHO cells. The impact of including cMAR and iMAR with the above three promoters was studied as well. The information collected would benefit those choosing promoters and MAR elements during plasmid vector designs for generating cell lines with both high expression level and long-term expression stability.

Materials and Methods

Vector Construction

The CMV containing bicistronic vector without MAR was constructed based on a previously described IRES-mediated tricistronic vector [40] with the LC-IRESwt-HC region replaced with EGFP cDNA. The remaining bicistronic vectors with or without MAR were then constructed by replacement of the CMV promoter with either EF1α or SV40 promoter and insertion of cMAR or iMAR immediately upstream of the promoter and downstream of the SpA. The CMV and SV40 promoter were cloned from pcDNA3.1(+) (Life Technologies, Carlsbad, CA). The EF1α promoter with sequence corresponding to the region from −463 to +1,010 (relative to the transcription start site of +1) of the CHO EF1α gene (NCBI: AY188393.1) was isolated from CHO K1 cells. The iMAR and cMAR were cloned from a pEPI-1 vector [41] and a pPAG1 vector [37], respectively. All restriction enzymes used in the vector construction were purchased from New England Biolabs (Ipswich, MA). The DH5α^{TM} competent cells were purchased from Life Technologies.

Cell Culture and Media

CHO K1 cells (American Type Culture Collection, Manassas, VA) were maintained in medium consisting of Dulbecco's modified Eagle's medium (DMEM) + GlutaMax^{TM} (Life Technologies) supplemented with 10 % fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO). Regular passaging was carried out every 3–4 days by diluting cells to 2×10^5 cells/mL. Cell density and viability were measured using the trypan blue exclusion method on an automated Cedex counter (Innovatis, Bielefeld, Germany).

Generation of Stably Transfected Cell Lines

Triplicate transfections were performed for each vector using Nucleofector I system according to manufacturer's instructions (Lonza, Cologne, Germany). 5×10^6 cells were transfected with 5 μg of linearized plasmids in each transfection. The transfected cells were then resuspended in 2 mL of pre-warmed maintenance medium in 6-well tissue culture plates. At 24 h post-transfection, maintenance medium was removed, and 2 mL of maintenance medium containing 800 μg/mL of G418 was added into each well for selection of stable transfectants. Stably transfected pools were obtained after 2–4 weeks depending on the vectors. Six clones were randomly isolated from each pool to obtain a total of 18 clones for each vector using limiting dilution method in 96-well tissue culture plates.

Stability Testing

The clones isolated for each vector were passaged in 6-well tissue culture plates in the absence of G418 for 8 weeks. MFI for each clone before and after stability testing was measured with the FACS Calibur. Retention of EGFP

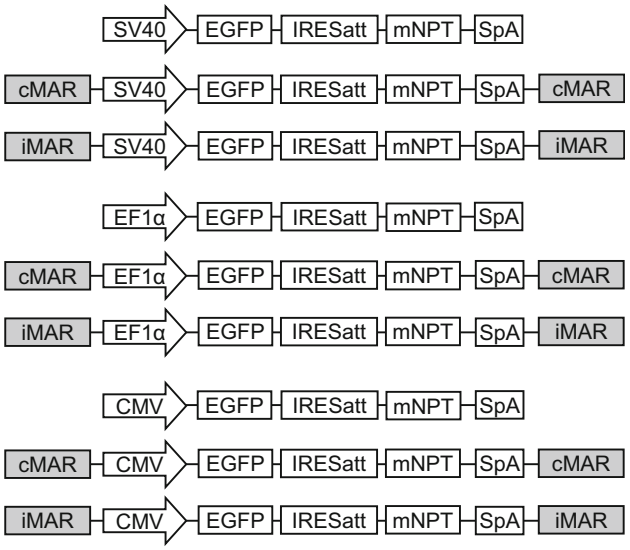

Fig. 1 Schematic representation of vectors for evaluating promoters and the impact of MAR on recombinant protein expression level and stability in CHO cells. SV40, simian virus 40 promoter; EF1α, Chinese hamster elongation factor-1α promoter; CMV, human cytomegalovirus IE gene promoter; iMAR, human interferon β matrix attachment region (MAR) element; cMAR, chicken lysozyme MAR element; IRESatt, attenuated encephalomyocarditis virus (EMCV) internal ribosome entry site with reduced translation efficiency; SpA, simian virus 40 early polyadenylation signal; EGFP, enhanced green fluorescence protein cDNA; mNPT, mutated neomycin phosphotransferase cDNA with amino acid D at 261 changed to G [43]

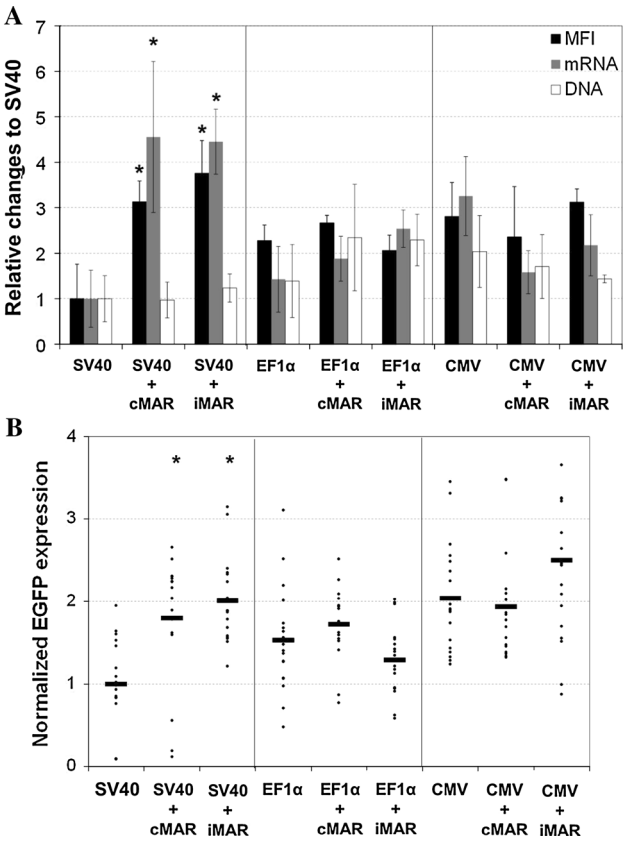

Fig. 2 Use of different promoters and the impact of MAR on gene expression level in stably transfected pools (**a**) and clones (**b**). Three stably transfected pools were generated for each vector. To characterize each stably transfected pool, 2 mL of cultures at a density of 2×10^5 cells/mL were seeded into each well of 6-well plates. Cells were collected at day 3 and measured for the EGFP MFI with the FACS Calibur and EGFP gene copies and mRNA levels using quantitative real-time PCR (qRT-PCR). Results in **a** were presented as the EGFP MFI (*black bar*), mRNA levels (*gray bar*), and gene copies (*white bar*) normalized to those from the SV40 promoter. Each value represents the average and standard deviation of three independent stably transfected pools. Six clones each were isolated from three separately transfected pools for a total of eighteen clones for each vector. Each *dot* in **b** represents the MFI of each clone normalized to the average MFI of the eighteen clones generated using the SV40 promoter. *Horizontal bars* signify the average value of the 18 clones for each vector. Mean values significantly different (two-tailed Student's *t* test) between the vectors containing MAR and the vector without MAR for each promoter indicated by $*p < 0.05$

expression for a clone was calculated as the ratio of MFI of the clone measured at the end of stability testing to the intensity at the start of stability testing.

Analysis of Relative Gene Copies and mRNA Levels

The relative EGFP gene copies and mRNA levels were determined using real-time quantitative PCR (qRT-PCR) as described previously [42]. Genomic DNA and total RNA were isolated from 5×10^6 cells using the Gentra Puregene Cell Kit (Qiagen, Hilden, Germany) and the RNAqueous-4PCR kit (Ambion, Austin, TX), respectively. EF1α and β-actin were used to normalize the variation in input amount and quality of RNA and DNA, respectively.

Results

Evaluating Recombinant Protein Expression Level Using Different Promoters and Impact of Including MARs

We first evaluated SV40, EF1α, and CMV promoters and their use with cMAR and iMAR for expression level in stably transfected CHO cells. Bicistronic vectors expressing an enhanced green fluorescence reporter protein (EGFP) and a mutated neomycin phosphotransferase (mNTP) selection marker under the control of SV40, EF1α, or CMV promoter were first constructed (Fig. 1). Copies of cMAR and iMAR were then inserted at the 5′ and 3′ flanking regions of the expression cassette in each vector. This configuration was shown to be more effective in enhancing stable transgene expression than using only a

single copy of MAR in previous studies [30, 35]. Use of the mNTP with reduced enzyme activity is for enhancing the selection stringency for high producing cells as only clones with greater transcriptional activity or more copies of the integrated vector can survive the selection process [40, 43, 44].

CHO K1 cells were transfected with each vector followed by drug selection for stable transfectants. Only tens of clones survived the selection in transfected pools generated using the vectors without MAR, and stably transfected pools were obtained in about four weeks. In contrast, hundreds of clones survived the selection in transfected pools generated using vectors containing MAR elements, and stably transfected pools were obtained in about 2 weeks. EGFP expression in stably transfected pools was quantified by measuring the mean fluorescence intensity (MFI). The SV40 promoter only vector gave the lowest EGFP expression, 2- and 3-fold less than that from the EF1α and CMV only vectors, respectively (Fig. 2a). Application of cMAR and iMAR on the SV40 promoter (SV40+cMAR and SV40+iMAR) enhanced EGFP expression by about 3- and 4-fold, respectively. Inclusion of MARs failed to enhance EGFP expression for EF1α and CMV promoters. Stably transfected pools generated using SV40+cMAR/iMAR vectors exhibited higher EGFP expression than those generated using any of the EF1α and CMV vectors. qRT-PCR analysis indicated that application of MARs on different promoters did not change the integrated EGFP gene copies compared to the use of each promoter alone. However, MARs enhanced EGFP mRNA levels when they were applied on the SV40 promoter but had no effect on the EGFP mRNA levels when they were applied on the other two promoters.

Comparison of the different promoters and MAR elements on EGFP expression were subsequently repeated in stably transfected clones (Fig. 2b). MFI varied dramatically between clones generated using the same vectors for all pools. Consistent with the results in stably transfected pools, both the average MFI and maximal EGFP expression in the SV40 promoter only clones were less than those generated using the EF1α and CMV promoters. Similar to the stable pools, application of cMAR and iMAR on the SV40 promoter enhanced both average and maximal EGFP expression levels in clones but exhibited no effect when used with EF1α and CMV promoters.

Evaluating Long-Term Recombinant Protein Expression Stability Using Different Promoters and Impact of Including MARs

We next evaluated the long-term transgene expression stability of the three promoters and MAR elements by determining the retention of EGFP expression after eight

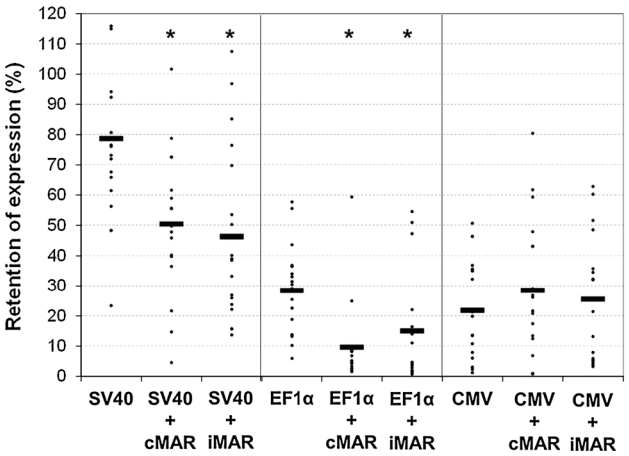

Fig. 3 Retention of EGFP expression in stably transfected clones generated using different vectors. Each *dot* represents the percentage of retention of EGFP expression for one clone. The *horizontal bars* signify the average value of 18 clones for each vector. Mean values significantly different (two-tailed Student's *t* test) between the vectors containing MAR and the vector without MAR for each promoter indicated by $*p < 0.05$

weeks of culturing in the absence of selection pressure. A precise definition of stable production varies depending on application but typically clones which maintain above 70 % of their starting productivity are considered to be stable [45]. Using 70 % retention as a cutoff, 11 out of 18 clones generated using a SV40 promoter were stable after eight weeks of passaging (Fig. 3). The 18 clones retained an average of 79 % of their starting EGFP expression level. A reverse correlation between the expression level and stability was observed for these SV40 clones ($R^2 = 0.6574$, Supplementary Fig. 1). All stable clones had relatively low EGFP expressions. In contrast, none of clones generated using EF1α and CMV promoters were stable, retaining an average of only 28 and 22 % of their starting levels, respectively. Addition of cMAR and iMAR on any promoters did not result in enhanced expression stability but instead decreased expression stability in clones for the SV40 and EF1α promoter. On average, clones generated using SV40+cMAR/iMAR vectors maintained about 50 % their original expression level and 30 % less than those generated using SV40 only but still greater than all the EF1α and CMV clones. Among 18 clones generated using the SV40+cMAR and SV40+iMAR vectors, four and five clones respectively were still stable after the eight weeks. In contrast to those clones generated using SV40 only, no correlation between expression level and stability was observed for clones generated using other vectors ($R^2 = 0.0002$ to 0.2428, Supplementary Fig. 1). Some stable clones generated using SV40+cMAR/iMAR also exhibited high EGFP expressions. For instance, the highest producing clone generated using the SV40+cMAR vector and the second highest producing clone generated using the

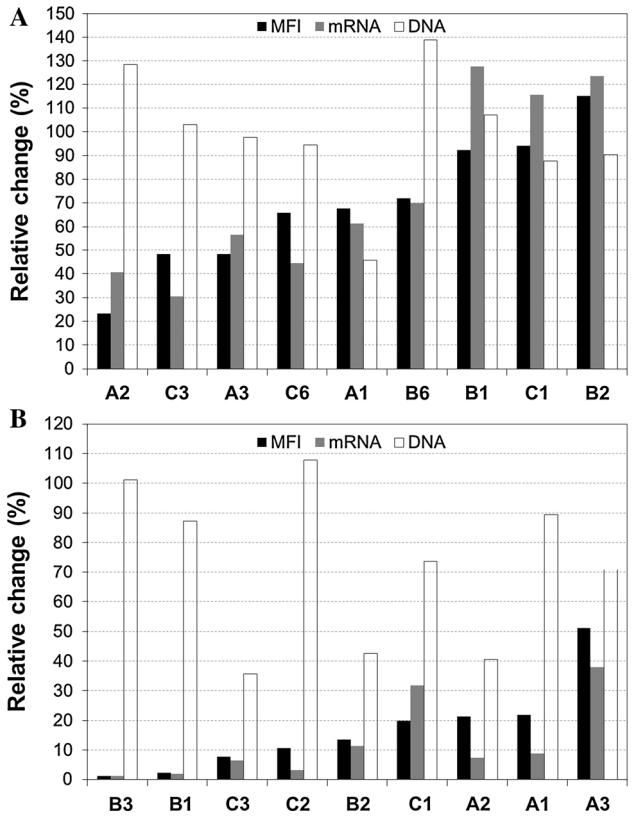

Fig. 4 Relative changes in EGFP expression (*black bar*), mRNA levels (*gray bar*), and gene copies (*white bar*) in SV40 and CMV clones during stability testing. The relative changes in EGFP expression, mRNA levels, and gene copies were calculated as the ratio of EGFP MFI, mRNA levels, and gene copies of a clone measured at week 8 to the starting level for the same clone measured at week 0. Each value represents the average of two measurements

SV40+iMAR vector had 73 % and 107 % retention of expression after 8 weeks culturing.

To understand why the SV40 promoter gave more stable expression than other promoters, nine SV40 clones and nine CMV clones of varying EGFP retention were sorted based on their retention levels and analyzed for changes in EGFP mRNA levels and gene copies. The SV40 clones retained EGFP expression ranging from 23 to 115 % (Fig. 4a), and the CMV clones had EGFP retention ranging from 1 to 51 % (Fig. 4b). At the end of stability testing, only the SV40 clone A1 had a significant drop in EGFP gene copies to below 50 %. Of the remaining eight clones, four clones maintained over 70 % EGFP expression and mRNA levels. None of the CMV clones retained over 70 % of EGFP expression or mRNA levels, despite six clones still retaining more than 70 % of their gene copies. The relative changes in EGFP expression levels were correlated to the changes in EGFP mRNA levels for all clones. These results suggest that the SV40 promoter is more resistant to transcriptional silencing than the CMV promoter.

Discussion

Previous studies indicated that endogenous mammalian promoters could provide more stable gene expression than the viral promoters. Interestingly, we found that the SV40 promoter gave the highest expression stability in stably transfected CHO cell clones, while none of eighteen clones generated using EF1α and CMV were stable. Analysis of the changes in EGFP gene copies and mRNA levels in SV40 and CMV clones suggested that the SV40 promoter was more resistant to transcriptional silencing. DNA methylation is one of mechanisms causing transcriptional silencing. Both CMV and EF1α are crowded with CG dinucleotides containing 31 and 141 CGs, respectively, while the SV40 promoter has only 6 CG dinucleotides (Supplementary Fig. 2). The lower number of CGs may be one reason why the SV40 promoter is more resistant to transcriptional silencing due to DNA methylation. Another possible reason is that SV40 contains two SP1 transcription factor binding sites (Supplementary Fig. 2), which was previously proposed to inhibit DNA methylation [46]. Mutation of CGs in the retroviral vector long terminal repeat had repressed promoter silencing in embryonic stem cells [47]. We attempted to remove the CGs within the SV40 promoter to enhance its resistance to transcriptional silencing. The CG-free SV40 promoter had lower expression than the wild-type SV40 in transient transfections and failed to survive the selection process for stable expression (unpublished data). The mutant SV40 we generated was likely too weak for our application, and further efforts are currently underway to develop CG-free SV40 promoters without compromising the gene expression levels.

Addition of either cMAR or iMAR enhanced the stable gene expression level for the SV40 promoter but had no effect on EF1α and CMV. Addition of the two MAR elements did not enhance long-term expression stability for any promoters. Among all the tested vectors, SV40+cMAR and SV40+iMAR were the best for obtaining both high expression level and stability in CHO cells. All previous studies based on SV40 promoter demonstrated that MARs were able to enhance stable gene expression [27, 32–37, 48]. Fewer studies of MAR were performed on the CMV promoter, and the results were conflicting [31, 35, 49]. Our results were consistent with the study done by Lonza in which the inclusion of cMAR into their glutamine synthetase expression vector, which is commonly used for mammalian expression in the industry, did not enhance monoclonal antibody expression levels [49]. A recent study demonstrated that the activity of MAR elements was most pronounced for the chromosomal positions with low expression potential but had negligible effects in the case of highly active chromosomal sites [5]. We speculate that effect of MAR on transgene expression may be less

effective when strong promoters and/or vectors with more stringent selection of high expression levels are used. Inclusion of MARs to a previously well-optimized vector design would likely yield less benefit compared to adding to a simpler basic vector. Further studies are needed to investigate and identify the optimal plasmid vector design and context to maximize the effect of including MAR elements to improve expression level and stability.

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