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Insertion of Core CpG Island Element into Human CMV Promoter for Enhancing Recombinant Protein Expression Stability in CHO cells

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Abstract (200 words)

The human cytomegalovirus promoter (hCMV) is susceptible to gene silencing in CHO cells, most likely due to epigenetic events, such as DNA methylation and histone modifications. The core CpG island element (IE) from the hamster adenine phosphoribosyltransferase (APRT) gene has been shown to prevent DNA methylation. A set of modified hCMV promoters was developed by inserting one or two copies of IE in either forward or reverse orientations either upstream of the hCMV enhancer, between the enhancer and core promoter (CP), or downstream of the CP. The modified hCMV with one copy of IE inserted between the enhancer and core promoter in reverse orientation (MR1) was most effective at enhancing expression stability without compromising expression level when compared with the wild-type (WT) hCMV. A third of 18 EGFP expressing clones generated using MR1 retained 70% of their starting expression level after eight weeks of culture in the absence of selection pressure, while none of 18 WT hCMV generated clones had expression above 50%. MR1 also improved antibody expression stability of methotrexate (MTX) amplified CHO cell lines. Stably transfected pools generated using MR1 maintained 62% of their original monoclonal antibody titer after eight weeks of culture in the absence of MTX, compared to only 37% for WT hCMV pools. Low levels of CpG methylation within both WT hCMV and MR1 were observed in all the analyzed cell lines and the methylation levels did not correlate to the expression stability, suggesting IE enhances expression stability by other mechanisms other than preventing methylation.

Keywords: CHO, hCMV promoter, core CpG island element (IE), gene silencing, expression stability, monoclonal antibody

Introduction

Maintaining high gene expression level is critical when producing therapeutic recombinant protein using mammalian cells. A substantial loss of productivity during the 2-3 months long scale up process can affect both product yield and quality, compromising regulatory approval of the therapeutic product[1]. Stability should be maintained without selection reagent for mass production of therapeutic protein as the reagents are toxic and expensive[1]. A precise definition of stable production varies depending on application but typically clones which can maintain 70% of their start productivity are considered to be stable[2]. Recombinant protein expression stability of a cell line is influenced by the vector integration site on the chromosome and the plasmid vector composition[3-5]. The promoter is an essential component of the plasmid vector which initiates gene transcription, and is able to affect both gene expression level and stability[4]. The human cytomegalovirus MIE gene (hCMV) promoter is commonly used for recombinant protein expression in mammalian cells[6-8]. Although the hCMV promoter gives high gene expression levels, there are many reports of declining production levels during long-term culture of the cell lines generated using this promoter[2, 9-18].

hCMV promoter silencing is largely attributed to the epigenetic events of promoter DNA methylation and histone modification[14, 15, 19-23]. Removing CpGs in the promoter is one approach to overcome silencing due to methylation[24]. Endogenous mammalian promoters which are less susceptible to silencing effects than viral promoters can also be used[25-30]. Another commonly used approach is to introduce DNA elements like insulators, locus control region (LCR), matrix attachment region (MAR), stabilizing anti-repressor element (STAR), and ubiquitous chromatin region opening elements (UCOE) into the vectors[3, 31-37]. CpG islands isolated from the mouse

and hamster adenine phosphoribosyltransferase (APRT) genes are alternative DNA elements which are shown to be effective at preventing DNA methylation[38-40]. Deletion analysis of the hamster APRT island identified a 120 base-pair core CpG island element (IE)[41, 42], making IE easier to use than the other DNA elements which can span several thousand base pairs. Inclusion of IE into the retroviral long terminal repeat (LTR) promoter led to efficient protection of the integrated viral vectors from silencing in NIL-2, HEK293, and QT6 cells[41]. Functions of DNA elements could be dependent on the cells and vector context[43]. For instance, UCOE was observed to exhibit promoter specificity and empirical testing was required to identify optimal UCOE-promoter combinations that gave higher expression level and stability[44]. It is still unclear whether IE can prevent silencing of the hCMV promoter and enhance recombinant protein production stability in CHO cells, the predominant host cell for industrial production of therapeutic recombinant proteins[45].

In this work, we studied the effect of inserting either one or two copies of IE in forward and reverse orientations into different locations on the hCMV promoter on recombinant gene expression levels and stability in CHO cells during long term culture. The modified hCMV promoter with IE inserted between the hCMV enhancer and core promoter (CP) enhanced expression stability and had comparable expression level to the wild-type (WT) hCMV. Besides generation of recombinant cells lines for stable productivity, this modified hCMV promoter can also be applied in other fields like cell engineering and gene therapy.

Materials and Methods

Cell Culture and media

Adherent CHO K1 cells (American Type Culture Collection, Manassas, VA) were grown in Dulbecco's modified Eagle's medium (DMEM) + GlutaMax™ (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO), hereinafter referred to as serum medium, in 75 cm^2 T-flasks in a humidified incubator at 37°C and 5% CO_2. Suspension dihydrofolate reductase (DHFR)-deficient CHO DG44 cells (Life Technologies) were grown in a protein-free medium consisting of HyQ PF (HyClone, Logan, UT) and CD CHO (Life Technologies) at a 1:1 ratio and supplemented with 1g/L sodium bicarbonate (Sigma), 6 mM glutamine (Sigma), 0.05% Pluronic F-68 (Life Technologies), and 1% hypoxanthine and thymine (HT) (Life Technologies) in 125 mL shake flasks in humidified Kuhner shaker incubator (Adolf Kühner AG, Birsfelden, Switzerland) with 8% CO_2 at 37 °C. Regular passaging of both adherent CHO K1 cells and suspension CHO DG44 cells was carried out every 3 to 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).

Construction of vectors

The wild-type (WT) hCMV promoter (NCBI: M60321) used in this work consists of an enhancer (-599 to -218 relative to the transcription start site of +1) and a core promoter (CP, -217 to -14). The bicistronic enhanced green fluorescence protein (EGFP) expressing vector containing the WT hCMV was constructed by replacing the LC-IRESwt-HC region in a previously described monoclonal antibody (mAb) expressing tricistronic vector with an EGFP cDNA (Fig. 1A)[46]. The EGFP cDNA was cloned from the pIRES-EGFP Vector (Clontech, Mountain View, CA). Two unique restriction sites, *XhoI* and *NheI,* were introduced between the enhancer and CP and

downstream of CP in the bicistronic vector respectively using QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Thirteen modified hCMV promoters were generated by insertion of one or two copies of IE in either forward or reverse orientations upstream of the enhancer using *MluI* site, in the middle of the enhancer and CP using *XhoI* site, and downstream of CP using *NheI* site. They were designated as UF1, UF2, UR1, UR2, MF1, MF2, MR1, MR2, DF1, DF2, DR1, DR2, and UR2+MR1, respectively, based on the location, orientation, and copy number of IE (Fig. 1B). For instance, UF1 would denote one IE in the forward orientation inserted upstream of the enhancer, MR1 would denote one IE in the reverse orientation inserted in the middle of the enhancer and CP and DR2 would denote two reversely orientated IEs inserted downstream of the CP respectively. The mAb expressing tricistronic vector containing the WT hCMV was constructed as described previously (Fig. 1A) [47]. The tricistronic vector containing the MR1 was constructed by replacing the WT hCMV with MR1 using *MluI* and *BamHI* sites. IE elements were synthesized using overlapping PCR based on the previously described sequence[41]. All restriction enzymes used were from New England Biolabs (Ipswich, MA).

Transient transfections

Transient transfections were carried out in 6-well tissue culture plate using Fugene 6 (Roche, Indianapolis, IN). 2 mL of adherent CHO K1 cells at exponential phase were seeded at a density of 3×10^5 cells/mL into each well 24 h prior to transfection. Duplicate transfections were performed for each vector using a recipe of Fugene 6 to plasmid ratio of 6 µL:2 µg. At 48 h post-transfection, cells were collected and analyzed for EGFP fluorescence intensity using a FACS

Calibur (Becton Dickinson, MA). Each experiment was repeated once using independently prepared plasmids and cultures.

Generation of stably transfected cell lines

Comparison of the WT and modified hCMV promoters for EGFP expression level and stability was performed in adherent CHO K1 cells for easy isolation of clones. Triplicate transfections were performed for each vector using Nucleofector I system (Lonza, Cologne, Germany). In each transfection, 5×10^6 cells were transfected with 5 µg of linearized plasmids. The transfected cells were then resuspended with 2 mL of serum medium into 6-well tissue culture plates. At 24 h post-transfection, selection of stable transfectants was started in 2 mL of serum medium containing 800 µg/mL of G418. Non-transfected cells died after 7-10 days of selection and stably transfected pools were obtained after 4 weeks. Either three or six clones were randomly isolated from each pool by limiting dilution in 96-well tissue culture plates.

Comparison of the WT and modified hCMV promoters for mAb expression level and stability was performed in suspension CHO DG44 cells. Duplicate transfections were carried out for each vector using Nucleofector I system. In each transfection, 1×10^7 cells were transfected with 5 µg of linearized plasmids. The transfected cells were resuspended in 2 mL of protein-free medium in 6-well suspension culture well plates for 24 h recovery. Cell suspension was next centrifuged at ~100×g for 5 min and then resuspended in the protein-free medium with HT removed in 125 mL shake flasks to select for stable transfectants for 2 to 3 weeks. When the stably transfected pools recovered with viability greater than 95%, stepwise amplification was then carried out with methotrexate (MTX) concentrations of 50 nM, 100 nM, 250 nM, 500 nM, 750 nM, and 1000

nM. Each step of amplification was deemed complete when viability of cells recovered greater than 95%.

Stability analysis of stably transfected cell lines

Expression stability of EGFP expressing clones were determined in 6-well tissue culture plates. Two sets of clones generated by each promoter were banked before stability testing. One set of clones were thawed and passaged in the absence of G418 for 8 weeks. They were banked at the end of stability testing. The other set of clones banked before stability testing and the set of clones which had gone through stability testing were both thawed and passaged in the presence of and absence of G418 for 3 times, respectively. 2 mL of cultures at a density of 3×10^5 cells/mL for each clone were seeded into each well of 6-well plates. Cells were collected at day 4 and measured for the percentage of EGFP expressing cells and geometric mean fluorescence intensity with the FACS Calibur. The retention of EGFP expression level for each clone was calculated as the EGFP geometric mean fluorescence intensity measured at the end of stability testing divided by that before stability testing for the same clone. Both EGFP expressing and non-expressing cells were taken into account during calculation.

Expression stability of mAb expressing stably transfected pools were determined in 125 mL shake flask cultures. To determine the titer of each pool before stability testing, 25 mL of culture at a cell density of 2×10^5 cells/mL were seeded into the shake flasks. Supernatant was collected at the end of culture when viability declined below 50%. mAb concentration was measured using a nephelometric method on an IMMAGE 800 immunochemistry system (Beckman Coulter, Buckinghamshire, England). To test expression stability, stably transfected pools were passaged

in the absence of MTX for 8 weeks. The titer of each pool at the end of stability testing was determined as described above for pools before stability testing.

Analysis of relative gene copy numbers and mRNA levels

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. Analysis of the relative EGFP gene copy numbers and mRNA levels were determined by using real-time quantitative PCR (qRT-PCR) as described previously[10]. β-actin and eukaryotic translation elongation factor-1 alpha-1 (EF-1α) served as the internal controls to normalize the variation in input amount and quality of DNA and RNA, respectively. Primer sequences for amplification of EGFP, β-actin, and EF1α are listed in Table S1.

Analysis of promoter methylation

2 µg of purified genomic DNA prepared with Gentra Puregene Cell Kit was bisulfite treated using EpiTect Bisulfite Kit (Qiagen) according to manufacturer's protocol. The purified bisulfite converted genomic DNA was used as template for PCR to amplify the WT hCMV and MR1 promoter sequences using Platinum PCR SuperMix HighFidelity (Life Technologies) and primers as listed in Table S2. 1 µL of bisulfite-treated DNA was mixed with 10 pmol forward primer, 10 pmol reverse primer, 45 µL Platinum PCR SuperMix, and nuclease-free water to a total volume of 50 µL. The mixture was subjected to PCR conditions: 2 min at 95 °C (initial denaturation); 45 cycles with 30 s at 94 °C, 1 min at 55 °C, and 1.5 min at 68 °C; 10 min at 72 °C (final extension). The PCR products were gel purified using QIAQuick gel extraction kit (Qiagen) followed by ligation into pCR®2.1-TOPO TA cloning vectors (Life Technologies,

Carlsbad, CA). Six PCR products were sequenced and analysed for cytosine methylation on the promoters for each cell line. The percentage of methylation level within a promoter for a cell line was calculated as the ratio of methylated CG sites to the total CG sites contained in a promoter for the measured six PCR products.

Results

Effect of IE location, orientation and copy number within a hCMV promoter on EGFP expression level and stability

We first compared the transient EGFP expression levels of the WT and twelve modified hCMV promoters with either one or two copies of IE inserted at a single location in both forward and reverse orientations (Fig. 2A). Three modified hCMV promoters, DF2, DR1, and DR2, with IE inserted downstream of the CP expressed EGFP at levels significantly lower ($p<0.05$) than WT hCMV. All other modified hCMV promoters exhibited similar expression levels. EGFP expression levels of stably transfected clones were subsequently compared (Fig. 2B). Three stably transfected pools were generated using each promoter. The transfection efficiency was around 50% for all transfections (data not shown). Three clones were randomly isolated from each pool to obtain a total of nine clones for each promoter. EGFP expression level varied dramatically between clones generated using same promoters. Consistent with the transient transfections, IE decreased expression in stably transfected clones when inserted downstream of the CP. Introduction of IE into other locations of hCMV also decreased expression depending on the orientation and copy number. Inserting a single copy of IE upstream of the enhancer (UF1 and UR1) decreased expression while having two copies (UF2 and UR2) had no significant

effect regardless of orientations. When IE was inserted between the enhancer and CP, only the promoter with one copy inserted in reverse orientation (MR1) did not decrease EGFP expression.

The ability of modified hCMV promoters to sustain gene expression during long term culture was first evaluated by measuring the percentage of cells still expressing EGFP in a clonal population at the end of stability testing using FACS (Fig. 2C). All cells were verified to be EGFP expressing at the start of stability testing. Each clone was subsequently passaged for eight weeks in the absence of selection reagents. After eight weeks of culture, the percentage of EGFP expressing cells in all clones generated using the WT hCMV declined. The average percentage of EGFP expressing cells of the nine clones was 69% with the worst clone only having 23% of the population still expressing EGFP. Similar to the effect on expression level in stably transfect clones, no trends were observed between IE's position and anti-silencing effect. DR2 and MR1 maintained the highest percentage of EGFP expressing cells. All clones generated using these two promoters had close to 100% EGFP expressing cells at the end of stability testing. UF2, UR2, MF1, MF2, MR2 also increased the percentage of EGFP expressing cells compared with the WT hCMV. The average percentage of EGFP expressing cells in clones generated using these promoters was greater than 90% at the end of stability testing. The increase from other modified hCMV promoters was not statistically significant compared to the WT hCMV ($p<0.05$).

Simply maintaining protein expression is insufficient for recombinant protein production and the expression needs to stay at a high level. The modified promoters were further evaluated for the retention of EGFP expression level after eight weeks of culture. This was calculated as the ratio

of geometric mean fluorescence intensity of a clone measured at the end of stability testing to the starting level measured before stability testing (Fig. 2D). EGFP expression in all nine clones generated using the WT hCMV declined after long term culture. The best clone only had retained 41% of EGFP expression. On average, the nine clones retained 16% of EGFP expression. Eight modified hCMV promoters increased retention of EGFP expression levels compared to the WT hCMV. DR2 clones retained the highest EGFP expression levels, followed by MR1, MR2, DF2, UR2, UF1, MF1, and UF2. Clones generated using DR2 retained 69% of their EGFP expression on average, with the worst clone still retaining greater than 40% of expression. MR1, MR2, DF2, UR2, UF1, MF1, and UF2 increased the average retention of EGFP expression level to 27% to 50%. The percentage of EGFP expressing cells did not correlate to the retention of EGFP expression level (Supplementary Fig. S1). For instance, MR1 and DR2 clones maintained EGFP expression in all cells during stability testing (Fig. 2C) but expression levels in more than half the clones still decreased by more than 30% (Fig. 2D). The strength of each modified hCMV promoter for expression of EGFP in transient transfections and stably transfected clones and their ability to enhance EGFP expressing cells and retention of expression level are summarized in Fig. 1B. MR1 and UR2 were the only two modified hCMV promoters which had greater proportion of EGFP expressing cells and better retention of EGFP expression level without decreasing expression level compared to the WT hCMV when IE was inserted into a single site.

Effect of inserting IE into multiple locations of hCMV on EGFP expression level and stability

We next investigated if inserting IE into multiple locations of the hCMV promoter improved the effectiveness of enhancing expression and stability. As UR2 and MR1 are effective at enhancing expression stability without compromising expression level (Fig. 1B), we generated a modified

hCMV promoter with two copies IE inserted upstream of the enhancer in reverse orientation and one copy of IE inserted between the enhancer and CP in reverse orientation (UR2+MR1). Expression level and stability of UR2+MR1 promoter was evaluated as before. UR2 and MR1 were included in the testing to examine whether there were any cumulative effects. In transient transfections, UR2+MR1 expressed comparable EGFP level as the WT hCMV (Fig. 3A). Eighteen stably transfected clones were isolated from three stably transfected pools for each promoter instead of nine to obtain a more comprehensive dataset. Consistent with the previous results (Fig. 2), the 18 UR2 and MR1 clones each still had comparable EGFP expression with the WT hCMV but with enhanced expression stability (Fig. 3B, 3C, and 3D). The UR2+MR1 promoter resulted in a decrease in average EGFP expression by more than 50% compared to the clones generated using the WT hCMV (Fig. 3B). Expression stability of UR2+MR1 generated clones were enhanced when compared to WT hCMV but no improvements when compared to UR2 and MR1 (Fig. 3C and 3D).

Analysis of changes in EGFP gene copies, EGFP mRNA levels and promoter methylation in WT hCMV and MR1 generated clones

MR1 was most effective at enhancing expression stability among the modified hCMV promoters which exhibited comparable expression levels with the WT hCMV (Fig. 1B and Fig.2). While all clones generated using MR1 maintained almost complete EGFP expressing cell populations at the end of stability testing (Fig. 2C and 3C), two-thirds of MR1 clones retained less than 70% of EGFP expression level (Fig. 2D and 3D). To investigate why MR1 promoter exhibited varied degrees of enhancement for maintenance of expression level across clones, nine MR1 clones with retention of EGFP expression level ranging from 14% to 95% were analyzed for changes in

the relative EGFP gene copies and mRNA levels before and after stability testing (Fig. 4). Nine WT clones with EGFP expression level retained ranging from 1% to 41% were also analyzed for comparison. The clones were sorted in descending order based on their retention of EGFP expression levels. At the end of stability testing at week 8, EGFP gene copies decreased by more than half in WT clones A2, B2, and C3, decreased by less than 30% for A3, A1, C1, B1 and remained similar for clones C2 and B3. No significant decrease of EGFP gene copies was observed in MR1 clones. The retention of EGFP gene copies did not correlate to the retention of EGFP expression in both WT (R^2=0.042) and MR1 (R^2=0.115) clones. Higher correlation was observed between the retention of EGFP mRNA level and the retention of EGFP expression for both WT (R^2=0.452) and MR1 (R^2=0.494) clones (Fig. 4B). MR1 clone A2 which retained 95% of EGFP expression level maintained mRNA levels at 93% and the remaining unstable WT and MR1 clones with decreased EGFP expression levels had decreased mRNA levels.

To determine if the observed transcriptional silencing was caused by promoter DNA methylation, we compared CpG methylation patterns of WT hCMV and MR1 using bisulfite sequencing. WT hCMV and MR1 has 32 and 41 CpGs, respectively (Fig. 5A). Six PCR products were analyzed for each EGFP expressing clone at both week 0 and week 8. We did not observe any consistency in the methylation patterns (data not shown). The methylated CpGs were spread throughout the entire hCMV promoter and IE element for all clones. Methylation distribution of MR1 A2 clone is shown in Fig. 5B as an example. Using the PCR results, we determined the methylation level of a promoter by calculating the ratio of methylated CpGs on the six tested PCR products to the total CpGs contained in the promoter. Relatively low levels of methylation (<30%) occurred in all WT and MR1 clones at both week 0 and week 8 regardless

of their retained EGFP expressions except the WT clone A3 which had no detectable levels of promoter methylation (Fig. 5C). Even in the most stable MR1 A2 clone with 95% retention of EGFP expression, 11.8% and 15.9% of CpGs were methylated at the start and end of stability testing respectively. Increased methylation was observed in all WT clones and seven of the MR1 clones at the end of stability testing. Changes in methylation levels between week 0 and week 8 did not correlate to the retained EGFP expression in both WT (R^2=0.006) and MR1 (R^2=0.019) clones.

Comparison of wild type hCMV and MR1 for mAb expression level and stability

MR1 was further evaluated for generating stably transfected cell lines expressing monoclonal antibodies (mAb), the best-selling class of biopharmaceuticals[45]. Two tricistronic vectors containing the WT hCMV and MR1 respectively (Fig. 1A) were transfected into CHO DG44 cells and selected with stepwise increases of MTX concentrations from 50 nM to 100 nM to 250 nM to 500 nM to 750 nM and ending at 1000 nM. Antibody titers of the stably transfected pools generated using the WT hCMV increased from 3.8 mg/L to 263.5 mg/L when MTX concentrations increased from 0 nM to 250 nM but did not increase further when MTX concentrations were further increased (Fig. 6A). Titers of the stably transfected pools generated using MR1 peaked at MTX 500 nM, reaching 344.0 mg/L, which was 30% higher compared to the peak titer of pools generated using the WT hCMV. Two pools generated by each of the WT and MR1 hCMV promoters at MTX 500 nM were passaged for eight weeks in the absence of MTX to compare expression stability. The stable pools generated using the WT hCMV maintained 37% of their original titers at the end of stability testing while the stable pools generated using the MR1 promoter maintained 62% (Fig. 6B).

Discussion

In the previous study, IE was inserted into the viral LTR and expression stability was evaluated using the percentage of GFP expressing cells in clonal cultures[41]. The authors found that inserting one IE between the viral enhancer and promoter in both forward and reverse orientations ensured GFP expression in all cells during long term culture, while insertion of one IE upstream of the viral enhancer or downstream of the promoter had only minor anti-silencing effect regardless of orientation. We had similar observations that inserting one copy of IE between the hCMV enhancer and promoter in both orientations were effective in maintaining EGFP expressing cells while having only a single copy into other locations had no effect (Fig. 2C). We further explored using two copies of IE and observed that having two copies of IE upstream of the hCMV enhancer in both orientations (UF2 and UR2), between the enhancer and core promoter in both orientations (MF2 and MR2), and downstream of the promoter in reverse orientation (DR2) all enhanced the percentage of EGFP expressing cells after eight weeks of culture. We also observed no correlation between the proportion of cells maintaining EGFP expression and the retained expression level (Fig. S1). The location, orientation, and copy number of IE also affected expression stability and expression level differently. DR2 modified promoter was most effective at enhancing expression stability but had decreased EGFP expression level. MR1 also enhanced expression stability but did not impair expression level (Fig. 2). These results suggest that the mechanism of IE in transgene expression and stability involves complex interactions of the location, orientation, and copy numbers of IE within a promoter. Careful designing and evaluation is needed to realize IE's potential of enhancing expression stability and obtaining high expression levels.

Results from our analysis of promoter DNA methylation in MR1 EGFP clones were in conflict with previous reports that IE protected itself and sequences close to it from DNA methylation[41, 42]. It is clear that MR1 with IE inserted between the hCMV enhancer and CP enhanced the percentage of EGFP expressing cells and retention of EGFP expression level during long term culture (Fig. 2 and Fig. 3). However, we did not observe reduced DNA methylation for the MR1 modified promoter compared to the WT hCMV (Fig. 5). The two previous studies on IE were carried out in transgenic embryos using plasmid vector and in mammalian cells using viral vectors, respectively[41, 42]. The number of vectors integrated into the chromosome in those experiments could be very low compared to our cell lines, as we transfected a large amount of plasmid, 5 μg and used impaired NPT selection markers for obtaining high expression levels[46]. We have observed several hundred gene copies in some of our cell lines during CHO genome sequencing (data not published). The decreased EGFP expression during stability testing could be either due to mean gradual silencing of all integrated vectors or only a subgroup of vectors. As such, bisulfite sequencing of six 6 PCR products or even more may not be comprehensive to detect the difference in methylation level between the WT hCMV and MR1 promoters. Another factor which complicates the study of IE's mechanism is that plasmid vectors are randomly integrated into chromosome. Multiple copies of plasmid vectors could integrate into different sites of chromosome in the same clones and across different clones. The function of IE may be dependent on the integration sites. This is supported by the fact that MR1 exhibited varying degrees of enhancement for expression stability in different clones. By using bisulfite sequencing, we were unable to discriminate the effect of integration sites and IE on DNA methylation. A clearer understanding how IE enhances expression stability in CHO cells might be available

from cell lines with only a single gene copy integrated into predetermined sites on chromosome using technologies such as viral vectors and/or site directed integrations.

It is still arguable whether promoter DNA methylation is the cause of transcriptional silencing as it has been shown silencing of transgene transcription precedes promoter DNA methylation[48]. Our results also support that DNA methylation may not be the cause of transcriptional silencing as methylation level did not correlate to expression stability (Fig. 5). A recent study also found that cell lines with stable production exhibited high level promoter methylation at both early and late stages of stability testing[14]. Emerging evidence indicates that histone modification occurs during the same window of time as transgene inactivation and prior to DNA methylation[48]. It has been also reported that histone modifications occurred in parallel with decreased antibody expression during long-term culture of CHO cells. Analysis of histone modification may be carried out in future to understand if it either contributes to IE's ability to enhance expression stability or is the reason for transcriptional silencing of some MR1 clones during long term culture.

As both expression level and stability are important for recombinant therapeutics production, MR1, which enhanced expression stability without compromising expression level, is most favorable for generation of stably transfected cell lines for therapeutics production. For applications in which expression stability is most important, such as cell engineering, DR2 could be a better choice. Application of MR1 in the development of cell lines for therapeutic protein production would simplify the clone selection process as less clones need to be screened to isolate ones which are stable. Using the criteria of stability as maintaining 70% of gene

expression through the culture period, none of 18 EGFP expressing clones generated using the WT hCMV were stable, while one third of EGFP clones generated using MR1 were stable. We also demonstrated that MR1 effectively enhanced production stability of stably transfected pools expressing mAb, suggesting that the enhancement of expression stability by MR1 is not gene specific. Although we did not isolate mAb expressing clones, we expect it will be easier to isolate stable clones from the MR1 generated pools than from the WT hCMV generated pools due to the higher retention of mAb titer in the MR1 pools.

We evaluated the MR1 promoter for mAb expression using IRES linked multicistronic vectors where the product/reporter genes and selection marker genes are under the control of one promoter. Co-transfection of two separate vectors and a single vector containing multiple promoters are commonly used for industrial production of mAb. Each of the LC, HC, and selection marker genes is driven by its own independent promoter and transcribed separately. Although it is unclear whether the expression stability of the selection marker gene affects the expression stability of linked gene(s) of interest in a cell lines, we recommend the use MR1 or other IE modified promoters on each gene so that all genes including the selection marker have increased stability. Silencing of the selection marker gene could result in changes to the chromatin structure around the selection marker and the region close to it may also be affected, resulting in silencing of mAb genes. Moreover, as the size of IE is small, application of IE on all promoters will not increase the size of a plasmid vector.

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Fig. 1. Schematic representation of enhanced green fluorescence protein (EGFP) and monoclonal antibody (mAb) expressing vectors (A) and comparison between the wild-type (WT) and modified hCMV promoters for expression level and stability (B). Expression level between the WT and each modified hCMV promoter were compared in transient transfection and stably transfected clones using EGFP as reporter gene. Expression stability in stably transfected clones generated using the WT and each modified hCMV promoter was compared using the percentage of EGFP expressing cells and retention of EGFP expression level at the end of stability testing. IE, core CpG island element; Enhancer, hCMV enhancer; CP, hCMV core promoter; IRESwt, wild type encephalomycocarditis virus (EMCV) internal ribosome entry site (IRES); IRESatt, mutated EMCV IRES with attenuated translation efficiency; SpA, simian virus 40 early polyadenylation signal; LC, light chain cDNA; HC, heavy chain cDNA; mNPT, mutated neomycin phosphotransferase cDNA with amino acid D at 261 changed to G.

Fig. 2. Comparison of the wild-type (WT) and twelve modified hCMV (UF1, UF2, UR1, UR2, MF1, MF2, MR1, MR2, DF1, DF2, DR1, and DR2) promoters for EGFP expression level and stability. (A) EGFP expression in transient transfections. EGFP expression was measured at 48-h post transfection and normalized to the WT hCMV. Each value represents the average and standard error of measurements from four independent transfections. (B) EGFP expression in stably transfected clones before stability testing. (C) Percentage of EGFP expressing cells in different clones at the end of stability testing. (D) Retention of GFP expression level in different clones at the end of stability testing. Three clones each were isolated from three separately transfected pools for a total of nine clones for each promoter. Each dot represents values measured for one clone. The horizontal bar and error bars represent the average and standard error of values of nine clones. Mean values significantly different (two-tailed Student's t-test) from the WT are indicated by asterisks "*" ($p < 0.05$).

Fig. 3. Comparison of the wild-type (WT) and three modified hCMV (UR2, MR1, and UR2+MR1) promoters for EGFP expression level and stability. (A) EGFP expression in transient transfections. EGFP expression was measured at 48-h post transfection and normalized to the WT hCMV. Each value represents the average and standard error of measurements from four independent transfections. (B) EGFP expression in stably transfected clones at the start of stability testing. (C) Percentage of EGFP expressing cells in different clones at the end of stability testing. (D) Retention of EGFP expression level in different clones at the end of stability testing. Eighteen clones, six from each pool, were isolated from three pools generated by each promoter. Each dot represents values measured for one clone. The horizontal bar and error bars represent the average and standard error of values of eighteen clones. Mean values significantly different (two-tailed Student's t-test) from the WT are indicated by asterisks "*" ($p < 0.05$).

Fig. 4. Changes in relative EGFP gene copies (A) and EGFP mRNA levels (B) in WT and MR1 clones during stability testing. The retention of EGFP DNA, mRNA and expression level are calculated as the ratios of relative EGFP gene copies, mRNA levels and geometric mean fluorescence intensity of a clone measured at week 8 to the starting level for the same clone measured at week 0.

Fig. 5. Analysis of CpG methylation within wild-type (WT) hCMV and MR1 promoters during stability testing. (A) Sequence of MR1 promoter with CG highlighted in gray. (B) Methylated CG sites within MR1 promoter in clone A2 at week 0 (W0) and week 8 (W8). Genomic DNA was isolated from the cells at W0 and W8, bisulfite treated, PCR amplified, and cloned into pCR®2.1-TOPO TA cloning vectors. For each EGFP expressing clone, six PCR

products were isolated and sequenced. Every row represents one DNA clone, every column represents one CG site, starting from 5'-site. Methylated sites are shown in gray. (C) Changes in methylation level of WT hCMV and MR1 promoters. EGFP expressing clones generated using the WT hCMV and MR1 promoters were passaged in the absence of G418 for eight weeks. The percentage of methylation level within the WT or MR1 promoter in a clone is calculated as the ratio of methylated CG sites to the total CG sites within a promoter measured from six PCR products. The changes in methylation level in a clone are calculated as the difference between the percentage of methylation level within a promoter measured at week 8 and week 0.

Fig. 6. Comparison of the wild type (WT) and modified hCMV (MR1) promoter for monoclonal antibody (mAb) expression level and stability. (A) Titer of stably transfected pools generated using two tricistronic vectors with genes under the control of WT hCMV and MR1 promoters, respectively. The transfected cells were selected in medium without hypoxanthine and thymine (HT) and then underwent amplification at different concentrations of methotrexate (MTX). (B) Retention of mAb titer of stably transfected pools after stability testing. Stably transfected pools generated using the WT and MR1 vectors at 500 nM MTX were passaged for eight weeks in the absence of MTX for stability testing. Retention of mAb titer was calculated as the titer of stably transfected pool at the end of stability testing divided by the titer determined at the start of stability testing. Each value presented in figures represents the average and standard error of measurements from two independently transfected pools.

Fig. S1. Relationship between the percentage of EGFP expressing cells and retention of EGFP expression at the end of stability testing for modified hCMV promoters with IE

inserted upstream of the hCMV enhancer (A), between the hCMV enhancer and core promoter (B), and downstream of the hCMV core promoter (C).

Table S1. Primers used for analysis of relative gene copy numbers and mRNA levels by real-time quantitative PCR (qRT-PCR)

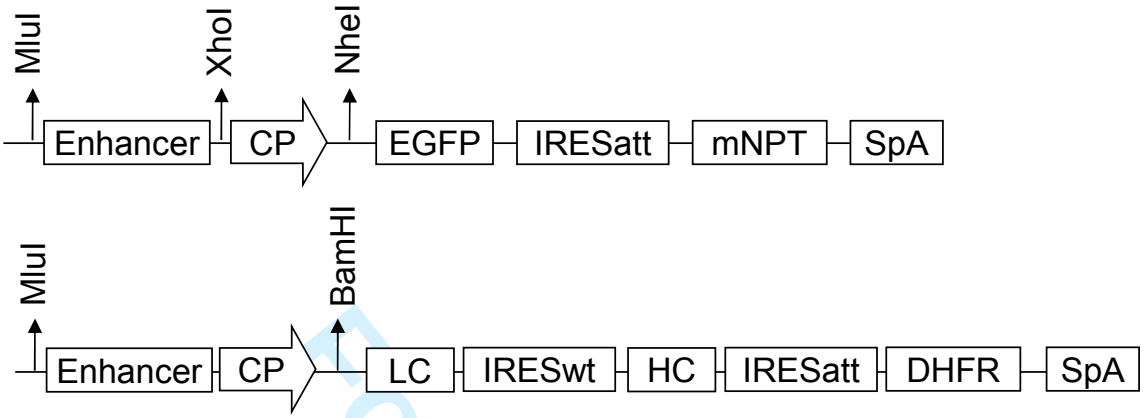
Gene targeted	Forward primer (5'-3')	Reverse primer (5'-3')
EGFP	CAAGCAGAAGAACGGCATCAA	GGACTGGGTGCTCAGGTAGTG
β-actin	AGCTGAGAGGGAAATTGTGCG	GCAACGGAACCGCTCATT
EF1α	TGGAAGATGGCCCTAAATTC	AACGACCCAGTGGAGGATAG

Table S2. Primers used for cloning of WT hCMV and MR1 promoters

Promoter	Forward primer (5'-3')	Reverse primer (5'-3')
WT hCMV	TATGAAGAATTTGTTTAGGGTTAG	TAACCAACTTAAATCTCCCTATAA
MR1	TATGAAGAATTTGTTTAGGGTTAGG	ACTCRACCAAAATAAACACCA

Fig. 1

(A)

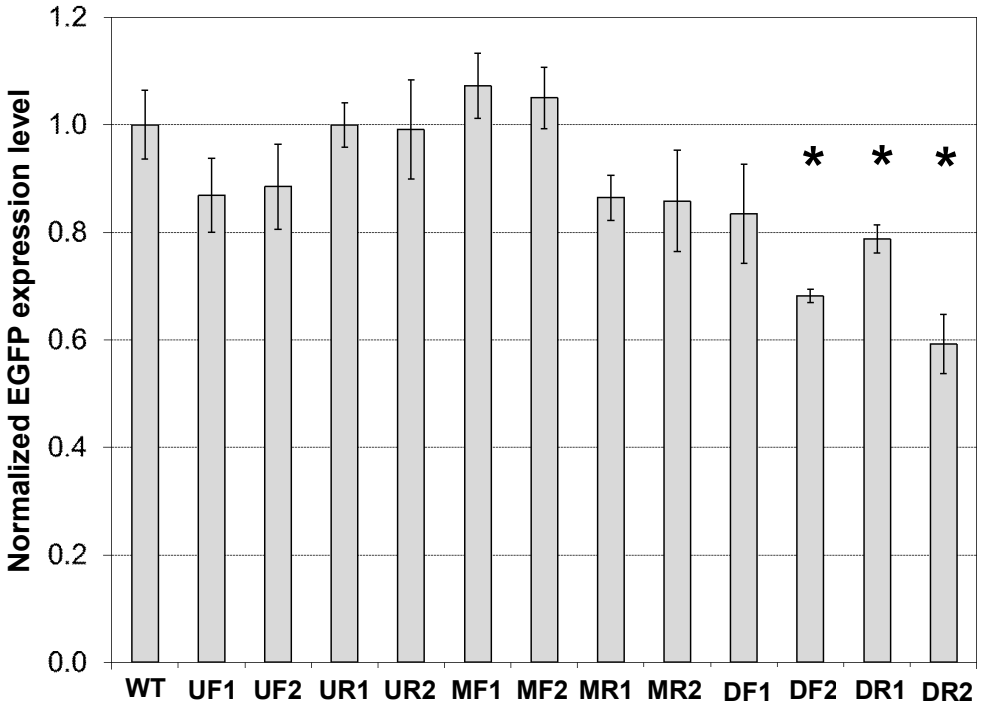


(B)

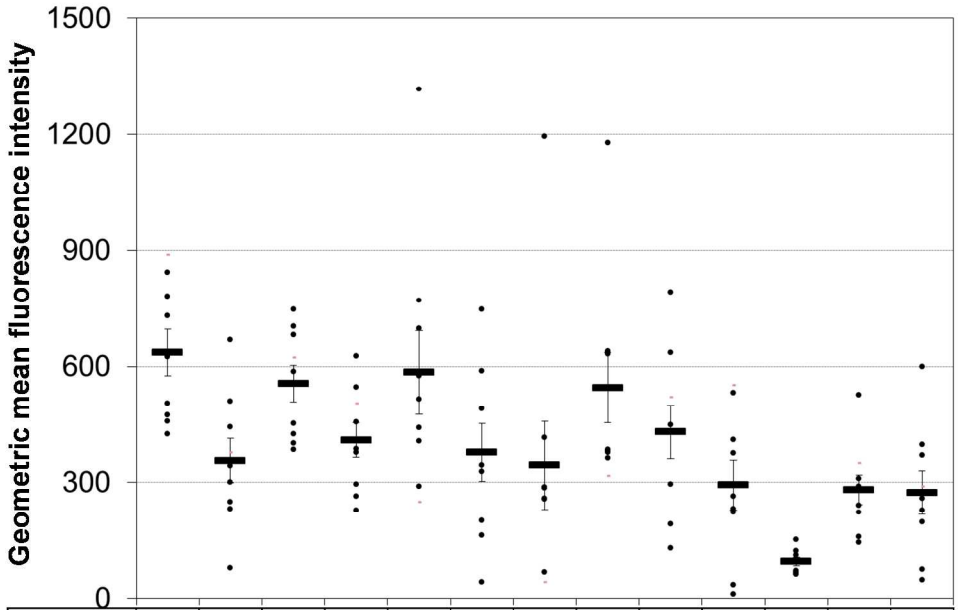
ID	Promoter structure	Transient expression	Stable expression	EGFP expressing cells (%)	Retention of EGFP expression level (%)
WT	Enhancer CP	–	–	–	–
UF1	IE Enhancer CP	No change	Down	No change	Up
UF2	IE IE Enhancer CP	No change	No change	Up	No change
UR1	∃I Enhancer CP	No change	Down	No change	No change
UR2	∃I ∃I Enhancer CP	No change	No change	Up	Up
MF1	Enhancer IE CP	No change	Down	Up	Up
MF2	Enhancer IE IE CP	No change	Down	Up	No change
MR1	Enhancer ∃I CP	No change	No change	Up	Up
MR2	Enhancer ∃I ∃I CP	No change	Down	Up	Up
DF1	Enhancer CP IE	No change	Down	No change	No change
DF2	Enhancer CP IE IE	No change	Down	No change	Up
DR1	Enhancer CP ∃I	Down	Down	No change	No change
DR2	Enhancer CP ∃I ∃I	Down	Down	Up	Up
UR2+MR1	∃I ∃I Enhancer ∃I CP	Down	Down	Up	Up

Fig. 2

(A)

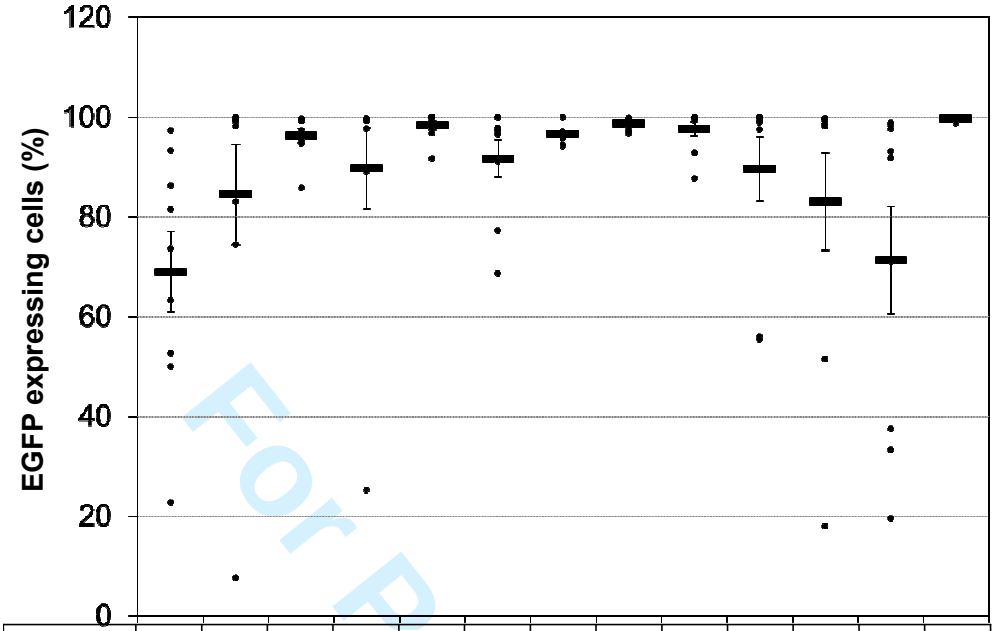


(B)



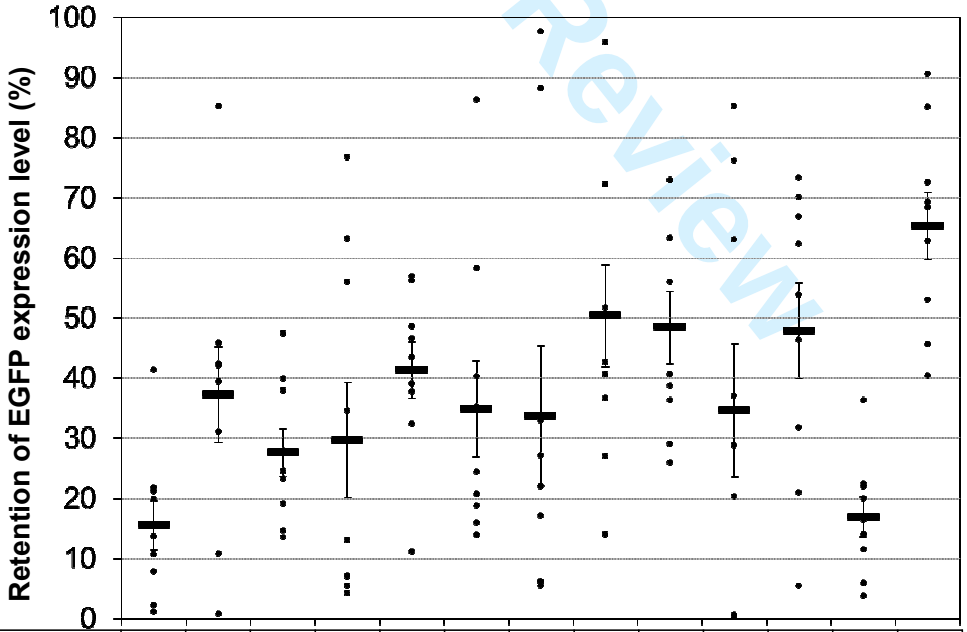
	WT	UF1	UF2	UR1	UR2	MF1	MF2	MR1	MR2	DF1	DF2	DR1	DR2
$p<0.05$		*		*		*	*		*	*	*	*	*

(C)



	WT	UF1	UF2	UR1	UR2	MF1	MF2	MR1	MR2	DF1	DF2	DR1	DR2
$p<0.05$			*		*	*	*	*	*				*

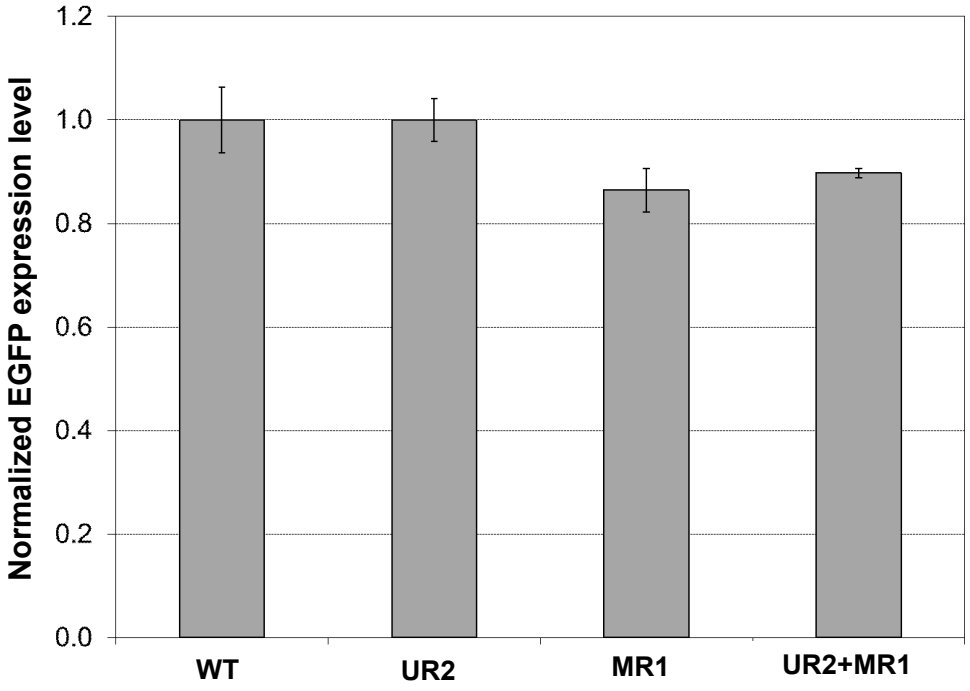
(D)



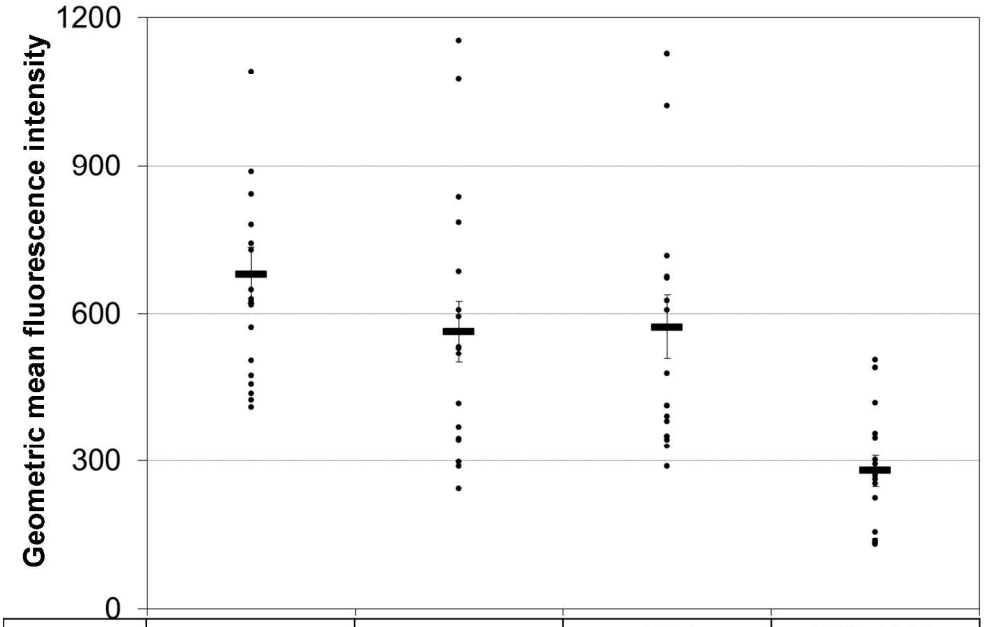
	WT	UF1	UF2	UR1	UR2	MF1	MF2	MR1	MR2	DF1	DF2	DR1	DR2
$p<0.05$		*	*		*	*		*	*		*		*

Figure 3

(A)

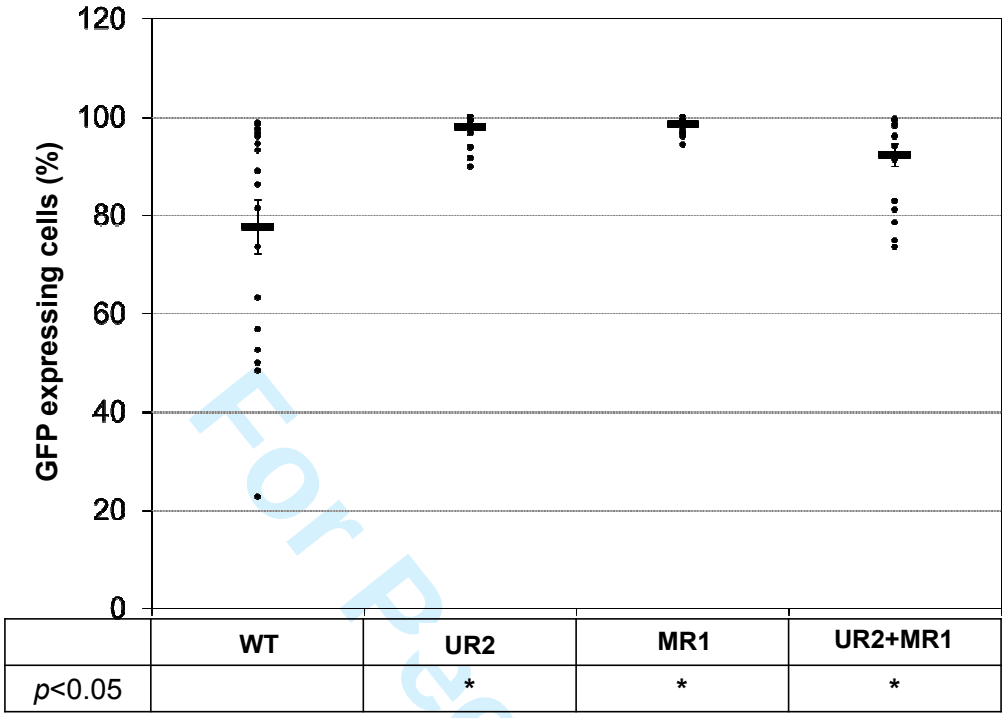


(B)



	WT	UR2	MR1	UR2+MR1
$p<0.05$				*

(C)



(D)

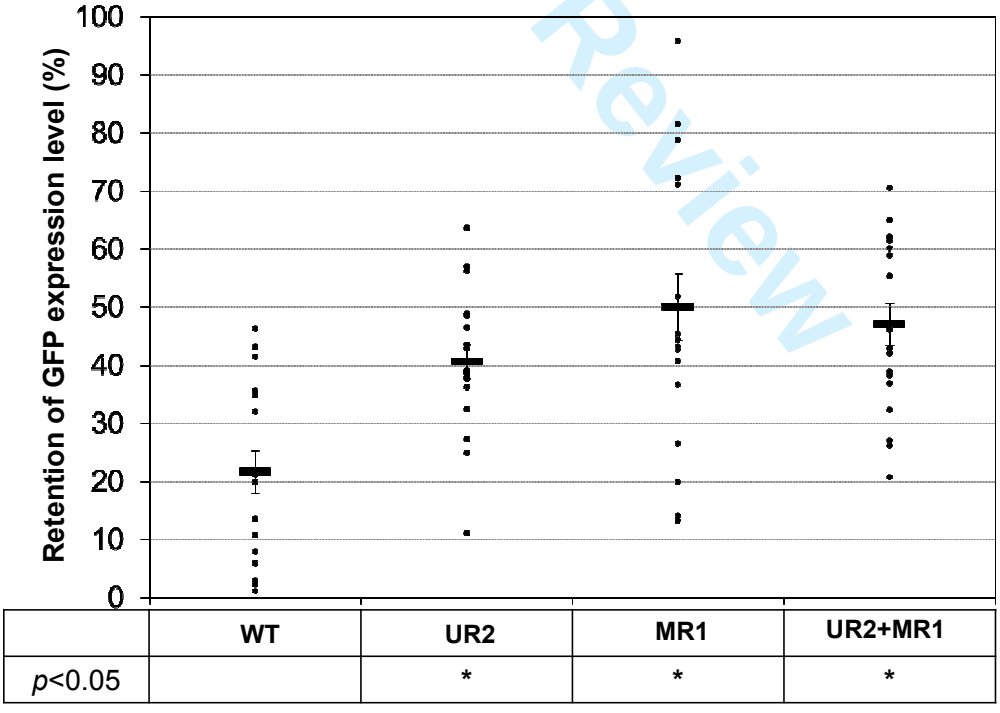
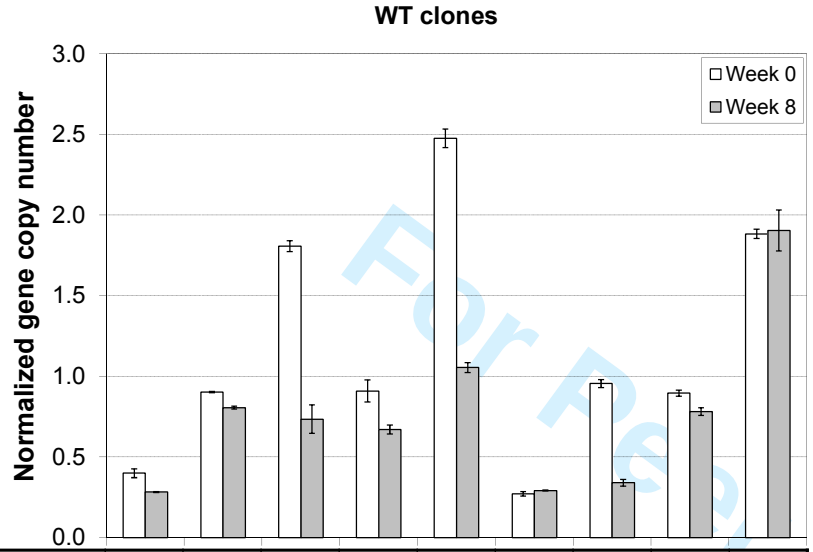
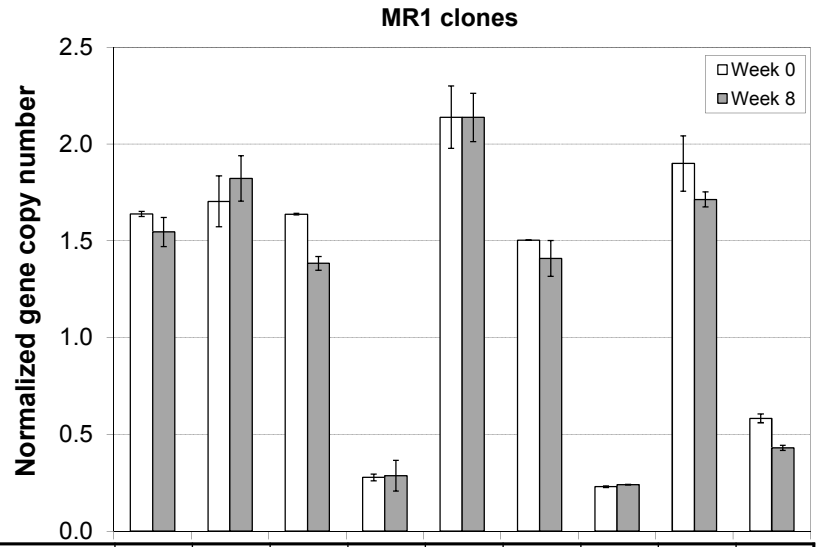


Figure 4A

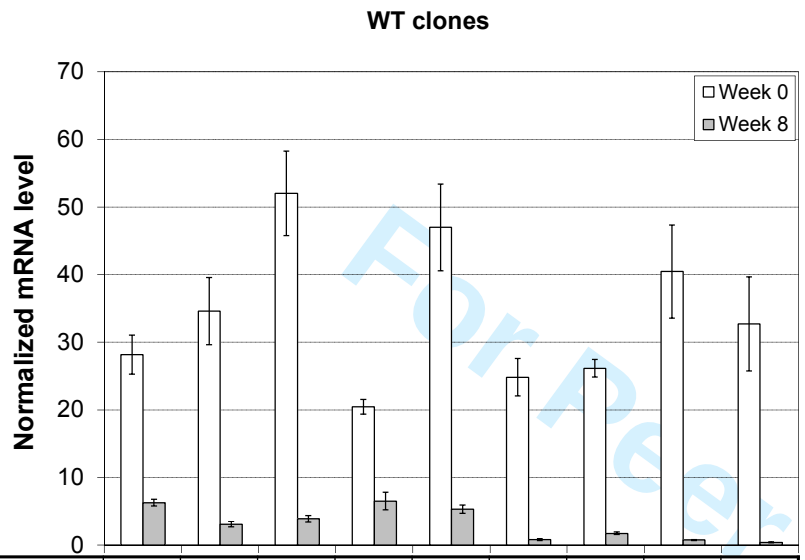


	A3	A1	A2	C1	B2	C2	C3	B1	B3
Retention of EGFP DNA (%)	71	89	41	74	43	108	36	87	101
Retention of EGFP expression (%)	41	22	21	20	14	11	8	2	1

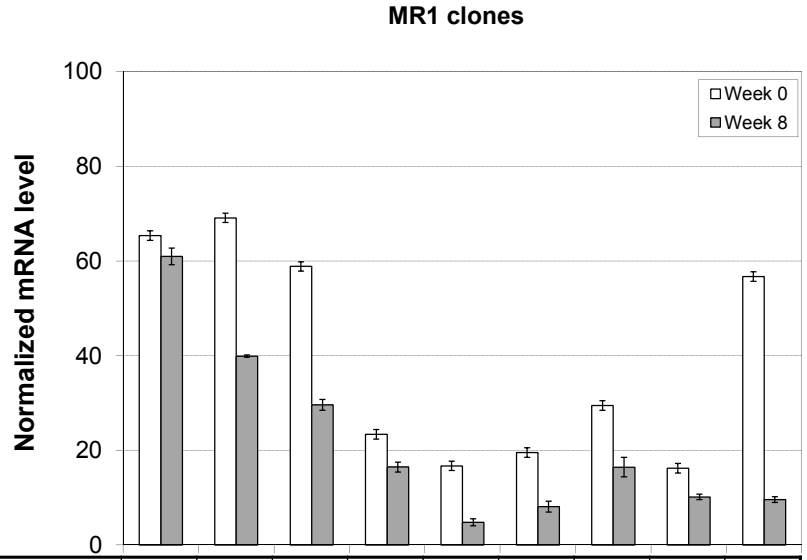


	A2	B3	B1	C1	A3	B2	C2	A1	C3
Retention of EGFP DNA (%)	94	107	84	103	100	94	105	90	74
Retention of EGFP expression (%)	95	72	72	52	43	41	37	27	14

Figure 4B



	A3	A1	A2	C1	B2	C2	C3	B1	B3
Retention of EGFP mRNA (%)	22	9	7	32	11	3	7	2	1
Retention of EGFP expression (%)	41	22	21	20	14	11	8	2	1



	A2	B3	B1	C1	A3	B2	C2	A1	C3
Retention of EGFP mRNA (%)	93	58	50	70	29	41	56	63	17
Retention of EGFP expression (%)	95	72	72	52	43	41	37	27	14

Fig. 5A

hCMV enhancer

```
                                                                  1
-623 TGACATTGAT TATTGACTAG TTATTAATAG TAATCAATTA CGGGGTCATT
                                    2 3                   4
-673 AGTTCATAGC CCATATATGG AGTTCCGCGT TACATAACTT ACGGTAAATG
        5               6       7       8           9
-623 GCCCGCCTGG CTGACCGCCC AACGACCCCC GCCCATTGAC GTCAATAATG
      10                   11                          12
-573 ACGTATGTTC CCATAGTAAC GCCAATAGGG ACTTTCCATT GACGTCAATG
                    13
-523 GGTGGAGTAT TTACGGTAAA CTGCCCACTT GGCAGTACAT CAAGTGTATC
                   14             15        16           17
-473 ATATGCCAAG TACGCCCCCT ATTGACGTCA ATGACGGTAA ATGGCCCGCC

-423 TGGCATTATG CCCAGTACAT GACCTTATGG GACTTTCCTA CTTGGCAGTA
           18          19
-373 CATCTACGTA TTAGTCATCG CTATTA
```

XhoI

```
       20
-347 CTCGAG
```

IE in reverse orientation

```
                 21          22          23          24   24
-341 TCCTTAGGGA GCGATCCAGC ACGAGGAGAG GCCGGGAGGG GGCGGGACGG
       26                          27
-291 GGCGGGGCCT CTGGGAGAGT GGGTTGCGGG GAGGCTGGCT TTTGGCAGGA
          28
-241 AGTAACGCAT TTGCTGGA
```

XhoI

```
       29
-223 CTCGAG
```

hCMV core promoter

```
          30                          31        32
-217 TGATGCGGTT TTGGCAGTAC ATCAATGGGC GTGGATAGCG GTTTGACTCA
     33                              34
-167 CGGGGATTTC CAAGTCTCCA CCCCATTGAC GTCAATGGGA GTTTGTTTTG
                   35                    36          37
-117 GCACCAAAAT CAACGGGACT TTCCAAAATG TCGTAACAAC TCCGCCCCAT
        38         39       40    41
 -67 TGACGCAAAT GGGCGGTAGG CGTGTACGGT GGGAGGTCTA TATAAGCAGA

 -17 GCTC
```

Fig. 5B

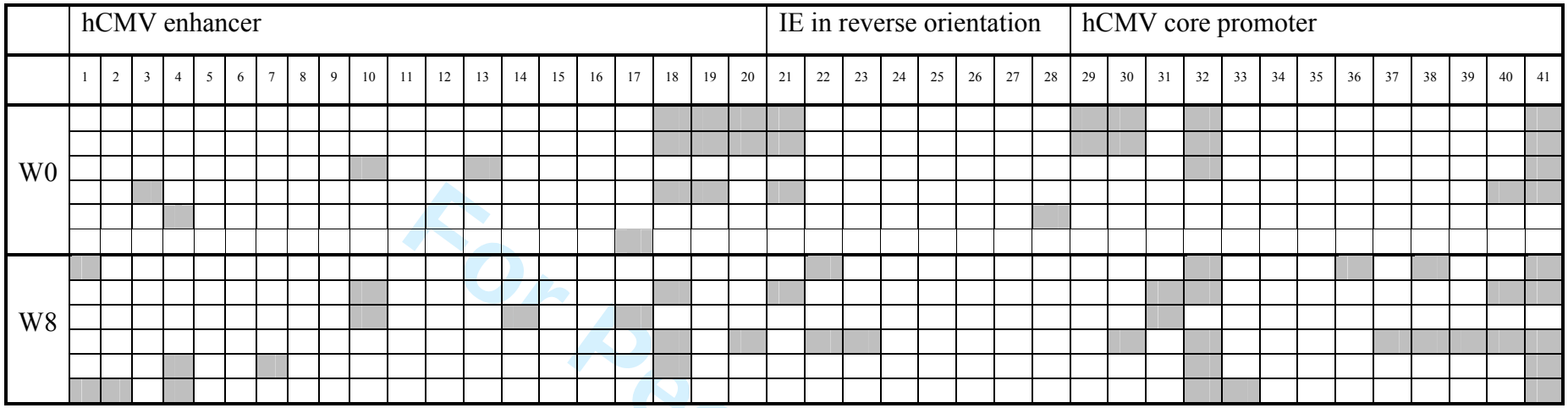
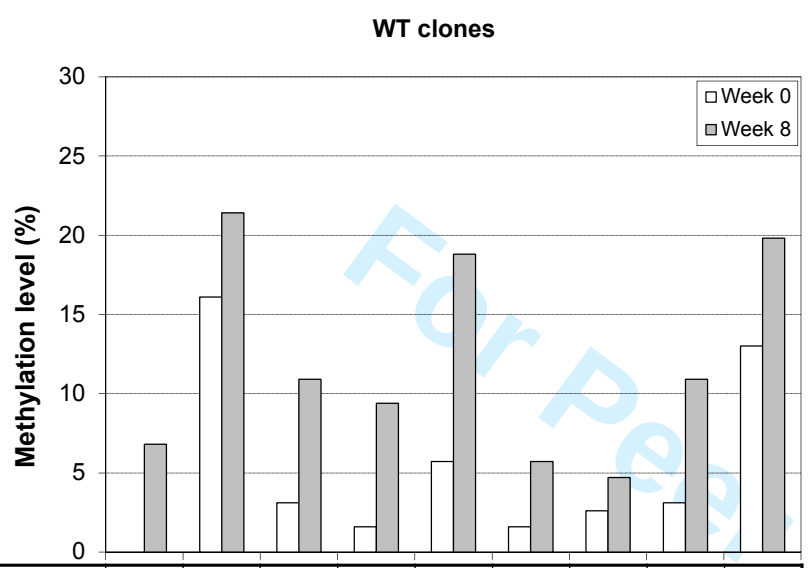
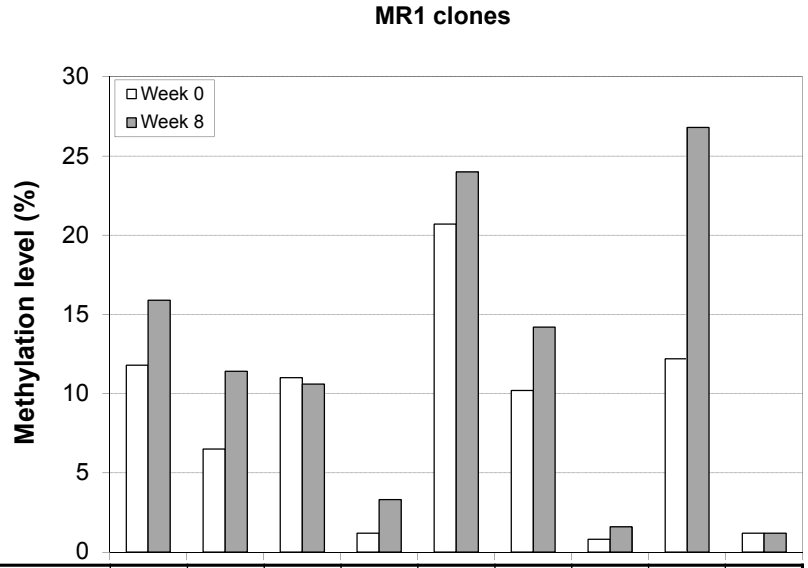


Fig. 5C.



	A3	A1	A2	C1	B2	C2	C3	B1	B3
Changes in methylation (%)	6.8	5.3	7.8	7.8	13.1	4.1	2.1	7.8	6.8
Retention of EGFP expression (%)	41	22	21	20	14	11	8	2	1



	A2	B3	B1	C1	A3	B2	C2	A1	C3
Changes in methylation (%)	4.1	4.9	-0.4	2.1	3.3	4.0	0.8	14.6	0
Retention of EGFP expression (%)	95	72	72	52	43	41	37	27	14

Fig. 6A

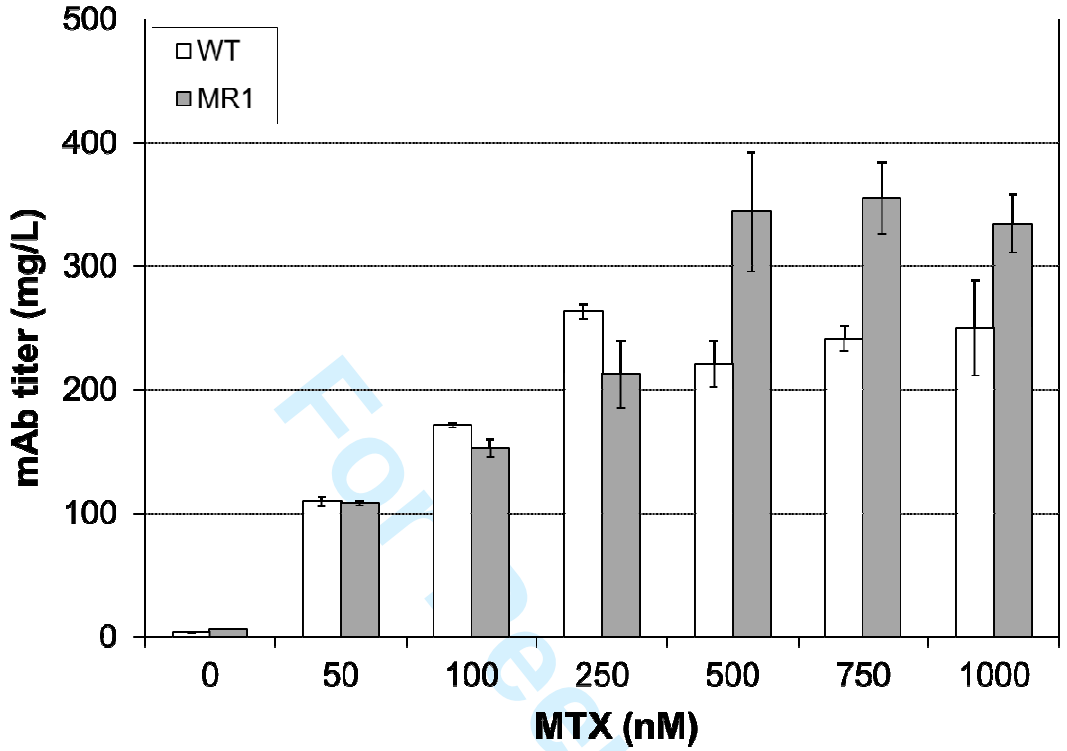


Fig. 6B

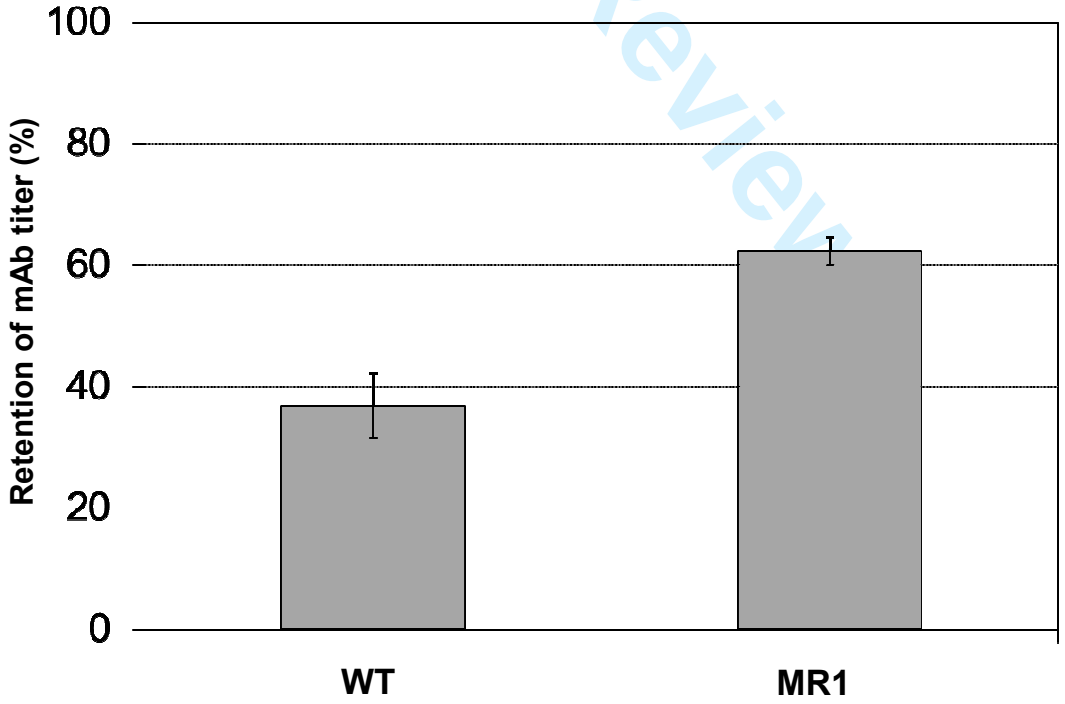


Fig. S1A.

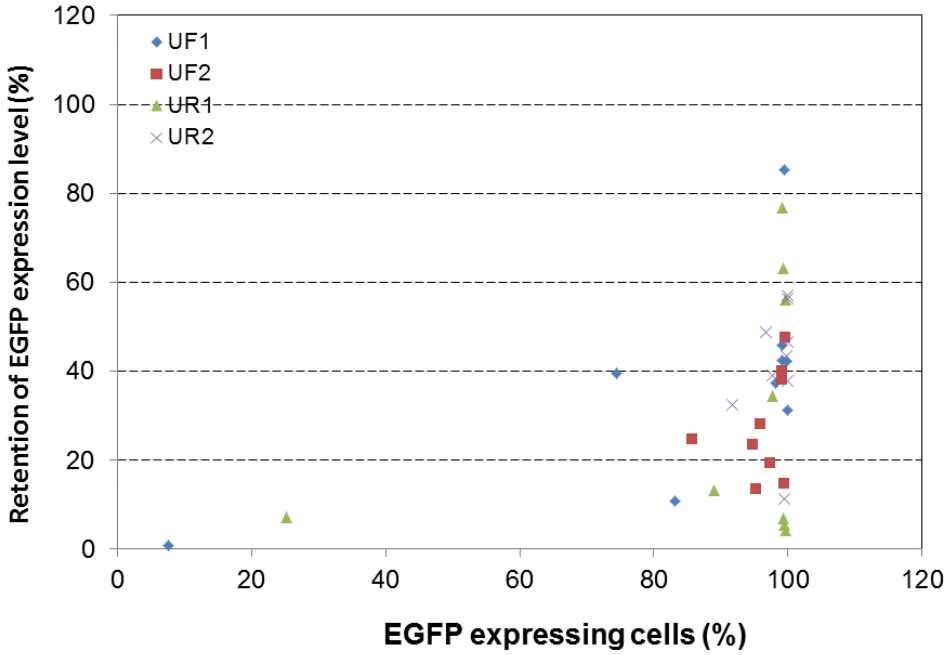


Fig. S1B

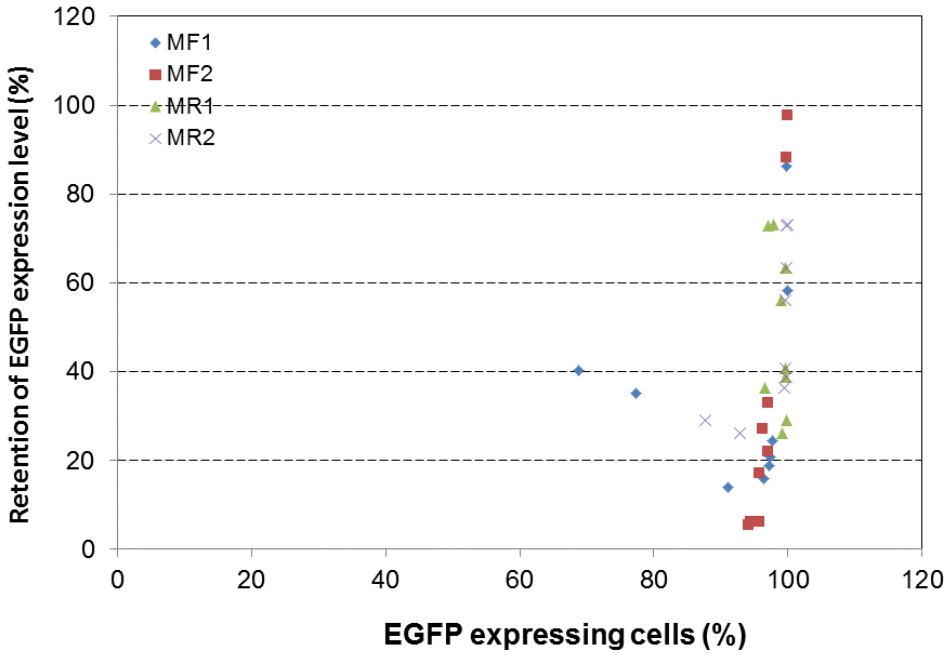
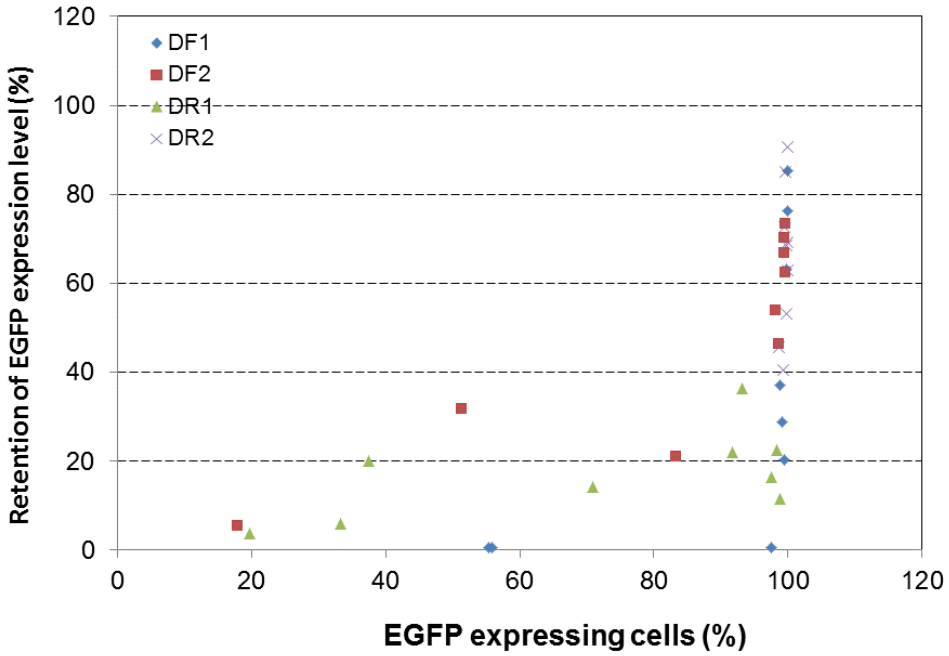


Fig. S1C



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