



## DNA methylation contributes to loss in productivity of monoclonal antibody-producing CHO cell lines

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### ABSTRACT

Production instability currently limits the use of mammalian cells for industrial production of therapeutic proteins. We have previously reported that the loss of productivity in recombinant monoclonal antibody producing Chinese Hamster Ovary (CHO-mAb) cell lines is mainly due to a decrease in heavy chain (HC) and light chain (LC) transcripts. Molecular analysis indicates that the decreased mRNA levels are not due to a loss in gene copies and change of integration sites. In this work, we further demonstrate that impaired *trans*-acting factors and spontaneous mutations to the DNA are not responsible for the reduced HC and LC transcription. Examination of two CpG sites by methyl-assisted quantitative real-time PCR assay revealed an increase in methylation of the human cytomegalovirus major immediate-early enhancer and promoter (hCMV-MIE) controlling the expression of LC and HC in cells which exhibited loss in productivity. Treatment of these cells with a DNA methylation inhibitor, 5-Aza-2'-deoxycytidine, partially restored the lost specific mAb productivity. The increase in productivity correlated to the increase in mRNA levels of HC and LC and the demethylation of hCMV-MIE promoter. This finding, which indicates that DNA methylation contributes to production instability, will be beneficial for generation of high-producing cell lines with stable productivity.

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### 1. Introduction

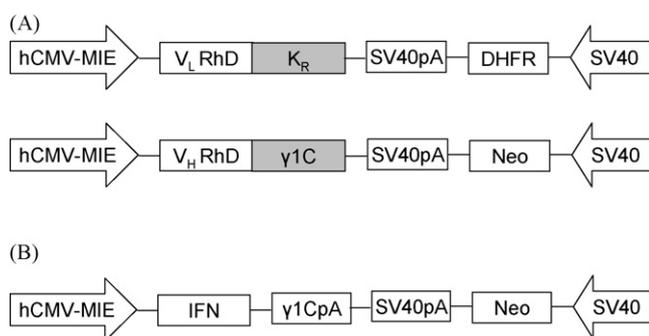
Production stability during long term culture is a major concern for industries producing therapeutic proteins using mammalian cell lines (Barnes et al., 2003; Wurm, 2004). During the time required for a typical industrial process scale up (about 2–3 months), substantial loss of productivity has been reported in cell lines generated by commonly used expression systems, such as glutamine synthetase (GS) (Barnes et al., 2001; Jun et al., 2006) and dihydrofolate reductase deficient Chinese Hamster Ovary (DHFR<sup>-</sup>CHO) (Fann et al., 2000; Kim et al., 1998; Strutzenberger et al., 1999). This loss of production affects both productivity and product consistency, compromising regulatory approval of the product (Barnes et al., 2003; Fann et al., 2000). Extensive studies have indicated that the causes of production instability are either due to a loss of transgene copies (Fann et al., 2000; Kim et al., 1998; Strutzenberger et al., 1999), or a decrease in transcripts (Barnes et al., 2004; Kim et al., 1998), or both (Jun et al., 2006). Cytogenetic analyses indicated that the loss of gene copies might be due to chromosome instability caused by gene amplification (Derouazi et al.,

2006; Kim and Lee, 1999). The underlying molecular mechanisms for the decrease in transcripts without loss of gene copy number have not been elucidated.

We previously generated a panel of monoclonal antibody (mAb)-producing cell lines using DHFR<sup>-</sup> CHO system (Chusainow et al., 2009). Consistent with literature reports, we found that the productivities of these cell lines gradually dropped during long term passaging without the selection reagent, methotrexate (MTX), but remained relatively constant in the presence of MTX (Fann et al., 2000; Kim et al., 1998). Cell lines which maintain stable production without MTX are preferred for mass production of therapeutic proteins as MTX is toxic and expensive. Molecular analysis of our cell lines had indicated that the copy numbers of heavy chain (HC) and light chain (LC) genes remained unchanged despite the mRNA levels decreasing substantially in cell lines which exhibited loss in productivity. In mammalian cells, gene transcription is regulated by the interaction between *trans*-acting factors and DNA elements. Impaired *trans*-acting factors and spontaneous DNA mutations can result in reduced transcripts. In stably transfected cells, transcription efficiency of a transgene is also determined by the integration site. Due to the fluidic nature of the CHO chromosome (Derouazi et al., 2006), the transgene may move from a transcriptionally active site to an inactive site during long term culture. Besides DNA rearrangement, the chromatin structure around

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**Fig. 1.** Schematic representation of vectors. (A) Structure of vectors expressing monoclonal antibody (mAb). (B) Structure of vector expressing interferon  $\gamma$  (IFN). hCMV-MIE, human cytomegalovirus major immediate-early enhancer and promoter; V<sub>L</sub>RhD, kappa light variable region; K<sub>R</sub>, genomic human kappa constant region; SV40pA, simian virus 40 early polyadenylation signal; DHFR, dihydrofolate reductase; Neo, neomycin resistant gene; SV40, simian virus 40 promoter; V<sub>H</sub>RhD, gamma heavy variable region;  $\gamma$ 1C, genomic human  $\gamma$ 1 constant region;  $\gamma$ 1CpA, polyadenylation signal derived from human  $\gamma$ 1 constant region.

the integration site can also be changed by epigenetic processes such as histone modifications and DNA methylation (Bird, 2007; Klose and Bird, 2006; Newell-Price et al., 2000; Prokhortchouk and Defossez, 2008; Ptashne, 2007; Richards and Elgin, 2002). Promoter methylation has been reported to be a common mechanism which can lead to gene silencing at transcription levels (Dang et al., 2000; Hejnar et al., 2001; Newell-Price et al., 2000; Swindle et al., 2004).

In our previous work (Chusainow et al., 2009), we did not observe any changes to transgene integration site in our CHO-mAb cell lines during long term passage, suggesting that other mechanisms were responsible for the decreased mRNA levels. Here we further investigated which of the other factors played a role in silencing the transcription of HC and LC genes in our CHO-mAb cell lines.

## 2. Materials and methods

### 2.1. Cell lines and media

CHO cell lines expressing a recombinant monoclonal IgG against RhD antigen were generated by co-transfection of LC and HC expression vectors into CHO DG44 cells and subsequent multiple step amplification in MTX-containing medium (Chusainow et al., 2009). Both LC and HC genes were under the control of human cytomegalovirus major immediate-early enhancer and promoters (hCMV-MIE). The two selection genes, DHFR in LC vector and neomycin in HC vector, were under the control of simian virus 40 (SV40) promoters (Fig. 1A). The cell lines generated were cultured in a mixture of HyQ PF (HyClone, Logan, UT) and CD CHO (Invitrogen, Carlsbad, CA) at 1:1 ratio and supplemented with 1 g/L sodium bicarbonate (Sigma, St. Louis, MO), 6 mM glutamine (Sigma), 0.1% Pluronic F-68 (Invitrogen), 600  $\mu$ g/mL G418 (Sigma), and the appropriate concentrations of MTX were applicable (Sigma).

### 2.2. Transient expression of interferon $\gamma$ (IFN) in CHO-mAb cell lines

$2 \times 10^6$  mAb-producing CHO cells passaged about 10 times in the presence of MTX, designated as Early(+MTX), and those passaged about 30 times without MTX, designated as Late(-MTX), were separately co-transfected with 2  $\mu$ g of plasmid encoding IFN (Fig. 1B) and 0.4  $\mu$ g of pMax-GFP (Amaxa, Gaithersburg, MD) encoding green fluorescence protein (GFP) by electroporation using Nucleofector (Amaxa) according to the manufacture's instructions. The GFP-encoding vector was used as an internal control to nor-

malize transfection efficiency. At 48-h post-transfection, cells were collected to measure the GFP fluorescence intensity using a FACS Calibur (Becton Dickinson, MA, USA) and supernatant was collected to measure the IFN concentration using a kit from Hycult Biotechnology (Uden, Netherlands).

### 2.3. Treatment of cells with 5-Aza-2'-deoxycytidine

25 mL of cell cultures were seeded into 125 mL shaking flasks (Corning, NY) at an initial density of  $4 \times 10^5$  cells/mL. A daily dose of 4  $\mu$ M of 5-Aza-2'-deoxycytidine (Aza, Sigma) dissolved in phosphate buffer saline (PBS, Sigma) was added to each culture for three consecutive days, while a control culture was treated with only PBS in parallel. Cell density and viability were monitored using trypan blue exclusion and culture supernatants were collected daily for later analysis of secreted antibody. Cells were harvested at day 3 (inoculation day designated as day 0) and stored in  $-80^\circ\text{C}$  freezer for later analysis of HC and LC mRNA levels and DNA methylation.

### 2.4. Analysis of mRNA levels

Relative mRNA levels of HC and LC were determined using quantitative real-time PCR (qRT-PCR) as described previously, except that Eukaryotic Translation Elongation Factor-1 Alpha-1 (EF1A1) was used instead of  $\beta$ -actin as the internal control to normalize for variation in RNA input between samples (Chusainow et al., 2009). It has been reported that the mRNA level of EF1A1 is more stable than that of  $\beta$ -actin in CHO cell lines at different culture conditions (Lee et al., 2007).

### 2.5. Analysis of antibody concentration and calculation of specific productivity

The concentrations of secreted mAb in the culture supernatant were measured by using enzyme-linked immunosorbent assay (ELISA) (Chusainow et al., 2009). The specific mAb productivity (qmAb) measured in  $\text{pg cell}^{-1} \text{day}^{-1}$  (pcd) was determined as the slope of mAb concentration versus integrated viable cell density from day 1 to day 3.

### 2.6. Methyl-assisted qRT-PCR (MAP)

hCMV-MIE (between -1141 and +73 relative to the transcription start site of +1), which contains 72 CpG dinucleotides, was derived from the 5' region of human cytomegalovirus immediate early gene (Genebank accession no. M60321). Methylation of CpGs at -924 (within BstUI restriction sequence, CCGG) and +53 (within HpaII restriction sequence, CCGG) were examined by a modified methyl-assisted qRT-PCR (MAP) assay (Hong et al., 2001). The two methyl-sensitive restriction enzymes (REs), BstUI and HpaII, were ordered from New England Biolabs (Ipswich, MA).

4  $\mu$ g of genomic DNA, isolated using the PureGene DNA purification kit (Gentra Systems, Minneapolis, MN), were digested overnight using 30 units of REs. An equal amount of DNA was treated with buffers without addition of any REs. In contrast to Hong's method in which the digested DNA was directly measured by qRT-PCR, we purified both the digested and non-digested DNA samples using ethanol precipitation as described in the handbook of the PureGene DNA purification kit. These purified DNA samples were then analyzed on an ABI PRISM<sup>®</sup> 7000 qRT-PCR Detection System (Applied Biosystems, Foster City, CA). The recipe for a 25  $\mu$ L PCR reaction was 30 ng of DNA,  $1 \times$  iTaq SYBR<sup>®</sup> Green Supermix (Bio-Rad, Hercules, CA), 364 nM of appropriate forward and reverse primers (Research Biolabs, Singapore), and HPLC water (Merck, San Diego, CA). Primers for amplifying the regions around sites of BstUI and HpaII in the hCMV promoter and in the internal control gene,

**Table 1**  
List of primers for MAP assay of methylated hCMV-MIE promoter.

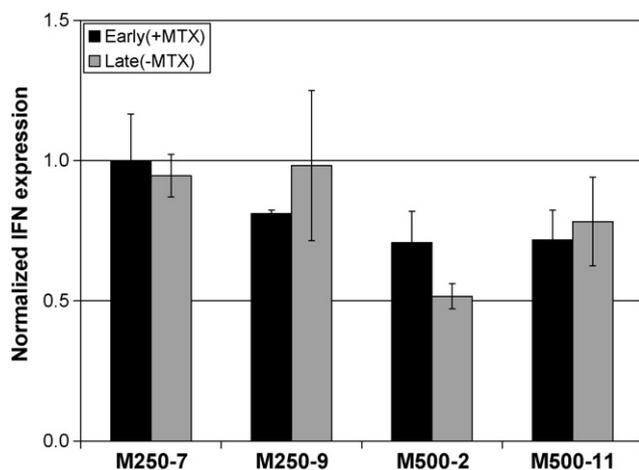
Target	Forward primer (5'–3')	Reverse primer (5'–3')
BstUI	ATGTCGCCGATGTGAGTTTCT	CCATCCCCGTAACGATATAA
HpaII	GCTCGTTTAGTGAACCGTCAGA	ATATTCATAAGCTTGGCGAGG
β-Actin	AGCTGAGAGGGAAATTGTGCG	GCAACGGAACCGCTCAIT

β-actin were manually designed (Table 1) to give amplicons of between 100 and 150 bases. The PCR program used included an initial denaturing step of 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. The data collected from the qRT-PCR detection system were analyzed using the  $2^{-\Delta\Delta C_t}$  method (Livak and Schmittgen, 2001).

### 3. Results

#### 3.1. *Trans-acting factors required for transcription were not impaired*

Four representative CHO-mAb clones, M250-7, M250-9, M500-2, and M500-11, were used in this study. Their productivities remained constant in the presence of MTX, but gradually dropped from 6% to 35% of original productivities after approximately 30 passages in the absence of MTX (Chusainow et al., 2009). To test whether *trans-acting* factors required for transcription of HC and LC genes were impaired in cells which exhibited loss in mAb productivities, a vector having same regulatory elements as the HC vector was constructed by replacing the HC gene in the HC vector with an IFN gene (Fig. 1). This modified vector was transiently transfected into Early(+MTX) cells with unchanged high productivity and Late(–MTX) cells with lowered productivity. As shown in Fig. 2, cells which lost mAb productivity after long term passage did not show lower transient IFN expression levels compared to those which maintained high productivity. This observation suggests that the *trans-acting* factors required for transcription of HC and LC genes were not impaired in cells which exhibited loss of productivity.



**Fig. 2.** Comparison of transient IFN expression levels in CHO-mAb cell lines which had been propagated in the presence of MTX for about 10 passages, designated as Early(+MTX), and in the absence of MTX for about 30 times, designated as Late(–MTX). Results represent changes in IFN expression normalized to an internal control, GFP expression, viable cell density, and IFN expression in an early passage number clone M250-7. Each point represents the average and standard deviation of four measurements of two samples from two transfections.

#### 3.2. *Methylation levels of hCMV-MIE were increased in cells that exhibited loss in productivity*

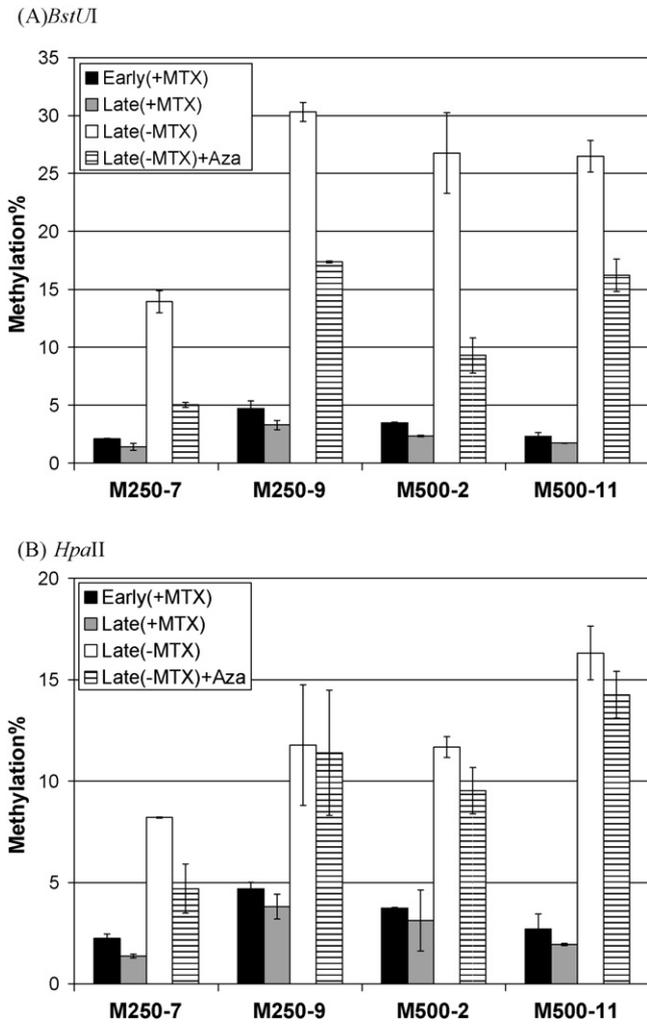
To investigate whether DNA methylation contributed to the reduced HC and LC transcripts, the methylation status of hCMV-MIE was examined in cells which exhibited loss of productivity using the MAP assay. As compared to Hong's method (Hong et al., 2001), the assay used in this work was improved in two aspects. Firstly, we purified the digested and non-digested DNA before analysis by qRT-PCR as we found that amplification efficiencies were affected by the components in the RE digestion solution, increasing variations between digested and non-digested DNA samples. Secondly, a house keeping gene, β-actin, was used as an internal control to normalize the variation in DNA input between samples. The improved assay reduced the standard deviation of duplicate measurements to less than 5%.

Genomic DNA was isolated from Early(+MTX) cells, Late(–MTX) cells, and cells which had been passaged in the presence of MTX for about 10 times, designated as Late(+MTX). They were analyzed for the percentage of DNA methylated at BstUI or HpaII sites located in the hCMV-MIE promoter. As shown in Fig. 3, less than 5% of DNA was methylated in Early(+MTX) cells for all four clones. There was also no significant changes to the methylation level in Late(+MTX) cells, corresponding well with the unchanged high productivities compared to Early(+MTX) cells. In contrast, Late(–MTX) cells that were derived from the same initial clones as Late(+MTX) had 15–30% of DNA molecules methylated at the BstUI site (Fig. 3A). This corresponds well to the decreased productivities of Late(–MTX) cells. Increased DNA methylation was also observed for a second CpG in the HpaII site in Late(–MTX), although the magnitudes were not as high as those at BstUI site (Fig. 3B).

#### 3.3. *The lost productivity was partially recovered by reversing DNA methylation*

In order to ascertain the role of DNA methylation in repressing the transcription of HC and LC, Late(–MTX) cells that exhibited loss in productivity were treated with a DNA methylation inhibitor, Aza. The concentration of Aza used in this study inhibited cell growth by 44–63% and killed less than 10% of cells. As shown in Fig. 4A, cells exposed to Aza exhibited increases in specific productivities as compared to the control (treated with only PBS). More than 2-fold increases were observed for clones M250-9 and M500-11, while the increases for clones M250-7 and M500-2 were 1.3-fold and 1.7-fold, respectively (Fig. 4B). The increase in specific productivity correlated with the increase in mRNA levels of HC and LC, suggesting that the increased specific productivities had resulted from demethylation reactivating transcription (Fig. 4B). Similar Aza treatment was also administered to Early(+MTX) cells. Besides the high-producing clones M250-7, M250-9, M500-2, and M500-7, three additional low-producing clones M250-3, M250-6, and M500-6 were also tested. Aza treatment resulted in growth inhibition of Early(+MTX) cells by 14–53%, however, no significant increase in productivity was observed (Fig. 5). These results suggested that the increased productivity in the Late(–MTX) was not due to growth suppression.

Although Aza treatment increased the mAb expression of Late(–MTX) cells that exhibited loss in productivity, the increased productivities only reached 12–50% of that in cells which had been passaged with MTX selection maintained (Fig. 4A). To investigate why the productivities were only partially restored, the methylation levels of the hCMV-MIE promoter in Late(–MTX) cells treated with Aza were directly measured. As shown in Fig. 3A, Aza decreased the methylated DNA at BstUI site by 9–17% as compared to those without Aza treatment. Slight decreases in methylation at HpaII site were also observed (Fig. 3B). Even after Aza treat-

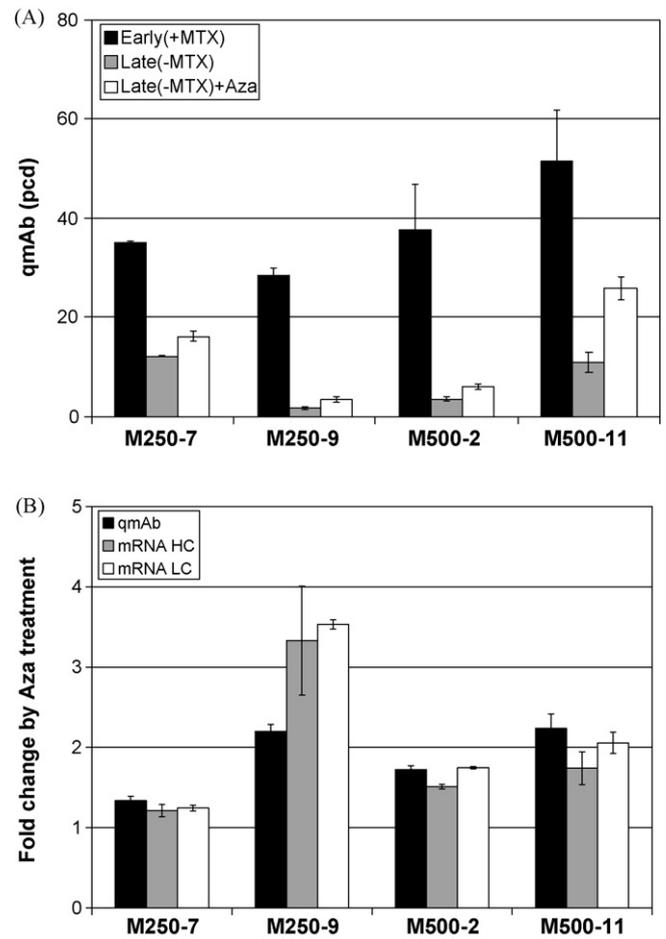


**Fig. 3.** Methylation status of hCMV promoter in mAb-producing CHO cells which had been passaged in the presence of MTX for about 10 times, designated as Early(+MTX), in the presence of MTX for about 30 times, designated as Late(+MTX), in the absence of MTX for about 30 times, designated as Late(-MTX), and in the absence of MTX for about 30 times and then treated with Aza, designated as Late(-MTX)+Aza. The graphs represent the methylation level at *Bst*UI site (A) and *Hpa*II site (B). Each point in the figure represents the average and standard deviation of four measurements from two samples.

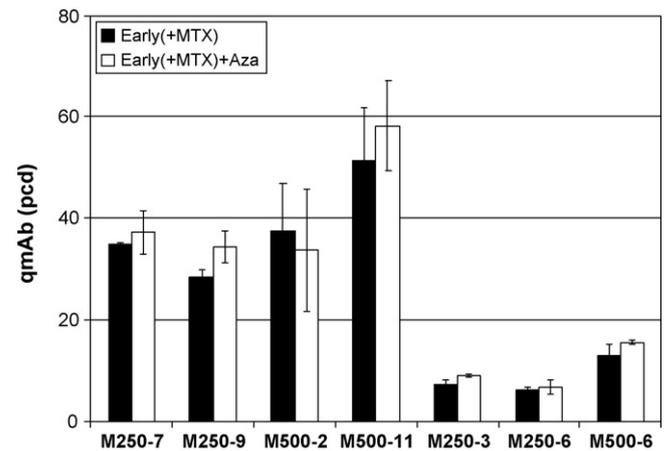
ment, the methylation levels at both sites in Late(-MTX) cells were still greater than in the Late(+MTX) cells which maintained high productivity in the MTX-containing medium (Fig. 3). This incomplete demethylation may explain why the productivities were not completely restored by Aza treatment. Increasing the concentration of Aza or extending the exposure time did not achieve further demethylation, mainly due to severe cell deaths (data not shown).

**4. Discussion**

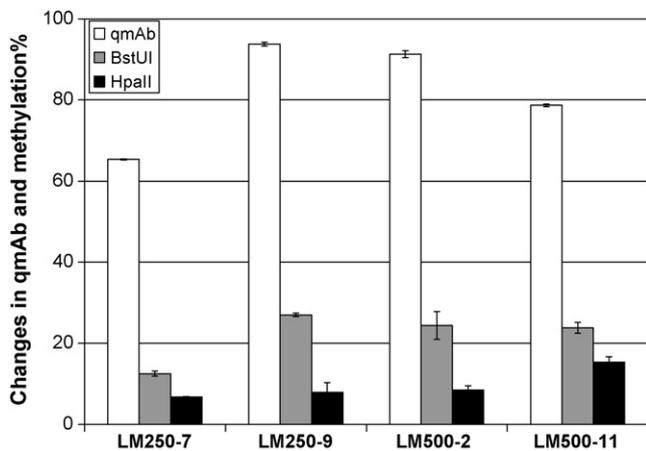
In our previous study, changes in transgene integration sites had been excluded as the cause of production instability in our CHO-mAb cell lines (Chusainow et al., 2009). In this work, we demonstrated that impaired *trans*-acting factors were also not the cause for instability, by studying the transient expression of an analogous reporter under the control of the same regulatory elements of HC gene. The lost productivity of Late(-MTX) cells was partially restored by treatment with Aza, a DNA methylation inhibitor, eliminating the possibility of permanent DNA mutation. Examination of two CpG sites by MAP assay revealed substantial increase in methylation of hCMV-MIE in Late(-MTX) cells which



**Fig. 4.** Effect of Aza on mAb expression in CHO cells which had been passaged in the absence of MTX for about 30 times, designated as Late(-MTX). The graph represents the effect of Aza on the specific productivity of mAb (qmAb) (A) and the correlation between relative changes in qmAb and levels of heavy chain (HC) and light chain (LC) mRNA induced by Aza (B). Each point represents the average and standard deviation of four measurements from two sets of samples.



**Fig. 5.** Effect of Aza on the specific productivity of mAb-producing CHO cells which had been passaged in the presence of MTX for about 10 times, designated as Early(+MTX). Each point represents the average and standard deviation of four measurements from two sets of samples.



**Fig. 6.** Correlation between the relative decreases in qmAb and increases in hCMV-MIE promoter methylation at BstUI and HpaII sites in the Late(-MTX) cells which have been passaged 30 times without MTX as compared those in the Early(+MTX) cells which are maintained in MTX for about 10 passages. Each point represents the average and standard deviation of measurements from two samples.

exhibited loss in productivity. Additionally, partial demethylation of the hCMV promoter by Aza restored some of the lost mAb productivity. These results suggested that DNA methylation contributed to the reduced HC and LC transcription. However, the relationship between changes in mAb expression and methylation of promoter is still not clear. The productivities of four clones investigated dropped by 65–94%, but methylation of the hCMV-MIE promoter at BstUI or HpaII sites was only observed in 8–30% of cells in populations of cells derived from these four clones (Fig. 6). Only two of the 72 CpG dinucleotides in the hCMV-MIE promoter were examined due to limitations in the availability of methylation sensitive restriction sites. It is unclear whether CpG sites other than those at BstUI and HpaII were methylated. Based on the fact that the CpGs at BstUI and HpaII sites were not methylated at equal level in a same cell population (Fig. 3), we speculate that hCMV-MIE promoters have different CpG methylation patterns in different cells. Examination of two or even a few more CpGs by MAP in fact, may not represent the methylated cells in a population. A more conclusive, in-depth understanding of the role of DNA methylation in production instability will require a comprehensive view of methylated CpGs in the entire hCMV promoter in individual cells and statistical assessments of populations of cells.

Another two questions still open for further investigation are how DNA methylation is targeted to the hCMV-MIE promoter and reasons why cells lost production in the absence of MTX but maintained high productivity in the presence of MTX. To date, the mechanism of how cells direct DNA methylation to a specific region has not been clearly elucidated (Bird, 2007; Miranda and Jones, 2007). Some evidences have indicated that tandem repeats of gene copies are more susceptible to DNA methylation (Hsieh and Fire, 2000; McBurney et al., 2002). This may explain why the productivity of most cell lines generated by DHFR- or GS-mediated gene amplification is not stable. With respect to the second question, it has been speculated that methylation of a specific DNA region is a stochastic process (Bird, 2007; Ptashne, 2007). There may be an equal chance of DNA being methylated, regardless of the presence or absence of MTX. When the product gene is methylated, the selection marker which is proximal to it may also be methylated and down regulated simultaneously. This is supported by the fact that the mRNA levels of both DHFR and mAb genes decreased correspondingly in cells which exhibited loss in productivity in our previous work (Chusainow et al., 2009). In the absence of MTX,

the cells with methylated DNA will lose productivity and may have growth advantage compared to the high-producing cells. They will gradually become dominant during long term culture in a cell population and finally result in a substantial drop of the overall productivity. While in the presence of MTX, cells that have methylated DNA and lose productivity will be killed due to loss of resistance to selection, leaving only high producing cells and thus high productivity is maintained.

The strategy of using chemicals, such as Aza, may not be feasible to alleviate DNA methylation for high productivity for a few reasons. Firstly, Aza is toxic which will inhibit cell growth and result in cell death. As observed in this work, although Aza increased the specific productivity, the titer was not increased correspondingly due to cell growth inhibition and cell death (data not shown). Secondly, the demethylation effect of Aza is non-specific. Besides the product gene, other genes which are harmful to cellular performance could be demethylated and activated as well. Thirdly, the effect of Aza varies depending on the cell lines used. It has been demonstrated that methylation in some cells cannot or is only partially reversed by Aza (Chiurazzi et al., 1999). This is consistent with our results that Aza only partially restored the lost productivity. Finally, silencing will be re-imposed when DNA methylation inhibitor is removed (McGarvey et al., 2006; Ptashne, 2007). Continuous addition of Aza, which is unstable in solution, to maintain its effect will inevitably increase the cost of production. As it may be too late to prevent DNA methylation once it occurs, a more effective strategy may be to prevent DNA methylation before it happens. It has been reported that insulators, matrix attachment region (MAR), and CpG island elements can prevent DNA methylation (Dang et al., 2000; Hejnar et al., 2001; Pikaart et al., 1998). Inclusion of such elements into vectors may be beneficial for the generation of high producing cell lines with stable productivity.

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