Strategies to improve recombinant protein production using CHO cell culture system

（CHO 細胞を用いた組換えタンパク質生産における生産性向上法に関する研究）

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Chapter 1 Introduction

1.1. Recombinant therapeutic antibody production in Chinese hamster ovary cells

At the end of the 1980s, the dominance of monoclonal antibody (mAb) approvals was about 10% of all biologic products approved, while recently they have represented almost 30% of all approvals and there is a large number of developed mAbs in clinical trials (Reichert 2015). Moreover, the market for biopharmaceutical products reached $140 billion in total cumulative sales in 2013 (Walsh 2014). These therapeutic proteins are produced by cultivation of recombinant cells. *Escherichia coli*, *Saccharomyces cerevisiae*, and mammalian cells are commonly used as host cells for industrial protein production systems. Among these host cells, mammalian cells, and especially Chinese hamster ovary (CHO) cell lines, are mainly used for therapeutic protein production. Recently, about 60–70% of all recombinant therapeutic proteins are produced by CHO cells (Jayapal et al. 2007). There are some advantages to using CHO cells as the host cells for therapeutic protein production. First, CHO cells are able to achieve appropriate folding and post-translational modification, and can thus produce recombinant proteins with the correct glycoforms for compatibility with humans (Koo et al. 2009; Li et al. 2005; Ono et al. 2003). Many therapeutic proteins require specific modifications that can only be

performed in higher eukaryotic cells (Maksimenko et al. 2015). Second, CHO cells have already been demonstrated to be safe host cells for clinical use, potentially making it easier to obtain approval from regulatory agencies. Third, CHO cells can easily be adapted to serum-free suspension culture, which is suitable for large-scale manufacture in bioreactors (Omasa et al. 2010). Since the first use of CHO cells for recombinant protein expression, CHO cell production systems have steadily improved by approaches through cell line development, clone screening and isolation, and optimization of basal media and feed supplements, as well as advances in cell culture process (Reinhart et al. 2015).

Medium optimization has been identified as an important key approach for increasing product concentrations (Zhang et al. 2013). Screening of new small molecules as medium additives and balancing of current medium components by studying their interactions have been reported to increase productivity (De Leon et al. 2007, Backliwal et al. 2008, Inoue et al. 2000). Bovine serum was a popular supplement in cell culture medium, because it improves cell growth and productivity. However, regulatory agencies encouraged the removal of animal-derived supplements because of safety concerns (Kishishita et al. 2015). Consequently, non-animal-derived hydrolysates from soy, wheat, and yeast, which can provide similar benefits

to serum, were widely used. Although hydrolysates can improve cell growth and product productivity, they led to process and product inconsistencies because of their compositional complexity and lot-to-lot variation (Luo et al. 2007, Zhang et al. 2003). Therefore, many biopharmaceutical companies are now shifting to develop chemically-defined (CD) medium formulations. The majority of the biopharmaceutical industry is currently using fed-batch cultures as a platform technology for biopharmaceutical protein production, because recent advances have resulted in higher final product concentrations (Li et al. 2010). Nevertheless, the demands of the ever-increased markets for therapeutic antibodies still require cells to be more highly productive and to be grown at higher cell densities. Several CD media for CHO cell cultures have been developed, however CD media that work in fed-batch culture processes and that support high cell density and high concentration of recombinant proteins are still unsatisfactory. In this study, we used nucleosides as components of cell culture medium and developed platform technology to improve product productivity in CHO cell culture system. The nucleosides addition strategy is expected to provide us simple and reproducible technology for antibody production. Optimized medium composition and development of an appropriate feed strategy is crucial approach, as they

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improve culture longevity and lead to a more efficient cell metabolism, which reduces the formation of metabolites that may negatively affect cell growth (Butler 2005).

Meanwhile, despite the progress of media development, the instability of specific production rate, which often occurs during long-term culture, is still crucial issue. During the media development study, some recombinant CHO cell lines showed instability of cellular productivity and it negatively affected the performance of nucleosides addition culture (data not shown). Therefore, overcoming instability of cellular productivity is important to maximize the effects of developed platform technology with CD media. Expressing vector engineering approach which modulates transcriptional activity is also known as an important approach (Barnes et al. 2003). Much attention has also been paid to construct-expressing systems represented by vector construction and developments in the generation of highly productive clones (Omasa 2002; Park et al. 2010). The structure of the expression vector is one of the most important aspects to improve productivity because it has a significant effect on the expression level, stability, and screening efficacy of high-producer cell lines. Conventional transfection of a constructed vector into a CHO cell line results in random integration of the gene of interest (GOI) into the host cell genome.

However, because the majority of the genome consists of transcriptionally non-permissive heterochromatin, there is a high probability that the GOI will be integrated into an area that is unfavorable for high level and stable expression. In addition, even if a GOI has integrated into a transcriptionally active region, expression may still be silenced by DNA methylation within the integrated transgene or its promoter region (Kwaks and Otte 2006; Saunders et al. 2015), which is called a "positional effect". Therefore, the expression level of the introduced gene varies depending on the integration site in the chromosome. These positional effects are the reasons for the variety of expression levels in transfected cell clones and the instability of cellular productivity during long term culture (Kim et al. 2004). To overcome these negative position effects, site-specific transgene integration methods have been developed recently. Site-specific integration is a target knock-in method using a known active site via the Cre/loxP or FRT/Flp-In™ system (Kim 2007). However, it is very difficult to determine a suitable active site for high and stable transgene expression in the host cell genome before target knock-in. Another approach is to use regulatory elements to prevent positional effects. Chromatin function-modifying elements, namely ubiquitous chromatin opening elements (UCOEs), scaffold or matrix attachment regions (S/MARs), stabilizing anti-repressor

(STAR) elements, and insulators, reduce or negate epigenetic processes from negatively affecting transgene expression even if the gene has been integrated into an area of heterochromatin (Saunders et al. 2015). In this study, we isolated DNA regulatory motifs from CHO genome sequence for stable protein production. In previous study, a bacterial artificial chromosome (BAC) library from stable and highly productive CHO cell line was constructed for genome-wide analysis. BAC library has provided a useful tool for obtaining sequence data for gene discovery and functional sequence annotation. Although the vector engineering and cell engineering to CHO cell culture has various benefits, the limited success in its application may be caused by the insufficient genomic information of CHO cells. In this regard, our approach has advantage and would be a powerful tool for therapeutic protein manufacturing.

1.2. Overview of the present study

Overview of the present study is as follows;

The purpose of the present study is improving recombinant protein production using CHO cell culture system. In chapter 2 and chapter 3, medium development approaches to increase product productivity are described. During the media development study, some recombinant CHO cell lines showed instability of specific production rate, which often occurs during long-term culture. To maximize the effects of developed media, expression vector engineering approach for stable product production is described in chapter 4. The structure of this study was summarized in Figure 1.

Chapter 2 describes the investigation of deoxyuridine addition to CHO cell culture in order to increase the product productivity. The results showed addition of deoxyuridine significantly increased the cell growth and the mAb concentration in fed-batch culture.

Chapter 3 describes the investigation of deoxyuridine, thymidine and deoxycytidine addition to CHO cell culture. Deoxyuridine, thymidine, and deoxycytidine are categorized as pyrimidine nucleosides and they are metabolized by related pyrimidine salvage pathways. The results suggest that synergistic effects could exist among these three combined nucleosides

for the cell growth and mAb concentration. Furthermore this chapter describes the investigation whether the enhancement of cell growth by nucleotide addition is a general phenomenon. The nucleotide addition effects were investigated using Fab fragment expressing CHO cell line.

Chapter 4 describes investigation of functional analysis of the genomic sequence derived from highly stable CHO cell line and isolation regulatory motifs which can act as an insulator and have positive effects for stable transgene expression.

The final chapter, Chapter 5, discusses implications of the obtained results and future research aspects.

Strategies to improve recombinant protein production using CHO cell culture system

Chapter 1
Background and objectives of this study

$$P = \rho \int X_v dt$$

P ; product concentration
ρ ; specific production rate
Xv ; viable cell concentration

inactivation

Regulatory motif   Recombinant protein expression gene

Chapter 4
Expression vector engineering for stable product production

Nucleoside addition

Viable cell density

Time

Chapter 2, 3
Medium development to increase the cell density and the product concentration

Chapter 5
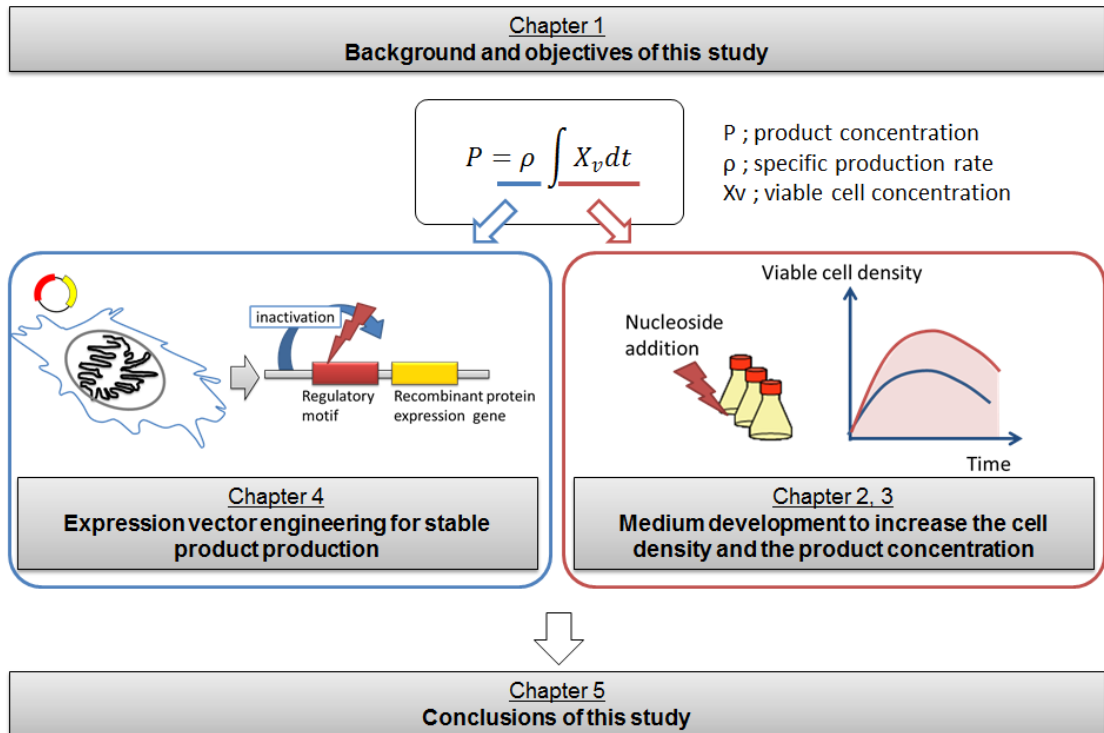**Conclusions of this study**

Figure 1 Structure of this study for improving recombinant protein production using CHO cell culture system

Chapter 2 Effects of deoxyuridine on cell growth and antibody production

2.1. Introduction

Recently, with the dramatic increase in demand for therapeutic proteins, manufacturing platforms for recombinant protein production using CHO cell culture systems have made significant progress (Omasa et al. 2010). Over the past two decades, recombinant protein productivity has been improved by more than 100-fold (Wurm 2004). Several efforts, including cell engineering, optimization of cell line development, cell culture medium optimization, and process development for scaling up or down, have led to significant improvements in recombinant protein production (Omasa 2002; Omasa et al. 2008; Nakamura et al. 2014). Consequently, CHO-based mAb production processes can achieve 1–10 g/L productivity in fed-batch cultures (Kunert et al. 2016).

It is well known that optimization of nutrient concentrations, such as amino acids, vitamins, and trace metals, is very important for improving protein production (Takagi et al. 2001; Kim et al. 2005; Chaderjian et al. 2005). However, few reports have described the application of exogenous nucleoside addition for protein production processes. Purine and pyrimidine nucleotides are essential cellular compounds involved in many biochemical processes. They are indispensable for cell growth because they are required not only for nucleic acid synthesis as building blocks, but also

for energy metabolism (Yamaoka et al. 1997). The *de novo* and salvage pathways are the principal pathways for nucleotide synthesis (Lane et al. 2015). Nucleotide biosynthesis via the salvage pathway induced by exogenous nucleosides is more efficient than *de novo* biosynthesis (Austin et al. 2012). Hence, it is important to investigate the effects of nucleosides on cell growth and protein production. Several studies have reported the influence of nucleosides on CHO cell growth (Chen et al. 2012a; Carvalhal et al. 2003). However, much more attention has been paid to the effects on product quality, especially glycosylation (Gramer et al. 2011; Wong et al. 2010), and little is known about the use of extracellular nucleosides for optimization of recombinant protein production as an effective tool to elevate antibody production. Furthermore, the impact of pyrimidines on CHO cell cultures has been less commonly reported in the literature compared with that of purines (Carvalhal et al. 2011).

In this chapter, we report an approach to increase the final product concentration through addition of pyrimidine nucleosides, especially deoxyuridine. It could be a beneficial platform technology for antibody-based therapeutic protein production systems.

2.2. Materials & methods

2.2.1. Cell lines and media

The three CHO cell lines used as the model cell lines in this study were generated from CHO-K1SV cells (Lonza Biologics, Slough, UK), which had been adapted to suspension culture and can grow in serum-free medium. The GS Gene Expression System (Lonza Biologics) was used as the expression system for CHO-K1SV cells (GS-CHO system). The expression vectors are constructed by cloning protein coding gene into GS expression vectors (Lonza Biologics) (Barnes et al. 2000). The GS coding sequence is under the control of a weak promoter; meanwhile the protein coding sequence is under the control of a powerful promoter (Bebbington et al. 1992). The two cell lines were transfected with plasmids encoding different protein molecules and established by single-cell cloning. CHO cell line A and CHO cell line B produced human mAb A and mAb B, respectively.

The basal and feed media used in this study were fully in-house CD media. The CD basal and feed media contained trace elements, inorganic salts, energy source, amino acids, vitamins, fatty acids, lipids, and others. Nevertheless, the glucose and other nutrient concentrations should be maintained at appropriate levels during the cell culture process to supply

sufficient amounts of energy source and cellular components. Several report described how much nutrient would be needed for GS-CHO culture (Carinhas et al. 2013, Kyriakopoulos et al. 2013, Sellick et al. 2011). Further nucleoside components were added as indicated.

2.2.2. Nucleosides

Deoxyuridine and deoxycytidine hydrochloride were purchased from Alfa Aesar (Heysham, UK). Deoxycytidine hydrochloride was used as the form for addition of deoxycytidine. Deoxyadenosine monohydrate and deoxyguanosine monohydrate were used as the form for addition of deoxyadenosine and deoxyguanosine. Deoxyadenosine monohydrate, deoxyguanosine monohydrate, and uridine were purchased from Wako Pure Chemical Industries (Osaka, Japan). All nucleosides were dissolved in water and sterilized by filtration through a 0.22-µm filter before addition.

2.2.3. Cell culture methods

Cells were routinely maintained in suspension culture using 125-mL shaking flasks (Corning, Corning, NY, USA) on a rotary shaker–incubator (Kuhner, Birsfelden, Switzerland) controlled at 36.5°C, 5% CO2, and 140 rpm.

Batch cultures were initiated at a cell concentration of 0.3×10^6 cells/mL with in-house CD media in 125-mL shaking flasks. Production culture was carried out in the fed-batch culture mode using the 125-mL shaking flasks. Cells were seeded at 1.0×10^6 cells/mL, and subjected to different nucleoside addition strategies. Feeding was operated with glucose and in-house CD media to provide sufficient nutrients to the cells. The feeding of in-house CD media was operated every day from day 2 to the end of the culture.

Cell suspension samples were taken at appropriate times, and the cell concentrations, cell viabilities, and protein product concentrations were analyzed.

2.2.4. Analytical methods of cell concentration and mAb concentration

The viable cell concentration (VCC) and cell viability were measured in an automated cell counting device (ViCell-XR; Beckman Coulter, Indianapolis, IN, USA) using the trypan blue exclusion method. The concentrations of mAb A in the culture supernatants were measured using an HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a UV detector and a protein A affinity column (Thermo Fisher Scientific, Waltham, MA, USA).

2.2.5. Calculation of specific growth rate and specific production rate

In fed-batch cultures, the mass balance of total cells, viable cells, and mAb were described by equations (1) and (2) in a previous report (Omasa et al. 1992). Briefly, V, X_t, μ, X_v, F_0, P, and ρ are the culture volume, total cell concentration, specific growth rate, viable cell concentration, drawing rate via sampling, mAb concentration, and specific production rate, respectively. These parameters are measurable during the culture.

$$\frac{dVX_t}{dt} = \mu VX_v - F_0 X_t \qquad equation\ (1)$$

$$\frac{dVP}{dt} = \rho VX_v - F_0 P \qquad equation\ (2)$$

Equations (3) and (4) are led by the integrals of equations (1) and (2). The specific growth rate μ and specific production rate ρ are calculated from equations (3) and (4).

$$\ln(VX_t) + \int \frac{F_0}{V} dt = \mu \int \left(\frac{X_v}{X_t}\right) dt + \ln(VX_t)_0 \qquad equation\ (3)$$

$$(VP) + \int F_0 P\, dt = \rho \int VX_v dt + (VP)_0 \qquad equation\ (4)$$

2.3. Results & discussions

2.3.1. Effects of nucleosides on cell growth in batch culture

To evaluate the effects of purine and pyrimidine nucleosides addition, batch cultures were initiated at a cell concentration of 0.3×10^6 cells/mL in 125-mL shaking flasks using CHO cell line B. Deoxyuridine, deoxycytidine, deoxyadenosine, and deoxyguanosine were added at the initial time at final concentrations of 5, 25, 100, 500, and 1000 mg/L. Control cultures were performed without addition of any nucleosides. Batch cultures in shaking flasks with different concentrations of nucleosides were carried out for 5 days, and the results of cell growth are summarized in Figure 2.
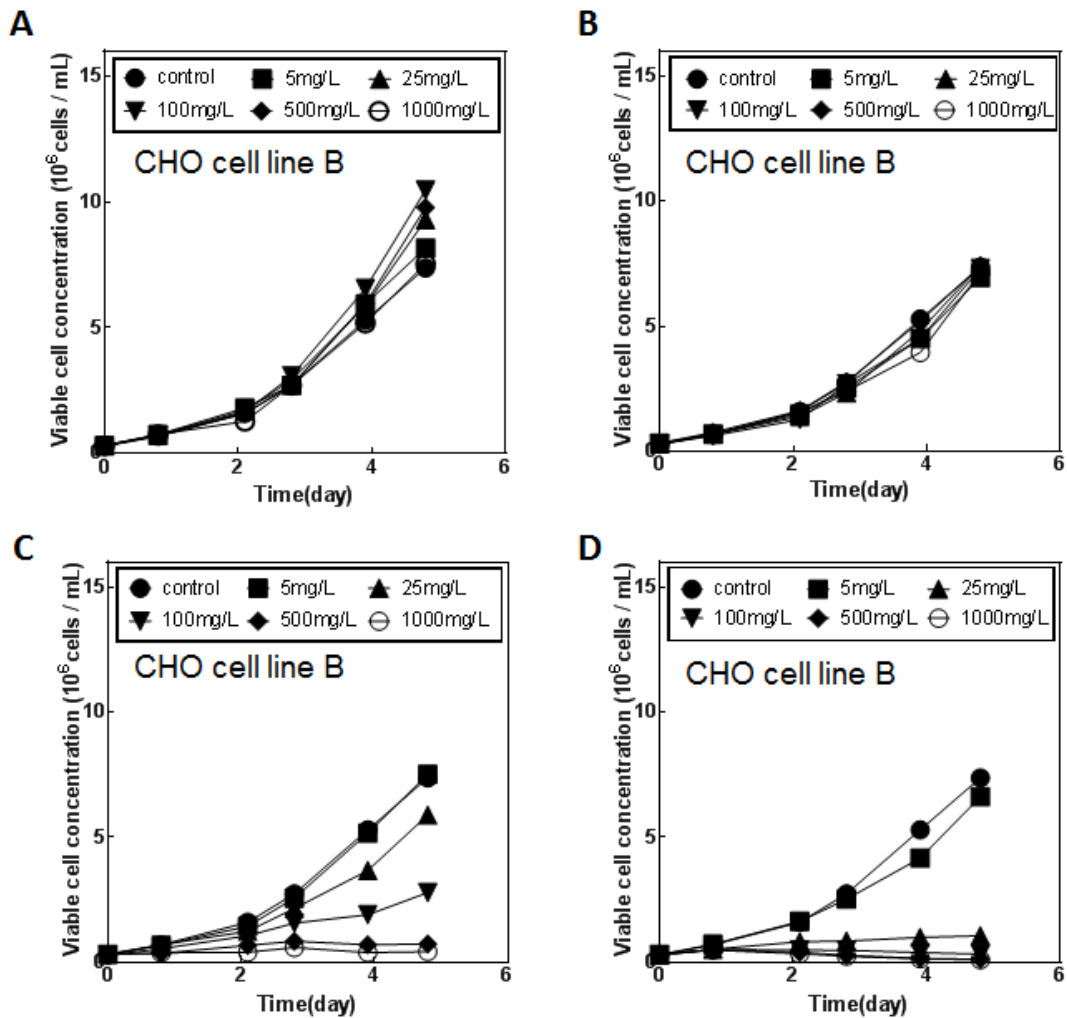
Figure 2 Effects of addition of different nucleosides on cell growth in CHO cell line B cultures. (A) Viable cell concentration of deoxyuridine addition culture. (B) Viable cell concentration of deoxycytidine addition culture. (C) Viable cell concentration of deoxyadenosine addition culture. (D) Viable cell concentration of deoxyguanosine addition culture. Control cultures were performed without nucleoside addition. Each nucleoside was added at the initial time of culture.

As shown in Figure 2A, addition of deoxyuridine significantly increased the VCC at day 5. In particular addition of 100 mg/L deoxyuridine, the VCC at day 5 (10.5×10^6 cells/mL) was 42% higher than that in the control culture (7.4×10^6 cells/mL). As shown in Figure 2B, no significant differences in the VCC at day 5 were observed in deoxycytidine addition culture. However, addition of deoxyadenosine or deoxyguanosine significantly decreased the VCC at day 5(Figure 2C, Figure 2D). In deoxyadenosine addition culture, it seemed that deoxyadenosine concentration-dependently arrested CHO cell growth. The results showed that deoxyadenosine and deoxyguanosine negatively affect the CHO cell growth. Deoxyuridine and deoxycytidine are categorized as pyrimidine nucleosides while deoxyadenosine and deoxyguanosine are categorized as purine nucleosides. It was reported that addition of purine nucleoside to cell culture media arrested the CHO cell growth (Carvalhal et al. 2011). As they described, our results also showed addition of purine nucleosides caused CHO cell growth arrest. These observations suggested that deoxyuridine had a potential to increase CHO cell growth in fed-batch culture.

2.3.2. Effects of deoxyuridine on cell growth and antibody production

Fed-batch cultures were initiated at a cell concentration of 1.0×10^6 cells/mL in 125-mL shaking flasks using CHO cell line A. Control cultures were performed without deoxyuridine addition. To determine the effects of deoxyuridine addition on CHO cell growth and mAb production, deoxyuridine was added on day 2 at final concentrations of 10, 25, 50, 100, and 200 mg/L. Fed-batch cultures in shaking flasks with different concentrations of deoxyuridine were carried out for 14 days, and the results are summarized in Figure 3 and Table 1.
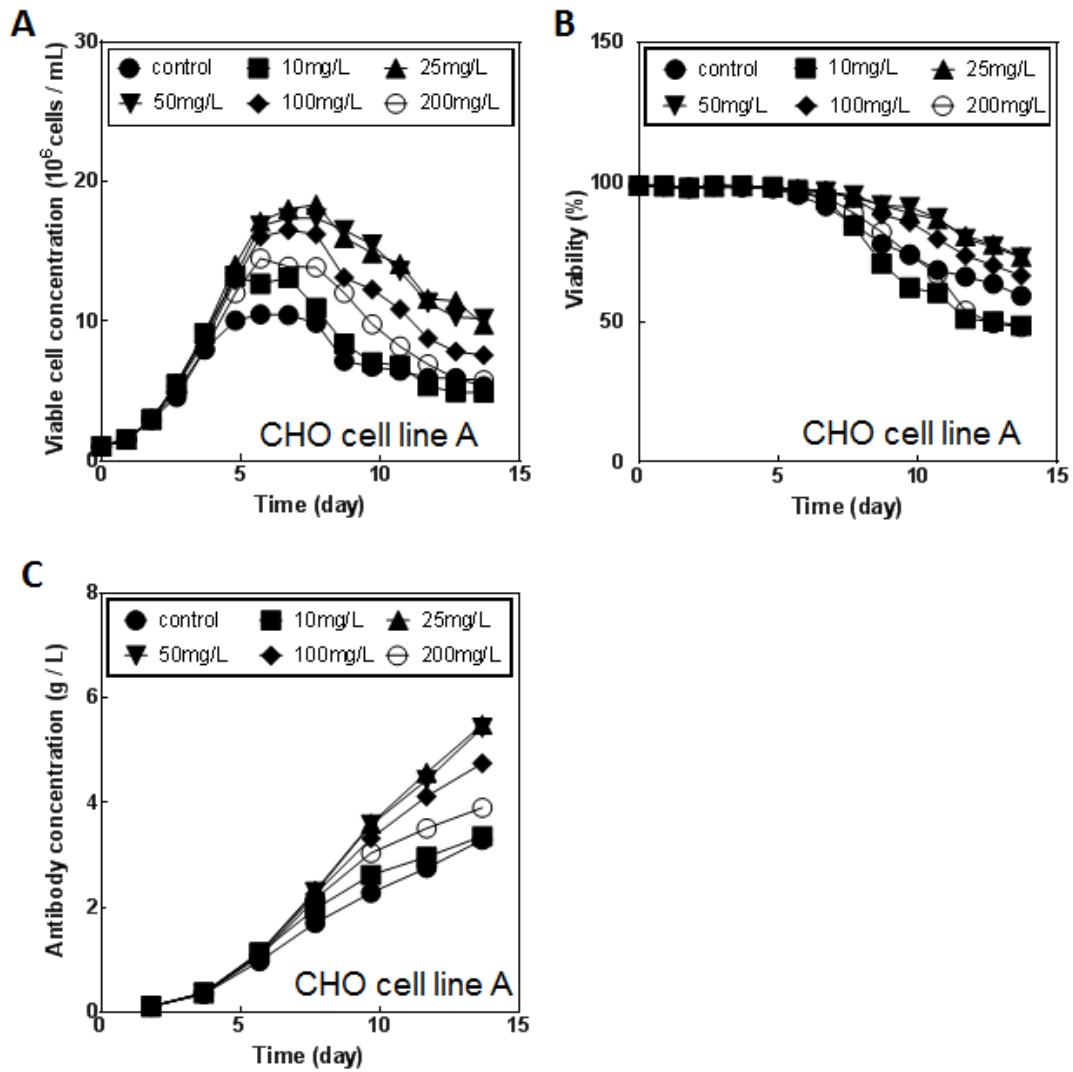
Figure 3 Effects of addition of different concentrations of deoxyuridine on cell growth, cell viability, and mAb concentration in CHO cell line A cultures. (A) Viable cell concentration. (B) Cell viability. (C) mAb concentration. Control cultures were performed without deoxyuridine addition. Deoxyuridine was added at the initial time of culture.

Table 1 Experimental conditions and kinetic parameters for different concentrations of deoxyuridine addition culture

Fed-batch condition	Peak VCC ($\times$ 10^6 cells/mL)	Viability at day 14 (%)	IVCC at day 14 ($\times$ 10^6 cells·h/mL)	mAb concentration at day 14 (g/L)	μ (1/h)*	ρ (pg/cell/h)**
Control	10.5	59.4	2226.9	3.3	0.024±0.0015	38.2±3.7
10 mg/L	13.2	48.7	2498.9	3.4	0.027±0.0018	35.6±2.6
25 mg/L	18.4	73.4	3827.4	5.5	0.027±0.0021	36.8±3.6
50 mg/L	17.4	73.1	3738.3	5.4	0.026±0.0018	37.0±3.2
100 mg/L	16.6	66.6	3278.2	4.7	0.026±0.0022	37.6±3.7
200 mg/L	14.5	48.2	2814.9	3.9	0.025±0.0009	37.0±2.7

The specific growth rate μ is calculated from the data in Figure 3 in the exponential phase

The specific production rate ρ is calculated from the data in Figure 3 in the whole process time

*With standard error

**With 95% confidence interval

As shown in Table 1, no significant differences in the specific growth rate were observed in the various deoxyuridine concentration cultures. However, addition of deoxyuridine at all concentrations from 10 mg/L to 200 mg/L significantly increased the peak VCC. In particular, addition of 25 mg/L deoxyuridine produced a peak VCC of 18.4×10^6 cells/mL, representing a 75% increase compared with that of the control culture (10.5×10^6 cells/mL). Furthermore, there were differences in cell viability. In the control culture, the cell viability gradually dropped off toward the end of the culture period. As shown in Figure 3, the viability after day 6 was not decreased following addition of 25 mg/mL to 100 mg/L deoxyuridine. The increases in peak VCC and viability led to higher values of the integrated viable cell concentration (IVCC). The increased final product concentrations could be primarily linked to the increased cell growth. In all cases with addition of deoxyuridine, increases in the mAb concentration were observed compared with no addition of deoxyuridine. The final mAb concentration in the control condition was 3.3 g/L, while the final mAb concentration in the deoxyuridine addition cultures was dramatically improved. For example, in the 25 mg/L deoxyuridine culture, the mAb concentration was 5.5 g/L, representing a 67% higher concentration compared with that in the control culture. Thus, addition of deoxyuridine

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caused significant increases in the peak VCC of CHO cells and the final product concentration. Furthermore, as shown in Table 1, there were no significant differences in the specific production rate. The elevation of the final product concentration seemed to be mainly caused by the increased IVCC. The final product concentration in cultures was reported to equal the specific production rate multiplied by the IVCC over the culture duration (Birch et al. 2006). Our findings showed that cells were able to increase their IVCC, without significant loss of the specific production rate.

However, the peak cell concentration decreased after addition of more than 100 mg/L deoxyuridine. It was estimated that the optimal range of deoxyuridine concentration to maximize the effects was 25 mg/L to 50 mg/L. To simplify further evaluations, 25 mg/L was selected as the added concentration.

2.3.3. Effects of uridine on cell growth and antibody production

To investigate whether uridine had a similar effect of deoxyuridine on cell growth, fed-batch cultures were performed. The fed-batch cultures were initiated at a cell concentration of 1.0×10^6 cells/mL in 125-mL shaking flasks using CHO cell line A. Uridine was added at the initial time at final concentrations of 25, 100, 200, and 1000 mg/L. Deoxyuridine was also added at the initial time at final concentrations of 25 mg/L. Control cultures were performed without addition of any nucleosides. Fed-batch cultures in shaking flasks with different concentrations of nucleosides were carried out for 14 days, and the results of cell growth are summarized in Figure 4.
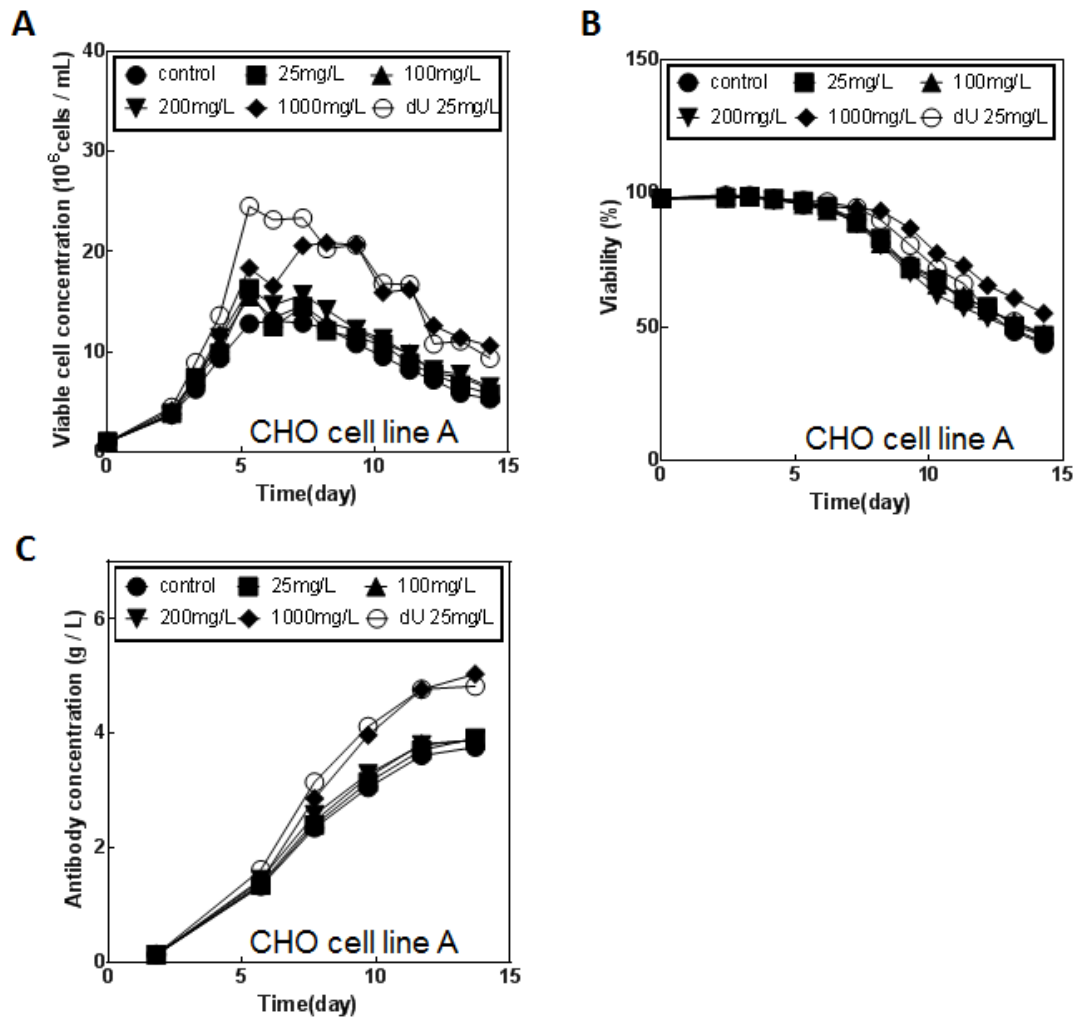
Figure 4 Effects of addition of different concentrations of uridine on cell growth, cell viability, and mAb concentration in CHO cell line A cultures. (A) Viable cell concentration. (B) Cell viability. (C) mAb concentration. Control cultures were performed without uridine or deoxyuridine addition. Uridine and deoxyuridine was added at the initial time of culture. dU 25 mg/L means cell culture condition in addition of deoxyuridine at 25 mg/L.

As shown in Figure 4, addition of deoxyuridine significantly increased the peak VCC. As a result of increases in cell growth, final product concentration was increased compared with no addition of deoxyuridine. Meanwhile, no significant differences were observed in less than 1000 mg/L uridine addition culture and addition of 1000 mg/L uridine could increase cell growth. The peak VCC of 25 mg/L deoxyuridine addition culture (24.5×10^6 cells/mL) was higher than that in 1000 mg/L uridine addition culture (20.9×10^6 cells/mL). However, slight increases in viability were observed after day 6 in the 1000 mg/L uridine addition culture. As a result of the increases in the peak VCC and viability, the final product concentrations were increased in the 1000 mg/L uridine addition culture. The final product concentrations were very similar between 25 mg/L deoxyuridine addition culture and 1000 mg/L uridine addition culture. From these results, 1000 mg/L uridine addition showed similar effect on cell growth and product concentration with 25 mg/L deoxyuridine addition.

Deoxyuridine is categorized as deoxyribonucleoside and uridine is categorized as ribonucleoside. The strategy of uridine addition to cell culture media was reported to investigate or control the quality of produced antibody quality, especially glycosylation (Gramer et al. 2011; Wong et al. 2010). In these reports, addition of 1 mM (244 mg/L) or 5 mM (1221

mg/L) of uridine were evaluated, however no significant effects on process performance were reported. It seemed that uridine addition had a capability to increase the product concentration, while, from the point of view of cost, the strategy of deoxyuridine addition was considered to be a superior technology. The necessary concentration of uridine was 40 fold higher than that of deoxyuridine. Therefore, deoxyuridine was selected as the added nucleoside for further evaluation.

2.4.Summary

We evaluated the effects of nucleosides addition in batch culture. Based on the results, CHO cell growth was increased in deoxyuridine addition culture, meanwhile, addition of deoxyadenosine or deoxyguanosine arrested CHO cell growth. We also evaluated the effects of deoxyuridine addition to fed-batch cultures of antibody-expressing CHO cell line. As a result of the improvement in cell growth and prevention of decrease in viability in the death phase, we could achieve high product concentrations.

Chapter 3 Synergistic effects of deoxyuridine, thymidine and deoxycytidine on cell growth and antibody production

3.1. Introduction

The ever-increased market demands for therapeutic antibodies require optimization of CHO cell culture process to improve manufacture efficiency (Chen et al. 2012). Development of advanced culture medium is considered as one of the key contributors to the increase of product concentration.

Previously, we showed addition of deoxyuridine significantly increased peak VCC and final product concentration in fed-batch culture. However, addition of deoxyuridine at more than 25 mg/L could not further enhance the increase in cell growth. It was supposed that the content of the intracellular deoxyuridine pool might be a limiting factor for this effect. It has been reported that cells have intracellular pools for individual nucleosides (Staub et al. 1988; Russell et al. 1980; Carvalhal et al. 2011). Deoxyuridine, thymidine, and deoxycytidine are categorized as pyrimidine nucleosides. These compounds are metabolized by related pyrimidine salvage pathways (Traut 1994). Thymidine and deoxycytidine were expected to have synergistic effects with deoxyuridine.

It is also known that different recombinant CHO cell lines show different responses to external culture environments (Porter et al. 2010; Kim et al. 2005). Furthermore, recombinant CHO cell lines expressing different types

of protein show different productivity. Therefore, it is important to confirm the effects of nucleoside addition using different CHO cell lines that produce different types of protein. To investigate whether the enhancement of cell growth by nucleoside addition is a general phenomenon, CHO cell line C expressing Fab fragment C was evaluated using fed-batch cultures. It was worth evaluating a protein with different characteristics for general application.

 In this chapter, we show the approach to further increase the final product concentration through combined addition of deoxyuridine, thymidine, and deoxycytidine. Moreover the efficacy of this strategy is also demonstrated with a Fab fragment expressing cell line to investigate whether this nucleosides addition strategy is a general platform technology.

3.2. Materials & methods

3.2.1. Cell lines and media

The three CHO cell lines used as the model cell lines in this study were generated from CHO-K1SV cells (Lonza Biologics, Slough, UK), which had been adapted to suspension culture and can grow in serum-free medium. The GS Gene Expression System (Lonza Biologics) was used as the expression system for CHO-K1SV cells (GS-CHO system). The expression vectors are constructed by cloning protein coding gene into GS expression vectors (Lonza Biologics) (Barnes et al. 2000). The GS coding sequence is under the control of a weak promoter; meanwhile the protein coding sequence is under the control of a powerful promoter (Bebbington et al. 1992). The two cell lines were transfected with plasmids encoding different protein molecules and established by single-cell cloning. CHO cell line B produced human mAb B, while CHO cell line C produced Fab fragment C.

The basal and feed media used in this study were fully in-house CD media. The CD basal and feed media contained trace elements, inorganic salts, energy source, amino acids, vitamins, fatty acids, lipids, and others. Nevertheless, the glucose and other nutrient concentrations should be maintained at appropriate levels during the cell culture process to supply

sufficient amounts of energy source and cellular components. Several report described how much nutrient would be needed for GS-CHO culture (Carinhas et al. 2013, Kyriakopoulos et al. 2013, Sellick et al. 2011). Further nucleoside components were added as indicated.

3.2.2. Nucleosides

Deoxyuridine and deoxycytidine hydrochloride were purchased from Alfa Aesar (Heysham, UK). Thymidine was obtained from Wako Pure Chemical Industries (Osaka, Japan). Deoxycytidine hydrochloride was used as the form for addition of deoxycytidine. All three nucleosides were dissolved in water and sterilized by filtration through a 0.22-µm filter before addition.

3.2.3. Cell culture methods

Cells were routinely maintained in suspension culture using 125-mL shaking flasks (Corning, Corning, NY, USA) on a rotary shaker–incubator (Kuhner, Birsfelden, Switzerland) controlled at 36.5°C, 5% CO2, and 140 rpm.

Production culture was carried out in the fed-batch culture mode using the 125-mL shaking flasks. Cells were seeded at 1.0×10^6 cells/mL, and subjected to different nucleoside addition strategies. Feeding was operated with glucose and in-house CD media to provide sufficient nutrients to the cells. The feeding of in-house CD media was operated every day from day 2 to the end of the culture. Cell suspension samples were taken at appropriate times, and the cell concentrations, cell viabilities, and protein product concentrations were analyzed.

3.2.4. Analytical methods of cell concentration and mAb concentration

The viable cell concentration (VCC) and cell viability were measured in an automated cell counting device (ViCell-XR; Beckman Coulter, Indianapolis, IN, USA) using the trypan blue exclusion method. The concentrations of mAb B in the culture supernatants were measured using an HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a UV detector and a protein A affinity column (Thermo Fisher Scientific, Waltham, MA, USA). The concentration of Fab fragment C was measured with a reverse-phase column (Imtakt Corp., Kyoto, Japan) using the same HPLC system.

3.2.5. Calculation of specific growth rate and specific production rate

In fed-batch cultures, the mass balance of total cells, viable cells, and mAb were described by equations (1) and (2) in a previous report (Omasa et al. 1992). Briefly, V, X_t, μ, X_v, F_0, P, and ρ are the culture volume, total cell concentration, specific growth rate, viable cell concentration, drawing rate via sampling, mAb concentration, and specific production rate, respectively. These parameters are measurable during the culture.

$$\frac{dVX_t}{dt} = \mu VX_v - F_0 X_t \qquad equation\ (1)$$

$$\frac{dVP}{dt} = \rho VX_v - F_0 P \qquad equation\ (2)$$

Equations (3) and (4) are led by the integrals of equations (1) and (2). The specific growth rate μ and specific production rate ρ are calculated from equations (3) and (4).

$$\ln(VX_t) + \int \frac{F_0}{V}\,dt = \mu \int \left(\frac{X_v}{X_t}\right) dt + \ln(VX_t)_0 \qquad equation\ (3)$$

$$(VP) + \int F_0 P\,dt = \rho \int VX_v dt + (VP)_0 \qquad equation\ (4)$$

3.3. Results & discussion

3.3.1. Synergistic effects of deoxyuridine, thymidine and deoxycytidine on cell growth and antibody production

To evaluate the combined effects among the three nucleosides, fed-batch cultures were performed at an initial cell concentration of 1.0×10^6 cells/mL with CHO cell line B. The combinations of nucleosides and culture results are summarized in Table 2. To evaluate the effects of combined addition of the nucleosides, fed-batch cultures in shaking flasks were carried out for 14 days. Control culture was performed without addition of any nucleosides. To determine the synergistic effects of deoxyuridine, thymidine, and deoxycytidine, the nucleosides were added at the initial time at final concentrations of 25 mg/L in various combinations.

Table 2 Experimental conditions and kinetic parameters for various combinations of nucleoside addition culture.

Fed-batch condition	Nucleoside			Peak VCC ($\times$ 10^6 cells/mL)	Viability at day 14 (%)	IVCC at day 14 ($\times$ 10^6 cells·h/mL)	mAb concentration at day 14 (g/L)	μ (1/h)*	ρ (pg/cell/day) **
	Deoxyuridine	Thymidine	Deoxycytidine						
Control	-	-	-	15.7	72.9	3417.0	5.1	0.026±0.004	46.2±6.8
dU	25 mg/L	-	-	21.7	73.1	4900.3	7.0	0.027±0.004	43.1±5.9
dT	-	25 mg/L	-	20.1	78.5	4481.7	6.8	0.028±0.004	46.7±6.8
dC	-	-	25 mg/L	16.3	74.8	3409.7	5.2	0.026±0.006	47.0±7.4
dU-dT	25 mg/L	25 mg/L	-	20.5	69.4	4741.2	6.9	0.027±0.004	45.3±5.7
dU-dC	25 mg/L	-	25 mg/L	22.1	77.1	4920.8	7.2	0.028±0.005	44.8±7.2
dT-dC	-	25 mg/L	25 mg/L	21.5	75.0	4527.2	6.3	0.029±0.004	42.4±6.2
dU-dT-dC	25 mg/L	25 mg/L	25 mg/L	23.5	86.3	5638.3	8.2	0.028±0.007	44.0±7.7

The specific growth rate μ was calculated from the data in Figure 5, Figure 6, and Figure 7 in the exponential phase

The specific production rate ρ was calculated from the data in Figure 5, Figure 6, and Figure 7 in the whole process time

*With standard error
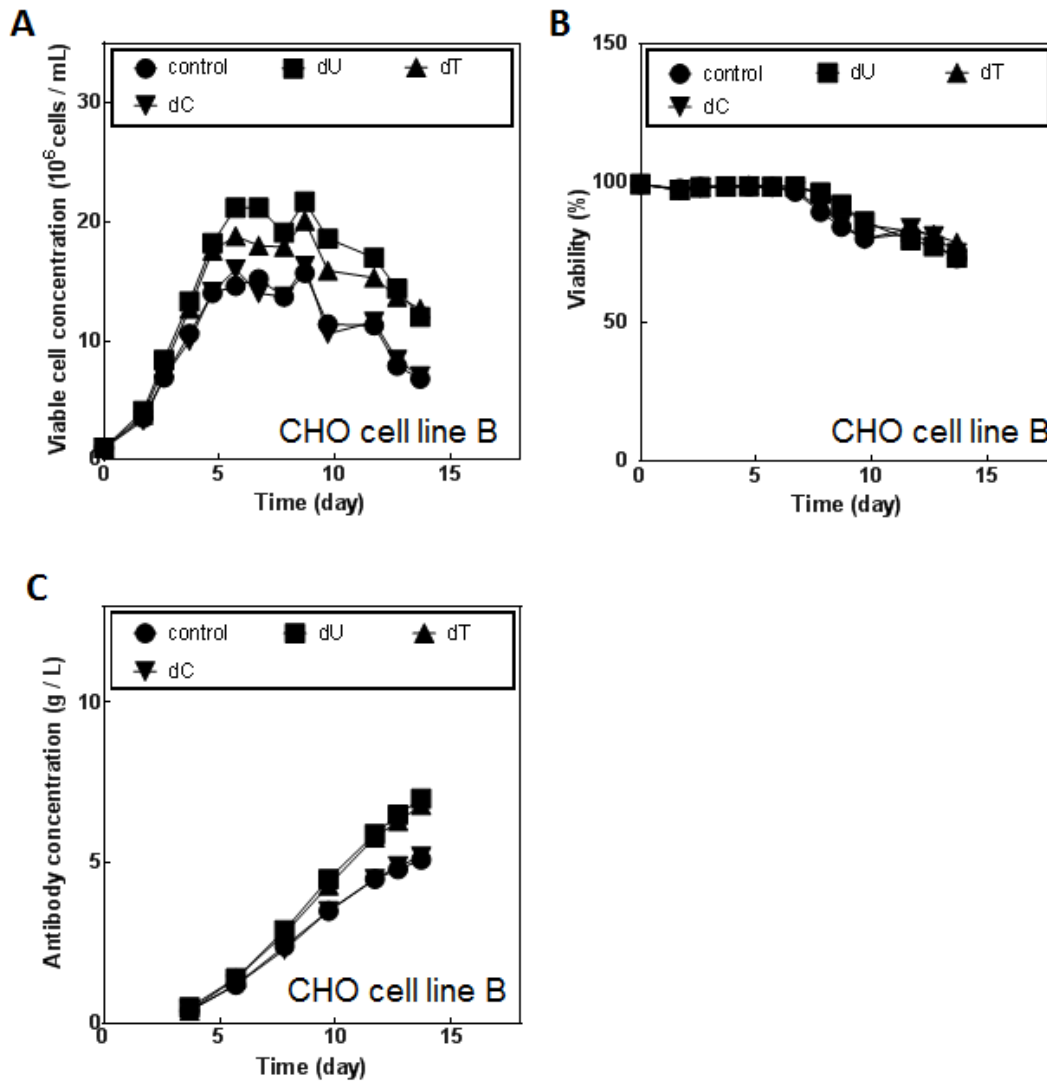
**With 95% confidence interval

Figure 5 Effects of addition of different single nucleosides on cell growth, cell viability, and mAb concentration in CHO cell line B cultures. (A) Viable cell concentration. (B) Cell viability. (C) mAb concentration. Control cultures were performed without nucleoside addition. Each nucleoside was added at the concentration of 25 mg/L at the initial time of culture. dU, dT, and dC indicate deoxyuridine, thymidine, and deoxycytidine, respectively

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As shown in Table 2 and Figure 5, no significant differences in the specific growth rate were observed between the single nucleoside addition cultures (dU, dT, and dC) and the control culture. However, deoxyuridine or thymidine addition increased the peak VCC and final product concentration. After addition of 25 mg/L deoxyuridine, the peak VCC (21.7×10^6 cells/mL) was 38% higher than that in the control culture (15.7×10^6 cells/mL). Moreover, after addition of 25 mg/L thymidine, the peak VCC (20.1×10^6 cells/mL) was 28% higher than that in the control culture (15.7×10^6 cells/mL). Slight increases in viability were observed from day 6 to day 10 in the deoxyuridine and thymidine addition cultures. As a result of the increases in the peak VCC and viability, the final product concentrations were increased in the deoxyuridine and thymidine addition cultures. The final mAb concentration in the deoxyuridine addition culture (7.0 g/L) was 37% higher than that in the control culture (5.1 g/L). Similarly, the final mAb concentration in the thymidine addition culture (6.8 g/L) was 33% higher than that in the control culture. As increases in the specific production rate were not observed in these conditions (Table 2), the increases in the final product concentration seemed to be mainly caused by the increases in the IVCC.
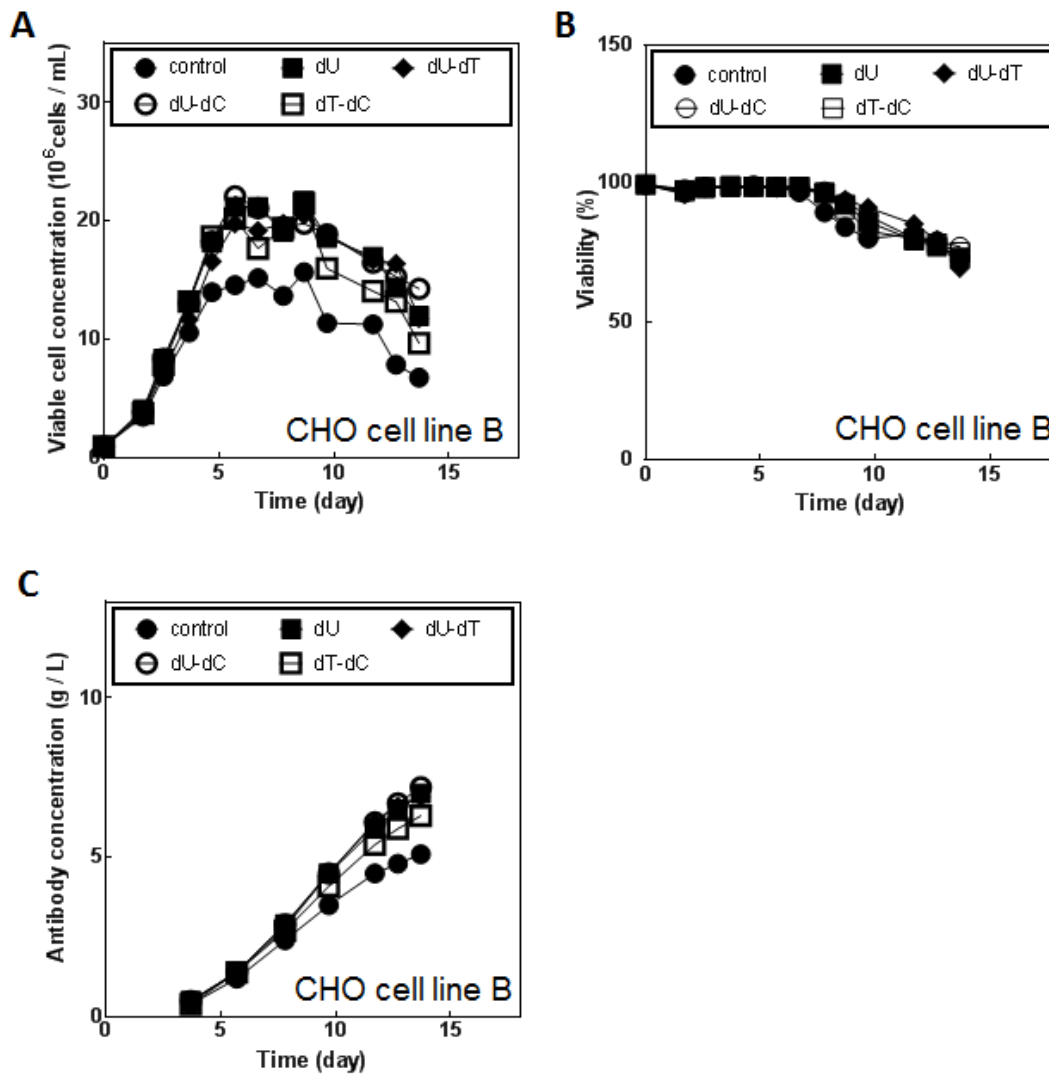
Figure 6 Effects of addition of two combined nucleosides on cell growth, cell viability, and mAb concentration in CHO cell line B cultures. (A) Viable cell concentration. (B) Cell viability. (C) mAb concentration. Control cultures were performed without nucleoside addition. Each nucleoside was added at the concentration of 25 mg/L at the initial time of culture. dU, dT, and dC indicate deoxyuridine, thymidine, and deoxycytidine, respectively

As shown in Table 2 and Figure 6, no significant differences in the specific growth rate were observed among the cultures with combined addition of two nucleosides (dU-dT, dU-dC, and dT-dC) and the deoxyuridine addition culture (dU). Moreover, none of the two combined nucleoside cultures showed higher peak VCC, viability, specific production rate, and mAb concentration than the deoxyuridine addition culture. Nevertheless, all two combined nucleoside cultures showed a higher peak VCC and mAb concentration than the control culture. Hence, these results showed no synergistic effects among the cultures with two combined nucleosides on the cell growth, viability, and mAb concentration.
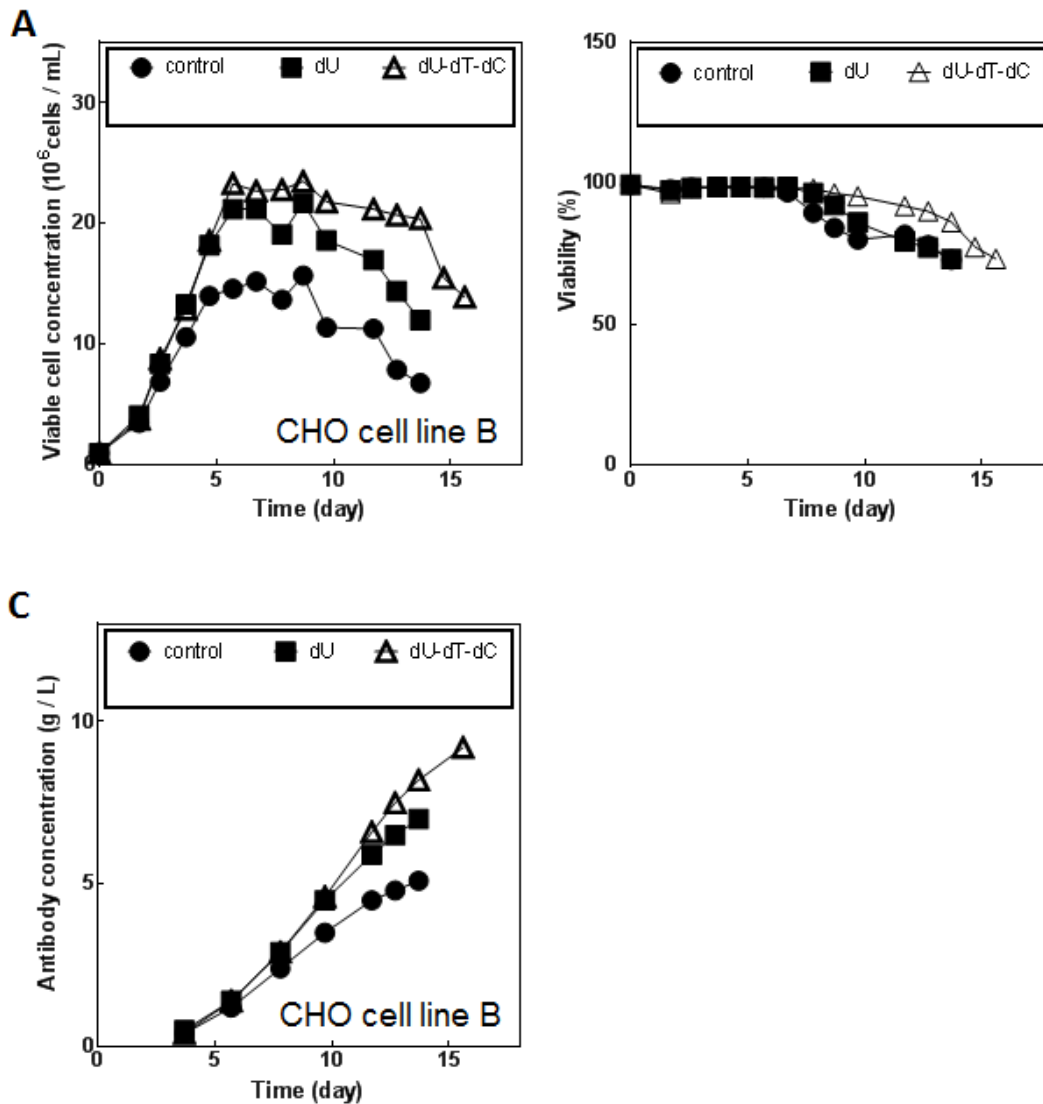
Figure 7 Effects of addition of three combined nucleosides on cell growth, cell viability, and mAb concentration in CHO cell line B cultures. (A) Viable cell concentration. (B) Cell viability. (C) mAb concentration. Control cultures were performed without nucleoside addition. Each nucleoside was added with 25 mg/L at the initial time of culture. dU, dT, and dC indicate deoxyuridine, thymidine, and deoxycytidine, respectively

As shown in Table 2 and Figure 7, the three combined nucleoside culture (dU-dT-dC) showed higher viability after day 8 compared with the deoxyuridine addition culture (dU). The viability at day 14 was 86.3% in the three combined nucleoside culture, while that in the deoxyuridine addition culture was 73.1%. The specific growth rates were similar between these two cultures. In addition, the peak VCC was slightly higher in the three combined nucleoside culture. Consequently, the IVCC was higher than that in the deoxyuridine addition culture. There was no significant difference in the specific production rate between these two cultures. As a result of the IVCC increase, the final product concentration (8.2 g/L) was increased by 17% compared with that in the single deoxyuridine addition culture (7.0 g/L). Because of the increased viability at day 14, the three combined nucleoside culture achieved a mAb concentration of 9.2 g/L at day 16. Thus, we were able to attain high mAb production with this nucleoside addition strategy using the GS-CHO system. These observations support the notion that synergistic effects could exist among these three combined nucleosides for the cell growth and mAb concentration. Overall, the mAb concentration at the final culture point (9.2 g/L) was dramatically increased by 80% compared with that in the control culture (5.1 g/L) following the addition of the three nucleosides.

Thymidine was previously reported to have an enhancement effect on cell growth (Chen et al. 2012b). However, this is the first report to identify that deoxyuridine has impacts on the cell growth and final product concentration, and that deoxyuridine, thymidine, and deoxycytidine have synergistic effects on the cell growth and product concentration. The observed effects suggested that the pyrimidine salvage pathway could be one of the key metabolic pathways for improvement of mAb production. The strategy of exogenous nucleoside addition might have advantages for cell growth by supplying precursors of nucleic acids via the salvage pathway. The reason why synergistic effects were only observed for the combination of deoxyuridine, thymidine, and deoxycytidine remains unclear. Further investigations are necessary to understand the mechanism underlying these effects.

3.3.2. Verification of nucleoside addition effects using a Fab fragment-expressing CHO cell line

The fed-batch cultures were carried out at an initial cell concentration of 1.0×10^6 cells/mL. Control cultures were performed without nucleoside addition. To investigate the effects of the nucleosides, two types of fed-batch culture with 25 mg/L deoxyuridine addition and with deoxyuridine, thymidine, and deoxycytidine addition at 25 mg/L each were performed. Nucleosides were added at the initial time of culture. Fed-batch cultures in shaking flasks were carried out for 14 days. The results are summarized in Figure 8.
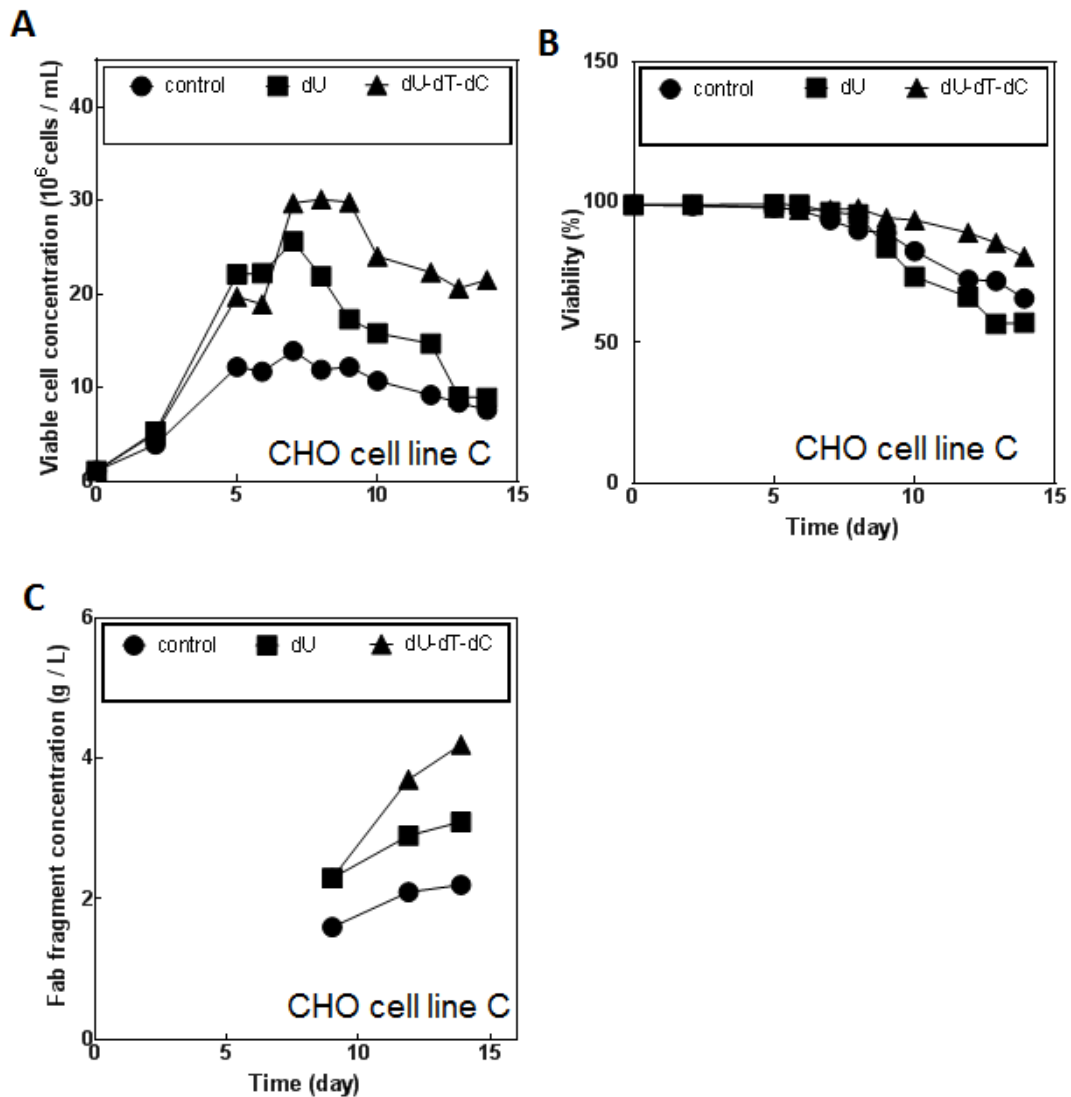
Figure 8 Effects of addition of three combined nucleosides on cell growth, cell viability, and mAb concentration in CHO cell line C cultures. (A) Viable cell concentration. (B) Cell viability. (C) Fab fragment concentration. Control cultures were performed without nucleoside addition. Each nucleoside was added with 25 mg/L at the initial time of culture. dU, dT, and dC indicate deoxyuridine, thymidine, and deoxycytidine, respectively

As shown in Figure 8A and Figure 8C, 25 mg/L deoxyuridine addition increased the peak VCC and also increased the final product concentration. In the deoxyuridine addition culture, the peak VCC (25.6×10^6 cells/mL) was 84% higher than that in the control culture (13.9×10^6 cells/mL). However, in the deoxyuridine addition culture, a gradual decrease in viability was observed after day 6 (Figure 8B). As a result of the increase in the peak VCC, the final product concentration was increased in the deoxyuridine addition culture. The Fab fragment concentration in the deoxyuridine addition culture (3.1 g/L) was 40% higher than that in the control culture (2.2 g/L). Moreover, the three combined nucleoside culture showed an 18% higher peak VCC (30.1×10^6 cells/mL) than that in the single deoxyuridine addition culture (25.6×10^6 cells/mL). Interestingly, the viability after day 8 remained higher compared with that in the single deoxyuridine addition culture. Finally, the viability at day 14 in the three combined nucleoside culture was 80.6%, while that in the deoxyuridine addition culture was 57.1%. As a result of the peak VCC and viability increases, the final product concentration (4.2 g/L) was increased by 35% compared with that in the control culture (3.1 g/L). The synergistic effects of the three nucleosides were also observed in these results. Overall, the enhancement effect of deoxyuridine addition on CHO cell line C growth

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was confirmed, and the final Fab fragment concentration was increased in CHO cell line C cultures. Furthermore, synergistic effects of deoxyuridine, thymidine, and deoxycytidine were also observed. These results suggest that the effects of deoxyuridine, thymidine, and deoxycytidine addition can form a general strategy for increasing product concentrations among recombinant CHO cell lines. Moreover, the effects of three combined nucleoside addition were also confirmed in CHO cell line A culture. The three combined nucleoside culture showed a 27 % higher peak VCC (26.4×10^6 cells/mL) than that in the single deoxyuridine addition culture (20.7×10^6 cells/mL) and 124 % higher peak VCC than that in the control culture (11.8×10^6 cells/mL). The viability after day8 was also higher than that in the single deoxyuridine addition culture. As a result of the IVCC increase, the final product concentration (5.9 g/L) was increased by 28 % compared with that in the single deoxyuridine addition culture (4.6 g/L) and increased by 99 % compared with that in the control culture (2.9 g/L) (data not shown). The significant increase in cell growth and product concentration were observed in CHO cell line A, CHO cell line B and CHO cell line C from independent experiments.

3.4.Summary

We investigated the effects of combined addition of deoxyuridine, thymidine, and deoxycytidine to fed-batch cultures of antibody-expressing CHO cell line. Our results suggest that addition of these pyrimidine nucleosides can increase CHO cell growth, with no significant change in the specific production rate. As a result of increased cell growth, the antibody concentration was elevated and we were able to achieve more than 9 g/L during 16 days of culture. Similar effects of nucleoside addition were observed in fed-batch cultures of a Fab fragment-expressing CHO cell line and the final Fab fragment concentration was more than 4 g/L. This nucleoside addition strategy could be a beneficial platform technology for antibody-based therapeutic protein production.

Chapter 4 Isolation and function analysis of regulatory

motifs in CHO genome sequence

4.1. Introduction

With the recent dramatic increase in the demand for therapeutic proteins, establishment of efficient production systems, including expression, cultivation, and purification, is required to increase manufacturing capacity. To meet this expectation, cell engineering, culture medium modification, scale-up/down, and process development have been carried out in CHO cell manufacturing systems (Omasa 2002; Omasa et al. 2008). Among these approaches, an insulator is fairly effective for protection from surrounding DNA influences. An insulator is a regulatory element that has enhancer-blocking activity that interferes with enhancer-promoter communication when interposed between them. In addition, some insulators can serve as boundary elements between transcriptionally active chromatin and heterochromatin (Maksimenko et al. 2015). Many insulators have been identified in various species, but the most well-characterized element is chicken β-globin $5'$ hypersensitive site 4 (cHS4), a potent insulator that has been shown to combine both enhancer-blocking and barrier activities (Majocchi et al. 2014). Recently, the cHS4 insulator was used for GOI expression in the CHO-K1 cell line. However, cHS4 has poor efficacy in the CHO-K1 cell line (Izumi and Gilbert 1999; Pikaart et al. 1998). It has

been suggested that this inefficacy might be due to the origin of the insulator sequence.

In our previous study, we established numerous *Dhfr* gene-amplified CHO cell lines from the CHO DG44 cell line by methotrexate (MTX) selection and clarified the relationships between the productivity and stability of gene-amplified cells and the location of the amplified gene (Yoshikawa et al. 2000b). We established the stable CHO DR1000L-4N cell line containing more than 160 copies of mouse *Dhfr* and the human granulocyte-macrophage colony stimulating factor gene (Yoshikawa et al. 2000a). It was hypothesized that the chromosomal location of the exogenous gene-amplified region in the CHO DR1000L-4N genome was closely related to the stable productivity and high ability to maintain amplified genes (Omasa et al. 2009). For further investigation of the genomic sequence derived from this region, we established a CHO genomic BAC library from CHO DR1000L-4N cells, which was estimated to cover five times the CHO genome size (Omasa et al. 2009). To obtain the genomic sequence of this specific chromosomal region, BAC clone Cg0031N14 was selected for detailed sequencing analysis, which contained the exogenous gene-amplified region. We have also reported the complete

nucleotide sequence of the insert in the Cg0031N14 BAC clone by shotgun sequencing (Park et al. 2010).

In this chapter, we performed functional analysis of the genomic sequence derived from BAC clone Cg0031N14. It was hypothesized that the chromosomal location of the exogenous gene-amplified region in the CHO DR1000L-4N genome contains regulatory motifs for stable protein production. Therefore, we isolated DNA regulatory motifs from the CHO DR1000L-4N cell line and determined whether these motifs act as an insulator.

4.2. Materials & methods

4.2.1. Regulatory motif analysis

The CCCTC-binding factor (CTCF) binding site database (CTCFBSDB) is a comprehensive collection of experimentally determined and computationally predicted CTC-binding sites (CTCFBSs) from the literature (Bao et al. 2008). The database can be accessed via http://insulatordb.uthsc.edu/ (formerly http://insulatordb.utmem.edu/). Most CTCFBSs were found to share a 20 bp motif (Kim et al. 2007). Recent studies have identified core motifs for CTCFBS sequences, and these motifs are represented by a positional weight matrix (PWM) (Xie et al. 2007). The PWM score corresponds to the log-odds of the observed sequence being generated by the motif versus being generated by the background (Bao et al. 2008). A large positive score of PWM suggests a good match for the CTCFBS, and in this study, we selected a short sequence with a PWM score of >3.0 as a suggestive match for a CTCFBS.

4.2.2. CHO genome sequence from the *Dhfr*-amplified cell line

The complete nucleotide sequence of the insert in the Cg0031N14 BAC clone was determined by shotgun library sequencing (Omasa et al. 2009). In brief, to construct a shotgun library of a BAC clone, the BAC clone DNA was sheared mechanically. The resulting fragments were ligated into pUC118. The DNA fragments inserted into pUC118 were amplified by polymerase chain reaction (PCR) using the M13 primer. The PCR fragments were used as template DNA for further sequence analysis. The obtained sequence data were processed with the Phred/Phrap/Consed package (http://www.phrap.com/) of base-calling, sequencing assembly, and finishing editing software (Ewing and Green 1998; Ewing et al. 1998; Gordon et al. 1998). To fill in approximately 10 gaps at the end of the shotgun phase, primer walking on the BAC and shotgun DNA was carried out. In this study, these shotgun library clones were used for evaluation of vector construction.

4.2.3. Construction of expression vectors containing regulatory motif sequences

To evaluate the efficacy of the predicted insulator sequences, an expression plasmid vector, pBS-cytomegalovirus (CMV)-SNAP26m, was constructed according to the scheme shown in Figure 9. In brief, the pELuc-CMV plasmid was constructed by introducing a CMV promoter into the Emerald Luc (ELuc) vector, pELuc-test, and the ELuc gene was digested out of the pELuc-CMV plasmid by EcoRI and NotI. The SNAP26m gene was amplified by PCR (primer atcgaattcaccatggacaaagactg and ctatgcggccgctcatggcgcgcctatacc) from the SNAPm expression plasmid pSNAPm (Covalys Biosciences AG) and ligated to the pELuc-CMV plasmid. The pSNAP26m-CMV plasmid was constructed from the SNAP26m gene amplified by PCR (primer ccggatccgatgtacgggccagatatacgcgttg and ctggagctcataccacatttgtagaggttttacttg) using the SNAPm expression plasmid pSNAPm (Covalys Biosciences AG) and pELuc-CMV plasmid. The CMV promoter/SNAP26m/SV40 polyA cassette in the pSNAP26m-CMV plasmid was amplified by PCR and introduced into the pBluescript II SK (-) plasmid (Agilent Technologies, Santa Clara, CA) digested by SacI and BamH1. As a result of SNAP26m cassette ligation into the pBluescript II

SK (-) plasmid, the pBS-CMV-SNAP26m vector was established. SNAP26m is SNAP-tag variant protein, and it is possible to detect SNAP26m expression using the photo-stable green fluorescent substrate SNAP-Cell-505.
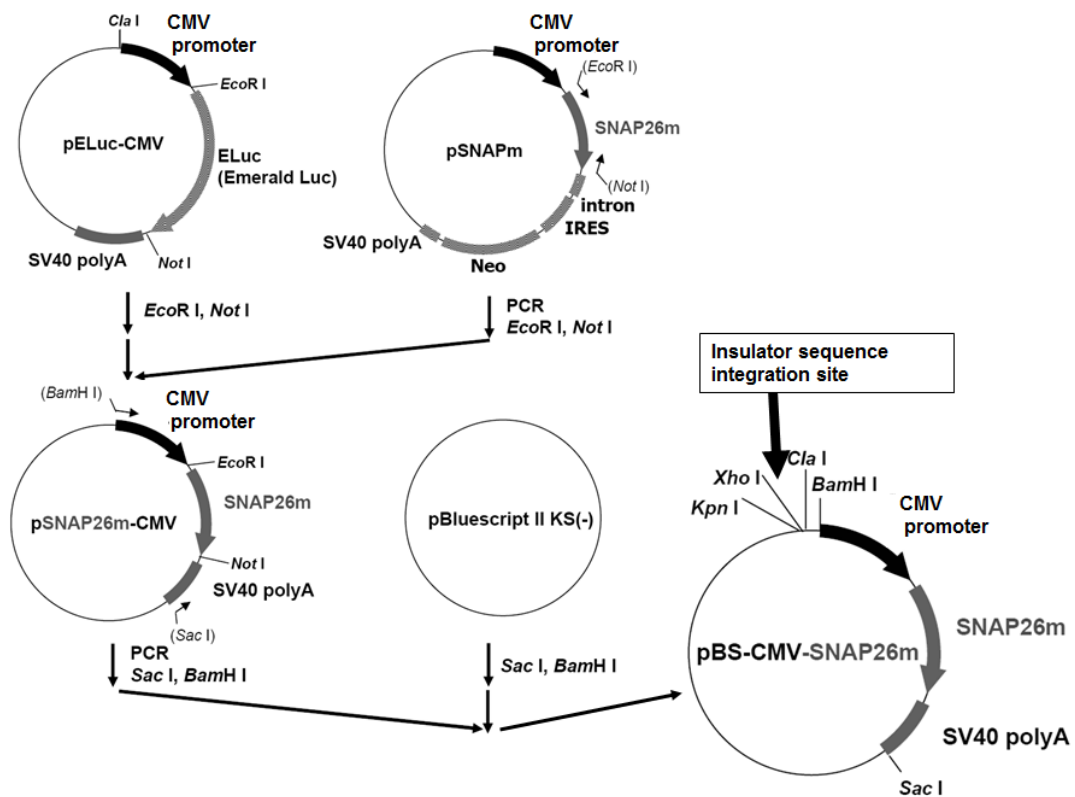

Figure 9 Construction procedure for the expression vector pBS-CMV-SNAP26m.

4.2.4. Transfection

CHO-K1 (derived from RIKEN BioResource Center cell bank, RCB0285) cells (2×10^5) were seeded into each well of a 12-well plate and cultivated for 24 h in Ham's F12 medium (Nissui Pharmaceutical, Tokyo Japan) supplemented with 10% fetal bovine serum (FBS). The constructed SNAP26m (SNAP-tag variant protein) expression vector and pPUR (Clontech, Palo Alto, CA) were linearized by digestion with AhdI (New England Biolabs, Ipswich, MA) and mixed at a ratio of 9:1. The mixed vectors (1 μg) were transfected into CHO-K1 cells using GeneJuice Transfection Reagent (Merck Millipore, Darmstadt, Germany) according to the manufacturer's instructions. After 24 h of transfection, the cells were detached with Trypsin-EDTA Solution (Nacalai Tesque, Kyoto, Japan) and transferred into 90-mm dishes. The culture medium was replaced with Ham's F12 medium supplemented with 10% FBS and 6 μg/mL puromycin (InvivoGen, San Diego, CA) for transfectant selection. The selection medium was changed every 3–4 days until puromycin-resistant clones were obtained at 3 weeks. The obtained puromycin-resistant cell pools were analyzed for further evaluation.

4.2.5. Flow cytometric analysis

Puromycin-resistant cells (2×10^5) were seeded into each well of a 12-well plate and cultivated for 24 h. The medium was changed to 0.5 mL/well Ham's F12 medium containing 2 μL SNAP-Cell-505 (Covalys Biosciences AG, Witterswil, Switzerland), followed by incubation for 60 min at 37°C. The medium was removed, and the cells were washed three times with Ham's F12 medium to remove unreacted fluorescent dye.

The cells were detached with Trypsin-EDTA Solution, and the collected cells were resuspended in D-PBS(-) (Nacalai Tesque). Flow cytometric analysis was performed using a FACSCalibur flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ).

4.3. Results & discussion

4.3.1. Prediction of regulatory motifs in the CHO genome sequence and construction of expressing vector

It was estimated that the chromosomal location of the integrated plasmid DNA in the CHO DR1000L-4N cell line was close to the telomere region based on our previous study (Yoshikawa et al. 2000b). Even though the telomere region on a chromosome is typically enriched with heterochromatin (Blasco 2007; Gonzalo et al. 2006), the DR1000L-4N cell line maintained high productivity throughout long-term cultivation in a previous study (Yoshikawa et al. 2000b). In addition, it is known that the telomere region has positional effects that influence the neighboring gene expression levels (Perrod and Gasser 2003; Wakimoto 1998). CHO DR1000L-4N cells, which were resistant to 1 μM MTX, had over 100 copies of the amplified gene in the genome and maintained high productivity during more than 50 days of cultivation without MTX. Another 1 μM MTX-resistant CHO cell line did not have a high copy number and could not maintain productivity without MTX. It was hypothesized that there were insulator elements near the transgene-integrated site in the CHO DR1000L-4N genome, which stabilize transgene expression.

Insulators are DNA sequence elements with enhancer-blocking and chromatin-bordering functions (Bell et al. 2001; Scott et al. 2006; West et al. 2002). CTCF is the only identified trans-acting factor that binds to insulators in vertebrates (Kim et al. 2007). CTCF is a general transcription regulator that is conserved in the genome from fruit flies to humans (Moon et al. 2005). CTCFBSDB enables identification of insulator candidates in determined genome sequences (Bao et al. 2008; Herold et al. 2012; Xie et al. 2007). To identify the insulator candidate in the CHO cell genome, 168 kb sequence data of BAC clone Cg0031N14 was applied to CTCFBSDB. As a result of CTCF-binding motif retrieval, four candidates were obtained from the sequence data of BAC clone Cg0031N14 via CTCFBSDB (Table 3) (Figure 10).

Table 3 Candidate motif sequences predicted by CTCFBSDB.

| ID	Motif sequence	Initial position	Size (bp)	Direction	PWM score
REN_20	TCCACCACTAGGGG GCGCGC	41821	20	-	20.8898
MIT_LM2	TAGCCAGAAGAGG GCATCA	45182	19	+	19.3604
MIT_LM7	CATCCAGCAGAGGG AGATGG	91094	20	+	17.6996
MIT_LM23	CCACCACTAGGGGG CGCGCT	41820	20	-	15.4348

Initial position means starting sequence number of motif sequence in Cg0031N14 BAC clone sequence (DDBJ accession number, AB370295)

Direction +, - means forward and reverse direction from initial number, respectively.

PWM (positional weight matrix) score was obtained from CTCFBSDB (Bao et al. 2008).
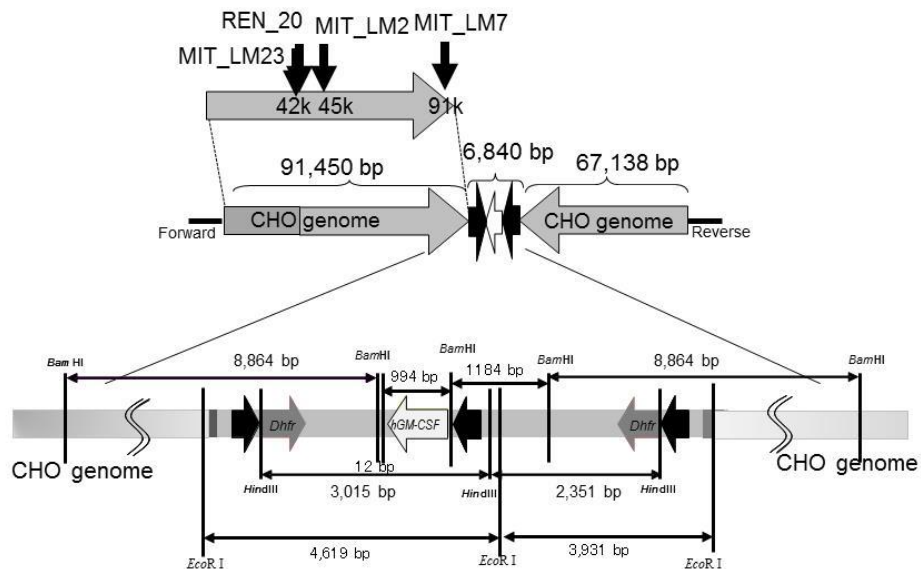
Figure 10 Structure of BAC clone Cg0031N14 and the position of the predicted CTCF-binding site motif.

Among four candidates, REN_20 and MIT_LM23 showed almost the same position. Assuming that the CHO genome size was about 3 Gb and the number of genes in the genome was about $2–10\times10^4$, the number of genes in the non-redundant Cg0031N14 sequence (90 kb) was estimated to be 0.6–3. Furthermore, considering the number of CTCF-binding sites in the human genome is estimated to be 1×10^4 by ChIp-chip analysis (Herold et al. 2012), about 0.4 CTCF-binding sites may exist in the 90 kb sequence. Consequently, it seems that three retrieval candidates, REN_20, MIT_LM2, and MIT_LM7 motifs are predicted insulator sequences.

To evaluate the insulator effect of the predicted insulator sequences, we selected REN_20 (position 41820) and MIT_LM2 (position 45182) motifs for further study among three retrieval candidates, REN_20, MIT_LM2, and MIT_LM7 motifs. It seems that MIT_LM7 motif is potential candidate motif but it is located near central inverted region (Figure 10), we did not select it for further study. Furthermore, it is not sure that only CTCF-binding site is effective or neighborhood area is also necessary for stabilizing effect. Thus, to introduce the CHO genome sequence containing the selected motifs into the integration site between KpnI and XhoI or XhoI and ClaI in the pBS-CMV-SNAP26m vector, we selected suitable five shotgun clones from Cg0031N14, which contained the candidate motifs.

These shotgun clones (insert size is about 5,000bp) contains CTCF-binding site and long neighbor CHO genomic region.

The five selected shotgun clones containing 41820 and/or 45182 locations are shown in Figure 11. Six insert sequences from the selected shotgun clones were amplified by PCR and introduced into the pBS-CMV-SNAP26m vector. Finally, we obtained six expression constructs, CHO1–6 (Table 4). CHO4 and CHO5 had the same expression constructs and were duplicates.
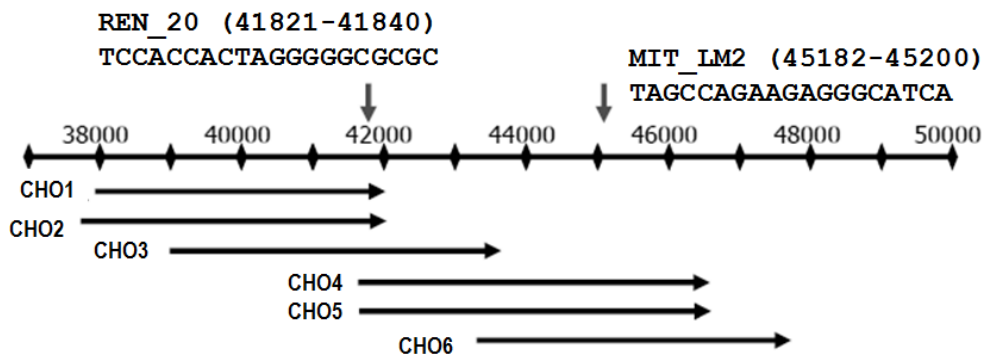

Figure 11 Position map of constructs CHO1–6 derived from the CHO genome on BAC clone Cg0031N14. CHO 4 and 5 are the same construct. Number means sequence number in Cg0031N14 BAC clone sequence (DDBJ accession number, AB370295).

Table 4 SNAP26m-expressing constructs.

Shotgun clone No.	Expression construct ID	Initial position	End position
1L24	CHO1	37738	42048
2O11	CHO2	37934	42043
2P05	CHO3	38934	43490
2B15	CHO4,5	41601	46746
1A02	CHO6	43232	47601

Initial position means starting sequence number of motif sequence in Cg0031N14 BAC clone sequence (DDBJ accession number, AB370295). End position is also end sequence number of this BAC clone sequence.

4.3.2. Confirmation of the stabilizing effect on transgene expression

The SNAP26m expression vector containing candidate motifs (CHO1–6 constructs) was transfected into CHO-K1 cells to confirm the stabilizing effect on transgene expression. To evaluate the transgene expression level, we conducted flow cytometric analysis. A histogram of fluorescence intensity in transfected CHO clones is shown in Figure 12. The fluorescence intensity of each construct was distributed but gradually shifted to high expression compared with the negative control. To analyze in detail the tendency of expression, we analyzed the ratio of higher expression clones for each construct. Based on the M2 gate in Figure 12, we evaluated the ratio of SNAP26m-positive cells. Figure 13 shows the percentage of SNAP26m-positive cells. Compared with the results of the mock vector (-) (negative control), cells transfected CHO1 or CHO2 constructs showed a low percentage of the expressing population. A significant increase in transgene expression was observed in cells transfected CHO4 or CHO5 constructs. The difference in the results between CHO4 and CHO5 was considered to be caused by experimental variability because they contained the same sequence. The experiments of CHO4 and CHO5 constructs were performed independently. It is estimated

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that the expression vector randomly integrated and transgene copy number were also not the same between CHO4 and CHO5 experiments. However, the significant increase in transgene expression was observed in both constructs experiments. In order to confirm this effect, single cell cloning from constructed hetero cell pool and comparing the productivity of several top clones are necessary for further confirmation. Desirably, duplicated transfection experiments and flow cytometry analysis are preferable in future. As a result, it estimated that CHO4 and CHO5 constructs had stabilizing or enhancing effects on transgene expression, which is considered to be an insulator-like function. The CHO4, CHO5 and CHO6 constructs contains same MIT_LM2 motif but the effect of CHO6 is not the same of CHO4 and CHO5. It is estimated that the combination of the CTCF-binding site and specific neighbor CHO genomic region are important for stabilizing or enhancing effects.
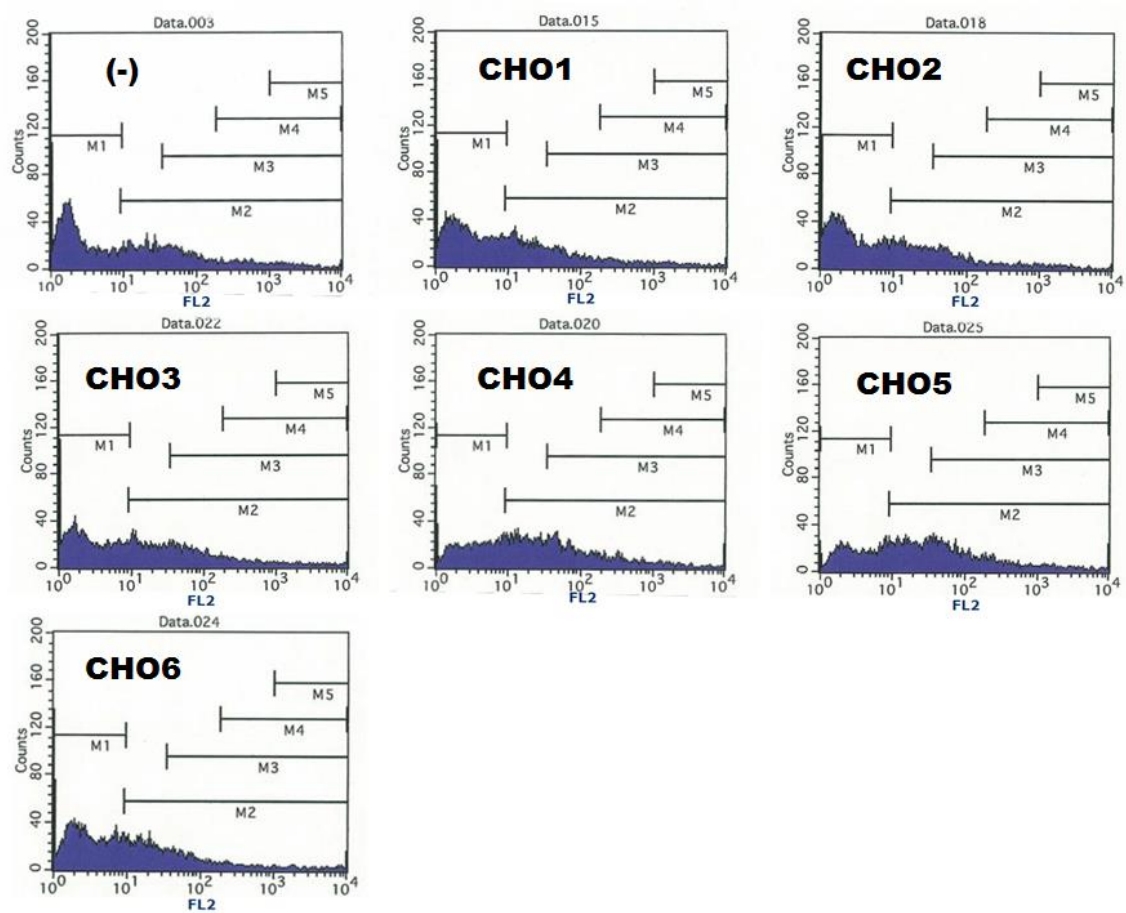
Figure 12 The histogram of fluorescence intensity of transfected cells using flow cytometry analysis. (-): negative control (transfection of mock vector without containing CHO genome), CHO1 to CHO6 corresponded to each construct.
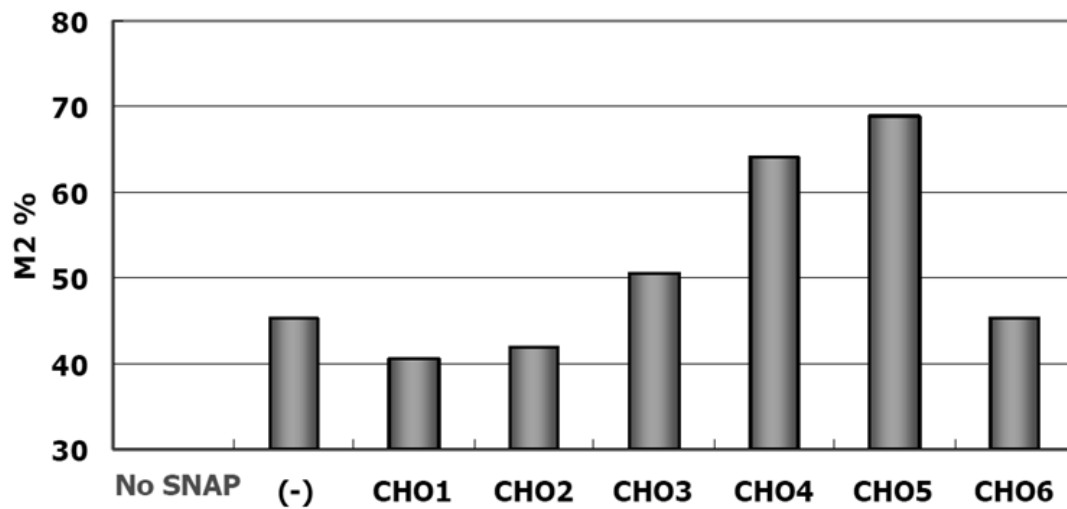
Figure 13 Ratio of positive cells obtained from M2-gated data in flow cytometric analysis.

Based on the evaluation results, we demonstrated that stable transgene expression could be promoted by the CHO genome sequence from Cg0031N14. Telomere and centromere regions are a major part of the heterochromatin. In particular, the telomere region consists of repetitive sequences of TTAGGG, and has a three-dimensional structure (Blasco 2007; Gonzalo et al. 2006). The phenomenon known as the telomere position effect is a well-known reason for decreases in expression of an introduced gene near telomeres (Gonzalo et al. 2006; Wakimoto 1998)). DNA methylation is the main cause of gene silencing in telomere regions. It has been reported that an insulator can protect the DNA methylation-

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dependent repressive effect in telomere regions (Rincon-Arano et al. 2007). Despite the transgene in the DR1000L-4N cell line being near the telomere region, its productivity was maintained for a long time without being affected by inactivation. Therefore, our hypothesis was proved to be correct, stating that the boundary elements, which could block the progression of strong inactivation, existed in the CHO DR1000L-4N telomere region of the CHO cell genome to stabilize gene expression. This insulator sequence could contribute to reducing the time and labor required for cell line development by stable expression of a transgene. In this study, we did not investigate the effect of MIT_LM7 motif on transgene expression. This motif is located quite near inverted region, it seems that this could be another candidate for stabilizing element. Recently, long-term stability is also important factor for cell engineering (Kim et al. 2011). The instability of specific production rate is explained by epigenetic effect and genetic loss of transgene in gene-amplified recombinant CHO cells (Kim et al. 2011). This CHO DR1000L-4N cell shows quite stable transgene expression more than 50 days during maintain cultivation (Yoshikawa et al. 2000a). Long-term stability investigation using this element is necessary for further study.

4.4.Summary

We performed functional analysis of genomic sequence derived from CHO DR1000L-4N cell line, a stable and high copy number *Dhfr* gene-amplified cell line. As a result of CTCF-binding motif retrieval, four candidates were obtained from the sequence data. On the basis of evaluation results, we demonstrated that stable expression of a transgene can be promoted by the genome sequence from CHO DR1000L-4N cell line. We found that regulatory motifs isolated from the recombinant CHO genome sequence can act as an insulator and have positive effect for high and stable transgene expression. We isolated the insulator sequence in the CHO genome sequence, which was expected to have effective activity in a CHO cell expression system.

Chapter 5 CONCLUSIONS

The clinical and commercial success of monoclonal antibodies has led to the need for large-scale production in CHO cell culture. This has resulted in an increased effort to improve process efficiency with minimizing the cost of goods. In this study, we evaluated nucleotides addition to fed-batch production culture to increase the product productivity. Our nucleoside addition strategy is a simple and reproducible methodology that resulted in an increase in the final product concentration. Table 5summarizes the current high productive CHO cell culture processes in the literature. Our study showed a remarkably high product concentration compared with other previous studies. As our new strategy is based on a fed-batch culture system, it is easy to apply common cell culture processes. In particular, among CHO cell culture processes using GS-CHO systems with CD culture media, our data showed a significantly high product concentration. Although the mechanisms of the enhancement are unclear, the nucleoside addition strategy will be a powerful platform for the efficient production of proteins. To elucidate the mechanism underlying the effects of nucleoside addition, further detailed analyses of metabolites will be necessary in future studies.

Table 5 Process-relevant data from different CHO production systems for antibody production

Process type	Culture scale	Host cell / gene expression system	Culture media	Peak VCC ($\times 10^6$ cells/mL)	Final product concentration (g/L)	Reference
Fed-batch	100-L pilot	CHO DG44 / DHFR	With hydrolysate	25.0–30.0	13.0 (day 18)	Huang et al. 2010
Fed-batch	10-L bioreactor	CHO-K1SV / GS	CD	10.0–15.0	3.2 (day 15)	Porter et al. 2010
Fed-batch	2-L bioreactor	DUK-XB11 / DHFR	CD	8.0	7.5 (day 20)	Yu et al. 2011
Perfusion	Not described	CHO DHFR⁻ / not described	With hydrolysate	127	3.0 (day 12)	Clincke et al. 2013
Fed-batch	500-mL shake flask	CHO DG44 / Not described	CD	21.3	5.8 (day 16)	Reinhart et al. 2015
Fed-batch	24-deep well plate	CHO-K1SV GS⁻ / GS*	Not described	Not described	7.6 (day 16)	Rajendra et al. 2016
Fed-batch	125-mL shake flask	CHO-K1SV / GS	CD (nucleoside addition)	23.5	9.2 (day 16)	This study

The final product concentration is shown with the process run time

DHFR: *Dhfr*-gene amplification system; GS: GS Gene Expression System from Lonza Biologics; CD: chemically-defined medium

*This study used the original engineered GS gene expression system derived from the GS-CHO system with the CHO K1SV GS knockout cell line

In a previous report, the IVCC and peak VCC were identified as important factors for predicting final product concentrations (Golabgir et al. 2016). Another report showed enhancement effects of thymidine and hypoxanthine addition on the mAb production process, but their performance was insufficient for current protein production processes (Chen et al. 2012b). Those authors demonstrated elevation of the final product concentration (from 0.18 mg/L to 0.63 mg/L) as a result of increasing the peak VCC from 3.80×10^6 cells/mL to 6.45×10^6 cells/mL. They optimized additional compounds and concentrations in lower peak VCC cultures than the current process shown in Table 5. A metabolomics analysis showed that metabolism varied in CHO cultures with different growth characteristic (Dietmair et al. 2012). Therefore, it is important to investigate the effects of nucleoside addition in the current high VCC culture process. Recently, a continuous bioprocess based on perfusion technology has been dramatically developed for biopharmaceutical production. To attain this highly-effective continuous bioprocess, maintenance of a high cell concentration during perfusion cell culture is necessary. In the high cell concentration process, the requirements for optimal addition of nutrients and nucleosides might be increased. In the

future, our new strategy will be scaled up to bioreactor use, and the quality of the produced proteins will be analyzed.

As described in this report, we were able to attain high mAb concentration with combined nucleoside addition strategy using the GS-CHO system. Despite the fact that the GS-CHO system can be very effective for the production of therapeutic proteins, it was reported that some transfectants were unstable with regard to long-term productivity as we have seen in our study (Dorai et al. 2012, Bailey et al. 2012). The potential impact of instability issues can be enormous at every stage of the biotherapeutic development process from cell line development to commercial manufacturing. However, the extensive stability studies using the GS-CHO cell lines are lacking in published literature. The GS Gene Expression System was developed and distributed by Lonza Biologics under license; there are some limitations for modification or reverse engineering. Therefore we used a CHO genome sequence derived from stable and highly productive CHO cell line established in previous study to find out regulatory motifs for stable product production. Although CHO cells are a well-used mammalian host cell line for the production of therapeutic proteins, DNA regulatory elements in CHO cells, which could be used in combination with strong viral promoters, are not as well studied as those in

human cells (Kang et al. 2016). The use of strong viral promoters such as the CMV promoter for cell line development is effective but easy associated with transgene instability due to DNA methylation (Kim et al. 2011). Therefore, various regulatory DNA elements have been identified, which protect the transgene from positional effects and improve its expression levels (Palazzoli et al. 2011). Our CHO genome-derived insulator element is expected to be used to generate stable recombinant CHO cell lines with high transgene expression. Furthermore, it could be a powerful tool for therapeutic protein manufacturing. The technologies developed in this study covers monoclonal antibody manufacturing from stable cell line development to production culture. As a future work, the new-finding genome sequence should be used to generate highly stable expressing CHO cell lines, and established CHO cell lines should be performed fed-batch culture with nucleosides addition condition. It could be beneficial platform technology for recombinant protein production in CHO cell culture system.

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