Identification of regulatory motifs in the CHO genome for stable monoclonal antibody production

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Abstract Chinese hamster ovary (CHO) cell lines are widely used for therapeutic protein production. When a transgene is integrated into the genome of a CHO cell, the expression level is highly dependent on the site of integration because of positional effects such as gene silencing. To overcome negative positional effects and establish stable CHO cell lines with high productivity, several regulatory DNA elements are used in vector construction. Previously, we established the CHO DR1000L-4N cell line, a stable and high copy number *Dhfr* gene-amplified cell line. 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. Our results suggest that stable expression of a transgene can be promoted by the CHO genome sequence, and it would be a powerful tool for therapeutic protein manufacturing.

Keywords Chinese hamster ovary cells · Therapeutic antibody · Insulator · Epigenetics

Introduction

The market for therapeutic proteins such as monoclonal antibodies (mAbs) is ever growing, and there is a large number of developed mAbs in clinical trials (Reichert 2015). 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 because of their proper folding and post-translational modifications (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).

With the recent dramatic increase in the demand for therapeutic proteins, establishment of efficient production systems, including expression, cultivation,

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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 developments, much attention has 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^{TM} 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 (UCOE), 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). 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 bacterial artificial chromosome (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 study, we performed functional analysis of this genomic sequence derived from BAC clone Cg0031N14. This sequence was derived from the

highly stable cell line DR1000L-4N and may contain the effective sequence that can cancel the positional effect from the surrounding DNA. We also found that regulatory motifs isolated from the recombinant CHO genome sequence can act as an insulator and have positive effects for high and stable transgene expression.

Materials and methods

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.

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.

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 Fig. 1. 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 *NorI*. The SNAP26m gene was amplified by PCR (primer atcgaattcaccatggacaaagactg and ctatcgggccgctcatg-gcgcgcctatacc) from the SNAPm expression plasmid pSNAPm (Covalys Biosciences AG, Witterswil, Switzerland) and ligated to the pELuc-CMV plasmid. The pSNAP26m-CMV plasmid was constructed from the SNAP26m gene amplified by PCR (primer ccg-gatccgatgtacgggccagatatacgcgttg and ctggagctcacacattttagaggttttacttg) 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, USA) digested by *SacI* and *BamHI*. 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.

Transfection

CHO-K1 (derived from RIKEN BioResource Center cell bank, Tsukuba, Ibaraki, Japan, RCB0285) cells (2.9 × 10⁵) 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, USA)

Fig. 1 Construction procedure for the expression vector pBS-CMV-SNAP26m

were linearized by digestion with *AhdI* (New England Biolabs, Ipswich, MA, USA) 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, USA) 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.

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), 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, Kyotot, Japan). Flow cytometric analysis was performed using a FACSCalibur flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

Results and discussion

Prediction of regulatory motifs in the CHO genome sequence and construction of expression 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 μ M 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 μ M 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-integration site in the CHO DR1000L-4N genome, which stabilized 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 1; Fig. 2).

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 $2\text{--}10 \times 10^4$, 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 (initial position 41820) and MIT_LM2 (initial 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 (Fig. 2). 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 *Clal* 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 5000 bp) contains CTCF-binding site and long neighbor CHO genomic region.

The five selected shotgun clones containing 41820 and/or 45182 locations are shown in Fig. 3. 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 2). CHO4 and CHO5 had the same expression constructs and were duplicates.

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 Fig. 4. 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.

Table 1 Candidate motif sequences predicted by CTCFBSDB

ID	Motif sequence	Initial position	Size (bp)	Direction	PWM score
REN_20	TCCACCACTAGGGGGCGCGC	41,821	20	—	20.8898
MIT_LM2	TAGCCAGAAGAGGGCATCA	45,182	19	+	19.3604
MIT_LM7	CATCCAGCAGAGGGAGATGG	91,094	20	+	17.6996
MIT_LM23	CCACCACTAGGGGGCGCGCT	41,820	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)

CTCF-binding site motif

BAC clone : Cg0031N14 (168 kb)

Fig. 2 Structure of BAC clone Cg0031N14 and the position of the predicted CTCF-binding site motif

Based on the M2 gate in Fig. 4, we evaluated the ratio of SNAP26m-positive cells. Figure 5 shows the percentage of SNAP26m-positive cells. Compared with the results of the mock vector (—) (negative control), cells transfected with CHO1 or CHO2 constructs showed a low percentage of the expressing population. A significant increase in transgene expression was observed in cells transfected with 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 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

Fig. 3 Position map of constructs CHO1–6 derived from the CHO genome on BAC clone Cg0031N14. CHO4 and 5 are the same construct. Number means sequence number in Cg0031N14 BAC clone sequence (DDBJ accession number, AB370295)

Table 2 SNAP26m-expressing constructs

Shotgun clone no.	Expression construct ID	Initial position	End position
1L24	CHO1	37,738	42,048
2O11	CHO2	37,934	42,043
2P05	CHO3	38,934	43,490
2B15	CHO4,CHO5	41,601	46,746
1A02	CHO6	43,232	47,601

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

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 was estimated that CHO4 and CHO5 constructs had stabilizing or enhancing effects on transgene expression, which was considered to be an insulator-like function. The CHO4, CHO5 and CHO6 constructs contained the same MIT_LM2 motif but the effect of CHO6 was not the same of CHO4 and CHO5. It was estimated that the combination of the CTCF-binding site and specific neighbor CHO genomic region were important for stabilizing or enhancing effects.

Based on the evaluation results, we demonstrated that stable transgene expression could be promoted by the CHO genome sequence from Cg0031N14. We isolated the insulator sequence in the CHO genome sequence, which was expected to have effective activity in a CHO cell expression system. 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-dependent repressive effect in telomere regions (Rincon-Arango 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 proven 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 the 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

Fig. 4 Histograms of fluorescence intensities in transfected cells using flow cytometric analysis. (–): negative control (transfection of a mock vector without an insert); CHO1–6 correspond to each construct

Fig. 5 Ratio of positive cells obtained from M2-gated data in flow cytometric analysis

cells (Kim et al. 2011). This CHO DR1000L-4N cell shows quite stable transgene expression more than 50 days during maintenance cultivation (Yoshikawa

et al. 2000a). Long-term stability investigation using this element is necessary for further study.

The use of strong viral promoters such as the CMV promoter for cell line development is effective but easily 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). 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). 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.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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