A regulatable selective system facilitates isolation of heterologous protein hyper-producing mammalian cells without gene amplification

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Abstract

In this article, we describe a new method that facilitates to isolate mammalian cells inducible hyper-producing heterologous proteins. This method uses the tetracycline-inducible system to express both the selection marker and the heterologous gene, therefore, allows to increase the selection pressure by reducing the transcription of the selection marker gene. Using this method, we were able to isolate recombinant Chinese hamster ovary cells with a high efficiency. One of established clones produced the recombinant bovine β -lactoglobulin as heterologous protein at a peak rate of $12 \mu\text{g } 10^{-6}$ cells/day with an inducibility of about 100-fold. This clone was over expressed the mRNA of β -lactoglobulin and the drug resistant gene but did not amplify their genes. When cultured in a hollow fiber bioreactor, the cells were able to secrete β -lactoglobulin over $300 \mu\text{g ml}^{-1}$. This method is applicable to a broad range of eukaryotic systems and is of general value to technology for recombinant protein production.

Abbreviations: β -LG, β -lactoglobulin; BS, blasticidin S; CHO, Chinese hamster ovary; CMV, cytomegalovirus; *dhfr*, dihydrofolate reductase; DMEM, Dulbecco's modified Eagle's medium; Dox, doxycycline; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; MEM, minimum essential medium; MTX, methotrexate; rtTA, reverse tetracycline transactivator; tTA, tetracycline transactivator.

Introduction

Since animal cells can produce completely functional and active proteins by post translation modification, the animal cells have been known to be superior to prokaryotic cells for production of mammalian recombinant bioactive proteins. However, the low production rate of animal cells still remains a major obstacle for recombinant protein production in industrial fields. Many approaches have been tested to enhance cellular productivity. The most successful approach is the well-known gene amplification system using a dihydrofolate reductase (*dhfr*) gene on CHO cells (Kemball-Cook et al., 1994). This method utilizes the selection pressure of methotrexate (MTX) to increase the copy number of the *dhfr* gene. However, use of

this method to establish high producing cell lines is a laborious and time-consuming task. Another approach to increase cellular productivity is to improve the efficiency of transcription for each gene copy by utilizing potent promoters (Wang et al., 1984; Takebe et al., 1988; Le et al., 1988), inducible promoters (Friedman et al., 1989) or effective promoter activation (Yano et al., 1994; Teruya et al., 1995; Shirahata et al., 1995).

In this report, we describe a novel approach to improve cellular productivity without gene amplification. This method is based on the assumption that reducing the gene expression of the selection marker should also lead to the increase of selection pressure. There have been two reports attempting to apply stringent selection pressure by restricting the selectable marker level. One is an employment of the weak

promoter (herpes simplex virus thymidine kinase promoter without the polyoma enhancer) to drive the expression of a *neo* gene carrying a mutation reducing the resistance to G418 (Niwa et al., 1991). The other is using a *cis*-DNA sequence to diminish the stability of the *dhfr* mRNA (Miloux et al., 1994). The tetracycline inducible expression system using tetracycline transactivator (tTA) seems ideal for a desirable control of selection pressure (Gossen and Bujard, 1992). Gossen et al. described a modified version of the system using reverse tetracycline transactivator (rtTA) in which the transcription from the tetracycline responsive promoter could be induced over 3–4 orders of magnitude by doxycycline (Dox), which is a tetracycline derivative (Gossen et al., 1995). We used the modified tetracycline inducible system to regulate the expression of blasticidin S (BS) resistant gene, *bsr* (Izumi et al., 1991), and demonstrated that the method efficiently works in CHO cells.

Materials and methods

Vector construction

Plasmids related to tetracycline-responsive expression, pUHD10-3 carrying tetracycline responsive promoter PhCMV*-1 and pUHD172-1neo carrying the rtTA gene under the control of ordinary CMV promoter, were generous gifts from Dr. H. Bujard (Gossen and Bujard, 1992; Gossen et al., 1995). Plasmids pUBsr9 and pTD4 were vectors carrying blasticidin B deaminase (*bsr*) or dihydrofolate reductase (*dhfr*) genes driven by PhCMV*-1 promoter, respectively (Zhang et al., 1997). Briefly, the plasmid pUBsr9 (Figure 1A) was constructed with a *bsr* gene derived from pSV2-*bsr* (Kaken Pharmaceuticals, Tokyo, Japan) was inserted into pUHD10-3. The plasmid pTD4 (Figure 1B) was composed of pUHD10-3 and a mouse *dhfr* gene derived from pSV2-*dhfr* (ATCC, Rockville, MD). To construct the bovine β -lactoglobulin (β -LG) expression vector, a *Sall* fragment containing the β -LG cDNA (a gift from Dr. S. Kaminogawa) was inserted into the *Sall* site of pSBC1 (a gift from Dr. H. Hauser) (Dirks et al., 1993). The *XhoI*-*EcoRI* region upstream of the β -LG in above plasmid was replaced by an *XhoI*-*EcoRI* fragment of PhCMV*-1 promoter from pUHD10-3. The *XhoI*-*HindIII* fragment containing the PhCMV*-1 promoter and the β -LG gene was recovered and inserted into the corresponding sites in pBluescript II KS+ (Stratagene, La Jolla, CA).

The insert digested with *XhoI*-*SpeI* was replaced to the *XhoI*-*XbaI* region in pUHD10-3, and termed pUL5 (Figure 1C).

Cell lines

Chinese hamster ovary cells lacking *dhfr* gene (CHO-D⁻ cells) were cultured in MEM- α medium (GIBCO BRL Gaithersburg, MD) supplemented with hypoxanthine (100 nM), thymidine (16 nM) and 10% fetal bovine serum (FBS; Biowhittaker, Walkersville, MD) (10% FBS/MEM- α HT+).

To establish TA5 cells, CHO-D⁻ cells were transfected with pUBsr9 and pUHD172-1neo by the calcium phosphate co-precipitation method (Chen and Okayama, 1987). The cells were selected with 5 μ g ml⁻¹ of blasticidin S (BS; Kaken Pharmaceuticals) in the presence of 1 μ g ml⁻¹ doxycycline-HCl (Dox; SIGMA, St. Louis, MO), and then transfectants were cloned by the limiting dilution method. Ten clones were randomly chosen to examine whether the *bsr* gene was induced by Dox. The established clone TA5 indicated that the *bsr* expression was indeed in a Dox-dependent manner. To apply stringent selection pressure, TA5 cells were cultured in the medium containing 5 μ g ml⁻¹ of BS and a lower concentration of Dox (1 ng ml⁻¹). Then D1A was cloned under this stringent condition. To isolate DL1 and ER/B cells, TA5 cells were transfected with pUL5 and pTD4. The cells were selected with Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% dialyzed FBS (10% dFBS/DMEM) and 1 μ g ml⁻¹ of Dox. A clone displaying Dox-dependent *dhfr* and β -LG expression was named DL1. To apply the stringent selection pressure, DL1 cells were cultured in 10% FBS/ERDF medium (Kyokuto Pharmaceutical, Tokyo, Japan) which is non-selective to a *dhfr* maker. The medium was supplemented with 5 μ g ml⁻¹ of BS but without Dox. The pool of clones that survived in this medium was named ER/B (Figure 2A).

To establish 16A and 16B cells, CHO-D⁻ cells were transfected with pTD4 and pUHD172-1neo, and cultured in 10% dFBS/DMEM containing 500 μ g ml⁻¹ of G418 (GIBCO BRL) to select *dhfr* positive and G418 resistant cells. A pool of *dhfr* positive and G418 resistant clones were transfected with pUL5 and pUBsr9 in 10% FBS/MEM- α HT+. The transfected cells were selected with 5 μ g ml⁻¹ of BS in the presence of 1 μ g ml⁻¹ Dox followed by limiting dilution cloning. The β -LG producing clones were divided into two equal portions. One was plated into

Figure 1. Plasmids used in the experiments. (A) pUBsr9 was constructed so that a *bsr* gene derived from pSV2-*bsr* was inserted into pUHD10-3. (B) pTD4 was composed of pUHD10-3 and a mouse *dhfr* gene derived from pSV2-*dhfr*. (C) pUL5 was bovine β -lactoglobulin expression plasmid.

10% FBS/MEM- α HT+ containing $5 \mu\text{g ml}^{-1}$ of BS and $1 \mu\text{g ml}^{-1}$ of Dox, and the other in the same medium but without Dox. 16A was a clone secreting high level of β -LG with Dox, and 16B was the counter-part of 16A surviving in the Dox-free medium containing $5 \mu\text{g ml}^{-1}$ of BS (Figure 2B).

Southern and Northern analysis

To isolate genomic DNA, about 5×10^6 cells were suspended with 200 mM EDTA containing 0.5% SDS and $100 \mu\text{g ml}^{-1}$ of Proteinase K (Wako Pure Chemical Industries, Osaka, Japan), then incubated at 50°C for 1 h and at 37°C overnight. The lysates were

Figure 2. Establishment of recombinant CHO cell lines. Shown is a schematic flow of the CHO cell lines employed in this report.

then extracted with phenol, phenol/chloroform and chloroform/isoamyl alcohol. After precipitation with ethanol, the DNA was dissolved in TE containing RNase ($50 \mu\text{g ml}^{-1}$) and incubated at 37°C overnight. For the Southern analysis, the DNA samples were digested with *EcoRI* and *BamHI* overnight then purified with phenol/chloroform and ethanol precipitation. After digested again with *EcoRI* and *BamHI* for 4 h, the DNA concentration was determined with UV spectrophotometer. $0.5 \mu\text{g}$ of each samples were applied onto 0.7% agarose gel electrophoresis. The separated DNAs were transferred to Hybond- N^+ membrane (Amersham Pharmacia Biotech, Buckinghamshire, U.K.) with the capillary method. The hybridization and detection were done using the GENE IMAGE kit (Amersham Pharmacia Biotech) according to the manufacture's recommendation. In the analysis, the probes of rtTA, *bsr* or β -LG genes were a 1.1 kbp of *EcoRI*-*BamHI* fragment from plasmid pUHD15-1 (a gift from Dr. H. Bujard) (Gossen and Bujard, 1992), a 0.49 kbp *EcoRI*-*BamHI* fragment from plasmid pUBsr9 or a

0.6 kbp *SaII* fragment, respectively. The β -LG fragment was also used as a standard to estimate the copy number of the introduced β -LG gene.

Total RNAs were isolated with TRIzol reagents (GIBCO BRL). 5×10^6 cells were lysed with TRIzol reagents, precipitated with 2-propanol after chloroform addition. The pellets were dissolved in $300 \mu\text{l}$ of 4 M LiCl on ice, and then extracted with chloroform/isoamyl alcohol (24:1). The RNAs were then precipitated with ethanol and dissolved in RNase-free water. Ten μg of each RNA samples were applied onto a 1.2% agarose gel electrophoresis. The hybridization and detection were done using the GENE IMAGE kit.

Protein analysis

The β -LG produced from recombinant CHO cells was determined with the enzyme-linked immunosorbent assay (ELISA), which was previously described (Zhang et al., 1997). A pair of anti-bovine β -LG mouse monoclonal antibody was briefly employed. One was 62A6 for the solid-phased antibody and

another was 61C1 with biotinylation for the detection antibody (Totsuka et al., 1990). The β -LG was then detected using streptavidin-horseradish peroxidase conjugate (Amersham Pharmacia Biotech), ABTS diammonium salt (Wako) and H_2O_2 as substrate. Native β -LG purified from bovine milk was used as a standard.

Electrophoresis of the protein samples was carried on a 12% polyacrylamide gel as described (Laemmli, 1970). The total protein was detected by staining it with Coomassie brilliant blue R-250. For Western blotting, the separated proteins were transferred onto the nitrocellulose membrane, and then detected with biotinylated 61C1 antibody, streptavidin-HRP conjugate and ECL detection system (Amersham Pharmacia Biotech).

β -LG production in Tecnomouse bioreactor

The hollow fiber based Tecnomouse bioreactor (Integra Biosciences, Germany) was used to produce β -LG protein as an example for mass-production. High producing clone 16B (total 7.8×10^7 cells) were recovered from culturing in Dox-free medium and suspended in 5 ml of 10% FBS/ERDF medium containing $1 \mu\text{g ml}^{-1}$ of Dox, then inoculated into the incubation cassette with a surface area of about 400 cm^2 . The cells were incubated at 37°C in 5% CO_2 atmosphere. The serum free ERDF medium containing $1 \mu\text{g ml}^{-1}$ of Dox was circulated in a recycling manner. The culture medium was changed in intervals of ten days and spent medium was harvested in intervals of five days to evaluate β -LG production.

Results

Regulatable selection pressure could establish the clone with highly expressing transcripts

We first determined the window of Dox concentration that was capable of applying appropriate selection pressure to CHO cells. The TA5 clone was isolated after co-transfection with pUBsr9 and pUDT172-1neo, which carries an rtTA gene under the control of a constitutive CMV promoter (Gossen et al., 1995). Dox-dependent *bsr* expression in TA5 cells was confirmed by comparing the cell growth in 10% FBS/MEM- α medium (HT+) containing $5 \mu\text{g ml}^{-1}$ of BS (BS-selective medium) supplemented with or without $1 \mu\text{g ml}^{-1}$ of Dox (data not shown). About

300 cells were plated into 35 mm diameter dishes containing BS-selective medium supplemented with $1 \mu\text{g}$, 100 ng , 10 ng , 1 ng and 0 ng ml^{-1} of Dox, respectively. The cells in the presence of $1 \mu\text{g}$ and 100 ng ml^{-1} of Dox indicated resistance to the BS, but no clone appeared in the absence of Dox. Only one clone appeared in the dishes supplemented with 10 and 1 ng ml^{-1} Dox, suggesting that the cells were subjected to a high stringent selection under these conditions. D1A was cloned from the stringent condition from parent TA5 cells treated with 1 ng ml^{-1} of Dox. To evaluate the further inducement by Dox, stringent-selected clone D1A (which was established in the presence of 1 ng ml^{-1} of Dox) was cultured in the presence of $1 \mu\text{g ml}^{-1}$ of Dox. The mRNA level of *bsr* and the co-transfected rtTA gene in TA5 and D1A cells were examined by the Northern hybridization analysis. Figure 3A shows that D1A cells cultured with $1 \mu\text{g ml}^{-1}$ of Dox synthesized significantly higher levels of both transcripts than parent TA5 cells. However, the copy numbers of these genes in both clones were not significantly different (Figure 3B). It was indicated that the higher level of transcripts in D1A cells was not resulting from gene amplification.

High expressing cells for heterologous protein by regulatable selection pressure

As increased rtTA level should be capable of enhancing the transcription from the tetracycline-responsive promoter, over-expression of a heterologous gene might be achieved by introducing the gene under the control of the tetracycline responsive promoter into such cells. To examine this possibility, TA5 cells were co-transfected with pUL5 carrying β -LG gene driven by the tetracycline responsive promoter PhCMV*-1 and pTD4 carrying a *dhfr* gene driven by the same promoter. After selection, a DHFR-positive clone was isolated and named DL1. We first confirmed that the β -LG production in this clone was Dox-dependency by comparing the production rate in the non-selective medium (10% FBS/MEM- α HT+) supplemented with or without Dox (data not shown). DL1 cells were then cultured in 10% FBS/ERDF medium containing $5 \mu\text{g ml}^{-1}$ BS without Dox to apply high stringent selection pressure. A mixture of the clones (named ER/B) that were capable of growing under this condition were pooled and further analyzed. It was indicated that the *bsr* and rtTA expression was significantly higher in ER/B cells than parent DL1 cells (Figure 3C). The expression of the *bsr* gene was further induced by

Figure 3. Comparison of mRNA levels in parent cells with the stringently selected cells (A and C), and the transfected exogenous gene integrated into chromosomal DNA of cells (B and D). (A) Total RNAs were isolated from TA5 and D1A cells cultured in the presence of Dox ($1 \mu\text{g ml}^{-1}$), then the Northern hybridization was performed. (B) Genomic DNA was isolated from TA5 and D1A cells, then the Southern hybridization was performed. (C) Total RNAs were isolated from DL1 and ER/B cells cultured with ($1 \mu\text{g ml}^{-1}$) or without Dox, then the Northern hybridization was performed. (D) Genomic DNAs were isolated from DL1 and ER/B cells, then the Southern hybridization was performed. Details were described in the Materials and Methods Section.

Dox (comparison ER/B with Dox and ER/B without Dox in Figure 3C). In the case of these cells, gene amplification was not also observed (Figure 3D). Although the β -LG expression was enhanced in ER/B cells (Figure 3C), the degree is not as great as we expected.

Establishment of β -LG high producing CHO clone

We employed the selection drug BS as a stringent selection pressure to cells. To regulate effectively selection pressure, the CHO cells were first transfected with pUHD172-1neo and pTD4. A pool of DHFR-positive and G418-resistant cells were then transfected with pUL5 and pUBsr9. Five clones were obtained expressing β -LG with resistant to BS in the presence of $1 \mu\text{g ml}^{-1}$ of Dox. Each of the clones was then

plated equally into two dishes. One dish was supplemented with both BS ($5 \mu\text{g ml}^{-1}$) and Dox ($1 \mu\text{g ml}^{-1}$) and the other with only BS. The cells from the Dox-free indicated a higher production rate than their counter-parts cultured with $1 \mu\text{g ml}^{-1}$ of Dox (Data not shown).

The clone 16A (isolated from culture with $1 \mu\text{g ml}^{-1}$ of Dox) and the clone 16B (isolated from culture without Dox) were further analyzed. 16B cells produced β -LG at a peak rate of $12 \mu\text{g } 10^{-6} \text{ cells day}^{-1}$ when fully induced with $1 \mu\text{g ml}^{-1}$ of Dox. However, in the absence of Dox, they only produced β -LG at a rate about $122 \text{ ng } 10^{-6} \text{ cells day}^{-1}$ (Figure 4A). The nearly 100-fold inducibility indicates that the excellent performance of the tetracycline inducible system is retained after stringent selection. The β -LG production of 16B is several-folds higher than that by 16A (Figure 4A). In an attempt to elucidate the reasons, we compared the copy number of the corresponding genes (β -LG, *bsr* and *rtTA*) in these clones by Southern hybridization. As shown in Figure 4C, no apparent differences were found. These results suggested that gene amplification might not be the main aspect in these experiments. The copy number of β -LG in 16A and 16B is estimated around 30 copies per cell (data not shown). This multi-copy integration might be a result of calcium phosphate transfection.

Large-scale production of heterologous β -LG in Tecnomouse system

We then examined whether the cells isolated with this method could be used in mass production. For this purpose, we used a hollow fiber based bioreactor, Tecnomouse system, to culture 16B cells. The cells that were recovered from the Dox-free medium were inoculated into the culture cassette and induced with $1 \mu\text{g ml}^{-1}$ Dox. The supernatants were harvested at five-day intervals and the β -LG protein was quantified with ELISA. As shown in Figure 5A, 16B cells were able to secrete high levels of β -LG protein reaching to $325 \mu\text{g ml}^{-1}$. This was confirmed by Coomassie staining of the SDS-PAGE gel (Figure 5B) and Western blotting (Figure 5C). It seemed that the amounts of β -LG of by Coomassie staining were larger than the standard one (Figure 5B). However, immunostained signals were indicated the expected levels of the recombinant β -LG (Figure 5C). These results indicate that our method should be of practical value to the technology of recombinant protein production.

Figure 4. β -LG production in parent 16A and the stringently selected 16B cells (A), comparison of mRNA levels in parent cells with the stringently selected cells (B), and the transfected exogenous gene integrated into the chromosomal DNA of cells (C). (A) β -LG production from 16A and 16B cells cultured with ($1 \mu\text{g ml}^{-1}$) or without Dox. The production rate was measured using ELISA. (B) The total RNAs were isolated from 16A and 16B cells cultured with ($1 \mu\text{g ml}^{-1}$) or without Dox, then the Northern hybridization was performed. (C) Genomic DNA was isolated from 16A and 16B cells and then the Southern hybridization was performed.

Discussion

To enhance the cellular productivity of recombinant animal cells, various approaches were developed and investigated. Most popular system for mass-production of recombinant protein in animal cells is gene amplification with *dhfr* gene in CHO-D⁻ cells (Schimke, 1988). However, this procedure is time consuming and laborious and also has several problems. Most of the gene markers for the gene amplification are genetically recessive, this means that effective amplification needs the marker gene deficient host cell line like as *dhfr*-lacking CHO cell line. In addition, single-step high-level MTX selection in *dhfr* gene amplification might result in cells with mutation of MTX resistant DHFR enzyme (Haber et al., 1981) or with altered MTX transport properties (Assaraf and Schimke, 1987).

In this report, we developed a new method facilitating to isolate mammalian cells inducible high producing recombinant protein in animal cells. The

stringent selected clones achieved hyper-production of recombinant protein subsequently the induction of Dox. This new method has some advantages; (1) stringent selection pressure was supplied to transfected cells as reducing the concentration of Dox, which resulted in a decrease the cellular resistance to BS, (2) the use of any genetically dominant gene for drug resistance was available, it meant application for various host cells, (3) this method allowed a single-round high stringent selection, and (4) the powerful inducibility of tetracycline inducible system created a broad window for selection pressure. The commercial tetracycline inducible systems such as Tet-OffTM or Tet-OnTM (CLONETECH) were frequently used to investigate the interesting gene by 'turn-on' or 'turn-off' of the gene expression. We focused the different use of the tetracycline inducible system to enhance the selection pressure in the isolation of the heterologous protein hyper-producing cells.

Figure 5. β -LG production of 16B cells in Tecnomouse system. High producing clone 16B cells (7.8×10^7 cells) were inoculated in the reactor unit of the Tecnomouse system. STD; 1 μ g of purified bovine β -LG in 0.1% BSA/PBS. Tec1; supernatant harvested on Day 5. Tec2; harvested on Day 10. Tec3; harvested on Day 15. (A) Production rate of β -LG measured using ELISA. (B) SDS-polyacrylamide gel electrophoresis stained with Coomassie brilliant blue R-250. Each 10 μ l of Tec1, Tec2 and Tec3 harvested with supernatant samples were subjected to electrophoresis. (C) β -LG protein detected by Western blotting. The blot was corresponding to the electrophoresis as shown in (B).

The established TA5 cells and derivative D1A were determined to enhance the inducible expression of the mRNA levels driven by PhCMV*-1 promoter. The inducibility and usefulness as the parent were confirmed by the Northern hybridization analysis (Figure 3A). Next, it was examined that both of the heterolog-

ous protein production and the stringent selection by decrease the Dox concentration. For practical recombinant protein production, DL1 was obtained by TA5 cells transfected with β -LG and *dhfr* expression vectors. We emphasize that *dhfr* gene was used one of the selective marker gene for *dhfr*-lacking CHO cells. In addition, we did not use MTX as the reagent for the gene amplification. DL1 and stringent selected ER/B were accessed mRNA levels in culture with inducible concentration of Dox. It was confirmed that this condition strongly induced the β -LG and *bsr* expressions in the ER/B cells with the treatment of Dox against the treatment without Dox. However, comparison of DL1 and Dox-induced ER/B cells did not show a great enhancement of the β -LG expression. Then, we tried to further enhance the expression level of β -LG. The CHO cells were transfected with rtTA and *dhfr* expression vectors, and selected by HT⁻ and G418. Survived cells were continuously transfected with β -LG and *bsr* expression vectors, and selected by BS with Dox. After the stringent selection without Dox, established clone 16B showed a high producing rate of the β -LG with induction of Dox. It was considered that the second transfection with *bsr* gene into the pool of inducible host candidates and the stringent selection with BS at the final selection step, were effective to establishing a highly inducible recombinant producing clone.

Selection for recombinant cells with stringent condition might facilitate the isolation of high expression clones, but the excess stringent condition might be difficult in obtaining stable clones. In our new method, varying the concentration of Dox made it possible to choose the appropriate selection pressure for any selection drug such as BS. Since both genes of drug resistance and recombinant protein were driven by PhCMV*-1 promoter, appropriate stringent selection lead to an isolation of the high producing clones for recombinant products. It remains unclear why the cells were able to survive in the stringent selection. All the Southern hybridization analyses showed that gene the amplification of the drug resistant gene did not occurred in spite of the increasing mRNA expression level augmentation (Figures 3 and 4). It was suggested that this new system enhanced the transcription level for the genes driven by PhCMV*-1 promoter without the gene amplification. Since gene amplification requires DNA replication and cell division, optimal amplification occurs when cells are subjected to severe, but not absolute growth-limiting conditions (Kaufman, 1990). The treatment with 5 μ g ml⁻¹ of BS

resulted in the killing of ordinary sensitive CHO cells within five days. This period was significantly shorter than the period required by MTX. Furthermore, the selection pressure was increased by single round step in our system. It was probably too short of a time period and too stringent selective condition to acquire the drug resistance through the gene amplification in the cells. It was reported that the transactivator rtTA showed slightly transcriptional activity even if in the absence of Dox (Zhu et al., 2001). We assume that the unknown transcriptional mechanisms acted on the tightly stringent selection in order to survive.

To demonstrate the availability of our new system, we isolated 16B cells inducible hyper-producing β -LG protein. 16B cells without Dox-induction displayed an ordinary production rate, but nearly 100-fold inducibility by Dox (Figure 4A). The production rate was enhanced into $12 \mu\text{g } 10^{-6} \text{ cells day}^{-1}$ by the induction of Dox. This productivity was higher than conventional expression system using CMV promoter and CHO cells. In the case of the recombinant human interleukin-6 production in the conventional system, the production rate was $5.6 \mu\text{g } 10^{-6} \text{ cells day}^{-1}$ (Katakura et al., 1999). The cells were examined in that culture and recombinant protein production in a high-density cell culture system, Tecnomouse system. The production level of β -LG protein produced over $300 \mu\text{g ml}^{-1}$ in Tecnomouse system, is one of the most successful examples achieved with other methods (Cockett et al., 1990; Friedman et al., 1989).

In summary, we developed a method that allows cells to isolate inducible hyper-producing heterologous proteins. This method is theoretically applicable to any eukaryotic systems and of general value to recombinant protein production technology.

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