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An approach to further enhance the cellular productivity of exogenous protein hyper-producing Chinese hamster ovary (CHO) cells

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Abstract

The cell line D29, which was easily and rapidly established by the promoter-activated production and glutamine synthetase hybrid system, secreted recombinant human interleukin-6 (hIL-6) at a productivity rate of 39.5 μg 10^{-6} cells day^{-1}, one of the highest reported levels worldwide. The productivity rate was about 130-fold higher than that of the cell line A7, which was established without both promoter activation and gene amplification. Although D29 cells had a high copy number and high mRNA level of the hIL-6 gene as well as a high secretion rate of hIL-6, large amounts of intracellular hIL-6 protein accumulated in D29 cells compared to A7 cells. Northern blotting analysis showed no change in the GRP78/BiP expression level in D29 cells. In contrast, an electrophoresis mobility shift assay revealed strong activation of NF-κB in D29 cells. These results suggest that large amounts of hIL-6 translated from large amounts of hIL-6 mRNA cause excess accumulation of intact hIL-6 in the endoplasmic reticulum (ER), and that subsequent negative feedback signals via the ER overload response inhibit hIL-6 protein secretion. To enhance the hIL-6 productivity rate of D29 cells by releasing the negative feedback signals, the effect of pyrrolidinedithiocarbamate, an inhibitor of NF-κB activation, was examined. Suppression of NF-κB activation in D29 cells produced a 25% augmentation of the hIL-6 productivity rate. Therefore, in highly productive cells like D29 cells, the release of negative feedback signals could increase the total amount of recombinant protein secretion.

Abbreviations: CHO – Chinese hamster ovary; dhfr – dihydrofolate reductase; DMEM – Dulbecco's modified Eagle's medium; ECL – enhanced chemiluminescence; ELISA – enzyme-linked immunosorbent assay; EMSA – electrophoretic mobility shift assay; EOR – endoplasmic reticulum overload response; ER – endoplasmic reticulum; FBS – fetal bovine serum; GS – glutamine synthetase; hIL-6 – human interleukin-6; MSX – methionine sulphoximine; MTX – methotrexate; PAP – promoter-activated production; PDTC – pyrrolidinedithiocarbamate; UPR – unfolded protein response

Introduction

Animal cells are known to be superior to prokaryotic cells for the production of recombinant proteins for clinical use, since they can produce completely functional and active proteins by post-translational modification. However, the low production rate of animal cells remains a major obstacle to the mass production of recombinant proteins, and several methods have been developed to enhance the cellular productivity. The most popular technique is a gene amplification system involving the dihydrofolate reductase (*dhfr*) gene in Chinese hamster ovary (CHO) cells (Schimke 1988; Kemball-Cook et al. 1994). This system utilizes the selection pressure of methotrexate (MTX) to increase the copy number of the *dhfr* gene. Another efficient gene amplification system was developed using the glutamine synthetase (GS) gene as a selectable and amplifiable marker, and methionine sulphoximine (MSX) as the selective agent. This system has been used successfully for the overexpression of a number of foreign genes, including a tissue inhibitor of metalloproteinase (Cockett et al. 1990). On the other hand, another approach to increasing 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) or inducible promoters (Friedman et al. 1989).

We previously developed a promoter-activated production (PAP) system to enhance recombinant protein productivity in cultured cells by activating specific promoters via transcriptional activators such as oncogenes (Yano et al. 1994; Teruya et al. 1995; Shirahata et al. 1995). This PAP system was shown to be very effective in CHO cells, in which the peak productivity reached 13.6 μg 10^{-6} cells day^{-1} (Katakura et al. 1999). In addition, we recently reported that a hybrid system using both promoter activation and gene amplification achieved a very high level of production (approximately 40 μg 10^{-6} cells day^{-1}) of a recombinant protein (Dong et al. 2003).

On the other hand, we found that mass production of recombinant proteins in CHO cells by PAP systems may lead to endoplasmic reticulum (ER) signaling with GRP78/BiP upregulation (Miura et al. 2001). Upregulation of GRP78/BiP is a well-known ER signaling pathway. In general, abnormal protein accumulation in the ER leads to upregulation of GRP78/BiP, known as the unfolded protein response (UPR), while an overload of normal proteins in the ER leads to the ER overload response (EOR) (Pahl and Baeuerle 1995; Pahl 1999; Cudna and Dickson 2002). Therefore, the observed GRP78/BiP upregulation is a typical UPR phenotype. In the current report, we investigated the accumulation of recombinant proteins in exogenous protein hyperproducing CHO cells using the PAP and GS hybrid system, as well as an approach to further enhance the recombinant protein productivity of this system.

Materials and methods

Cells and cell culture

The *dhfr*-deficient CHO cell line was maintained in Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceuticals, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; Irvine Scientific, Santa Ana, CA), 100 nM hypoxanthine and 16 nM thymidine. The establishments of *ras* clones I, I13, A7, Y13 and D29 were described previously (Katakura et al. 1999; Dong et al. 2003) and are summarized in Figure 1. Briefly, *ras* clone I was established by transfection of pCMVD-*ras* into the *dhfr*-deficient CHO cells, followed by selection with G418 (1 mg ml^{-1}) and amplification of the transfected *ras* gene with 50 nM MTX. I13, the model cell line of the PAP system, was established by co-transfection of pCMVP-hIL-6 and pSV2-*bsr* into the *ras* clone I cells. The *ras* clone I and I13 cells were maintained in DMEM supplemented with 5% dialyzed FBS and 50 nM MTX. The conventional human interleukin (hIL)-6-producing recombinant CHO cell line A7 was established by transfection of pCMVP-hIL-6 and pSV2-*bsr* into the *dhfr*-deficient CHO cells, and the cells were cultured in DMEM supplemented with 5% FBS. The clones Y13 (GS system) and D29 (PAP and GS hybrid system) were established from the *dhfr*-deficient CHO and *ras* clone I cells by transfection with pEE14-IL6 and pSV2-*bsr*, respectively.

The medium for the GS gene amplification system was composed of glutamine-free DMEM (Bio Whittaker, Walkersville, MD) supplemented

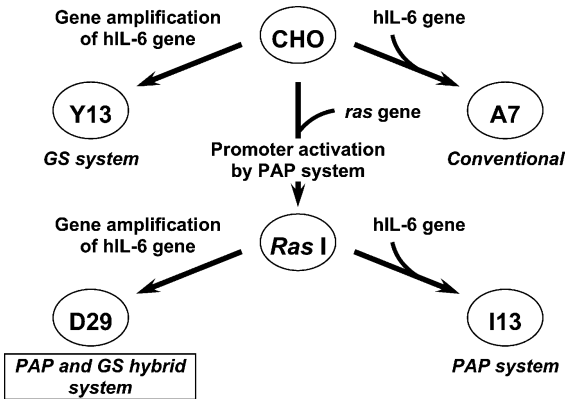

Figure 1. Establishment of recombinant CHO cell lines. A schematic diagram for the establishment of the CHO cell lines used in this study is shown.

with 30 μM each of adenosine, guanosine, cytidine and uridine, 10 μM thymidine, 500 μM each of glutamic acid and asparagine, and 100 μM of other non-essential amino acids, and was termed G-DMEM. Y13 and D29 cells were cultured in G-DMEM supplemented with 5% dialyzed FBS. All cells were cultured in a humidified atmosphere of 5% CO_2/95% air at 37 °C.

Southern and northern analyses

After isolation of genomic DNA using a WAKO DNA Extractor WB according to the manufacturer's recommendations (Wako Pure Chemical Industries, Osaka, Japan), the DNA was precipitated with ethanol and dissolved in TE buffer (10 mM Tris–Cl, 1 mM EDTA, pH 8.0). For Southern analysis, the DNA samples were digested with *Eco*RI and *Hin*dIII overnight, purified with phenol/chloroform and precipitated with ethanol. After another digestion with *Eco*RI and *Hin*dIII for 4 h, the DNA concentration was determined using a UV spectrophotometer. Next, 3.5 μg of each sample was subjected to 0.8% agarose gel electrophoresis. The separated DNAs were transferred to a Hybond-N^+ membrane (Amersham Bioscience, Buckinghamshire, UK) by the capillary method. Hybridization and detection were carried out using a GENE IMAGE kit (Amersham Bioscience) according to the manufacturer's recommendations. In the analysis, the probe for the hIL-6 gene was a 0.7 kbp *Eco*RI-*Hin*dIII fragment from the pCMVP-hIL-6 plasmid (Teruya et al. 1995). The hIL-6 cDNA fragment was also used as a standard to estimate the copy number of the introduced hIL-6 gene.

For northern analysis, total RNAs were isolated from 5×10^6 cells using the TRIzol reagent (Invitrogen, Carlsbad, CA). Next, 10 μg of each total RNA was subjected to 1.2% agarose gel electrophoresis. The separated RNA was transferred to a Hybond-N^+ membrane, and hybridization and detection were carried out using the GENE IMAGE kit. In the analysis, the probe for the hIL-6 gene was the same as that used in the Southern analysis. After stripping the hIL-6 probe, the same membrane was rehybridized with a β-actin probe (Wako Pure Chemical Industries). In the evaluation of GRP78/BiP expression, the probe was a cDNA fragment amplified by PCR using the primers 5′-ACTGGAATTCCTCCTGCTCC-3′ and 5′-GTTCCTGGTATCAATGCGC-3′ (Miura et al. 2001). The band intensities were quantified using a Kodak EDAS system (Eastman Kodak Co., Rochester, NY).

Enzyme-linked immunosorbent assay (ELISA) of hIL-6

The amounts of hIL-6 produced by the recombinant CHO cell lines were measured by ELISA as previously described (Katakura et al. 1999). Briefly, a goat anti-hIL-6 antibody (R&D Systems Inc., Minneapolis, MN) was added to a microtitration plate (Nalge Nunc International K. K., Tokyo, Japan) that had been pre-coated with rabbit anti-goat IgG (H&L) antibodies (Zymed Laboratories Inc., South San Francisco, CA), and then supernatants from hIL-6-producing cell lines and their appropriate dilutions, as well as standard control samples, were added. After addition of an anti-hIL-6 monoclonal antibody (Genzyme Co., Cambridge, MA) which had been biotinylated using biotin-*N*-hydroxysuccinimide ester (Sigma-Aldrich Co., St. Louis, MO), hIL-6 was detected by incubation with a streptavidin-horseradish peroxidase conjugate (Amersham Bioscience) and subsequent reaction with 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (Wako Pure Chemical Industries). The absorbances at 405 nm with a reference of 492 nm were measured.

Western blotting

Secreted hIL-6 in the culture media and accumulated hIL-6 in the recombinant CHO cells were determined by western blotting. Briefly, 5×10^5 cells of each clone were inoculated into 60 mm culture dishes and cultured for 3 days, and then cell-culture supernatants or cell pellets corresponding to the equivalent cell number were prepared. The samples were denatured in 1 volume of 2×sample buffer (50 mM Tris–Cl, 10% glycerol, 2% SDS, 2% 2-mercaptoethanol, 0.1% bromophenol blue) by boiling for 5 min, and then subjected to 12% SDS-polyacrylamide gel electrophoresis. The separated proteins were electrotransferred onto a nitrocellulose membrane (Schleicher & Schuell Inc., Keene, NH). hIL-6 protein was detected with a biotinylated anti-hIL-6 antibody and a streptavidin-horseradish peroxidase conjugate and then visualized with an enhanced chemiluminescence (ECL) detection system (Amersham Bioscience).

Nuclear extraction and electrophoretic mobility shift assay (EMSA)

Nuclear extraction and EMSA were performed as described previously (Yamashita et al. 2001). Briefly, 1×10^8 cells were harvested and centrifuged for 5 min at $3000\times g$. Nuclear extracts were prepared from the resulting cell pellets as previously described (Dignam et al. 1983) with minor modifications. After washing with PBS, the cells were resuspended in 1 ml of buffer A (10 mM HEPES, pH 7.6, 15 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich) and 10 μg ml^{-1} leupeptin (Sigma-Aldrich)). After removing the soluble fraction by centrifugation at $800\times g$ for 1 min at 4 °C, the pellets were resuspended in 1 ml of buffer B (buffer A containing 0.2% NP-40). After removing the soluble fraction by centrifugation at $800\times g$ for 1 min at 4 °C, the pellets were resuspended in 1 ml of buffer C (0.25 M sucrose, 10 mM HEPES, pH 7.6, 15 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF and 10 μg ml^{-1} leupeptin). After removing the soluble fraction by centrifugation at $800\times g$ for 1 min at 4 °C, the pellets were resuspended in 20 μl of buffer D (50 mM HEPES, pH 7.9, 400 mM KCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 0.5 mM PMSF, 10 μg ml^{-1} leupeptin) and incubated for 30 min at 4 °C with agitation. After centrifugation at $600\times g$ for 15 min at 4 °C, the supernatants were collected and applied to EMSA. The protein concentrations were measured by the Bradford method (Bradford 1976) via a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Richmond, CA). A double-stranded oligonucleotide (40 pmol) containing the NF-κB consensus sequence was end-labeled with [$\gamma-{}^{32}$P]dATP (Amersham Bioscience) and used as a probe for the EMSA. The sequence of the sense strand of this oligonucleotide was 5′-AGTTG AGGGGACTTTCCCAGGC-3′. Binding reactions (10 μl total volume) were performed by incubating 0.8 μg of the nuclear extract with the [^{32}P]-labeled double-stranded oligonucleotide probe (1 pmol) in the reaction buffer (10 mM Tris–HCl, pH 7.5, 10 mM KCl, 1 mM DTT, 1 mM EDTA, 12.5% glycerol, 0.1% Triton X-100, 250 μg ml^{-1} BSA, 50 μg ml^{-1} Poly(dI-dC)·Poly (dI-dC) (Sigma-Aldrich)) in the presence or absence of a non-radioactive NF-κB competitor for 15 min at room temperature. The resulting DNA-protein complexes were resolved by 5% polyacrylamide gel electrophoresis. Bands were visualized using a BAS1000 radio-image analyzer (Fuji Photo Film, Tokyo, Japan).

Results

D29 cells producing large amounts of recombinant hIL-6 have a high copy number and high mRNA level of hIL-6, but hIL-6 protein accumulates in the cells

The cell line D29, which was easily and rapidly established by the PAP and GS hybrid system, secreted recombinant hIL-6 protein at a productivity rate of 39.5 μg 10^{-6} cells day^{-1} (Table 1), one of the highest levels reported worldwide. The productivity rate of D29 cells was about 130-fold higher than that of A7 cells, which were established using a conventional method without both promoter activation and gene amplification. The cell lines I13 using the PAP system and Y13 using GS gene amplification showed 10- and 24-fold higher rates than the A7 cell line, respectively. Since these 4 clones, D29, A7, I13 and Y13, did not indicate

extreme high production rate of the recombinant hIL-6 in each establishing methods (Katakura et al. 1999; Dong et al. 2003), we carried on the further investigation about the 4 clones. In order to determine the copy numbers and mRNA levels of the transfected hIL-6 gene, Southern and northern hybridization analyses were performed. The copy numbers and mRNA levels of the transfected hIL-6 gene in A7 (conventional), I13 (PAP), Y13 (GS) and D29 (PAP and GS hybrid system) cells are shown in Figures 2a and b. The results from the Southern and northern analyses were consistent with our expectations. The mRNA levels of hIL-6 were augmented in the cell lines using the PAP system, while the copy numbers of hIL-6 were amplified in the cell lines employing GS gene amplification. In particular, D29 cells using the PAP and GS hybrid system had both a high copy number and a high mRNA level contributed by the gene amplification and the promoter activation, respectively. To evaluate the hIL-6 protein secretion, western blotting analysis was performed to compare the secreted and intracellular hIL-6 protein levels. It is known that the molecular weight of hIL-6 is 23–30 kDa by diversity of glycosylation (Santhanam et al. 1989). We observed that large amounts of 26 kDa and small amounts of 30 kDa hIL-6 in the all lanes of western blotting. However, it was difficult to observe the signal of 30 kDa hIL-6 about the lane of A7 and I13 cells in Figure 2c. As shown in Figure 2c, accumulation of hIL-6 was observed in the high hIL-6-producing cells, such as the Y13 and D29 cells. In particular, large amounts of intracellular hIL-6 protein accumulated in D29 cells, compared to cells using a conventional system.

hIL-6 accumulates in D29 cells by the EOR

Abnormal protein accumulation in the ER leads to GRP78/BiP upregulation and decreased protein secretion, and this well-known type of ER signaling is called the UPR. Northern analysis revealed no change in the GRP78/BiP mRNA expression level in D29 cells compared with the conventional cells as a control (Figure 3a), suggesting that the accumulated recombinant hIL-6 in the D29 cells was not an abnormal protein. Another ER signaling pathway has been proposed in which normal protein accumulation causes ER overload, and this is known as the EOR. NF-κB activation is the typical phenotype of the EOR. Therefore, we evaluated the activation level of NF-κB in the hIL-6-producing CHO cell lines by EMSA, and found that NF-κB was highly activated in the high hIL-6-producing cells using the PAP and GS hybrid system (Figure 3b). The shifted band in Figure 3b was confirmed as specific band of NF-κB in the other experiments (data not shown). This result suggests that the large amounts of hIL-6 protein in the ER of D29 cells activate the negative feedback signals via NF-κB and inhibit the secretion of hIL-6 protein.

Release of the negative feedback signals via the EOR further enhance the hIL-6 productivity rate of D29 cells

The above results showed that the high hIL-6-producing D29 cells accumulated normal hIL-6 protein intracellularly by the EOR. We tried to further enhance hIL-6 secretion by D29 cells by EOR release via the inhibition of NF-κB activation. Therefore, we determined the effect of pyrrolidinedithiocarbamate (PDTC), an inhibitor of NF-κB activation by its antioxidant activity, on hIL-6 secretion by D29 cells using the PAP and GS hybrid system. The treatment of PDTC on other cell lines did not affected the productivity (data not shown). The cell viability or the cell growth was slightly inhibited by PDTC treatment on all cell lines (data not shown). As shown in Figure 4, PDTC treatment inhibited NF-κB activation in D29 cells, and their NF-κB

Table 1. Enhancement of hIL-6 production in various recombinant CHO cell lines established by promoter activation, gene amplification using GS, and hybrid systems.

Cell line	System	Productivity (μg 10^{-6} cells day^{-1})	Relative productivity
A7	Conventional system	0.3	1.0
I13	Promoter activation (PAP)	3.1	10.3
Y13	Gene amplification (GS)	7.3	24.3
D29	Hybrid system (PAP and GS)	39.5	131.2

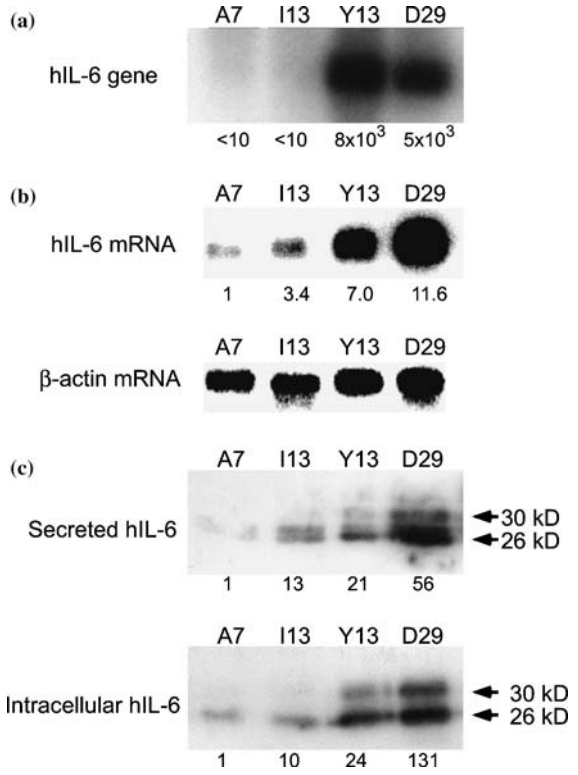

Figure 2. Comparisons of the hIL-6 gene copy numbers, hIL-6 mRNA levels, secreted hIL-6 and intracellular hIL-6 in the hIL-6-producing CHO cell lines. (a) The transfected hIL-6 gene in the genomic DNA of recombinant CHO cell lines (A7, I13, Y13 and D29) was detected by Southern blot analysis. (b) Expressions of hIL-6 mRNA in the hIL-6-producing cell lines. RNA blots were probed with a hIL-6 probe (top panel) and then with a β-actin probe as an internal control (bottom panels). (c) Evaluation of the amounts of secreted and intracellular hIL-6 protein in the hIL-6-producing cell lines. Secreted hIL-6 protein in the culture supernatant (top panel) and accumulated hIL-6 protein in the cells (bottom panel) were analyzed by western blot analysis. The signals in the cell lines were quantitatively determined using a Kodak EDAS system and normalized against the values of the A7 cells (lane 1).

activation level after PDTC treatment was the same as the normal ER status, such as that in A7 cells (conventional) in Figure 3b. This suggests that PDTC treatment blocked the EOR in D29 cells. The suppression of NF-κB activation in D29 cells brought about a 25% augmentation of the hIL-6 secretion rate (Table 2).

Discussion

In the field of mass production of recombinant proteins by animal cells such as CHO cells, quality

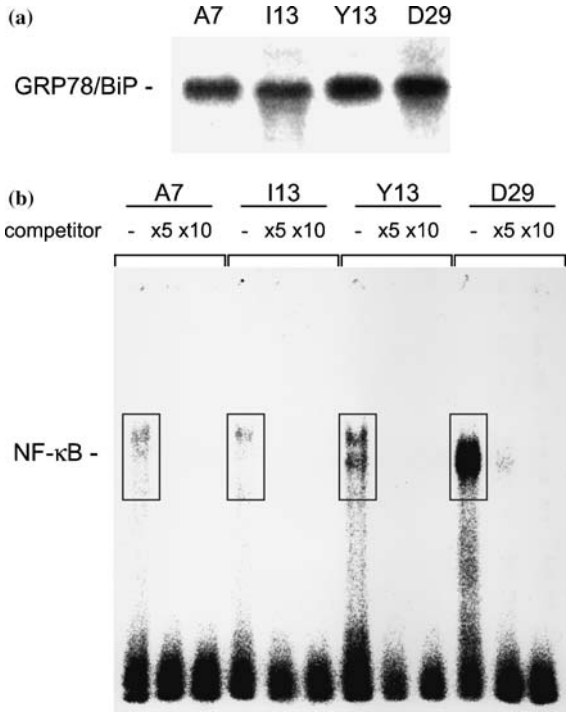

Figure 3. Comparisons of GRP78/BiP mRNA expression and NF-κB activation in the hIL-6-producing CHO cell lines. (a) Northern blot analysis of GRP78/BiP expression in the hIL-6-producing cell lines. Ten micrograms of each total RNA was subjected to 1.2% agarose gel electrophoresis and transferred to a membrane. After hybridization, the GRP78/BiP signals were detected using a GENE IMAGE kit. (b) Electrophoretic mobility shift assay (EMSA) of NF-κB in the hIL-6-producing cell lines. Nuclear extracts were incubated with a radio-labeled double-stranded oligonucleotide probe containing the NF-κB binding site, separated by electrophoresis and analyzed. A non-radioactive double-stranded oligonucleotide containing the NF-κB binding site was used as a competitor in the experiment.

control of the synthesized recombinant protein is important. In particular, abnormal protein accumulation can cause severe stress in the ER of the cells and/or induction of the UPR. In addition, it often induces apoptosis. We previously reported that hyper-production of recombinant human immunoglobulin λ light chain caused upregulation of GRP78/BiP and recombinant protein accumulation in the ER (Miura et al. 2001). On the other hand, normal hIL-6 protein accumulation in the ER by the EOR via NF-κB activation was observed in the present study. The EOR tendency of each CHO cell line we examined was relatively correlated to their mRNA level of the overexpressed hIL-6. The mechanism by which the EOR activates NF-κB is currently unclear (Jiang et al.

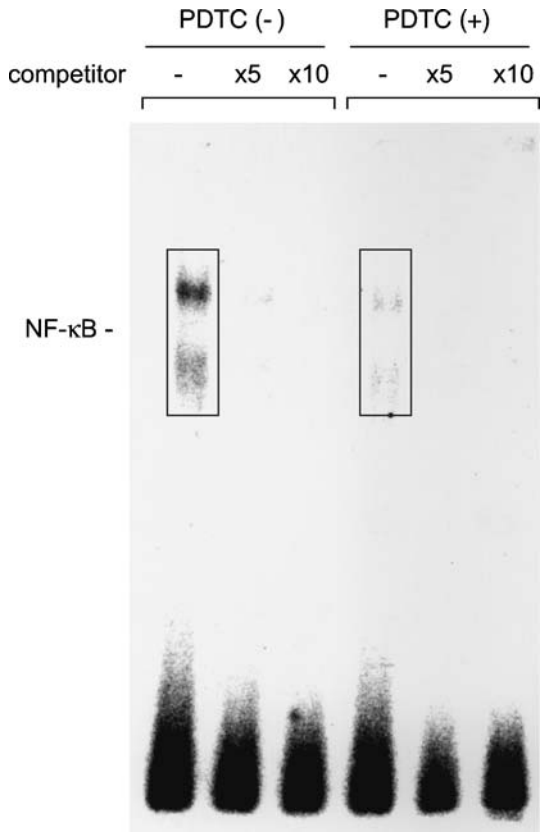

Figure 4. The cellular productivity of hIL-6 in D29 cells is augmented by inhibition of NF-κB activation. EMSA of NF-κB in D29 cells with or without pyrrolidinedithiocarbamate (PDTC), an inhibitor of NF-κB activation.

Table 2. PDTC treatment enhances hIL-6 productivity of D29 cells established by the PAP and GS hybrid system.

	PDTC (−)	PDTC (+)
Productivity (μg 10^{-6} cells day^{-1})	39.2 ± 1.3	48.8 ± 1.6
Relative productivity	1	1.25

2003). However, it was known that activated NF-κB in the EOR played the role of decrease of the protein synthesis or secretion (Pahl and Baeuerle 1995; Pahl 1999; Cudna and Dickson 2002). Since facilitation of hIL-6 secretion is effective for enhancing the protein production rate, we inhibited NF-κB activation by PDTC treatment. The subsequent EMSA revealed that inhibition of NF-κB activation led to augmentation of the hIL-6 production rate (Table 2).

Regarding the hyper-production of recombinant proteins in CHO cells, two different types of intracellular recombinant protein accumulation and ER signals have been observed. One was the previously reported UPR, while the other was the EOR in the present report. It is unclear why synonymic CHO-derived cell lines showed different phenotypes. In these cases, how to establish the hyper-producing clone and what recombinant protein was produced, were suggested as reasons for the differences. Since the approach was investigated as a way to enhance the mRNA level of the recombinant protein, it is therefore considered that the difference in the recombinant protein used is important for these phenotypes under ER signaling. In the previous study, we used the human immunoglobulin λ light chain of the HB4C5 antibody as a recombinant protein (Miura et al. 2001), whereas in the current study, we used the hIL-6 protein. Both of these proteins are *N*-glycosylated. hIL-6 is a typical glycoprotein similar to other bioactive proteins. On the other hand, the human immunoglobulin λ light chain of the HB4C5 antibody is unique. Although most immunoglobulin light chains do not have a sugar chain, the human immunoglobulin λ light chain of the HB4C5 antibody is *N*-glycosylated in the complementarity determining region 1. The structure of the sugar chain greatly contributes to appropriate antigen recognition and reactivity of the HB4C5 antibody (Tachibana et al. 1992, 1996, 1997). Thus, it is considered that this protein has a tendency to induce the UPR upon overexpression. The results from the overexpressions of these distinct recombinant proteins suggest that a novel assessment system needs to be developed for quality control of hyper-produced recombinant proteins in the ER for both the UPR and EOR.

In conclusion, the D29 cells had a high copy number and high mRNA level as well as a high productivity rate using the PAP and GS hybrid system. However, large amounts of hIL-6 protein accumulated in the cells, compared to the conventional cells. It is suggested that NF-κB activation transduced negative feedback signals from the ER to the nucleus in D29 cells via the EOR. Inhibition of NF-κB activation by PDTC treatment produced an augmentation of the productivity rate in the PAP and GS hybrid system. Therefore, hyper-producing cells, such as D29 cells, established by the PAP and GS hybrid system could show increased total amounts of recombinant protein secretion after release of negative feedback signals.

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