A hybrid system using both promoter activation and gene amplification for establishing exogenous protein hyper-producing cell lines

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Abstract

We previously developed a promoter-activated production (PAP) system using amplified *ras* oncogene to activate the cytomegalovirus (CMV) promoter controlling the foreign gene in mammalian cells. CHO cells were demonstrated to be suitable for the PAP system. Here, we show that very high-level production of a recombinant protein was achieved when the human CMV promoter was inserted into a glutamine synthetase (GS) minigene expression plasmid, pEE14. A highly productive host CHO cell line, *ras* clone I containing amplified *ras* oncogene, was further transfected with the plasmid expressing both hIL-6 gene and GS minigene, and selected with methionine sulfoximine. We were able to establish a hIL-6 hyper-producing cell line, D29, which exhibited a peak productivity rate of approximately 40 μg hIL-6 10^{-6} cells day⁻¹ through a combination of the PAP system and the GS gene amplification system. The cellular productivity of D29 cells was about 13-fold higher than control hIL-6-producing cells derived from CHO cells whose hIL-6 gene was amplified by the GS gene amplification system, and about 5-fold higher than the I13 cells established by the PAP system, which contains amplified *ras* oncogene and non-amplified hIL-6 gene. When D29 cells were cultured for a month, an accumulation rate of approximately 80 μg hIL-6 ml⁻¹ per 3 days was achieved on the 9th day. These results indicate that this PAP and GS hybrid system enables the efficient and rapid establishment of recombinant protein hyper-producing cell lines.

Abbreviations: CHO – Chinese hamster ovary; CMV – cytomegalovirus; *dhfr* – dihydrofolate reductase; DMEM – Dulbecco's modified Eagle's medium; ELISA – enzyme-linked immunosorbent assay; FBS – fetal bovine serum; GS – glutamine synthetase; MSX – methionine sulfoximine; MTX – methotrexate; PAP system – promoter-activated production system.

Introduction

In the field of recombinant protein production, animal cells have been shown to be superior to prokaryotic cells for the production of bioactive mammalian proteins. However, the low production rate of animal cells still remains a major obstacle for recombinant protein production in the industrial field. Several methods have been developed to enhance cellular productivity, such as promoter transactivation and gene amplification.

We have developed a promoter-activated production (PAP) system to enhance recombinant protein productivity in cultured cells by activating specific promoters with the transcriptional activator such as oncogenes. Briefly, the PAP system is constructed by transfecting host cells with an effector plasmid carrying the transcriptional activators and a reporter plasmid carrying the gene of interest under the control of a strong promoter. The products of the effector plasmid should stimulate the promoter of the reporter plasmid and enhance production of the foreign protein. The best combination of oncogene and promoter for efficient protein production was shown to be the *ras* oncogene and the cytomegalovirus (CMV) promoter (Yano et al. 1994; Teruya et al. 1995). Furthermore, when the E1A oncogene was introduced into a human interleukin-6 (hIL-6)-producing cell line that was already *ras*-amplified, productivity was boosted 10-fold (Shirahata et al. 1995). This PAP system was shown to be more effective in CHO cells, where the peak productivity reached $13.6 \mu\text{g } 10^{-6} \text{ cells day}^{-1}$ (Katakura et al. 1999).

Another widely used method for enhancing recombinant protein production is gene amplification. In the popular *dhfr* gene amplification system, sequential increases in the concentration of the selective agent, methotrexate (MTX), result in the cells carrying amplified copies of the *dhfr* gene and the gene of interest (Kemball-Cook et al. 1994). Although this method can enhance the production rate of recombinant proteins, it takes a long time, as several rounds of selection and cloning are required. 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 over-expression

of a number of foreign genes including a tissue inhibitor of metalloproteinases (Cockett et al. 1990).

In this report, we attempted to enhance the productivity in recombinant CHO cells generated with the PAP system and gene amplification of the GS gene. Here, we demonstrate that very high-level production of a recombinant protein was achieved using the hybrid system, combining the PAP system with the GS gene amplification system.

Materials and methods

Vector construction

The hIL-6 expression vector pEE14-IL-6 was constructed by inserting a *Hind*III-*Eco*RI fragment of the hIL-6 cDNA into the corresponding cloning sites in the pEE14 vector (LONZA Biologic plc, Slough, UK; Cockett et al. 1990), which carried a GS minigene driven by the SV40 late promoter (Figure 1). The hIL-6 gene was driven by the strong CMV promoter and amplified by the GS minigene amplification. The vectors pCMVP-hIL-6 (Yano et al. 1994) and pSV2-*bsr* (Izumi et al. 1991) were used for expression of hIL-6 by the CMV promoter and the blasticidin S resistance gene by the SV40 early promoter, respectively.

Figure 1. Vector construction used for GS gene amplification. CMV promoter: human cytomegalovirus immediate early promoter; hIL-6: human interleukin-6; poly A: poly A additional sequence; Amp^r: β -lactamase; SV40L: SV40 late promoter; GS-minigene: glutamine synthetase minigene (Cockett et al. 1990).

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, USA), 100 nM hypoxanthine and 16 nM thymidine. The establishments of *ras* clone I, I13 and A7 cells were described previously (Katakura et al. 1999) and are summarized in Figure 2. 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. The conventional 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.

The medium for the GS gene amplification system was composed of glutamine-free DMEM (Bio WHITTAKER, Walkersville, MD, USA) supplemented with 30 μM each of adenosine, guanosine, cytidine and uridine, 10 μM of thymidine, 500 μM each of glutamic acid and asparagine, and 100 μM of other non-essential amino acids, and termed G-DMEM. GS gene-transfected 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.

Figure 2. Establishment of recombinant CHO cell lines. A schematic diagram for the establishment of the CHO cell lines utilized in this study is shown.

Transfection and amplification of the GS gene

The *dhfr*-deficient CHO and *ras* clone I cells (3×10^5 cells per 60-mm culture dish) were transfected with 13.5 μg of pEE14-IL6 and 1.5 μg of pSV2-*bsr* using the calcium phosphate co-precipitation method (Chen and Okayama 1987). The transfected cells were selected with 5 $\mu\text{g ml}^{-1}$ of blasticidin S and 25 μM MSX. Individual clones were isolated by a limiting dilution method. The clones derived from CHO and *ras* clone I cells were named Y and D, respectively (Figure 2). The hIL-6-secreting clones were screened by enzyme-linked immunosorbent assay (ELISA).

Detection 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, goat anti-hIL-6 antibody (R&D, Minneapolis, MN, USA) was added to a micro-titration plate (Nunc, Roskilde, Denmark) pre-coated with rabbit anti-goat IgG (H&L) antibodies (Zymed, San Francisco, CA, USA), and then supernatants from hIL-6-producing cell lines, their appropriate dilutions, and standard control samples were added. After adding an anti-hIL-6 monoclonal antibody (Genzyme, Cambridge, MA, USA) which had previously been biotinylated using *N*-hydroxysuccinimidobiotin, hIL-6 was detected by incubation with streptavidin-horseradish peroxidase conjugate (Amersham, Buckinghamshire, UK) and subsequent reaction with 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (WAKO, Osaka, Japan). The absorbance at 405 nm with a reference of 492 nm was measured.

Results

Evaluation of hIL-6 productivity of GS gene amplification clones with or without the PAP system

We obtained twenty Y clones from the CHO cells transfected with pEE14-IL-6 and pSV2-*bsr* by one round of selection with both 5 $\mu\text{g ml}^{-1}$ of blasticidin S and 25 μM MSX. As shown in Figure 3, nine of the Y clones produced detectable levels of

Figure 3. Cellular productivity rate of hIL-6-producing CHO clones generated by one round of GS gene amplification. A7 was the CHO clone generated by the conventional method (Katakura et al. 1999). Y clones were generated by single step gene amplification of the transfected hIL-6 gene.

hIL-6. Among these clones, Y13 showed the highest peak productivity value of $3.1 \mu\text{g } 10^{-6} \text{ cells day}^{-1}$. In the case of recombinant hIL-6 expression without gene amplification in the CHO cells, the productivity rate was $0.3 \mu\text{g } 10^{-6} \text{ cells day}^{-1}$ (A7 cells in Figure 3). Compared to A7 cells as the conventional method, Y13 cells produced recombinant hIL-6 at a more than 10-fold higher level. This enhancement was achieved by only a single round of GS gene amplification. Therefore, a combination of gene amplification and the PAP system was examined.

By transfecting *ras* clone I with pEE14-IL-6 and pSV2-*bsr*, 43 D clones were obtained after one round of selection with both $5 \mu\text{g ml}^{-1}$ of blasticidin S and $25 \mu\text{M}$ MSX (Figure 4). The peak productivity rates of 11 clones were demonstrated to be more than $10 \mu\text{g } 10^{-6} \text{ cells day}^{-1}$, and the overall productivities of the D clones were demonstrated to be much higher than those of the Y clones. The hIL-6 hyper-producing D29 cells showed a peak productivity value of $39.5 \mu\text{g } 10^{-6} \text{ cells day}^{-1}$, which was over 13-fold higher than Y13, the highest hIL-6-producing cell line with only one round of GS gene amplification. D29 cells showed a higher productivity rate compared to I13 cells established by *ras*-primed CHO cells transfected with the hIL-6 gene as the PAP system. I13 cells produced recombinant hIL-6 at $7.3 \mu\text{g } 10^{-6} \text{ cells day}^{-1}$ (Figure 4).

The peak productivity rate of Y13 was 10-fold greater than that of A7, and that of D29 was 5.4-

fold greater than that of I13. These results indicated that the productivity rate of hIL-6 was markedly augmented by GS gene amplification in both the CHO cells and *ras* clone I. On the other hand, the peak productivity rate of D29 was 12.7-fold greater than that of Y13. This indicated that combination of the GS gene amplification and the PAP system was effective for establishing a recombinant protein hyper-producing cell line. In addition, the hIL-6 hyper-producing CHO cell lines were established within a month.

D29 cells produced large amounts of recombinant hIL-6 for a month

The cellular productivity of D29 cells in long-term culture was evaluated. D29 cells (1×10^5 cells) were inoculated in 35-mm culture dishes and cultured for a month with culture media changes at 3-day intervals. The amount of hIL-6 produced during each 3-day interval and the cell number were assessed. The productivity rates of hIL-6 were calculated from the above values. As shown in Figure 5, the hIL-6 productivity rate was almost $20 \mu\text{g } 10^{-6} \text{ cells day}^{-1}$. At around the 10th day, the maximum hIL-6 production of over $80 \mu\text{g ml}^{-1}$ per 3 days was observed. This period was also showed the greatest cellular productivity rate at $46 \mu\text{g } 10^{-6} \text{ cells day}^{-1}$. The cell number reached 10^6 cells within a week, and this value was retained during the experimental period.

Discussion

Various approaches have been developed to enhance the cellular productivity of recombinant protein production using animal cells. The most popular system for mass production of recombinant proteins in animal cells is gene amplification with the *dhfr* gene in *dhfr*-deficient CHO cells (Schimke 1988). However, this gene amplification method is laborious and time-consuming, since effective gene amplification of the target results from multi-step MTX selection over a long time to acquire the resistance to the selection drug by gene amplification. Many oncogene products are known to act as trans-activators of some promoters. We tried to enhance the cellular productivity of recombinant

Figure 4. Cellular productivity rate of hIL-6-producing CHO clones generated by combination of the PAP system and one round of GS gene amplification. I13 was the CHO clone generated by the PAP system only (Katakura et al. 1999). D clones were generated by the hybrid system, which combined the gene-amplified transfected hIL-6 gene (gene amplification) in the *ras*-primed CHO cells (PAP system).

animal cells for exogenous gene expression by promoter activation with oncogene products as trans-activators, termed the PAP system. To establish high recombinant protein-producing animal cell lines, the PAP system is composed of a reporter plasmid expressing a recombinant protein under the control of the potent CMV promoter and an effector plasmid expressing oncogenic *c-Ha-ras* also under the control of the CMV promoter. The recombinant BHK and CHO cell lines established with the PAP system can produce recombinant proteins in commercially viable amounts quickly and easily (Teruya et al. 1995; Katakura et al. 1999).

In this report, a hybrid system that combined the PAP system and GS gene amplification was constructed, and the effectiveness of the novel system was evaluated. We previously described that high hIL-6-producing CHO clones were obtained by the PAP system using *ras*-primed CHO cells (Katakura et al. 1999). The previously established hIL-6-producing CHO cell lines, A7 and I13, were employed in this study as recombinant protein-producing models of the conventional and PAP systems, respectively. First, we evaluated the cellular productivity rate of exogenous hIL-6 production by recombinant CHO cells with one round of GS amplification. The results

Figure 5. Evaluation of cellular productivity rate, 3-day accumulation and cell number for the D29 cell line established by the hybrid system. The hIL-6 accumulation (solid bars, upper graph), productivity rate (closed circles, lower graph) and total cell number (open circles, lower graph) are shown. D29 cells (1×10^5 cells) were inoculated in 35-mm culture dishes and cultured for a month with culture media changes at 3-day intervals. The amounts of hIL-6 were measured by ELISA, and the cell number was counted at 3-day intervals.

showed that one round of GS gene amplification effectively enhanced the productivity rate of exogenous hIL-6 production in the recombinant CHO clones (Figure 3). These results indicated that gene amplification was efficient for enhancing hIL-6 production in CHO cells. However, the production rate of Y13 was only half that of I13, a clone established with the PAP system. These results suggested that other methods were required to further enhance the productivity rate in the gene amplification system. Since the PAP system enhances the foreign gene expression by a different mechanism from the GS gene amplification system, a combination of the two systems was possible.

Next, enhancement of the cellular productivity of the reporter plasmid (hIL-6 encoding vector) by GS gene amplification in the PAP system was examined. The results showed that high hIL-6-producing recombinant cell lines could be established in a month by combination of the PAP

system and GS gene amplification (Figure 4). In fact, among the 43 D clones obtained after transfecting *ras* clone I cells with the pEE14-IL6 and pSV2-*bsr* vectors, 33 clones expressed hIL-6 at the rate of over $1 \mu\text{g ml}^{-1}$. The levels of hIL-6 expression in the D clones were significantly higher than those in the Y clones (Figures 3 and 4). More than half the D clones showed a higher production rate than that of I13 (Figure 4). In previous reports, the recombinant BHK cell lines producing hIL-6 showed a productivity rate at $5.5 \mu\text{g } 10^{-6} \text{ cells day}^{-1}$ by the PAP system (Shirahata et al. 1995), and recombinant CHO cell lines showed a productivity rate at $5.6 \mu\text{g } 10^{-6} \text{ cells day}^{-1}$ by a conventional system or $13.2 \mu\text{g } 10^{-6} \text{ cells day}^{-1}$ by the PAP system, respectively (Katakura et al. 1999). The highest hIL-6 producing clone, D29, displayed a production rate as high as $39.5 \mu\text{g } 10^{-6} \text{ cells day}^{-1}$. This level is 5-fold higher than that of I13, and is the highest productivity level ever reported for recombinant hIL-6.

Recently, some techniques for exogenous protein production in CHO cells were reported. Kito et al. (2002) developed high-level recombinant protein production CHO strains using gene amplification of the *dhfr* gene and gene targeting in the cells. Their most productive clone was able to produce 160 mg l^{-1} in 7 days in a low-protein medium in a spinner-flask. Another approach of ours to produce recombinant protein hyper-producing cells using the tetracycline-inducible system was developed. Hyper-production of bovine β -lactoglobulin in CHO cells with productivity of over $300 \mu\text{g ml}^{-1}$ in 15 days in a hollow fiber bioreactor was achieved using the tetracycline-inducible system (Teruya et al. 2002).

In this study, we have described a novel system named the PAP & GS hybrid system for establishing recombinant protein hyper-producing cell lines through activation of the promoter controlling the recombinant gene expression by oncogenes (PAP system) and GS gene amplification. The peak productivity value of the recombinant CHO cells generated with the PAP & GS hybrid system reached $39.5 \mu\text{g } 10^{-6} \text{ cells day}^{-1}$, which is sufficient to fulfill the commercial demand.

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