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Productivity enhancement of recombinant protein in CHO cells via specific promoter activation by oncogenes

Yoshinori Katakura[1,*], Perry Seto[1], Takumi Miura[1], Hideya Ohashi[2], Kiichiro Teruya[1] & Sanetaka Shirahata[1]

[1] *Graduate School of Genetic Resources Technology, Kyushu University, Hakozaki 6-10-1, Higashi-ku, Fukuoka 812-8581, Japan*

(Author for all correspondence, e-mail: katakura@grt.kyushu-u.ac.jp)*

[2] *Pharmaceutical Laboratory, Kirin Brewery Co. Ltd., Souja-machi, Maebashi, Gunma 371, Japan*

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Abstract

To construct a recombinant protein highly producing cell lines, we have previously developed the Oncogene Activated Production (OAP) system by using BHK-21 cells. Here we verified the availability of the OAP system in CHO cells. We firstly generated 'primed' *ras* amplified CHO cells, *ras* clone I, by introducing human c-Ha-*ras* oncogene into CHO cells. This *ras* clone I enables quick and easy establishment of recombinant protein hyper producing cell lines by introduction reporter gene of interest. Then we generated I13 by introducing human interleukin 6 (hIL-6) gene as a reporter gene, which showed enhanced productivity rate as compared to A7 established by conventional method. Furthermore, we found that hIL-6 production level of I13 was slightly improved by raising the CO_2 concentration from 5 to 8% possibly because of the enhanced growth rate. We further introduced the E1A oncogene, which has been shown to have a synergistic effect on the recombinant protein production of the *ras*-amplified BHK-21 cells, then evaluated the productivity. When culture in 5% CO_2 condition, only the slight effect can be seen. However when cultured in 8% CO_2 condition, not only cell number, but also productivity increased significantly, resulted in great augmentation of hIL-6 production, maximum production being 88.6 μg/ml/3 days. This study demonstrates that recombinant protein production level reached commercially desirable level by utilizing our OAP system in CHO cells and optimizing the culture condition.

Abbreviations: CMV, cytomegalovirus; hIL-6, human interleukin 6; OAP, oncogene activated production

Introduction

Animal cells are known to be superior to prokaryotic cells for producing mammalian recombinant proteins because animal cells can perform the post translational modifications necessary for complete function and activity of proteins. However, the low productivity of animal cells still remain a major obstacle for their acceptance as tools for mass production in industry. To overcome this problem, researchers have developed a variety of methods. For example, high expression vectors, which contain a heterologous strong promoter plus splicing and polyadenylation signals, have been constructed and utilized, but have not been totally successful in raising the productivity of animal cells (Kronman, 1992; Sasada, 1988; Takeda, 1988). The successful augmentation of recombinant protein production in this system depends upon the compatibility between the cell type and the promoter used (Wenger, 1994; Lin, 1994). To obtain high production of a recombinant protein, increasing the gene copy number by gene amplification has been widely used. One of the best known gene which was found to amplify the gene copy number was the dihydrofolate reductase (dhfr) gene (Kemball-Cook, 1994). A drawback to this system is the rather time consuming selection process

where a stepwise increase of methotrexate (MTX), the specific inhibitor of dhfr, is needed to sufficiently amplify a foreign gene to result in the generation of highly productive variants. Recently, glutamine synthetase gene was also found to be useful to amplify the gene copy number. After selecting the transfected cells harboring the multi-copy numbers of glutamine synthetase gene by gradually decreasing the concentration of glutamine in the medium, the target gene copy number can be amplified by adding methionine sulfoximine (MSX) to the medium. By using this system, productivity of the tissue inhibitor of metalloproteinases (TIMP) reached the level of 110 μg/10^6 cell/day (Cockett, 1990).

To construct a highly productive cell line, we developed the Oncogene Activated Production (OAP) system. This system uses the combinations of an effector plasmid, which consists of an oncogene, and a reporter plasmid that carries the expression gene of interest under the control of a strong promoter. The product of the effector plasmid should stimulate the promoter of the reporter plasmid to enhance production of the foreign protein. Previously we demonstrated in BHK-21 cells that of all the oncogenes (c-*fos*, c-*myc*, c-*jun*, c-*myb*, and c-Ha-*ras*) and promoters (β-actin, cytomegalovirus (CMV), metallothionein II_A, simian virus early (SV40E) and SRα) tested, the best combination for efficient protein production was found to be oncogene *ras* and CMV promoter (Yano, 1994). Furthermore, when the E1A oncogene was introduced into the recombinant protein producing BHK-21 cell clone that was already *ras* amplified, productivity was boosted 10 fold to a peak production rate of 5.34 μg/ml/day (Shirahata, 1995).

Here we report the establishment of recombinant protein hyper producing CHO cell lines by the OAP system, and further show the successful achievement of hyper production of recombinant protein by coexpression of *ras* and E1A oncogenes and by optimization of culture condition for recombinant CHO cells.

Materials and methods

Effector and reporter plasmids

The pRC/CMV vector (Invitrogen, San Diego, CA) was used to construct both effector and reporter plasmids as described previously (Yano, 1994). To construct the reporter plasmid, the human interleukin 6 (hIL-6) cDNA (British Bio-technology, U.K.) was inserted into the pCMVP vector which was constructed from the pRc/CMV by cutting out the neomycin resistance gene (Yano, 1994). The resulting plasmid was named pCMVP-hIL-6 (Figure 1A). Next we constructed the effector plasmid. The human activated c-Ha-*ras* oncogene fragment (Japanese Cancer Research Resources Bank: JCRB) was inserted into the pRc/CMV vector, and named pCMV-*ras*. Furthermore, the fragment containing SV40E promoter, dhfr gene and poly A region derived from pSV2-dhfr plasmid (ATCC, Rockville, MD) was inserted upstream from the CMV promoter of the pCMV-*ras*. The resulting plasmid was named pCMVD-*ras* (Figure 1B). The pSV2-gpt-E1A plasmid (Figure 1C), which carries the E1A gene derived from adenovirus type 12, was generously donated by Dr. K. Onodera at the University of Tokyo. The E1A gene fragment was excised from pSV2-gpt-E1A and cloned into the pRc/CMV. The resulting plasmid was named pCMV-E1A (Figure 1D). The selection markers pSV2-bsr and pSV2-hph were purchased from Kaken Pharmaceuticals (Tokyo, Japan) and ATCC, respectively.

Culture medium

Chinese Hamster Ovary (CHO) cell line DUKX-B11 which lacks the dhfr gene (CHO D^-) were cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 0.1 mM hypoxanthine (Wako, Kyoto, Japan) and 0.016 mM thymidine (Wako) (DMEM-HT) with 5% fetal bovine serum (FBS: Irvine Scientific, Irvine, CA, U.S.A.). Recombinant CHO cells which contains the dhfr gene are cultured in DMEM supplemented with 5% dialyzed FBS but without hypoxanthine and thymidine. Cells were incubated in the appropriate medium in 5 or 8% CO_2 at 37 °C.

Establishment of recombinant CHO cell lines

CHO D^- cells were cultured in DMEM-HT medium with 5% FBS. The reporter plasmid pCMV-hIL-6 and the selection marker pSV2-bsr were co-transfected into the CHO cells using the highly efficient calcium phosphate co-precipitation method (Chen, 1987). When confluency was reached, 5 μg/ml of blasticidin S (WAKO) was added to the medium to select recombinant CHO cells. Selected cells were cloned by limiting dilution method and screened for hIL-6 production using sandwich ELISA as described below. The clone with the highest productivity was named A7 (Figure 2).

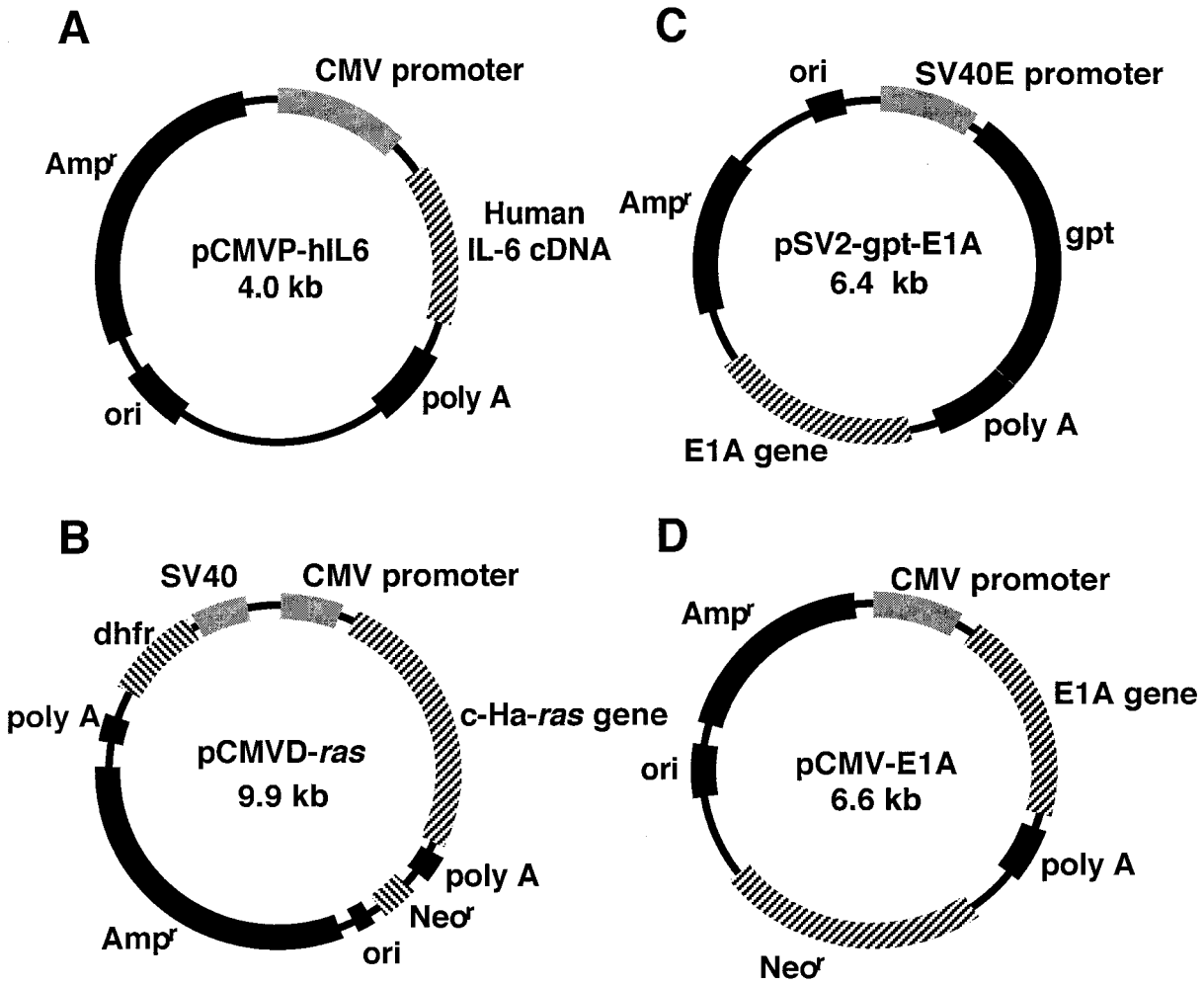

Figure 1. Plasmids used in the OAP system. A: Reporter plasmid for producing hIL-6. B, C and D: Effector plasmids for enhancing the transcription of hIL-6 gene under the control of CMV promoter.

To establish *ras* primed cells, CHO D$^-$ cells were transfected with the plasmid pCMVD-*ras* and selected with 1 mg/ml of G418 (WAKO). Gene amplification was further done using 50 nM MTX (Sigma, St. Louis, MI) in DMEM with 5% dialyzed FBS. Twelve single clones were then isolated and established. To identify the 'primed' *ras* clone with the highest potential for hyper productivity, the *ras* amplified clones were co-transfected with the reporter plasmids pCMVP-hIL-6 and the selection marker pSV2-bsr. After selection with 5 μg/ml of blasticidin S, transfectants were isolated using the limiting dilution method. Over thirty clones from each 'primed' clonal parent were screened for hIL-6 production. The 'primed' *ras* clone with the highest number of hyper producing subclone was named ras clone I, and the overall top producing clone found was I13 (Figure 2).

I13 was further transfected with either the pSV2-gpt-E1A or the pCMV-E1A plasmid along with the selection marker pSV2-hph. After selection with 200 μg/ml hygromycin B, over 80 clones from both lines were isolated and evaluated for hIL-6 production. Clone CMV from the pCMV-E1A transfected line, and clone SV2 from the pSV2-gpt-E1A line were demonstrated to be the top producers of their respective family lines (Figure 2).

Detection of hIL-6

The amount of hIL-6 produced by the recombinant cell lines was measured by the enzyme-linked immunosorbent assay (ELISA) as previously described (Ya-

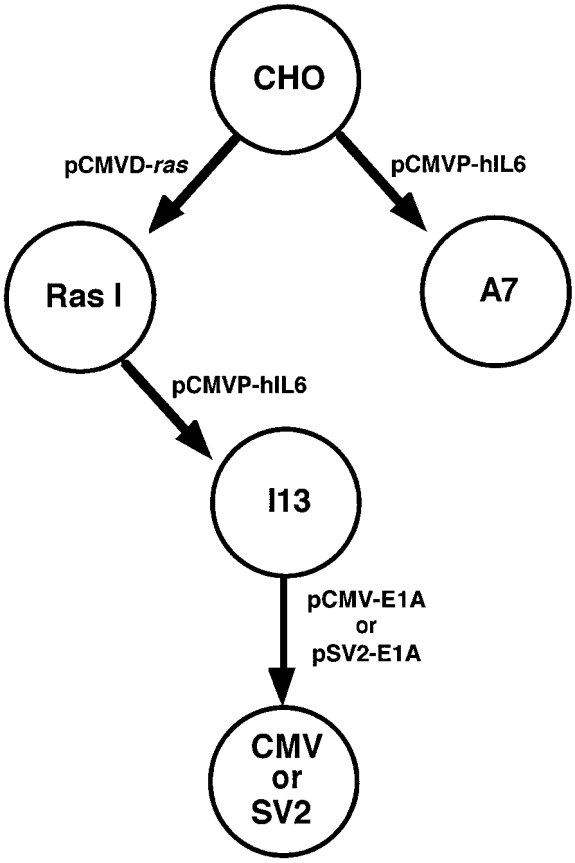

Figure 2. Establishment of recombinant CHO cell lines. Shown is a schematic diagram for the establishment of the CHO cell lines utilized in this study.

mada, 1989). Briefly, after 96 flat bottom well plates (Nunc, Roskilde, Denmark) were coated with appropriately diluted rabbit anti-goat IgG (H&L) (Zymed, San Francisco, CA) and incubated at 37 °C for 1 h. After washing three times with 0.05% Tween 20 (WAKO) in PBS (TPBS), appropriately diluted goat anti-hIL-6 antibody (R&D, Minneapolis, MN) in TPBS was added and incubated at 37 °C for 1 h. Again after washing three times with TPBS, supernatant from hIL-6 producing cell lines, their appropriate dilutions, and standard control samples were added and incubated at 37 °C for 1 h. After washing three times, 1000-fold diluted anti-hIL-6 monoclonal antibody (Genzyme, Cambridge, MA), which was previously biotinylated using N-hydroxysuccinimidobiotin (Sigma), was added and incubated at 37 °C for 1 h. After washing, 1000-fold diluted solution of streptavidin horseradish peroxidase conjugate (Amersham, Buckinghamshire, U.K.) in TPBS was added and incubated at 37 °C for 1 h. Again after washing, a solution of ABTS disodium salt (WAKO) diluted in 0.2 M citrate buffer with 1/10000 (w/v) of H_2O_2 was added to the plate. Measurement at the wavelength 405 nm with a reference at 492 nm was taken.

Results

Evaluation of hIL-6 productivity for A7 and I13 cells cultured in 5% CO_2

A7 and I13 were evaluated for their productivities over a thirty day period in 5% CO_2 (Figure 3, circles). Productivity and 3 day accumulation values for I13 were shown to be much greater than A7, although the peak cell numbers of these cell lines were not greatly different. Peak productivity value for I13 was 13.2 μg/10^6 cells/day at day 30 and that for A7 was 5.6 μg/10^6 cells/day at day 27 (Table 1). When comparing peak productivity, the value of I13 was 2.4 fold greater than that of A7. Peak 3 day accumulation values were reached on day 15 for I13 and day 18 for A7, and shown to be 17.3 μg/ml/3 day for I13 and 10.2 μg/ml/3 day for A7 (Table 1). This demonstrates that the peak 3 day accumulation value for I13 was 1.7 fold greater than A7. These data show that the *ras* primed cell line, specifically *ras* clone I from which I13 was generated, can produce recombinant cell lines with enhanced productivity.

In this study, we established primed *ras* amplified CHO cells, *ras* clone I. Recombinant protein hyper producing cell lines can be easily and quickly established by introducing any reporter gene. Clone I13 established by introducing hIL-6 cDNA into *ras* clone I were shown to have a productivity rate almost twice that of control A7 and over 9 times greater than the primed *ras* amplified recombinant BHK clone TBR 19 (Teruya, 1995), demonstrating that CHO cells was more suited for high level expression of recombinant protein by using OAP system than BHK cells.

Increased CO_2 concentration improved the accumulation of recombinant protein

For culturing CHO cells in high cell density to obtain large amount of recombinant protein, incubation of CHO cells in 8% CO_2 is thought to be more suited for preventing the rise in pH value of the culture medium during the culture. Microscopic analysis was done for I13 cultured in either 5 or 8% CO_2 (data not shown). Cells grown in 5% CO_2 remained in a monolayer and

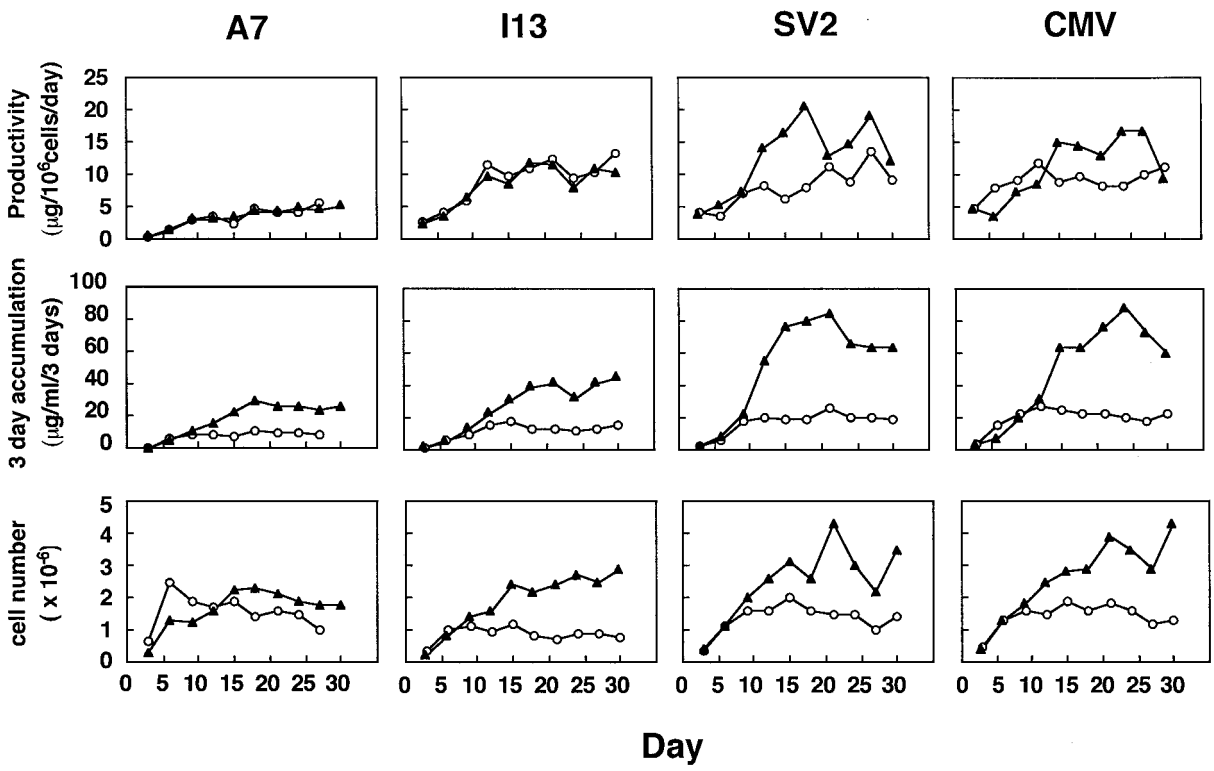

Figure 3. Evaluation of productivity, 3 day accumulation and cell number for recombinant CHO cell lines established by the OAP system. Shown are the productivity, 3 day accumulation, and cell number values for established CHO cell lines cultured in 5% CO_2 (○) or in 8% CO_2 (▲). A7 was established by the conventional method. I13 was established by introducing hIL-6 expressing plasmid into primed *ras* amplified CHO cell clone, *ras* clone I. SV2 and CMV were generated by introducing the E1A gene under the control of SV40E promoter and CMV promoter respectively, into I13.

the morphology of most of the cells were elongated although a few round shape cells can be observed. In contrast, cells cultured in 8% CO_2 grew exceeding the monolayer. The morphology of the cells seen were for the most part round and grow in aggregates. This data suggest that the increased cell number shown for cells cultured in 8% CO_2 may be result of the ability to form aggregates.

We then checked the productivity of recombinant CHO cells cultured in 8% CO_2 condition (Figure 3, triangles). For A7 and I13, productivity values between the two culture condition, 5 and 8% CO_2, did not significantly change. On the other hand, cell growth was dramatically augmented. Total cell numbers for A7 and I13 cultured in 8% CO_2 condition greatly increased as compared to those cultured in 5% CO_2 condition. This increased cell growth had a corresponding effect on the 3 day accumulation of hIL-6 production for both cell lines. Peak 3 day accumulation reached on day 18 for a value of 29.2 μg/ml/3 day for A7 and on day 30 for a value of 45.2 μg/ml/3 day for I13 (Table 1). Peak values increased 2.86 and 2.61 times respectively as compared to those cells cultured in 5% CO_2.

The effect of the E1A oncogene on the production of hIL-6 from ras amplified recombinant CHO cells cultured in 5% CO_2

Previously, we have reported that E1A and *ras* oncogenes synergistically enhance recombinant protein production under control of CMV promoter in BHK-21 cells (Shirahata, 1995). Thus, we tried to enhance production level of recombinant protein from the ras amplified recombinant CHO cell line, I13 by introduction of E1A. The E1A oncogene has been shown to be toxic in CHO cells. Therefore transfection of I13 was done using the E1A gene under the control of either the CMV promoter or the relatively weaker SV40E promoter as described in the Section 'Materials and methods'. Evaluation of the production of hIL-6 from the highest producing established cell

Table 1. Peak values for productivity, 3 day accumulation and cell number

Reporter plasmid	Effector plasmid	Host cell	Clone	Productivity (μg/10^6 cells/day)		Accumulation (μg/ml/3 days)		Cell number ($\times 10^5$)	
				5% CO_2	8% CO_2	5% CO_2	8% CO_2	5% CO_2	8% CO_2
pCMVP-hIL6		CHO	A7	5.6	5.1	10.2	29.2	25	23
pCMVP-hIL6		Ras I	I13	13.2	11.9	17.3	45.2	12	29
	pCMV-E1A	I13	CMV	11.9	16.9	26.8	88.6	19	43
	pSV2-E1A	I13	SV2	13.6	20.6	25.2	84.4	20	43

lines, named CMV and SV2 respectively, cultured in 5% CO_2 was performed as above (Figure 3, circles). When compared to I13, the peak productivity values did not change significantly for both E1A transfected cell line (SV2 and CMV). However, cell number values for SV2 and CMV slightly increased, suggesting that the E1A oncogene may affect cell growth although the effect is not great. This effect seems to have affected the 3 day accumulation of total hIL-6 secreted into the spent medium. The peak 3 day accumulation levels for SV2 and CMV were 25.2 μg/ml/3 day and 26.8 μg/ml/3 day, respectively (Table 1). The peak 3 day accumulation levels for SV2 and CMV were 1.45 and 1.55 times greater than I13. This result demonstrates that E1A and *ras* oncogenes can synergistically enhance hIL-6 production also in CHO cells.

Increased CO_2 concentration improved hIL-6 production from E1A transfected I13

While the E1A oncogene may not have had a significant effect when cultured in 5% CO_2, culturing E1A transfected I13 (SV2 and CMV) in 8% CO_2 had a significant effect on productivity, 3 day accumulation, and cell growth (Figure 3, triangles). Peak productivity was reached on day 18 for a value of 20.6 μg/10^6 cells/day for SV2 and on day 24 for a value of 16.9 μg/10^6 cells/day for CMV (Table 1). Furthermore, peak cell number for both cell lines reached 4.3×10^6 cells (Table 1). The effect of enhanced productivity and cell number greatly affected the 3 day accumulation values for both cell lines (Figure 3, triangles). Peak 3 day accumulation values for SV2 was 84.4 μg/ml/3 day and for CMV was 88.6 μg/ml/3 day (Table 1). Peak 3 day accumulation values increased 3.35 and 3.30 times respectively as compared to those cells cultured in 5% CO_2. Overall top 3 day accumulation level is 88.6 μg/ml/3 day for CMV cultured in 8% CO_2, which is 8.68 fold greater than the value for A7 established by conventional method cultured in 5% CO_2. These results demonstrate that E1A and *ras* oncogenes synergistically enhance the recombinant protein production in CHO cells, and that optimization of culture conditions for recombinant CHO cells such as CO_2 concentration enables further augmentation of production level for recombinant protein.

Discussion

Various systems have been developed to enhance the productivity of animal cells, but none have been totally satisfactory. Recombinant protein cDNA have been inserted into a vector containing strong promoter and/or enhancers, yet has not been successful in raising productivity to commercially desirable levels.

Gene amplification, using various methods, have also been tried, but the procedure is time consuming and laborious. A combination of oncogenes which stimulate certain promoters have been shown to be promising in raising productivity values (Yano, 1994; Teruya, 1995; Shirahata, 1995). Taken together, the Oncogene Activated Production (OAP) system which utilizes a reporter plasmid which contains a recombinant protein cDNA under the control of the CMV promoter and an effector plasmid which contains c-Ha-*ras* oncogene also under the control of the CMV promoter to generate recombinant animal cell lines. The recombinant BHK cell lines established by the OAP system can produce recombinant protein at commercially viable amounts quickly and easily (Shirahata, 1995).

In this report, CHO cells were utilized as host expression cells to validate the OAP system. We firstly generated primed *ras* amplified CHO cells. Although establishment of these reporter less cells was laborious and time consuming, once establishment is finished, any reporter gene can be introduced into these *ras*

primed cells and establishment of hyper producing cell lines is relatively quick and easy. *Ras* clone I was established followed by generation of clone I13. I13 were shown to be have a productivity rate almost twice that of A7 cells established by conventional method (Table 1). This result demonstrates that the OAP system can function also in CHO cells, indicating that Ras protein can be produced in CHO cells in a large amount and can be recruited to the CMV promoter which regulates hIL-6 gene in reporter plasmid, resulted in the enhanced hIL-6 production.

When I13 cells were cultured in 8% CO_2 condition instead of 5% CO_2, we observed that cells grown in 5% CO_2 remained in a monolayer while cells incubated in 8% CO_2 exceeded the monolayer and grew in aggregates. We thus evaluated for recombinant protein production. As expected, while the productivity of I13 did not significantly vary with between 5 and 8% CO_2, the cell number rose 2 fold. This increase in cell number had a corresponding effect on total production of hIL-6, the peak accumulation value for I13 cultured in 8% CO_2 being 45.2 μg/ml/3 days (Table 1). When cell supernatants were evaluated for pH, cultures in 8% CO_2 remained for the most part during long-term culture at pH 7.0 while the cultures in 5% CO_2 dropped to pH 6.6 (data not shown). Eight % CO_2 condition is thought to be more suited than 5% CO_2 for maintaining pH value of cultures at 7.0 during long-term culture, which may resulted in the enhanced cell growth.

The introduction of E1A oncogene was found to boost the productivity of recombinant BHK cells (Shirahata, 1995). When incubated in 5% CO_2 a slight increase was seen in only in cell growth which had a slight effect on 3 day accumulation. Interestingly, when cultured in 8% CO_2, not only cell number, but also productivity increased significantly, resulted in great augmentation of hIL-6 production from E1A transfected I13. Maximum hIL-6 production was shown to be 88.6 μg/ml/3 day (Table 1). This demonstrates that although the reason why the E1A oncogene is active in 8% but not in 5% CO_2 condition remains to be elucidated, recombinant protein production level reached commercially desirable level by utilizing our OAP system in CHO cells and optimizing the culture condition.

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