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Availability of oncogene activated production system for mass production of light chain of human antibody in CHO cells

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Abstract

We previously established a *ras*-oncogene amplified Chinese hamster ovary (CHO) cell line, named *ras* clone I, as an universal host cell line for oncogene activated production (OAP) system to mass-produce recombinant protein by activation of the cytomegalovirus immediate early (CMV) promoter with *ras* protein. The λ light chain (C5λ) of human monoclonal antibody HB4C5 is expected to be potentially useful for lung cancer targeting. We generated a C5λ hyper-producing cell line by transfecting *ras* clone I with the C5λ gene expression plasmid regulated by the CMV promoter, of which productivity was 5.3 times greater than the hyper productive CHO cell line generated by using conventional CHO cells. Introduction of the adenovirus E1A gene into the hyper-producing cell line derived from *ras* clone I resulted in further 9.5 times enhancement of the productivity, suggesting the synergistic effect of E1A and *ras* oncogenes on the recombinant protein production driven by the CMV promoter. In addition, intracellular accumulation of C5λ and upregulation of BiP was found in hyper-producing cell lines which were introduced E1A and *ras* oncogene. This result suggests that excessive intracellular accumulation of C5λ protein, which might be caused by that the amount of produced C5λ in ER is beyond the ability of CHO cells to secrete, might signal the BiP promoter. Our data imply that *ras* clone I is available as a general host cell for establishing the recombinant protein hyper-producing CHO cells by the OAP system, and suggest that further mass production of recombinant proteins in the OAP system can be possible by clarifying the accurate role of upregulated BiP protein.

Abbreviations: BBS, BES-buffered saline; BPB, bromophenol blue; CMV promoter, cytomegalovirus immediate early promoter; DHFR, dihydrofolate reductase; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; PAGE, polyacrylamide-gel electrophoresis; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate

Introduction

Mammalian cells are useful host cells for producing complex and/or glycosylated form of recombinant proteins for clinical applications. Chinese hamster ovary (CHO) dihydrofolate reductase (DHFR) cells (Urlaub et al., 1980) is a most popularly used host cell line and can mass-produce recombinant proteins by using amplifiable selectable marker gene of DHFR and selection drug of methotrexate (MTX) (Kemball-Cook et al., 1994; Lucas et al., 1996; Kim et al., 1998). Cockett et al. (1990) have reported glutamine synthetase gene as an another amplifiable marker. By using this marker, productivity of the tissue inhibitor of metalloproteinases (TIMP) reached the level of 110 μg/10^6 cells/day. However, even by using this method, it is laborious and time-consuming task to establish highly productive cell lines.

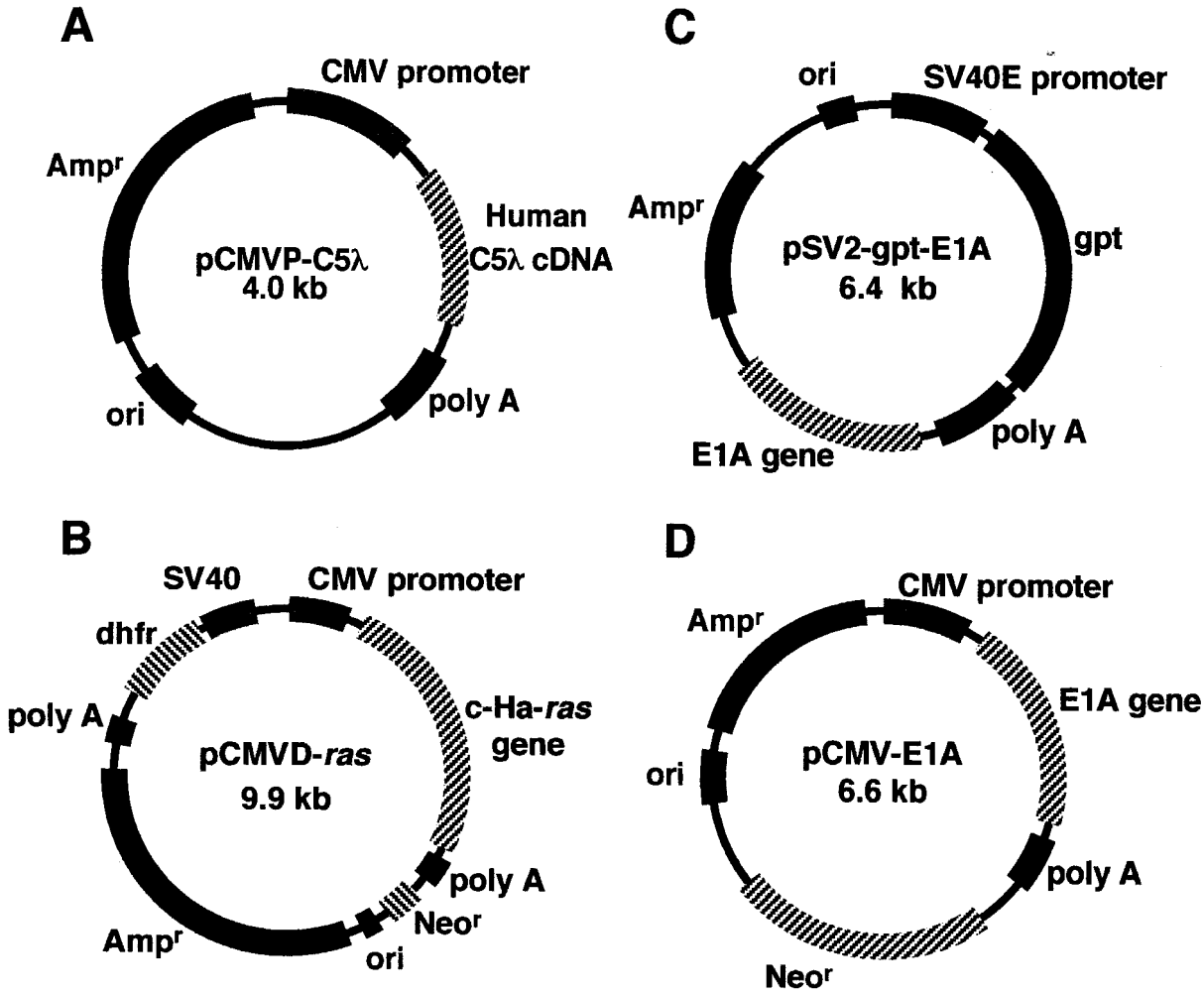

Figure 1. Plasmids used in the OAP system. (A) Reporter plasmid for producing C5λ. (B–D) Effector plasmids for enhancing the transcription of C5λ gene under the control of CMV promoter.

These backgrounds prompted us to development of an oncogene-activated production (OAP) system which enables rapid establishment of recombinant protein hyper-producing cell lines. The OAP system is constructed by introducing a reporter plasmid carrying a gene of interest and effector plasmids carrying oncogenes into host cells. Previously, we have optimized the combination between oncogenes and promoters used in the OAP system. Among the combinations tested, the most suitable combination for efficient protein production was found to be the c-Ha-*ras* oncogene and the cytomegalovirus immediate early (CMV) promoter (Yano et al., 1994). In this combination, c-Ha-*ras* oncoprotein is thought to affect and enhance the transcriptional ability of the CMV promoter which drives the transcription of the gene of interest. Based on this result, we established *ras*-oncogene amplified BHK-21 (TBR-19). When the human interleukin 6 gene was transfected into TBR-19, the productivity of human interleulin 6 reached 1.34 μg/10^6 cells/day, which was about 15 times higher than the control value (Teruya et al., 1995). Furthermore, to rapidly and easily establish recombinant proteins hyper-producing cell lines, we established *ras*-primed cell line (*ras* clone I) by introducing c-Ha-*ras* oncogene into CHO cells and further amplifying the copy number with MTX. Consequently, we have succeeded in establishment of the human interleukin 6 (hIL-6) hyper-producing cell lines by introducing hIL-6 expression plasmid whose expression was driven by the CMV promoter into the *ras* clone I. Moreover, when the adenovirus E1A oncogene was transfected into the hIL-6

Table 1. Enhancement of C5λ production by the OAP system

Cell lines	Introduced-oncogenes	Peak productivity (μg/10^6 cells/day)
wt-26	–	0.09±0.001
wtI-16	*ras*	0.48±0.03
wtIE-13	*ras* + E1A	4.60±0.35

hyper producing *ras* clone I, peak productivity of one of cloned cell line reached 20.6 μg/10^6 cells/day, which is comparable to the top productivities reached by using mammalian cells (Katakura et al., 1999). In this report, we evaluated the availability of this OAP system for producing other recombinant protein than hIL-6.

A human-human hybridoma clone, HB4C5 was generated by fusing human lymphoblastoid cells and lymphocytes from a lung cancer patient (Murakami et al., 1985). The HB4C5 cells secrete monoclonal antibody (mAb) which specifically recognizes lung cancer cells, especially adenocarcinoma (Yano et al., 1988). Even only the λ light chain of this mAb (C5λ) as well as the whole molecule can recognize lung cancer cells, and further has a higher affinity to cancer tissues than the original HB4C5 mAb (Nishimura et al., 1999). Thus, we constructed the reporter plasmid expressing C5λ protein and attempted to produce C5λ molecule, which can be used for diagnosis of human lung cancer by radioisotope imaging. As a result, C5λ hyper-producing cell line was rapidly and easily established from *ras* clone I, and the productivity was further increased by introducing E1A oncogene. These results suggest that the *ras* clone I can be used as a host cell line to rapidly and easily establish the hyper-producing cell lines for various recombinant proteins.

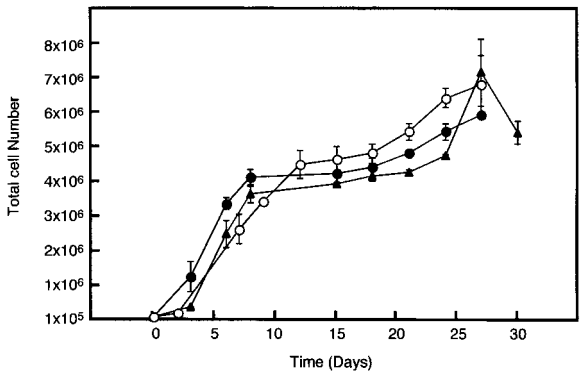

Figure 2. Growth curves of C5λ producing cell lines. Each cell lines were seeded into 35-mm dishes at 1×10^5 cells/dish. The number of cells was counted by using a cell counter (Sysmex F-300, TOA, Kobe, Japan) every 3 days. (▲), wt-26 (control); (●), wtI-16 (*ras*); (○), wtIE-13 (*ras* + E1A).

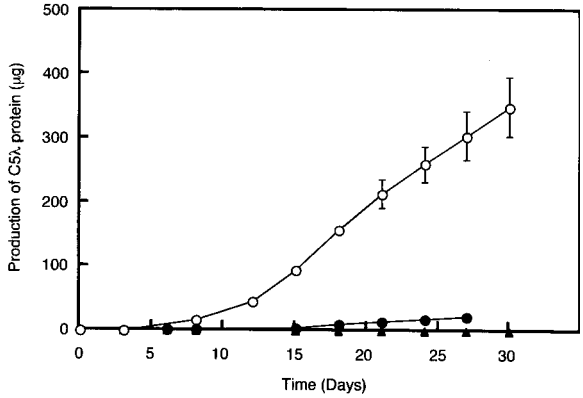

Figure 3. The accumulation of the C5λ protein in culture media. Cells were cultured in 35 mm dishes in 2 ml of DMEM supplemented with 5% serum. The amount of C5λ protein secreted into the medium was assayed by sandwich ELISA every 3 days and the total amount of C5λ secreted was calculated. (▲), wt-26 (control); (●), wtI-16 (*ras*); (○), wtIE-13 (*ras* + E1A).

Materials and methods

Construction of expression plasmids

A pRc/CMV vector was purchased from Invitrogen (San Diego, CA, USA). The human c-Ha-*ras* oncogene (pSK2) was obtained from Japanese Cancer Research Resources Bank (JCRB, Tokyo, Japan). The human c-Ha-*ras* gene fragment was excised from pSK2 by *Not*I digestion. The fragment was blunted and subcloned into the *Sma*I site of pBluescript II, and then excised by *Hin*dIII and *Not*I and cloned into the pRc/CMV, and named pCMV-c-Ha-*ras*. Next, pSV2-*dhfr* was digested with *Pvu*II and *Bam*HI to excise SV40-E-*dhfr*-polyA fragment. This SV40-E-*dhfr*-polyA fragment was blunted and inserted into the *Nru*I site upstream from the CMV promoter of the pCMV-c-Ha-*ras* plasmid, and named pCMVD-c-Ha-*ras* (Figure 1B). pSV2-gpt-gE1A (Figure 1C) carrying the E1A gene, which was derived from adenovirus type 12, was a generous gift from Dr K. Onodera (The University of Tokyo). The E1A gene was excised from pSV2-gpt-gE1A with *Hae*III and *Acc*I digestion. This E1A gene fragment was blunted and subcloned into *Sma*I site of pBluescript II, then inserted into the *Xba*I-*Apa*I site of pRc/CMV, and named pCMV-E1A (Figure 1D). The *neo* res-

istant gene was excised from pRc/CMV vector and named pCMVP. C5λ cDNA (Tachibana et al., 1993) was inserted downstream from the CMV promoter of pCMVP and named pCMVP-C5λ (Figure 1A).

Culture media

CHO cells lacking the *dhfr* gene were cultured in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 0.1 mM hypoxanthine (Wako, Osaka, Japan) and 0.016 mM thymidine (Wako) (DMEM-HT) with 5% fetal bovine serum (FBS, Whittaker Bioproducts, Walkersville, MD, USA). The CHO cells transfected with pCMVD-c-Ha-*ras* was cultured in DMEM with 5% dialyzed FBS (dFBS) and 50 nM methotrexate (MTX) (Sigma, St. Louis, MO, USA). dFBS was prepared by dialyzing FBS using the Spectra/Por membrane (Molecular weight cut: 1000: Specrum, Houston, TX, USA). Cells were cultured in 5% CO_2/95% air atmosphere at 37 °C.

Cell culture and DNA transfection

CHO cells were grown in DMEM supplemented with 5% FBS. Transfection into CHO cells was carried out by the calcium phosphate transfection protocol (Chen et al., 1987). Briefly, 3×10^5 cells were plated onto 60-mm plastic dishes and grown overnight. Fifteen μg of expression plasmid were mixed with 25 μl of 0.25 M $CaCl_2$ and 250 μl of 2 × BBS, and filled up to a final volume of 500 μl with water, then incubated at room temperature for 30–60 min. This calcium phosphate DNA solution was added dropwise onto the dishes, and swirled to mix completely. Cells were cultured for 15–24 h at 3% CO_2/97% air atmosphere. After the cells were washed twice with PBS, fresh medium was added.

Establishment of recombinant CHO cells

To establish stable cell lines expressing recombinant protein by conventional calcium phosphate coprecipitation method, CHO cells were cotransfected with 13.5 μg of pCMVP-C5λ and 1.5 μg of pSV2-*bsr*. When confluence was reached, 5 μg ml^{-1} of blasticidin S (Funakoshi, Tokyo, Japan) was added to the medium. Single clones were isolated using the limiting dilution cloning, then were evaluated for C5λ productivity to select the clone showing the highest productivity. We previously established a *ras* amplified CHO cell line named *ras* clone I, which was isolated from the CHO cells transfected with pCMVD-c-Ha-*ras* (Katakura et al., 1999). In this study, pCMVP-C5λ was introduced into the *ras* clone I as above. Then the clone showing the highest productivity of C5λ protein was selected by limiting dilution cloning. This clone was further transfected with pCMV-E1A plasmid along with the selection marker of pSV2-*hph*. Selection was performed using 200 μg ml^{-1} of hygromycin B (Wako). Single clone was isolated by limiting dilution cloning and assessed its productivity.

Determination of concentration of the secreted C5λ protein

Cell-culture supernatants were assayed with sandwich ELISA using goat anti-human λ polyclonal antibody (TAGO, Burlingame, CA, USA) as capturing antibody and peroxidase labeled goat anti-human λ polyclonal antibody (TAGO) as detecting antibody. The human λ chain (Gelco Diagnostic, Shreveport, LA, USA) was used as a standard for calibration of the assay.

Northern analysis for C5λ and E1A mRNA

Total RNA was isolated from each subconfluent culture using the TRIzol reagent (Gibco BRL, Gaithersburg, MD). Six μg of total RNA was subjected to 1.2% agarose gel electrophoresis under denaturing condition. Separated RNA was transferred to Hybond-N$^+$ membrane (Amersham, Buckinghamshire, UK), and hybridized with C5λ or E1A fragment labeled with fluorescein-dCTP by Random prime labeling module (Amersham). Subsequently, hybridized bands were visualized by *Gene Images* CDP-Star detection module (Amersham). After stripping the C5λ or E1A probe, the same membrane was rehybridized with β-actin probe (Wako).

Western blotting

Cell-culture supernatants or cell pellets corresponding to equivalent cell number were prepared. These samples were denatured in 1 volume of 2× sample buffer (50 mM Tris-HCl, 10% glycerol, 2% SDS, 2% 2-melcaptoethanol, 0.1% BPB) by boiling for 5 min, and then run on 10% SDS/PAGE denaturing gel. Separated proteins were transferred to nitrocellulose membrane (Schlicher and Shuell, Dassel, Germany) by electroblotting. C5λ protein was detected with the peroxidase-labeled anti-human λ chain antibody (TAGO) and visualized with enhanced chemiluminescence (ECL: Amersham).

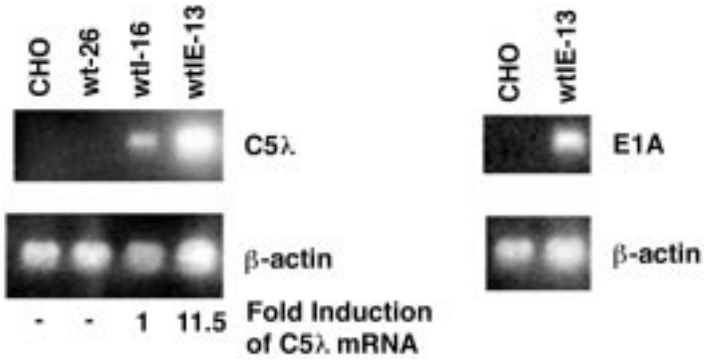

Figure 4. Expression of C5λ and E1A mRNA in each C5λ producing cell lines. RNA blots were probed with a C5λ or E1A probe (top panels) and with a *β*-actin probe as internal control (bottom panels). The amount of C5λ mRNA in wtIE-13 (*ras* + E1A) was quantitatively determined by Kodak EDAS system and normalized against that in wtI-16 (*ras*) (lane 2).

Northern analysis of BiP mRNA

mRNA was prepared by using a QuickPrep Micro mRNA Purification Kit (Pharmacia, Uppsala, Sweden). BiP cDNA fragment amplified by PCR using primers (5′-ACTGGAATTCCTCCTGCTCC-3′ and 5′-GTTCCTGGTATCAATGCGC-3′) was labeled with fluorescein-dCTP by using the Random prime labeling module (Amersham). Blotting, hybridization and detection procedures were performed as described above. Band intensities were quantified with the Kodak EDAS system (Eastman Kodak Co., Rochester, NY, USA).

Results and discussion

Expression of C5λ protein under a control of the CMV promoter activated by oncogene

We introduced pCMVP-C5λ which express C5λ protein under the control of the CMV promoter, along with the selection marker gene of pSV2-*bsr* into the *ras* clone I. After selection with blastcidin S, C5λ hyper-producing cell lines were obtained by limiting dilution cloning. Concentration of secreted C5λ protein was tested by sandwich ELISA. Productivities for recombinant CHO cell lines generated by conventional method or OAP system were listed in Table I. Compared with a control highest producing cell line (wt-26) generated by using CHO cells as host cells, the productivity of C5λ protein for a recombinant CHO cell line (wtI-16) generated by introducing C5λ expression plasmid into the *ras* clone I increased 5.3 fold. Furthermore, E1A oncogene was introduced into the wtI-16, and then the highest producing clone was selected by limiting dilution cloning. A highest producing recombinant CHO cell line (wtIE-13) which was generated by introducing E1A gene into the wtI-16 secreted C5λ protein 51 times greater than wt-26. Next, we assessed the proliferative potential of these recombinant CHO cells, because recombinant protein hyper-producing cells frequently show slower growth rates. However, the growth rate of wtIE-13 was nearly as high as that of wt-26 (Figure 2). Moreover, Figure 3 shows the accumulated production of C5λ protein by wt-26 (control), wtI-16 (*ras*) and wtIE-13 (*ras* + E1A) in long-term culture. In this figure, a noteworthy point is that the straight increase of the accumulated amount of C5λ protein in wtIE-13 demonstrates that hyper-producing cell lines stably can produce recombinant proteins even in long-term culture. Thus, these results indicate that wtIE-13 can be available for mass production of C5λ protein. In some expression systems, high overexpression of recombinant protein has been reported to lead to the production of inactive protein resulting from aggregation, misprocessing and misfolding, etc. We have analyzed the biological activity of C5λ produced from *ras* amplified CHO cells (*ras* clone I). As a consequence, tumor radioimmunoimaging experiments in LC6 (lung squamous cell carcinoma line)- xenografted nude mice by the injection of ^{125}I-labeled recombinant C5λ, which produced from *ras* clone I, gave tumor-specific and recombinant human λ light chain-dependent images on day 5 postinjection (manuscript in preparation). This result indicates that C5λ produced from *ras* clone I can be potentially useful for lung cancer targeting.

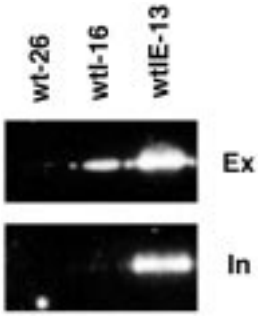

Figure 5. Evaluation of the extracellular and intracellular amounts of C5λ proteins. Secreted C5λ protein into the culture supernatant (Ex) and accumulated C5λ protein inside the cell (In) in C5λ producing cell lines (wt-26, wtI-16, wtIE-13) were analyzed by western blot analysis as described in the section Materials and Methods.

Evaluation for availability of the OAP system at the transcription level

In order to evaluate the availability of the OAP system at the transcription level, total RNA was prepared from these cell lines and subjected to Northern blot analysis (Figure 4). The results indicate that C5λ transcription was enhanced by primed *ras* in wtI-16 and further augmented by simultaneous introduction of *ras* and E1A in wtIE-13. This result is consistent with the result that C5λ productivity was enhanced by *ras*, and synergistically by *ras* and E1A (Table I). These results demonstrate that recombinant protein hyper-producing ability of the recombinant CHO cells generated by the OAP system can be explained by the transcriptional activation of the CMV promoter by oncogenes, leading to augmented transcription of the target gene inserted downstream from the CMV promoter. Furthermore, this result shows that *ras* and E1A synergistically enhance the transcriptional ability of the CMV promoter, suggesting that *ras*-signaling cascade can be enhanced by E1A, and that all of target genes inserted downstream from the CMV promoter can be overexpressed in the OAP system.

Evaluation for availability of the OAP system at the translation and translocation level

In order to evaluate the availability of the OAP system at the translation and translocation level, we examined the intracellular and extracellular amount of recombinant protein by Western blotting. As shown in Figure 5, the extracellular amount of C5λ protein increased by introduction of *ras*, and synergistically augmented by simultaneous introduction of *ras* and E1A, which is consistent with the result of sandwich ELISA (Table I). Furthermore, we tested for the amount of intracellular C5λ protein. Although C5λ protein can not be detected intracellularly in wt-26 and wtI-16, C5λ protein greatly accumulated intracellularly in wtIE-13. To evaluate the augmented production and its concurrent intracellular accumulation of C5λ, C5λ mRNA level shown in Figure 4 were quantified with the Kodak EDAS system. As a result, C5λ mRNA level in wtIE-13 was about 11.5 times higher than that in wtI-16. On the other hand, the productivity of C5λ protein in wtI-16 and wtIE-13 were 0.48 μg/10^6 cells/day and 4.6 μg/10^6 cells/day (Table I), and a ratio of the productivity of wtIE-13 to wtI-16 was 9.5 times. This induction value of the C5λ productivity was a little lower than the value of transcriptional induction level in wtIE-13. These results indicate that promoter activation by *ras*/E1A in the OAP system does not exactly lead to full augmentation of protein production. On the basis of these findings, we speculate that the amount of produced protein is beyond the ability of CHO cells to secrete, and then excess amounts of C5λ protein may be accumulated intracellularly in wtIE-13.

Accumulation of intracellular C5λ protein

We anticipated that large amount of intracellular accumulation of C5λ protein observed in wtIE-13 might induce expression of BiP (heavy chain binding protein) dependent upon the ER overload (Kozutsumi et al., 1988). To assess this possibility, we evaluated for the expression of BiP mRNA by Northern blotting. As shown in Figure 6, BiP transcript was shown to be approx. 2-times greater in wtIE-13 than other recombinant CHO cells. The mammalian BiP promoter is involved in stress-inducible BiP expression (Chang et al., 1989; Li et al., 1994, 1997). Thus, excessive intracellular accumulation of C5λ protein might signal the BiP promoter, resulted in the upregulated transcription of BiP. The stressed ER is linked to the UPR (unfolded protein response) pathway as well as to the activation of the NF-κB transcription factor complex. Pahl et al. has mentioned that the pathway mediated by NF-κB activation may sense the amount of protein processed through the ER and signal ER overload, and that such a signal could trigger increased ER biosynthesis to relieve the cognition in this organelle (Pahl et al., 1995). Thus, the accurate role of upregulated BiP protein with ER overload must be elucidated, which would lead to enable us to hyper-produce the recombinant protein in the OAP system.

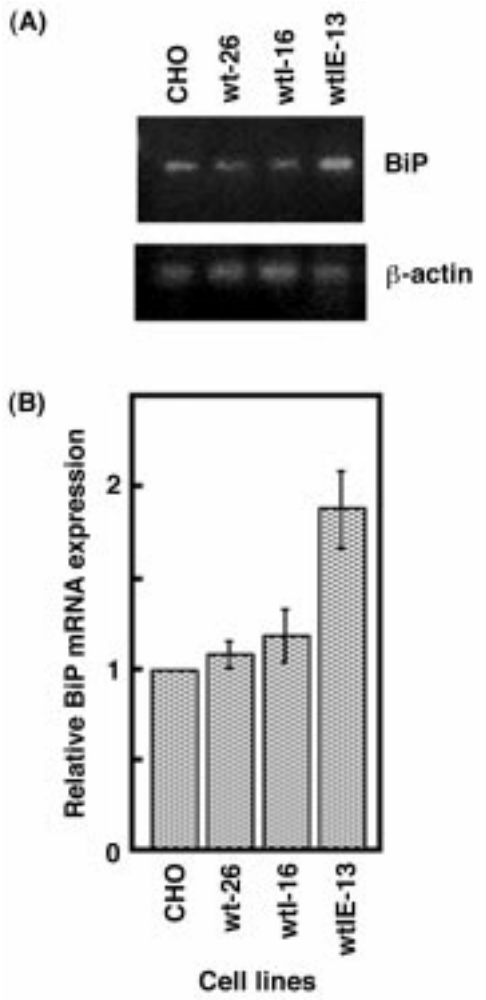

Figure 6. Northern Blot analysis of BiP mRNA in each C5λ producing cell lines. (A) mRNA (0.6 μg) was electrophoresed on a 1.2% agarose gel under denaturing condition, transferred to Hybond-N^+ membrane (Amersham), and hybridized with fluorescein-labeled BiP (top panel) or β-actin (bottom panel) probes. (B) The amount of BiP mRNA was quantitatively determined by Kodak EDAS system and normalized against that in CHO cells (lane 1).

Conclusion

In conclusions, we have established a useful host cell line termed *ras* clone I, which enable us to rapidly establish recombinant proteins hyper-producing cell lines. In this study, we clarified that C5λ protein can be produced at 5.3-fold higher levels when using *ras* clone I as host cells as compared to the conventional method. Furthermore, when E1A oncogene was introduced into the recombinant *ras* clone I expressing C5λ protein, the C5λ productivity was boosted up to 51-fold higher than the control. The growth potential of C5λ hyper-producing cell lines was maintained for at least 30 days, indicating that mass production of C5λ protein is possible by using this cell line. Moreover, in recombinant proteins hyper-producing cell lines such as wtIE-13, a large amount of proteins were accumulated inside the cells, which might be leading to upregulation of BiP. This suggests that further mass production of recombinant proteins in the OAP system can be possible by clarifying the accurate role of upregulated BiP protein and elucidating these speculations.

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