Gene, 108 (1991) 193–200

GENE 06132

Efficient selection for high-expression transfectants with a novel eukaryotic vector

(Recombinant DNA; β-actin promoter; bovine papilloma virus; transfection; interleukin-2; G418 selection; copy number)

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Received by A. Nakazawa: 27 June 1991
Revised/Accepted: 6 August/7 August 1991
Received at publishers: 3 September 1991

SUMMARY

We have developed a new expression vector which allows efficient selection for transfectants that express foreign genes at high levels. The vector is composed of a ubiquitously strong promoter based on the β-actin promoter, a 69% subregion of the bovine papilloma virus genome, and a mutant neomycin phosphotransferase II-encoding gene driven by a weak promoter, which confers only marginal resistance to G418. Thus, high concentrations of G418 (approx. 800 μg/ml) effectively select for transfectants containing a high vector copy number (> 300). We tested this system by producing human interleukin-2 (IL-2) in L cells and Chinese hamster ovary (CHO) cells, and the results showed that high concentrations of G418 efficiently yielded L cell and CHO cell transfectants stably producing IL-2 at levels comparable with those previously attained using gene amplification. The vector sequences were found to have integrated into the host chromosome, and were stably maintained in the transfectants for several months.

INTRODUCTION

In recent years, various techniques have been developed for efficient introduction of DNA into cultured eukaryotic cells, and have allowed expression of various genes cloned in an expression vector for the studies of gene regulation and protein biosynthesis (Okayama and Berg, 1982; Wong et al., 1985; Miyazaki et al., 1989). Although many experiments can be performed with cells transiently expressing foreign genes, it is often necessary to isolate cell lines that permanently express foreign genes. Especially when large quantities of a product are required for the experiments, the success of the experiments depends on the isolation of a cell line highly expressing the foreign gene.

The level of expression of the introduced gene depends mostly on the strength of the promoter and the copy number of the integrated DNA in the transfected cells. Therefore, expression vectors utilize very strong promoters, such as the SV40 early or late promoter, the CMV-IE promoter, the cytoplasmic *Ac* promoter, and the adenovirus major late promoter (Okayama and Berg, 1982; Wong et al., 1985; Foecking and Hofsteffer, 1986; Miyazaki et al., 1989). Two approaches have been undertaken to increase the vector copy number in stably transfected cells. One is to use co-amplification of introduced DNA mediated by some selectable marker genes, among which the *dhfr* gene is most widely used (Kaufman and Sharp, 1982; McCormick et al.,

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Abbreviations: *Ac*, gene encoding β-actin; *AG*, modified chicken β-actin promoter; bp, base pair(s); βGal, β-galactosidase; BPV, bovine papilloma virus; *CAG*, *AG* promoter with CMV-IE enhancer; CHO, Chinese hamster ovary; CMV, cytomegalovirus; *dhfr*, gene encoding dihydrofolate reductase; HMW, high molecular weight; HSV, Herpes simplex virus; IE, immediate early; IL-2, interleukin-2; kb, kilobase(s) or 1000 bp; LTR, long terminal repeat; MEM, modified Eagle's medium; *neo*, gene (Nm^R) encoding NPT II; Nm, neomycin; NPT II, Nm phosphotransferase II; nt, nucleotide(s); *ori*, origin of DNA replication; ^{R}, resistance; RSV, Rous sarcoma virus; SV40, simian virus 40; *tk*, gene encoding thymidine kinase; u, unit(s).

1984). Cells harboring more than 1000 copies of the *dhfr* gene could be selected by sequentially increasing concentrations of methotrexate. Although this system appears to be the most powerful technique for obtaining high levels of constitutive expression in mammalian cells, the disadvantages of this method include requirement of the cells deficient in *dhfr* expression for effective selection, prolonged time required for the repeated cloning of the resistant cells, and instability of the amplified genes when selective pressure is removed.

Another way to increase the vector copy number is to use expression vectors which contain all or part of the BPV genome. The BPV fragment allows the vector to replicate to 50–100 copies per cell, resulting in high levels of constitutive expression of the inserted genes (Sarver et al., 1982; DiMaio et al., 1982; Binetruy et al., 1982; Law et al., 1983). Recently, another expression vector based on the Epstein–Barr virus genome has been reported (Margolskee et al., 1988). However, selection of transformants is still required to achieve high levels of expression with these vectors.

In this report, we describe a new vector for stable expression with the following distinct features. First, this vector contains a ubiquitously strong promoter derived from the chicken *Ac* gene (Fregien and Davidson, 1986; Miyazaki et al., 1989). Thus, this vector can be utilized in a wide variety of cell lines. Second, the selective marker used in this vector is the *neo* gene with a mutation which lowers the resistance to G418 (Yenofsky et al., 1990). This marker gene is driven by a weak promoter. Therefore, only transfectants that contain a high copy number of the vector survive the selection by high concentrations of G418. This selection should work in most eukaryotic cells. Third, the vector contains the BPV 69% fragment which allows the vector to replicate autonomously. This sequence facilitates the production of high-copy-number transfectant clones by G418 selection. We demonstrate that the new expression vector is utilized to rapidly obtain transfectants constitutively producing human IL-2 at high level.

RESULTS AND DISCUSSION

(a) Promoter selection

Use of efficient promoter elements for construction of expression vectors enhances the levels of expression of the linked exogenous genes in the transfectants. We have previously shown that the chicken *Ac* promoter and its derivative, the *AG* promoter, are highly active in a wide range of cell types (Miyazaki et al., 1989). In order to confirm these observations, the strength of the promoters was evaluated by βGal activity after transient transfection into several cell lines of different cell types in comparison with the RSV LTR and the *CMV-IE* promoter, both of which are considered to be very active in various cell lines (Gorman et al., 1982; Foecking and Hofsteffer, 1986). As shown in Table I, the *Ac* and *AG* promoters are more active than the RSV LTR in all these cell lines. The *CMV-IE* promoter exhibited much higher activity than the other ones in L cells and CHO cells, although it showed only weak activity in F9 cells, an undifferentiated embryonal carcinoma cell line. With the hope of combining the advantages of both the *Ac* and *CMV-IE* promoters, we have engineered another promoter, designated the *CAG* promoter, by connecting the CMV-IE enhancer sequence to the AG promoter. This composite promoter produced the highest levels of βGal in all the four cell lines tested. These results suggest that the CMV-IE enhancer and the *Ac* enhancer additively stimulate tran-

TABLE I

Comparison of βGal activities directed by various promoters in different cell lines

lacZ constructs[a]	Relative βGal activity[b]			
	L	F9	CHO	HepG2
pAc-*lacZ*	1.0	1.0	1.0	1.0
pAG-*lacZ*	2.8	2.3	0.4	1.5
pCAG-*lacZ*	17.0	1.7	5.4	2.5
pCMV-*lacZ*	11.4	0.1	4.7	0.9
pRSV-*lacZ*	1.2	0.1	0.4	0.6
p*lacZ*	<0.01	<0.01	<0.01	<0.01

[a] Plasmids pAc-*lacZ*, pAG-*lacZ*, and pRSV-*lacZ* were described previously (Miyazaki et al., 1989). pCMV-*lacZ* was produced by replacing the *Xho*I-*Hin*dIII segment including the *Ac* promoter in pAc-*lacZ* with the approx. 660-bp *Hin*cII-*Hin*dIII CMV-IE enhancer/promoter derived from CDM8 plasmid (Seed and Aruffo, 1987). The *Hin*cII-*Nco*I region of the CMV-IV enhancer was also excised from CDM8, and was introduced using a *Sal*I linker into an *Xho*I site located directly 5′ to the *AG* promoter in pAG-*lacZ*, resulting in pCAG-*lacZ*. p*lacZ* is a *lacZ* plasmid lacking any eurkaryotic promoter.

[b] L *tk*⁻ cells, F9 *tk*⁻ cells (Gmür et al., 1980), and HepG2 cells (Aden et al., 1979) were grown in Dulbecco's MEM supplemented with 10% fetal bovine serum. CHO *dhfr*⁻ cells (Urlaub and Chasin, 1980) were grown in MEM α-medium with ribonucleosides and deoxyribonucleosides supplemented with 10% fetal bovine serum. For transient expression assay, transfection of DNA was performed by the Ca·phosphate coprecipitation procedure as described (Miyazaki et al., 1986; Chen and Okayama, 1987). Cells (1×10^5) were seeded in a 6-cm dish, and were transfected 20 h later with 5 μg of plasmid DNA. Cells were washed after 20 h and re-fed with 5 ml of fresh medium. They were harvested after 48 h incubation, and cell extracts were prepared and assayed for βGal activity by incubation with *o*-nitrophenyl-β-D-galactopyranoside as described (Herbomel et al., 1984). The βGal activity directed by these constructs was measured relative to that generated by pAc-*lacZ* (set at 1.0) for each cell line. The βGal activities by pAc-*lacZ* in these cell lines relative to that generated in L cells (set at 1.0) were 16.0 in F9, 2.3 in CHO, and 28.0 in HepG2. The values shown are the average of duplicate experiments.

scription from the *AG* promoter. Thus, we have chosen the *CAG* promoter for the construction of expression vectors.

(b) Construction of expression vectors for stable transfection

We constructed an expression vector by introducing the *CAG* promoter, rabbit β-globin gene sequences including a polyadenylation signal, and an SV40 *ori* into pUC13 (Fig. 1). This vector, designated pCAGGS, was designed for transient expression, and exhibited high-level production of βGal, IL-2, IL-5, leukemia inhibitory factor and others in various cell types (data not shown).

We engineered another expression vector for stable expression by introducing the BPV 69% fragment and a selectable marker gene into pCAGGS. The *BPV* sequence was used to allow the vector to replicate extrachromosomally in the transfected cells. In an attempt to develop an efficient means of selection for the transfectants containing a high copy number of the plasmid, we utilized a weak expression unit of the *neo* gene which confers resistance to an Nm derivative, G418, and has been widely used as a selectable marker for many kinds of cells. This *neo* expression unit derives from the pMC1neo polyA + plasmid (Thomas and Capecchi, 1987), and has a spontaneous mutation in the coding region of the *neo* gene, resulting in a severalfold reduction in the activity of NPT II (Yenofsky et al., 1990). Furthermore, the polyoma enhancer sequence was removed from the HSV *tk* promoter prior to integration into pCAGGS (see Fig. 1 legend). Thus, the *neo* expression unit used in the vector construction confers only marginal resistance to G418, so that only transfected cell containing many copies of the vector could survive in the presence of high concentrations of G418. Fig. 1 illustrates the final construct of the expression vector, which was designated pABWN.

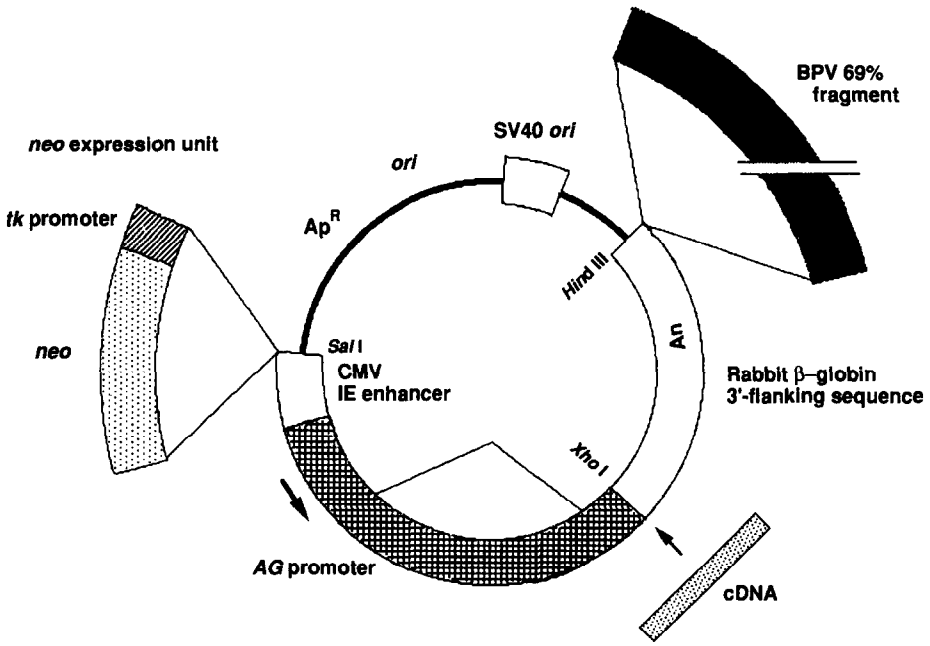

Fig. 1. Construction of pCAGGS and its derivatives. Plasmid pCAGGS was constructed from pUC13 (Messing, 1983). First, the *Xho*I-*Eco*RI segment of pAGS-3 including the *AG* promoter was inserted in place of the *Eco*O109-*Eco*RI region of pUC13 after changing the *Eco*O109 site to an *Xho*I site by an *Xho*I linker. Next, the *Eco*RI-*Sal*I region of this plasmid was replaced by the *Eco*RI-*Xho*I fragment from pKCR-3 (O'Hare et al., 1981) including a polyadenylation signal (An) and a 3′-flanking sequence of the rabbit β-globin gene. The *Bam*HI fragment from pAGS-*lacZ* including an SV40 *ori* was introduced into the *Pvu*II site of the above construct by using a *Bam*HI linker. Then, the *Sal*I CMV-IE enhancer fragment (see Table I) was inserted in place of the *Aat*II-*Xho*I region of this construct after changing the *Aat*II site to a *Sal*I site by a *Sal*I linker. Finally, a unique *Xho*I site was made in the *Eco*RI site by an *Xho*I linker, resulting in pCAGGS. A mutant *neo* gene was derived from pMC1neo polyA + (Thomas and Capecchi, 1987), which was purchased from Stratagene Inc. (La Jolla, CA), and had a point mutation in its coding region which reduces the activity of NPT II by one order of magnitude (Yenofsky et al., 1990). Plasmid pMC1neo polyA + has expression elements for the *neo* gene consisting of a mutant polyoma enhancer and the *tk* promoter of HSV, and an SV40 polyadenylation signal. The *Xho*I-*Sal*I fragment containing all these *neo* expression elements and the *Eco*RI-*Sal*I fragment lacking the mutant polyoma enhancer were excised from pMC1neo polyA +, and inserted into a *Sal*I site of pCAGGS by a *Sal*I linker. The resulting plasmids were designated pCXN and pCXWN. Plasmids pABN and pABWN were constructed by inserting the 5.4-kb *Hin*dIII-*Bam*HI fragment derived from pdBPV-MMTneo (342-12) (Law et al., 1983), that is the so-called 69% fragment of BPV-1, into a unique *Hin*dIII site of pCXN and pCXWN using a *Hin*dIII linker, respectively. A truncated form of the IL-2 cDNA (nt 41–542; Taniguchi et al., 1983) was introduced into a commonly unique *Xho*I site of pCXN, pCXWN, pABN and pABWN using an *Xho*I linker.

(c) IL-2 production by stable transfectants with the novel expression vector

We next evaluated the above vector by IL-2 production in L cells and CHO cells stably transfected by the pABWN vector containing the human IL-2 cDNA. These cells were chosen because they have commonly been used for expression of various genes or cDNAs. To assess the contribution of the BPV 59% fragment and the weak *neo* expression unit included in the vector, we made three additional vectors by modifying the pABWN plasmid as follows: pABN, which has the polyoma enhancer in the *neo* expression unit, pCXN, which also has this enhancer, but lacks the BPV sequence, and pCXWN, which lacks both the enhancer and the BPV sequence.

We introduced the above constructs containing the human IL-2 cDNA into L cells using the Lipofectin method (Felgner et al., 1987), and cultured these transfected cells in the presence of 200 μg/ml G418 for 18 days. Then, resistant colonies (>200) were pooled and subcultured at different G418 concentrations between 0 and 800 μg/ml. After one month and after three months, we assayed the culture supernatants for IL-2. As shown in Fig. 2, pABWN-IL2 could direct higher levels of IL-2 production than the other three constructs, especially after selection at higher G418 concentrations. A fivefold increase in IL-2 production was observed in pABWN-IL2 transfectants after selection at 800 μg G418/ml, as compared with that at 400 μg/ml. This expression level (4.3×10^4 u/10^6 cells/48 h) is comparable with the production by the stable expression systems so far reported which included the isolation of overexpressing clones (Conradt et al., 1986; Onomichi et al., 1987; Karasuyama and Melchers, 1988). Selection at higher concentrations of G418 (1200 or 1600 μg/ml) did not further enhance the IL-2 production from pABWN-IL2 transfectants (data not shown).

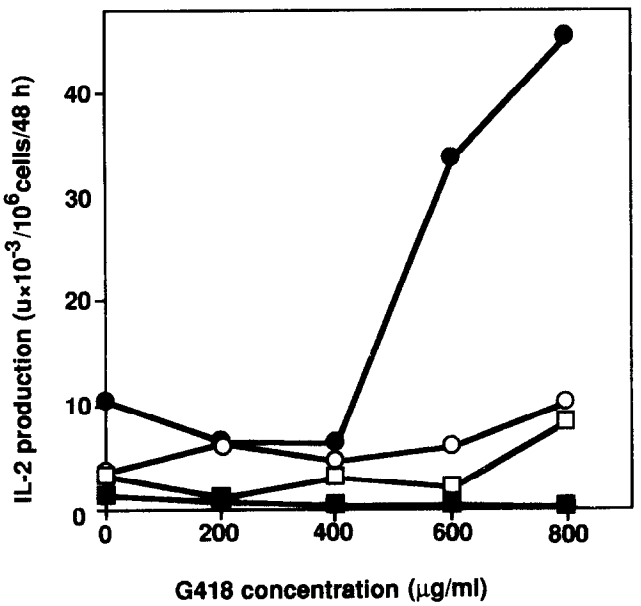

Fig. 2. Effects of G418 concentration on IL-2 production in L cell transfectants. L cells (2×10^5) were seeded in a 6-cm dish, and transfected 20 h later with 10 μg of pABWN-IL2 (blackened circle), pABN-IL2 (open circle), pCXWN-IL2 (blackened box), and pCXN-IL2 (open box) using Lipofectin reagent (Bethesda Research Laboratories, Gaithersburg, MD) (Felgner et al., 1987). Cells were washed after 6 h and re-fed with 5 ml of fresh medium. After 24 h, the medium was changed to selective medium containing 200 μg G418/ml (Geneticin; Sigma, St. Louis, MO). Selective medium was changed every two days. Pools of the stable transfectants were then subcultured in medium containing the indicated concentrations of G418. The cells were subcultured at 1:20 once a week. After one month, the cells were cultured in the absence of G418, and IL-2 assay of the culture supernatants was performed by enzyme-immuno assay using a commercially available assay kit (DuPont, Willington, DE). 1×10^3 u of IL-2 correspond to 0.33 μg of IL-2.

(d) DNA and RNA blot analyses of L cell transfectants

Southern-blot analysis of DNA from the L cell transfectants subcultured at different G418 concentrations revealed that the enhanced IL-2 production directed by pABWN-IL2 depended on the increase of the vector copy number which reached, on the average, 300 per diploid genome at 800 μg G418/ml (Fig. 3). In contrast, the cell pools from transfection with pABN-IL2 containing the BPV fragment and the polyoma enhancer for *neo* expression showed only a slight increase of the vector copy number (approx. 20) as the concentration of G418 increased. Northern-blot analysis of these subcultured pools indicated that the amounts of IL-2 and *neo* mRNA also increased in proportion as the selective pressure by G418 increased.

(e) IL-2 production in CHO cells with the expression vectors

We also examined the IL-2 productivity of these four constructs in CHO cells. The transfected cells were cultured in the presence of 200 μg G418/ml for 20 days. Then we picked five colonies from pABWN-IL2 and pCXWN-IL2 transfections, and established clones in 200 μg G418/ml (see below). The remaining colonies (>100) were pooled and subcultured at different G418 concentrations. We assayed these subcultured pools for their ability to secrete human IL-2 after one month. High-level IL-2 production by pABWN-IL2 was also obtained in CHO cells after selection at 800 μg G418/ml, while the other vectors showed low levels of expression (not shown). Southern-blot analysis of DNA from the pABWN-IL2 transfectants confirmed that this overproduction was also mediated by the selection of the transfectants with a high vector copy number (not shown).

BPV-based vectors have been shown to stay in the transfected cells as multicopy circular episomes (Zinn et al., 1982; Mitrani-Rosenbaum et al., 1983), which can be detected by Southern-blot analysis of the low-M_r DNA iso-

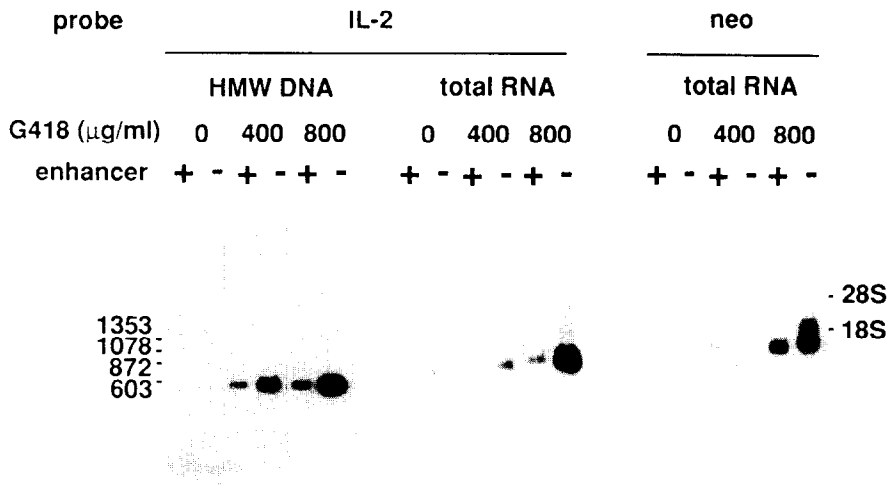

Fig. 3. Southern- and Northern-blot analysis of stably transfected L cells. L cells stably transfected with pABN-IL2 (enhancer +) and pABWN-IL2 (enhancer –), described in Fig. 2 legend, were further selected with the indicated concentrations of G418 for another month and subjected to Southern- and Northern-blot analysis. HMW DNA was prepared as described (Maniatis et al., 1982) and digested with *Eco*RI. Then 10 μg of each digest were electrophoresed in 1.2% agarose gel and transferred onto a nylon membrane (Biodyne B; Pall BioSupport, Glen Cove, NY). Total RNA was isolated from L cell transfectants using ribonucleoside-vanadyl complex (Berger and Birkenmeier, 1979). Total RNA (10-μg aliquots) was electrophoresed in 1% agarose gel containing 0.66 M formaldehyde and transferred onto a nylon membrane as above. Hybridization of the blots with probes labeled by random-priming was carried out in 50% formamide/1 M NaCl/5% dextran sulfate/1% sodium dodecyl sulfate for 16 h at 42°C. An IL-2 cDNA fragment and a *neo* gene fragment from pMC1neo polyA + were used to prepare ^{32}P-labeled IL-2 probe (middle and left panels) and *neo* probe (right panel), respectively. Size markers (in bp) are shown on the left.

lated from the transfected cells using the Hirt (1969) procedure. However, we could not find introduced vectors as episomes by Southern-blot analysis of Hirt supernatants from the transfected cells one month after transfection (data not shown). Considering the apparent effects of the BPV fragment on the increase of the vector copy number in the stable transfectants, the vector was probably present as a replicating episome in the early stage after transfection, but soon underwent integration into the host chromosome. With this regard, it is interesting to note that after entering a cell, BPV DNA transiently replicates to several hundred copies per cell (Lusky and Botchan, 1986). BPV-based vectors have been reported to be prone to rearrangements and to integration into the host chromosome (Ostrowski et al., 1983; Stenlund et al., 1983), and such instability has been considered a major disadvantage of the BPV-based

TABLE II

Copy numbers of integrated vectors and IL-2 productivity in isolated CHO cell transfectant clones

Vector[a]	Clone No.[a]	Approximate copy number of integrated vectors[b] (per diploid genome)	IL-2 production rate[c] (u × 10^{-3}/10^6 cells/48 h)
pABWN-IL2	1	500	74.4
	2	300	47.6
	3	100	33.8
	4	50	11.4
	5	10	0.8
pCXWN-IL2	6	100	24.2
	7	100	24.0
	8	50	12.2
	9	10	6.0
	10	10	5.6

[a] Plasmids pABWN-IL2 and pCXWN-IL2 were transfected into CHO cells as described in Fig. 2 legend. Five clones resistant to 200 μg G418/ml were established from each transfection.

[b] *Eco*RI + *Pst*I-digested HMW DNA (10 μg each) and standard plasmid DNA were electrophoresed, hybridized with an IL-2 probe and autoradiographed. Then the signal intensity for each clone was compared with that of the standard on the autoradiogram.

[c] Each clone was cultured in the absence of G418, and the culture supernatants were assayed for IL-2 production.

vectors. However, such rapid integration of the vector into the host chromosome does not deteriorate our system, but may rather stabilize the productivity of the transfected cells in long-term culture. In fact, the CHO transfectants selected at 800 μg G418/ml for a month maintained their IL-2 productivity for more than three months.

(f) Isolation of transfectant CHO cell clones

To examine the variation of the ability to produce IL-2 among the pABWN-IL2 transfectants, five clones (Nos. 1–5) from pABWN-IL2 transfection were analyzed for their IL-2 secretion and their copy numbers of the IL-2 gene (Table II). Similarly, we analyzed another five clones (Nos. 6–10) from transfection of pCXWN-IL2 lacking the BPV sequences. The results showed that the levels of IL-2 production by the transfectant clones correlated well with their copy numbers of the exogenous IL-2 gene, and that the presence of the BPV fragment appeared to increase the copy number of the vector integrated into the host chromosome. Clone No. 1 carried a very high copy number (approx. 500) of pABWN-IL2, and secreted IL-2 at a high level. It should be noted that, although clone No. 5 contained only about ten copies of vector sequences, this clone would be eliminated by a higher concentration of G418.

For rapid isolation of highly productive transfectants, we tested whether single-step selection of pABWN-IL2 transfectants at 800 μg G418/ml would give a similar enrichment of high producers of IL-2 as did the two-step selection (200 μg G418/ml followed by 800 μg/ml). The result showed that both selections were equally effective (data not shown). The single-step selection produced G418-resistant colonies of L cells or CHO cells within ten days due to rapid elimination of nontransformed cells, and allowed us to clone and identify highly expressing recombinant cell lines very rapidly. More importantly, a pool of pABWN transfectants obtained by single-step selection at 800 μg G418/ml yielded high-level expression of the inserted gene, which was sufficient for most purposes.

(g) Conclusions and discussion

(*1*) Plasmid pCAGGS exhibited strong transient expression in various cell lines, and especially in combination with COs7 cells (Gluzman, 1981) or BMT10 cells (Gerard and Gluzman, 1985) produced large quantities of proteins encoded by various genes and cDNAs (unpublished results). In addition, pCAGGS directed high-level expression of the ligated *lacZ* gene during the early stages of mouse embryos from fertilized eggs to blastocysts in vitro when microinjected into pronuclei of fertilized eggs (unpublished results). It also produced high-level expression of various ligated genes in all the tissues as far as examined in transgenic mice (unpublished results).

(*2*) Plasmid pABWN was constructed by introducing a weak *neo* expression unit and the BPV 69% fragment into pCAGGS and exhibited powerful expression not only in transient, but also in stable transfection. Under high selective pressure by G418, stably transfected L or CHO cells could be obtained within ten days, and even the pools of these cells stably exhibited expression as high as selected transfectant clones in previous reports (Onomichi et al., 1987; Conradt et al., 1986; Karasuyama and Melchers, 1988). So far, there have not been good selectable markers to rapidly enrich highly productive transfectants, but the weak *neo* expression unit described in this study can be used for such purpose in combination with the BPV 69% fragment. The elements which constitute the pABWN vector, the *CAG* promoter, the weak *neo* unit, and the BPV fragment have only low specificity to cell types or species. Therefore, this system can be applied not only to L cells or CHO cells, but to a wide variety of cell lines. The pABWN vector should be very useful for producing large amounts of valuable proteins in mammalian cells, for changing the phenotype of host cells by overexpression of some normal or mutated genes, and for disrupting the function of targeted genes by overproduction of anti-sense RNAs.

(*3*) It is conceivable that the weak *neo* unit may function as an amplifiable selectable marker in this system. To test this possibility, we examined whether low-copy-number transfectant clones with pABWN-IL2 show amplification of the *neo* gene after growth in the medium containing high concentrations of G418 for one month. The result showed that the copy numbers of the *neo* gene in these clones were not affected by this treatment (data not shown). High concentrations of G418 obviously retarded the growth of these cell clones, but were not lethal to them. Therefore, the enrichment of highly producing transfectants by high concentrations of G418 was mediated by the difference of the growth rates among the transfectants.

(*4*) Though this vector has the BPV 69% fragment, which contributes to rapid and efficient selection of highly producing cells in cooperation with the weak *neo* expression unit, the introduced vector integrates into the host genomes. Therefore, this vector cannot be used as a shuttle vector between mammalian cells and *E. coli*. However, such rapid integration of the vector into the host chromosome may help to increase the stability of its copy number. The weak *neo* unit may be used as a selectable marker to maintain vectors as high-copy-number episomes in combination with alternative autonomously replicating elements other than the BPV fragment, such as Epstein–Barr virus elements.

ACKNOWLEDGEMENTS

We are very grateful to Dr. T. Taniguchi (Osaka University) for kindly providing human IL-2 cDNA, and Ms.

S. Miyazaki for her excellent secretarial assistance. This work was supported by a Grant-in-Aid from the Japanese Ministry of Education, Science and Culture.

REFERENCES

Aden, D.P., Fogel, A., Plotkin, S., Damjanov, I. and Knowles, B.B.: Controlled synthesis of HBsAg in a differentiated human liver carcinoma-derived cell line. Nature 282 (1979) 615–616.

Berger, S.L. and Birkenmeier, C.S.: Inhibition of intractable nucleases with ribonucleoside-vanadyl complexes: isolation of messenger ribonucleic acid from resting lymphocytes. Biochemistry 18 (1979) 5143–5149.

Binetruy, B., Meneguzzi, G., Breathnach, R. and Cuzin, F.: Recombinant DNA molecules comprising bovine papilloma virus type 1 DNA linked to plasmid DNA are maintained in a plasmidical state both in rodent fibroblasts and in bacterial cells. EMBO. J. 1 (1982) 621–628.

Chen, C. and Okayama, H.: High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. 7 (1987) 2745–2752.

Conradt, H.S., Ausmeier, M., Dittmar, K.E.J., Hauser, H. and Lindenmaier, W.: Secretion of glycosylated human interleukin-2 by recombinant mammalian cell lines. Carbohydr. Res. 149 (1986) 443–450.

DiMaio, D., Treisman, R. and Maniatis, T.: Bovine papillomavirus vector that propagates as a plasmid in both mouse and bacterial cells. Proc. Natl. Acad. Sci. USA 79 (1982) 4030–4034.

Felgner, P.L., Gadek, T.R., Holm, M., Roman, R., Chan, H.W., Wenz, M., Northrop, J.P., Ringold, G.M. and Danielsen, M.: Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc. Natl. Acad. Sci. USA 84 (1987) 7413–7417.

Foecking, M.K. and Hofsteffer, H.: Powerful and versatile enhancer-promoter unit for mammalian expression vectors. Gene 45 (1986) 101–105.

Fregien, N. and Davidson, N.: Activating elements in the promoter region of the chicken β-actin gene. Gene 48 (1986) 1–11.

Gerard, R.D. and Gluzman, Y.: New host cell system for regulated simian virus 40 DNA replication. Mol. Cell. Biol. 5 (1985) 3231–3240.

Gluzman, Y.: SV40-transformed simian cells support the replication of early SV40 mutant. Cell 23 (1981) 175–182.

Gmür, R., Solter, D. and Knowles, B.B.: Independent regulation of H-2K and H-2D gene expression in murine teratocarcinoma somatic cell hybrids. J. Exp. Med. 151 (1980) 1349–1359.

Gorman, C.M., Merlino, G.T., Willingham, M.C., Pastan, I. and Howard, B.M.: The Rous sarcoma virus long terminal repeat is a strong promoter when introduced into a variety of eukaryotic cells by DNA-mediated transfection. Proc. Natl. Acad. Sci. USA 79 (1982) 6777–6781.

Herbomel, P., Bourachot, B. and Yaniv, M.: Two distinct enhancers with different cell specificities coexist in the regulatory region of polyoma. Cell 39 (1984) 653–663.

Hirt, B.J.: Replicating molecules of polyoma virus DNA. J. Mol. Biol. 26 (1969) 141–144.

Karasuyama, H. and Melchers, F.: Establishment of mouse cell lines which constitutively secrete large quantities of interleukin 2, 3, 4, or 5, using modified cDNA expression vectors. Eur. J. Immunol. 18 (1988) 97–104.

Kaufman, R.J. and Sharp, P.A.: Amplification and expression of sequences cotransfected with a modular dihydrofolate reductase complementary DNA gene. J. Mol. Biol. 159 (1982) 601–621.

Law, M.-F., Byrne, J.C. and Howley, P.M.: A stable bovine papillomavirus hybrid plasmid that expresses a dominant selective trait. Mol. Cell. Biol. 3 (1983) 2110–2115.

Lusky, M. and Botchan, M.: Inhibitory effect of specific pBR322 DNA sequences upon SV40 replication in simian cells. Proc. Natl. Acad. Sci. USA 83 (1986) 3609–3615.

Maniatis, T., Fritsch, E.F. and Sambrook, J.: Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982.

Margolskee, R.F., Kavathas, P. and Berg, P.: Epstein–Barr virus shuttle vector for stable episomal replication of cDNA expression libraries in human cells. Mol. Cell. Biol. 8 (1988) 2837–2847.

McCormick, F., Trahey, M., Innis, M., Dieckmann, B. and Ringold, G.: Inducible expression of amplified human beta interferon genes in CHO cells. Mol. Cell. Biol. 4 (1984) 166–172.

Messing, J.: New M13 vectors for cloning. Methods Enzymol. 101 (1983) 20–78.

Mitrani-Rosenbaum, S., Maroteaux, L., Mory, Y., Revel, M. and Howley, P.M.: Inducible expression of the human interferon beta 1 gene linked to a bovine papilloma virus DNA vector and maintained extrachromosomally in mouse cells. Mol. Cell. Biol. 3 (1983) 233–240.

Miyazaki, J., Appella, E. and Ozato, K.: Negative regulation of the major histocompatibility class I gene in undifferentiated embryonal carcinoma cells. Proc. Natl. Acad. Sci. USA 83 (1986) 9537–9541.

Miyazaki, J., Takaki, S., Araki, K., Tashiro, F., Tominaga, A., Takatsu, K. and Yamamura, K.: Expression vector system based on the chicken β-actin promoter directs efficient production of interleukin-5. Gene 79 (1989) 269–277.

O'Hare, K., Benoist, C. and Breathnach, R.: Transformation of mouse fibroblasts to methotrexate resistance by a recombinant plasmid expressing a prokaryotic dihydrofolate reductase. Proc. Natl. Acad. Sci. USA 78 (1981) 1527–1531.

Okayama, H. and Berg, P.: High efficiency cloning of full-length cDNA. Mol. Cell. Biol. 2 (1982) 161–171.

Onomichi, K., Eto, Y. and Shibai, H.: Expression of amplified cDNA and genomic sequences encoding human interleukin 2 in Chinese hamster ovary cells. J. Biochem. 102 (1987) 123–131.

Ostrowski, M.C., Richard-Foy, H., Wolford, R.G., Berard, D.S. and Hager, G.L.: Glucocorticoid regulation of transcription at an amplified, episomal promoter. Mol. Cell. Biol. 3 (1983) 2045–2057.

Sarver, N., Byrne, J.C. and Howley, P.M.: Transformation and replication in mouse cells of a bovine papillomavirus-pML2 plasmid vector that can be rescued in bacteria. Proc. Natl. Acad. Sci. USA 79 (1982) 7147–7151.

Seed, B. and Aruffo, A.: Molecular cloning of the CD2 antigen, the T-cell erythrocyte receptor, by a rapid immunoselection procedure. Proc. Natl. Acad. Sci. USA 84 (1987) 3365–3369.

Stenlund, A., Lamy, D., Moreno-Lopez, J., Ahola, H., Pettersson, U. and Tiollais, P.: Secretion of the hepatitis B virus surface antigen from mouse cells using an extra-chromosomal eucaryotic vector. EMBO J. 2 (1983) 669–673.

Taniguchi, T., Matsui, H., Fujita, T., Takaoka, C., Kashima, N., Yoshimoto, R. and Hamuro, J.: Structure and expression of a cloned cDNA for human interleukin-2. Nature 302 (1983) 305–310.

Thomas, K.R. and Capecchi, M.R.: Site-directed mutagenesis by gene targetting in mouse embryo-derived stem cells. Cell 51 (1987) 503–512.

Urlaub, G. and Chasin, L.A.: Isolation of Chinese hamster cell mutants deficient in dihydrofolate reductase activity. Proc. Natl. Acad. Sci. USA 77 (1980) 4216–4220.

Wong, G.G., Witek, J.S., Temple, P.A., Wilkens, K.M., Leary, A.C., Luxenberg, D.P., Jones, S.S., Brown, E.L., Key, R.M., Orr, E.C., Shoemaker, C., Golde, D.W., Kaufman, R.J., Hewick, R.M., Wang, E.A. and Clark, S.C.: Human GM-CSF: molecular cloning of the complementary DNA and purification of the natural and recombinant proteins. Science 228 (1985) 810–815.

Yenofsky, R.L., Fine, M. and Pellow, J.W.: A mutant neomycin phosphotransferase II gene reduces the resistance of transformants to antibiotic selection pressure. Proc. Natl. Acad. Sci. USA 87 (1990) 3435–3439.

Zinn, K., Mellon, P., Ptashne, M. and Maniatis, T.: Regulated expression of an extrachromosomal human β-interferon gene in mouse cells. Proc. Natl. Acad. Sci. USA 79 (1982) 4897–4901.