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Improvement of Transfection Efficiency of Epithelioma Papulosum Cyprini Carp Cells by Modification of Cell Cycle and Use of an Optimal Promoter

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Abstract: Several methods to improve transfection of epithelioma papulosum cyprini (EPC) carp cells have been tested and are reported here. By modifying the cell cycle state of EPC cell monolayers and selecting the best promoter for the plasmid to be transfected, we increased transfection efficiency from 12.8% to 55.1% and decreased the coefficient of variation among different experiments from 54.1% to 11.8%. Thus 2- to 3-fold higher transfection efficiencies were obtained when the EPC monolayers were treated with colchicine or thymidine before transfection. In addition, the plasmids pMOKβgal and its shorter derivative pMVC1.4βgal, both containing 218 bp of additional sequences upstream of the cytomegalovirus promoter contained in plasmid pCMVβ, consistently produced higher transfection efficiencies than pCMVβ. Combination of the two methods resulted in an improvement of both efficiency and reproducibility. These results should facilitate transfection of EPC cells to use as a model to obtain transgenics, to conduct quantitative transfected-cell fusion assays, to improve DNA-immersion-vaccination methods, or to obtain infectious cDNA from fish RNA viruses.

Key words: epithelioma papulosum cyprini (EPC) cells, carp, transfection.

INTRODUCTION

Because fish cells have longer cell cycles than mammalian cells and lower optimal temperatures for growth, commercial transfection reagents based on liposomes and developed for mammalian cells are not optimal for fish cells. However, attempts have been reported to optimize plasmid introns (Betancourt et al., 1993), promoters (Inoue et al., 1990; Moav et al., 1992; Sharps et al., 1992), enhancers (Friedenreich and Schartl, 1990), oncogenes (Hayasaka et al., 1990), or use of multipotent fish cells (Bejar et al., 1999) for transfection of fish cell lines (Hackett and Alvarez, 2000; Bearzotti et al., 1992).

Because the cell line epithelioma papulosum cyprini (EPC), isolated from carp (Fijan et al., 1983), was found to be the best predictor of plasmid activity in transgenic fish (Moav et al., 1992) and could be transfected (Bearzotti et al., 1992; Moav et al., 1992), we have used it as a first model to study possible methods to improve the previously

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reported liposome transfection efficiencies and low reproducibility of fish cell lines (Lopez et al., 2001).

Since the majority of the DNA-liposome complexes enter the cells (Tseng et al., 1999), but not all translocate throughout the endosome/lysosome/nuclear membranes before the DNA can be transcribed, we have studied methods to increase both transfection efficiencies and reproducibility by modifying those pathways through treatment of the EPC cells with chemical products. Release of the DNA from the endosome to the cytoplasm (before acidification to avoid DNA degradation) can be facilitated by the addition of lysosomotropic agents such as ammonium chloride, chloroquine, sucrose, or Ca^{2+} (Luthman and Magnusson, 1983; Lam and Cullis, 2000; Ciftci and Levy, 2001). Penetration of the nuclear membrane by the DNA after transfection by electroporation, liposomes (Brunner et al., 2000; Mortimer et al., 1999; Tseng et al., 1999), injection (Chan et al., 1998), or cell permeabilization (Escriou et al., 2001) is facilitated by the absence of nuclear membranes during mitosis. Inhibitors of cell cycle could thus be used to increase the number of cells in mitosis during the time of transfection. In addition, we have tested the effect of commercially available plasmid constructs using the β-galactosidase (β-gal) gene under the cytomegalovirus (CMV) promoter with different upstream sequences. The use of these methods singly or in combination improved both the efficiency and reproducibility of transfection of EPC cells.

Materials and Methods

Plasmids

The pCMVβ of 7.2 kb (Clontech Labs) and the pMOKβgal (6.9 kb) or the pMVC1.4βgal (5.9 kb) (Ready Vector, Madrid, Spain) plasmids were used. All of them contain the β-gal gene of *Escherichia coli* under the control of the minimal CMV early promoter. However, whereas pCMV contains the minimal CMV promoter (100 bp) and 687 bp upstream enhancer sequences, both pMOK and pMVC1.4 contained another 218 bp upstream of the 687 bp enhancer sequences of pCMV. pMVC1.4 was derived from pMOK by including a multiple cloning site and deleting approximately 1 kb of unnecessary bacterial sequences. The plasmids were used to transform *E. coli* DH5α by electroporation. Large amounts of plasmid were prepared from *E. coli* pellets by using the Wizar plus Megaprep DNA purification system (Promega). Plasmid solutions were adjusted to 1 mg/ml of total DNA by its absorbance at 260 nm and contained 80% to 100% of plasmid DNA, as shown by agarose gel electrophoresis, the rest being other bacterial DNA.

Transfection of EPC Cells and Additives

EPC cells (optimal growth at 28° to 30°C) (Fijan et al., 1983) were grown in wells of 24-well plates at 28°C in 400 μl of RPMI Dutch modified cell culture medium buffered with 20 mM HEPES (Flow, BioLink, Madrid, Spain), and supplemented with 10% fetal calf serum. EPC cells were plated at 500,000 cells/ml and incubated during 24 hours in a 5% CO_2 atmosphere. Plasmids (0.6 μg) complexed with 2 μl of Fugene 6 (Roche, Barcelona, Spain), during 15 minutes in 100 μl of RPMI without fetal calf serum, were added to the wells containing 300 μl of cell culture medium with 10% fetal calf serum. The cells were then incubated for 24 hours at 28°C in a 5% CO_2 atmosphere prior to the β-gal assays.

Other commercial transfection agents were obtained and tested as described in Table 1 (see results). Lysosomotropic agents such as ammonium chloride, chloroquine, sucrose, or $CaCl_2$ or colchicine and thymidine obtained from Sigma were added to the EPC cell monolayers when indicated in each experiment.

β-Galactosidase Quantitative Assays

To assay for β-gal activity, the Gal-screen gene assay system (Tropix, Applied Biosystems, Madrid, Spain) was used. After removing the cell culture medium, 200 μl of 0.025% Triton X-100 was added to the transfected EPC cells for best cell lysis, and the wells were agitated during 15 minutes. Then, 100 μl of the mixture of substrate and lysis enhancer buffer were pipeted to each of the wells, incubated during 60 minutes at room temperature, and 20-μl aliquots counted in a MiniLumat LB9506 apparatus (EG&G Berthold).

β-Galactosidase Staining Assays

To visualize β-gal activity by staining the EPC cells after transfection, the EPC cell monolayers were fixed during 5 minutes with cold methanol and stained with freshly made 2 mg/ml of 5-bromo-4-chloro-3-indoyl-β-D-galactopyr-

Table 1. Transfection Efficiencies with Several Commercially Available Transfection Reagents

Reagent	Origin	µl/µg DNA	X-gal-stained cells (%)	*N*
Fugene	Roche	3.3	30.1 ± 6.7	2
Chariot+NLS	Active Motif	4	0.2 ± 0.1	4
Polylisine, 1 mg/ml	Sigma	20	5.4 ± 2.1	2
MEG peptide, 1 mg/ml	Chiron	20	0.6 ± 0.1	2
Lipofectamina	GibcoBRL	3.3	7.6 ± 0.1	2
Lipofectamine 2000	InVitroGen	3.3	1.8 ± 1.1	2
GeneJuice	Novagen	3.3	19.0 ± 6.1	2
GenePORTER	GTS	5	1.6 ± 0.4	2
GeneJammer	Stratagene	5	3.1 ± 1.4	2
LipoTaxi	Stratagene	5	0.8 ± 0.8	2
Effectene	Qiagen	8enh+20	0.0 ± 0.0	1
Superfect	Qiagen	5	16.9 ± 3.2	1

[a]Transfections were performed by using 0.6 µg of plasmid DNA per well of a 24-well plate and following the indications in "Materials and Methods" described for Fugene. *N*, number of different experiments each by duplicates. Averages and standard deviations are presented. Effectene required a preincubation step with an enhancer (enh). NLS is nuclear localization peptide (CGGPKKKRKVG; [Liang et al., 2000]); it was employed at 4 µg/µg DNA. The DNA was first bound to the NLS peptides and transfected as it was the MEG peptide with Chariot, a protein transfection reagent. MEG peptide with DNA binding and translocation properties (GALFLGFLGAAGSTMGAWSQPKSKRKV; [Morris et al., 1999]). Partial EPC cell lysis occurred with the use of GeneJamer and Effectene.

anoside (X-gal) in 5 mM ferrocianide II, 5 mM ferrocianide III, 2 mM $MgCl_2$ in phosphate-buffered saline. After about 16 hours, the monolayers were washed with water and air dried. To estimate the percentage of X-gal-stained cells, 3 random and representative photographs per well were taken with an inverted microscope at ×100 provided with a digital Olympus camera. Total cells (700 to 1000 cells per well) and blue-stained cells were then counted in each photograph. Averages and standard deviations from 2 wells per experiment were then calculated.

Results

Transfection with Different Transfection Reagents, EPC Cell Concentrations, and Times

Table 1 shows the transfection efficiencies obtained when different commercial transfection reagents made available during recent years were applied to EPC cell monolayers. Maximal transfection efficiencies were obtained with Fugene, GeneJuice, and Superfect. Although the transfection efficiency (mean ± SD) obtained with Superfect (16.9 ± 3.2) was lower than that obtained with Fugene (30.1 ± 6.7), the β-gal activities estimated by luminescence were similar (results not shown), suggesting that the amount of β-gal activity expressed per cell was higher in the case of Superfect. Nevertheless, since Fugene, the reagent selected in a previous study against other earlier available transfection reagents (Lopez et al., 2001), showed the highest transfection efficiency, it continued to be used for the rest of the work.

After a few preliminary experiments, we first studied the dependence of the efficiency of transfection on the number of EPC cells plated. Transfection was performed 24 hours after plating, and β-gal assays were done 24 hours after transfection; these time variables were not previously controlled. In a typical experiment with an average maximal percentage of X-gal-stained cells of approximately 16%, Figure 1 (A) shows that maximal percentages of X-gal-stained EPC cells were obtained when plating 500,000 cells/ml. Higher or lower cellular plating concentrations decreased 3- to 4-fold the number of X-gal-stained cells. Similar results were obtained by estimating the total β-gal activity by luminescence (maximum of 17×10^6 cps). Since estimation of EPC doubling time under the conditions of the experiment was 44 hours at cellular concentrations between 300,000 and 800,000 cells/ml, and doubling time decreased at lower or higher cellular concentrations (not shown), those results suggested that actively dividing EPC cells were transfected best.

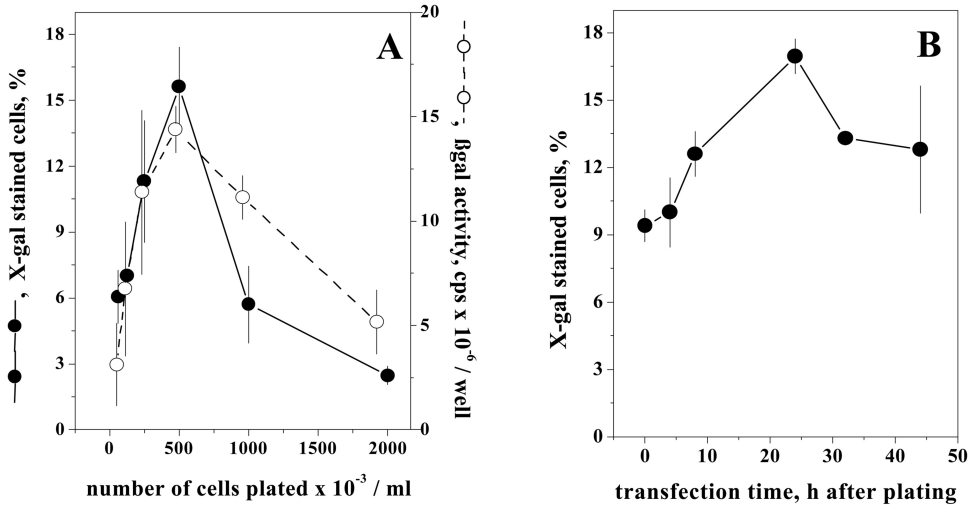

Figure 1. Dependence of X-gal-stained cells or β-gal activity with the number of cells plated (**A**) and the time of transfection after plating (**B**). A, Different amounts of EPC cells were seeded in 400 μl of RPMI, 10% fetal calf serum per well of a 24-well plate. Twenty-four hours later they were transfected with pCMVβ, and β-gal activity was assayed after 24 hours of incubation. B, About 500,000 cells/ml were plated per well of a 24-well plate. The cells were transfected at different times after plating, and the β-gal activity assayed 52 hours later. Averages and standard deviations from 2 experiments are represented.

We then tested the influence of the incubation time after transfection on the transfection efficiency. Although the first X-gal-stained cells could be detected as early as 10 hours after transfection, maximal X-gal-stained cell numbers were obtained after 24 hours, decreasing to about half the initial number after 48 hours, and showing near-background levels after more than 70 hours (data not shown).

To define the best moment for transfection relative to the age state of the EPC monolayer after plating, we added the Fugene-DNA complexes to cell monolayers at different times after plating and performed the β-gal assays 52 hours after plating. EPC monolayers maintained in the presence of the DNA-Fugene complex in the last 24 hours of culture (time of 20 hours in the Figure 1, B) showed the maximal amount of EPC cells stained (about 17% in this particular experiment; Figure 1, B). In contrast, EPC monolayers maintained between 42 and 52 hours (time 0 to 10 hours in Figure 1, B) in the presence of the DNA-Fugene complex, showed less than 13% of the cells stained with X-gal. Most probably this decrease in the transfection efficiency was due to detachment of cells with an excess of β-gal expression (picnotic cells highly stained with β-gal were abundant in those cultures), since Fugene was not toxic to the EPC cell monolayers up to a concentration of 20 μl/well (20% of dead cells after 48 hours).

Influence of Addition of Compounds Inhibiting the Cell Cycle

The increase in the efficiency of transfection with the time of culture, at least during the last 28 hours before the assay (time 24 hours in Figure 1, B), might be interpreted as being due to recruitment of mitotic cells during that time. Therefore, two results suggested that the percentage of X-gal-stained cells might be dependent on the cell cycle state: their dependence on cell concentration and the time of transfection after plating.

Experiments in the presence of 10 ng/ml of colchicine or 2.5 mM thymidine (compounds inhibiting mitosis and DNA synthesis, respectively) at different times before transfection, increased transfection efficiencies relative to those of nontreated cultures from firming the above mentioned hypothesis. Thus a maximal 2- to 3-fold increase in X-gal-stained cells was obtained when the EPC cells were incubated during 5 to 15 hours before transfection with 10 ng/ml of colchicine. A maximal 1.5- to 2-fold increase in X-gal-stained cells was also obtained with 5 hours of incubation before transfection with 2.5 mM thymidine. Increasing the time of incubation with colchicine or thymidine showed deleterious effects on the EPC cells, and β-gal activity decreased (Figure 2).

The effect of colchicine was optimal at 10 ng/ml (2-fold increase in the number of X-gal-stained cells compared to

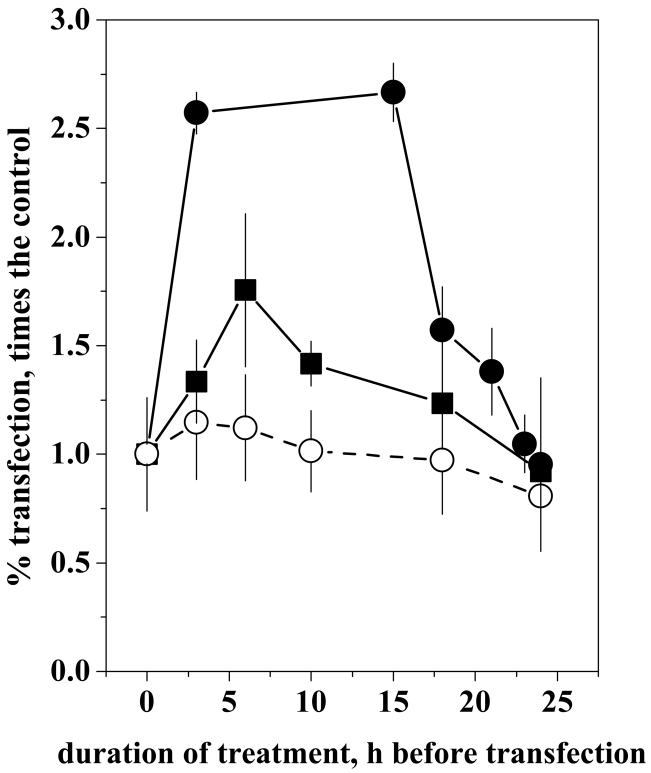

Figure 2. Effect of duration of treatment with colchicine or thymidine before transfection on the percentage of X-gal-stained EPC cells. About 500,000 EPC cells/ml were treated with 10 ng/ml of colchicine or 2.5 mM thymidine during different times before transfection. Just before transfection the EPC monolayers were washed, Fugene-DNA complexes added, and β-gal assayed 24hours later. The results are expressed in times the control by the formula, percentage of X-gal-transfected EPC cells with treatment divided by percentage without treatment. Averages and standard deviations from duplicates are represented.

nontreated cultures). Lower concentrations caused lower increases in the number of X-gal-stained cells, whereas higher concentrations were toxic to the cells (data not shown). The effect of colchicine was also dependent on the EPC cell concentration. Thus when the number of cells plated was increased to 1×10^6 ml, the stimulatory effect was difficult to detect (not shown).

Influence of the Addition of Lysosomotropic Compounds

To inhibit the low pH of the endosome to avoid possible damage on the transfected DNA, the addition of chloroquine (20 to 80 μM), $CaCl_2$ (0.1 to 4 mM), sucrose (20 to 80 mM), and NH_4Cl_2 (10 to 50 mM) and their effect on transfection were assayed. Only chloroquine showed a consistent but small 1.5-fold increase in the number of X-gal-stained cells ($n = 2$) (Table 2). However, an increase in β-gal activity of more than 5-fold could be estimated by measuring the β-gal activity by bioluminescence in parallel experiments, suggesting that inhibiting the low pH of the endosome might increase the level of β-gal expression per cell rather than the number of X-gal-stained cells (not shown).

Combination of chloroquine (20 μM) with several concentrations of colchicine, or of colchicine (10 ng/ml) with several concentrations of chloroquine, did not increase significantly the number of X-gal-stained cells with respect to controls in the absence of each of the compounds (not shown).

Use of β-Gal Under the Control of CMV Promoters with Different Upstream Sequences

Because previous studies demonstrated that transfection efficiencies in EPC were highest when the human CMV promoter was used, we compared the transfection efficiencies of several β-gal codifying plasmids that differ in the size of the sequences located upstream of the minimal CMV promoter (Figure 3, A).

Visual inspection of the plates containing EPC cell monolayers transfected with either pMOKβgal or pMVC1.4βgal (2 plasmids containing longer CMV promoters than pCMVβ) showed a blue background that was never seen before when pCMVβ was used (not shown). Figure 3 (A, B) shows that pMOKβgal and its derivative of lower molecular weight pMVC1.4βgal stained a 3- to 4-fold higher number of EPC cells than pCMVβ. Higher enhancements (about 10-fold) of β-gal activity (approx. 170×10^6 cps) by either pMOKβgal or pMVC1.4βgal were detected by assaying β-gal activity by bioluminescence (Figure 3, C). No significant differences in efficiency of transfection were found between these 2 plasmids in different experiments (Table 2). The β-gal activity was optimal at 1 to 3 μg of plasmid per milliliter. Higher or lower DNA concentrations showed a decrease in the amount of β-gal detected, to near-background levels (Figure 3, B, C), thus confirming results obtained previously (Lopez et al., 2001).

Variation of Transfection Efficiencies of EPC with Plasmids with Different Promoters and Cellular Treatments

Figure 4 shows EPC monolayers treated with colchicine and then transfected with pMVC1.4βgal after being fixed and

Table 2. Variation of Transfection Efficiencies of EPC with Plasmids with Different Promoters and Cellular Treatments

Plasmid	Treatment	Transfected cells % (No. of experiments)	CV, %	ITF
pCMVβ	—	12.8 ± 6.5 (24)	54.1	1
pMOKβ	—	29.1 ± 12.3 (6)	42.2	2.2
pMVC1.4β	—	30.7 ± 12.1 (13)	39.3	2.4
pCMVβ	Chloroquine	19.3 ± 5.6 (2)	29.0	1.5
pMVC1.4β	Chloroquine	34.7 ± 13.2 (3)	38.2	2.7
pCMVβ	Colchicine	17.9 ± 3.2 (3)	18.2	1.4
pMVC1.4β	Colchicine	55.1 ± 7.7 (3)	13.9	4.3
pMVC1.4β	Thymidine	34.3 ± 4.0 (3)	11.8	2.6

[a]CV, coefficient of variation expressed in percentage. Treatments were with 20 μM chloroquine during transfection, 10 ng/ml colchicine during 15 hours before transfection, and 2.5 mM thymidine during 5 hours before transfection. ITF, increase of transfection efficiency, was calculated by the formula, percentage of transfected cells divided by percentage of transfected cells with pCMVβ.

stained with X-gal. Cells with a large amount of β-gal appeared as intense blue spots, whereas other cells appeared with fainter blue stain. The number of X-gal-stained cells remained constant, and no diffusion of the blue color from the intense blue spots was observed when the fixed monolayers were incubated for 1 week (not shown).

However, the absolute value of the transfection efficiencies varied from experiment to experiment independently of the plasmid used (Figure 3, B, C; Table 2). The highest expression of β-gal after using pMVC1.4βgal for transfection among different experiments ($n = 13$) varied 30.7% ± 12.1% (coefficient of variation [CV], 39.3%), most probably because of the difficulty in reproducing the age state of the EPC cell monolayers.

Table 2 shows that by using pCMVβ, we obtained 12.8% ± 6.5% of transfected EPC cells ($n = 24$) with a CV of 54.1%. The use of pMOK or pMVC1.4, both of which utilize the larger CMV promoter, increased the transfection efficiencies 2.2- to 2.4-fold (approx. 30%) but only decreased the CV to 39.3%.

To further increase the percentage of X-gal-stained cells, the EPC cell monolayers were incubated with chloroquine (1.5- to 2.7-fold increase), colchicine (1.4- to 4.3-fold increase), or thymidine (2.6-fold increase) before the transfection. The treatment with the cell-cycle-inhibiting agents (colchicine or thymidine) decreased the CV to 11.8% to 18.2%, whereas the treatment with chloroquine showed higher CV values (29% to 38.2%).

The highest transfection efficiency (2.6- to 4.3-fold over the efficiency obtained with pCMVβ) with the lowest CV (11.8% to 18.2%) was thus obtained by using both the pMVC1.4 plasmid and colchicine- or thymidine-treated EPC cell monolayers (Table 2).

Discussion

Searching for the best conditions to transfect fish cells, we selected the EPC cell line as a model and Fugene as the best transfection agent, after selection from earlier available commercial transfection reagents in a previous study (Lopez et al., 2001). After using this system many times, a maximal efficiency of transfection of 37% was obtained; however, experiments with 5% of cells transfected were also found, and 12.8% ± 6.5% were the most frequent result ($n = 24$). The variation between experiments, a CV of 54.1%, indicates low reproducibility.

Because it seems likely that most of the liposome-transfected cells take up DNA, but only a few express the transgene (Scherman et al., 1998 2113), the relatively low efficiency could be due to the difficulties found in the pathway of the DNA inside the cell towards the nucleus. The permanence of the transfected DNA into the endosome/lysosome vesicles, or the inability to translocate the nuclear membrane owing to the low number of cells in mitosis (the duration of the EPC cell cycle was 44 hours), could lower the transfection efficiency. In addition, the difficulty of reproducing the age state of the EPC cell monolayers could cause further variation in the reproducibility between experiments.

Further experiments that showed the efficiency of transfection to be dependent on cell concentration and on time of addition of DNA relative to plating time also suggested a relation with the cell cycle state of the monolayers. Since the cell cycle state of the EPC cells, its plating cell concentration, and the timing of transfection were not strictly controlled during the first reported work (Lopez et al., 2001), we tried to improve their control. That the

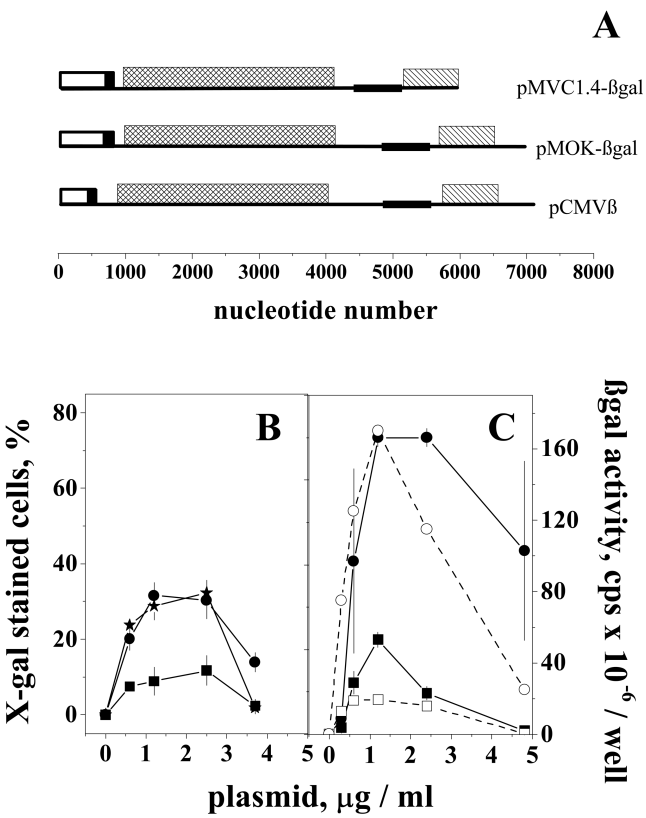

Figure 3. Scheme of the maps of pMVC1.4β-gal, pMOKβgal and pCMVβ plasmids and percentage of X-gal-stained EPC cells and β-gal activity obtained using those plasmids. **A:** Schematic maps of the linearized plasmids used are shown: ■, minimal CMV promoter; □, upstream regions; β-gal gene, ⊠, antibiotic-resistant gene; ▨, other bacterial regions common to the 3 plasmids, ■; **B,C**: About 500,000 EPC cells/ml were transfected with 0.6 µg of different plasmids after being complexed with 2 µl of Fugene in 100 µl of serum-free medium. Two extreme experiments (B and C) show the range of transfection efficiencies found among experiments. Averages and standard deviations from duplicates are represented. ■, X-gal-stained cells obtained with pCMVβ; ★, X-gal-stained cells obtained with pMOKβgal; ●, X-gal-stained cells obtained with pMVC1.4βgal; □, β-gal activity in cps obtained with pCMVβ; ○, β-gal activity in cps obtained with pMOKβgal or pMVC1.4βgal.

transfection efficiency could be increased by inhibitors of the cell cycle was confirmed by the 2- to 4-fold higher transfection efficiencies obtained when colchicine or thymidine were added to the EPC cell monolayers before transfection. Long treatments were not possible because of the toxic effects of colchicine or thymidine on the EPC monolayers. It is assumed that these agents acted by recruiting more EPC cells into the mitotic phase, in which nuclear membranes are absent. Similar increases in the expression of plasmid genes delivered by liposomes have been reported in mammalian cells after or during mitosis (Mortimer et al., 1999; Tseng et al., 1999; Brunner et al., 2000; Escriou et al., 2001).

Furthermore, treatment of the EPC cell monolayers with colchicine or thymidine decreased the variability of transfection efficiencies among different experiments to a CV of 11.8% to 18.2% (n = 9), suggesting that at least part of the variability might be due to the differences among the cell cycle state of the EPC cell monolayers in each experiment.

Because the use of lysosomotropic agents that avoid the lowering of the pH of the endosome had been reported to increase mammalian cell transfection (Morales et al., 1999), calcium (Haberland et al., 1999; Lam and Cullis, 2000), chloroquine (Bettinger et al., 2001; Luthman and Magnusson, 1983), and ammonium chloride (Ciftci and Levy, 2001) were tested to improve the efficiency of transfection by addition to the EPC monolayers during transfection. However, none of the added compounds were capable of significantly or reproducibly increase the percentage of X-gal-stained cells. Either the DNA-Fugene complexes did not use the endosome/lysosome pathway on EPC cells, or the added compounds had no effect on the escape of the transfected DNA from the lysosome in EPC cells.

Further improvements were then attempted by using plasmids with β-gal expression under the CMV promoter but with different upstream sequence lengths. Although many of the commercial CMV-promoter-based plasmids use the minimal CMV promoter of 100 bp, they differ in the size of the upstream sequences included. Two of those plasmids (pCMVβ and pMOKβgal), differing from pCMVβ in the size of the upstream sequences included, were tested on transfection of EPC cells. A consistent 3- to 4-fold increase in the efficiency of transfection was obtained when the plasmids with the longer upstream sequences were used (pMOKβgal or pMVC1.4βgal). The increase in transfection efficiency was such that it could be detected by the blue color of the transfected monolayers visible by the naked eye. The relative increase with respect to pCMVβ was independent of the efficiency of transfection of each particular experiment, suggesting an intrinsic mechanism related to transcriptional control in the EPC cells. The increase in β-gal activity estimated in parallel experiments by luminescence could be as high as 10-fold, showing that not only increased the number of cells expressing β-gal but also the amount of β-gal expressed by each transfected cell.

The comparison of sequences among the 3 plasmids used (Figure 3, A) indicated that the observed enhancement

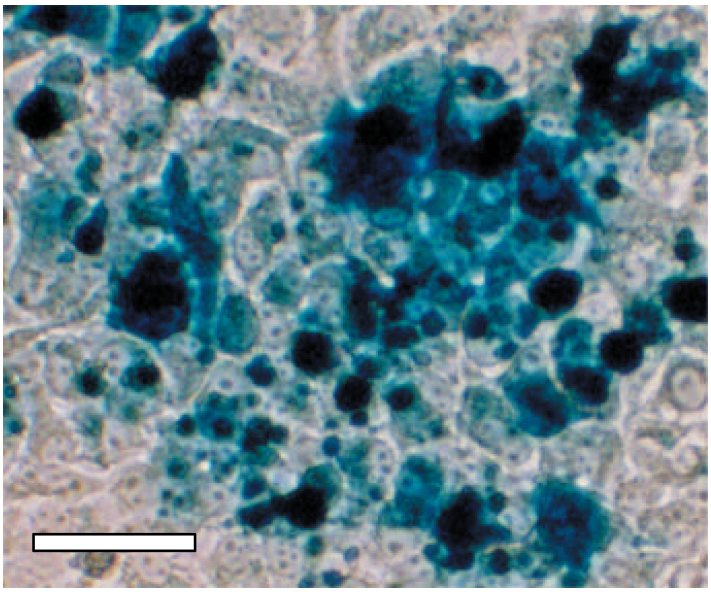

Figure 4. Morphology of colchicine-treated EPC cells stained with X-gal after transfection with pMVC1.4β: βgal. One day after transfection with pMVC1.4βgal, EPC monolayers were fixed and stained with X-gal and photographed. White bar is approximately 40 μm.

was likely due to the inclusion of the 218-bp upstream sequences found in pMOK and in pMVC1.4, although different introns or polyadenylation signals could also be involved. However, neither size (pMOK-βgal is about the same size as pCMVβ), nor the common bacterial sequences conserved among the 3 plasmids (Figure 3, A) were responsible for the differences observed.

By using the β-gal gene inserted into the pMOK or pMVC1.4 plasmids, the increases in both efficiency and in reproducibility of transfection were confirmed by treatment of the EPC cell monolayers with either colchicine or thymidine (Table 2). It remains to be seen if those enhancements are reproducible in other fish cell lines, although preliminary experiments in CHSE and RTG-2 (cell lines derived from salmonids) indicate this might be the case.

Studies of heterologous gene expression in transgenic fish (Chourrout et al., 1986; Chen et al., 1995; Walker et al., 1995) and the synthesis of infectious RNA fish viruses from cDNA copies (Estepa et al., 1999; Biacchesi et al., 2000a, 2000b, 2002), should benefit from the optimization of in vitro EPC cell transfection. A better promoter to increase transcription of foreign genes could also help to improve DNA vaccination methods by injection (Anderson et al., 1996; Lorenzen et al., 1998) or by immersion (Fernandez-Alonso et al., 1998, 1999).

Furthermore, quantitative assays of fish viral fusion with transfected rhabdoviral glycoproteins, as in mammals (Coll, 1999; Fernandez-Alonso and Coll, 1999; Nussbaum et al., 1994; Shokralla et al., 1999), are currently being used in our laboratory to complete an analysis of point mutants in protein G of viral hemorrhagic septicemia virus (VHSV) of salmonids, based on transfection with pMVC1.4 plasmids codifying the protein G gene mutants. We have observed similar transfection efficiencies and reproducibile expression of protein G gene mutants in the membranes of transfected EPC cells.

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