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IMPROVEMENT OF TRANSFECTION EFFICIENCY OF EPITHELIOMA PAPULOSUM CYPRINI CARP CELLS BY MODIFICATION OF THEIR CELL CYCLE AND BY USING AN OPTIMAL PROMOTER

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SUMMARY

Several methods to improve transfection of Epithelioma Papulosum Cyprini (EPC) 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 report an increased transfection efficiency from 12.8 to 55.1 % and a decreased coefficient of variation among different experiments from 54.1 to 11.8 %. Thus, 2-3-fold higher transfection efficiencies were obtained when the EPC monolayers were treated with colchicine or thymidine before transfection. In addition, the plasmids pMOKßgal and its shorter derivative pMVC1.4ßgal both containing 218 bp of additional sequences upstream of the cytomegalovirus (CMV) promoter of pCMVß, consistently produced higher transfection efficiencies than pCMVß. Combination of both methods resulted in an improvement of both maximal efficiency and reproducibility. These results should facilitate transfection of EPC cells to obtain transgenics, to make quantitative transfected-cell fusion assays, to improve DNA-immersion-vaccination methods or to obtain infectious cDNA from fish RNA viruses.

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. On the other hand, few studies have been reported to optimise 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 (Hackett and Alvarez, 2000) of fish cell lines (Bearzotti et al., 1992).

Because the cell line epithelioma papulosum cyprini (EPC), isolated from carp (Fijan et al., 1983), was the best predictor of plasmid activity in transgenic fish (Moav et al., 1992) and it was possible to transfect (Bearzotti et al., 1992; Moav et al., 1992), we have used the EPC as a first model of fish cell lines to study possible methods to improve their earlier reported liposome transfection efficiencies and their low reproducibility (Lopez et al., 2001).

Because the majority of the DNA-liposome complexes go into 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 by 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^{++} (Ciftci and Levy, 2001; Lam and Cullis, 2000; Luthman and Magnusson, 1983). 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 that occurs during mitosis. The use of 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 ßgal under the CMV promoter with different upstream sequences. The use of these methods singly or in combination improved both the efficiency of transfection and its reproducibility.

MATERIALS AND METHODS

Plasmids. The pCMVß of 7.2 Kb (Clontech Labs, Palo Alto, CA) and the pMOK ßgal (6.9 Kbp) or pMVC1.4ßgal (5.9 Kbp) (Ready Vector, Madrid, Spain), were used. All of them contain the ßgalactosidase (ßgal) gene of E.coli under the control of the minimal cytomegalovirus early promoter (CMV). However, whereas pCMV contains the minimal CMV promoter (100 bp) and 687 bp upstream enhancer sequences, both pMOK and pMVC1.4 contained other 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 ~ 1 Kbp of unnecessary bacterial sequences. The plasmids were used to transform E.coli DH5alpha by electroporation. Large amounts of plasmid were prepared from E.coli pellets by using the Wizar plus Megaprep DNA purification system (Promega, Madison, USA). Plasmid solutions were adjusted to 1 mg/ml of total DNA by its Absorbance at 260 nm and contained 80-100% of plasmid DNA, as shown by agarose gel electrophoresis, the rest being other bacterial DNA.

Transfection of EPC cells and additives. Epithelioma papulosum cyprini (EPC) cells (optimal growth at 28-30 ºC) (Fijan et al., 1983) were grown in wells of 24-wells plates at 28 ºC in 400 µl of RPMI Dutch modified cell culture medium buffered with 20 mM HEPES (Flow) and supplemented with 10 fetal calf serum. EPC cells were plated at 500.000 cells/ml and incubated during 24 h in a 5% CO_2 atmosphere. Plasmids (0.6 µg) complexed with 2 µl of fugene 6 (Roche, Barcelona, Spain) during 15 min in 100 µl of RPMI without fetal calf serum, were added to the wells containing 300 µl of cell culture medium with 10% of fetal calf serum. The cells were incubated for 24 h more at 28 ºC in a 5% CO_2 atmosphere prior to the ßgal assays.

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

ßgalactosidase quantitative assays. To assay for ßgal activity, the Gal-screen gene assay system (Tropix, Bedford, MA, USA) was used. After removing the cell culture medium, 200 µl of 0.025 % Triton x100 were added to the transfected EPC cells for best cell lysis and the wells agitated during 15 min. Then, 100 µl of the mixture of substrate and lysis enhancer buffer were pippeted to each of the

wells, incubated during 60 min at room temperature and 20 µl aliquots counted in a MiniLumat LB9506 apparatus (EG&G Berthold, Postfach, Germany).

ßgalactosidase staining assays. To visualise ßgal activity by staining the EPC cells after transfection, the EPC cell monolayers were fixed during 5 min with cold methanol and stained with 2 mg/mL of 5-bromo-4-chloro-3-indoyl-ß-D-galactopyranoside (X-gal) in 5 mM ferrocianide II, 5 mM ferrocianide III, 2 mM $MgCl_2$ in phosphate buffered saline, freshly made. After about 16 h, the monolayers were washed with water and air dried. To estimate the percentage of X-gal stained cells, three random and representative photographs per well were taken with an inverted microscope at x100 provided with a digital Olympus camera. Total number of cells (700-1000 cells per well) and blue-stained cells were then counted in each photograph after transfer to the computer. Averages and standard deviations from two wells per experiment were then calculated.

RESULTS

Transfection with various 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 obtained with Superfect (16.9 ± 3.2) was lower than the one obtained with Fugene (30.1 ± 6.7), the ßgal activities estimated by luminiscence were similar (results not shown), suggesting that the amount of ßgal 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 study the dependence of the efficiency of transfection with the number of EPC cells plated. Transfection was performed 24 h after plating and ßgal assays 24 h after transfection, since these time variables were not totally controlled before. In a typical experiment with an average maximal percentage of X-gal stained cells of ~ 16%, Figure 1A shows that maximal percentages of X-gal stained EPC cells were obtained when plating 500.000 cells per ml. Higher or lower cellular concentrations decreased 3-4-fold the number of X-gal stained cells. Similar results were obtained by estimating total ßgal activity by luminescence (maximal of 17 x 10^6 cps). Since estimation of EPC doubling time under the conditions of the experiment were of 44 h at cellular concentrations between 300.000 to 800.000 cells/ml and that doubling time decreased at lower or higher cellular concentrations (not shown), those results suggested that actively dividing EPC cells were transfected best.

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 h after transfection, maximal X-gal stained cell numbers were obtained after 24 h, decreasing to about half the initial number after 48 h and showing near to background levels after more than 70 h (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 ßgal assays 52 h after plating. EPC monolayers maintained in the presence of the DNA-fugene complex in the last 24 h of culture (time ₦20 h in the Figure 1B), showed the maximal number of cells stained (about 17 % in this particular experiment) (Figure 1B). In contrast, EPC monolayers maintained between 42-52 h (time 0 to 10 h in the Figure 1B) in the presence of the DNA-fugene complex, showed <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 ßgal expression (picnotic cells highly stained with ßgal were abundant in those cultures), since fugene was not toxic to the EPC cell monolayers up to a concentration of 20 µl per well (20 % of dead cells after 48 h).

Influence of the 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 h before the assay (time 24 h in the Figure 1B), might be interpreted as 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 of the time of transfection after plating.

According to this hypothesis, experiments made in the presence of 10 ng/ml of colchicine or 2.5 mM thymidine (two compounds inhibiting mitosis and DNA synthesis, respectively) at different times before transfection, increased transfection efficiencies respect to non treated cultures. Thus, a maximal of 2-3-fold increase in X-gal stained cells was obtained when cells were incubated during 5-15 h before transfection with 10 ng/ml of colchicine. A maximal of 1.5-2-fold increase in X-gal stained cells was also obtained with 5 h of incubation before transfection with 2.5 mM of thymidine. Increasing the time of incubation with colchicine or thymidine showed deleterious effects on the EPC cells and ßgal 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 non treated cultures). Lower concentrations did cause 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 concentrations. Thus when the number of cells plated was increased to 1 x 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 (20-80 mM) and NH_4Cl_2 (10-50 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, more than 5-fold increase in ßgal activity could be estimated by measuring ßgal activity by bioluminescence in parallel experiments (not shown), suggesting that inhibiting the low pH of the endosome might increase the level of ßgal 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 significatively the number of X-gal stained cells with respect to controls in the absence of each of the compounds (not shown).

Use of ßgal under CMV promoters with different upstream sequences. Because previous studies demonstrated that transfection efficiencies in EPC were highest when the human cytomegalovirus (CMV) promoter was used, we compared the transfection efficiencies of several ßgal codifying plasmids that differ on the size of the sequences located upstream of the CMV minimal promoter (Figure 3A).

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

Variation of transfection efficiencies of EPC with plasmids with different promoters and cellular treatments. Figure 4 shows the aspect of EPC monolayers treated with colchicine and then transfected with pMVC1.4ßgal after being fixed and stained with X-gal. Cells with a large amount of

ßgal appeared as intense blue spots whereas other cells appeared with more fainted blue stain. The number of X-gal stained cells remained constant and no diffusion of the blue colour from the intense blue spots was observed when the fixed monolayers were incubated during one week (not shown).

However, the absolute value of the transfection efficiencies varied from experiment to experiment independently of the plasmid used (Figure 3 BC; Table 2). The highest expression of ßgal after using the pMVC1.4ßgal for transfection among different experiments (n=13) varied 30.7 ± 12.1 % (CV of 39.3 %), most probably still due to the difficulty to reproduce the state of the EPC cell monolayers.

Table 2 shows that by using pCMVß, we obtained 12.8 ± 6.5 % of transfected EPC cells (n=24) with a variation coefficient (CV) of 54.1 %. The use of pMOK or pMVC1.4, both of which use the larger CMV promoter, increased the transfection efficiencies 2.2-2.4 fold (~ 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 (1.5-2.7 fold increase), colchicine (1.4-4.3-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 11.8-18.2 %, whereas the treatment with chloroquine showed higher CV (29-38.2 %).

The highest transfection efficiency (2.6 to 4.3-fold over the efficiency obtained with pCMVß) with the lowest CV (11.8-18.2 %), 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 commertial transfection reagents in a previous study (Lopez et al., 2001). After using this system many times, maximal efficiencies of transfection of 37 % were 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 was of a CV of 54.1 %, which means a 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 value of the 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 impossibility to translocate the nuclear membrane due to the low number of cells in mitosis (the duration of the EPC cell cycle was 44 h), could lower the transfection efficiencies. In addition, the difficulty to reproduce the age state of the EPC cell monolayers, could cause variation in the reproducibility between experiments.

Further experiments that showed the efficiency of transfection to be dependent on cell concentration and of 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 reprted work (Lopez et al., 2001), we tried to improve their control. That the transfection efficiency could be increased by inhibitors of the cell cycle was confirmed by the 2-4-fold higher transfection efficiencies obtained when colchicine or thymidine were added to the EPC cell monolayers before transfection. Longer treatments were not possible to perform because of the toxic effects of colchicine or thymidine on the EPC monolayers. It is assumed that this agents acted by recruiting more EPC cells into the mitotic phase where nuclear membranes are absent. Similar increases in the expression of plasmid genes delivered by liposomes, have been also reported in mammalian cells after or during mitosis (Brunner et al., 2000; Escriou et al., 2001; Mortimer et al., 1999; Tseng et al., 1999).

Furthermore, the treatment of the EPC cell monolayers with colchicine or thymidine decreased

the variability of transfection efficiencies among different experiments to a CV of 11.8-18.2 % (n=9), thus 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 were 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 significatively 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 there is no effect of the added compounds on the escape of the transfected DNA from the lysosome in EPC cells.

Further improvements were then searched by using plasmids with the ßgal expression under the CMV promoter but with different upstream sequence lengths. Although many of the commercial cytomegalovirus (CMV)-promoter-based plasmids use the minimal CMV promoter of 100 bp, they differed on the size of the upstream sequences included. Two of those plasmids (pCMVß and pMOKßgal) differing from pCMVß in the size of the upstream sequences included, were tested on transfection of EPC cells. A consistent 3-4-fold increase in the efficiency of transfection was obtained when the plasmids with the longer upstream sequences were used (pMOKßgal or pMVC1.4ßgal). The increase in transfection efficiency was such that it could be detected by the blue colour of the transfected monolayers visible at the naked eye. The relative increase with respect to pCMVß 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 ßgal activity estimated in parallel experiments by luminescence could be as high as 10-fold, showing that not only the number of cells expressing ßgal were increased but also the amount of ßgal expressed by each transfected cell.

Because of the comparison of sequences among the 3 plasmids used (Figure 3A), it is likely that the observed enhancement are due to the inclusion of the 218 bp upstream sequences found in pMOK and in pMVC1.4, although different introns or poly adenilation signals could also be involved. However,

nor size (pMOK-ßgal is about the same size than pCMVß), nor the common bacterial sequences which have been conserved among the 3 plasmids (Figure 3A) are responsible for the differences observed.

By using the ßgal gene inserted into the pMOK or pMVC1.4 plasmids, both the increase in 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 when using other fish cell lines, although preliminary experiments in CHSE and RTG-2 (two cell lines derived from salmonids) indicate this might be the case.

Studies of heterologous gene expression in transgenic fish (Chen et al., 1995; Chourrout et al., 1986; Walker et al., 1995) and the synthesis of infectious RNA fish viruses from cDNA copies (Estepa et al., 1999; Biacchesi etal., 2000a; Biacchesi et al., 2000b; Biacchesi et al., 2002), should benefit by the optimisation of in vitro fish cell transfection. On the other hand, a better promoter to increase transcription of foreign genes could help to improve DNA vaccination methods by injection (Anderson et al., 1996; Lorenzen et al., 1998) or by immersion (Fernandez-Alonso et al., 1999; Fernandez-Alonso et al., 1998). Furthermore, quantitative assays of fish viral fusion with transfected rhabdoviral glycoproteins as in mammalians (Coll, 1999; Fernandez-Alonso and Coll, 1999; Nussbaum et al., 1994; Shokralla et al., 1999), is currently being used in our laboratory to complete an analysis of point mutants in the protein G of viral haemorrhagic 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 reproducibility of the sucessful expresion of protein G gene mutants in the membranes of transfected EPC cells.

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 1mg/ml	Sigma	20	5.4 ± 2.1	2
MEG peptide 1mg/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

The transfections were performed by using 0.6 µg of plasmid DNA per well of a 24 well plate and following the indications in 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). Transfection reagents came from: Roche (Barcelona, Spain), Active Motif (Rixensart, Belgium), Sigma (St.Louis,Mi), Chiron Mimotopes (Victoria, Australia), GibcoBRL (Barcelona, Spain), InVitroGen (Barcelona, Spain), Novagen (Madison, WI, US), Stratagene (LaJolla, CA, US), GTS (S.Diego, CA, US), Qiagen (Hilden, Germany). NLS, 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 a protein with Chariot, a protein transfection reagent. MEG peptide with DNA binding and translocation properties: GALFLGFLGAAGSTMGAWSQPKSKRKV (Morris et al.,1999). Partial EPC cell lysis ocurred with the use of GeneJamer and Effectene.

Table 2.

Variation of transfection efficiencies of EPC with plasmids with different promoters and cellular treatments

Plasmid	Treatment	Transfected cells, % (number of experiments)		CV, %	ITF
pCMVß	-	12.8 ± 6.5	(24)	54.1	1
PMOKß	-	29.1 ± 12.3	(6)	42.2	2.2
pMVC1.4ß	-	30.7 ± 12.1	(13)	39.3	2.4
pCMVß	Chloroquine	19.3 ± 5.6	(2)	29.0	1.5
pMVC1.4ß	Chloroquine	34.7 ± 13.2	(3)	38.2	2.7
pCMVß	Colchicine	17.9 ± 3.2	(3)	18.2	1.4
pMVC1.4ß	Colchicine	55.1 ± 7.7	(3)	13.9	4.3
pMVC1.4ß	Thymidine	34.3 ± 4.0	(3)	11.8	2.6

CV, coefficient of variation expressed in percentage. Treatments were with 20 µM chloroquine during transfection, 10 ng/ml colchicine during 15 h before transfection and 2.5 mM thymidine during 5 h before transfection. ITF, Increase of transfection efficiency was calculated by the formula, percentage of transfected cells / percentage of transfected cells with pCMVß.

Figure 1.- Dependence of X-gal stained cells or ßgal activity with the number of cells plated (A) and the time of transfection after plating (B). Different amounts of EPC cells were seeded in 400 µl of RPMI 10% FCS per well of a 24-wells plate. Next day, 24 h later they were transfected with pCMVß and ßgal activity assayed after 24h of incubation (A). About 500.000 cells per ml were plated per well of a 24-wells plate. The cells were transfected at different times after plating and in all the wells, the ßgal activity was assayed 52 h later (B). Averages and standard deviations from two experiments each by duplicates are represented in the figure.

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 per ml were treated with 10 ng/ml of colchicine or 2.5 mM of thymidine during different times before transfection. Just before transfection the EPC monolayers were washed, fugene-DNA complexes added and ßgal assayed 24 h later. The results are expressed in times the control by the formula, percentage of X-gal transfected EPC cells with treatment / percentage of transfected EPC cells without treatment. Averages and standard deviations from duplicates are represented.

Figure 3.- Scheme of the maps of pMVC1.4ßgal, pMOKßal and pCMVß plasmids (A) and percentage X-gal stained EPC cells and ßgal activity by using those plasmids (B,C). In the insert A, the schematic map of the linearized plasmids used are shown. ■, minimal CMV promoter. □, upstream regions. ☒, ßgal gene. ◫, antibiotic resistant gene. ▬, other bacterial regions common to the 3 plasmids. B,C) About 500.000 EPC cells per ml were transfected with 0.6 µg of different plasmids after being complexed with 2 µl of fugene in 100µl of serum-free medium. Two extreme experiments B and C show the range of efficiencies found among experiments. Averages and standard deviations from duplicates are represented. ■, X-gal stained cells obtained with pCMVß. ●, X-gal stained cells obtained with pMOKßgal. ★, X-gal stained cells obtained with pMVC1.4ßgal. ○, ßgal activity in cps obtained with pCMVß. □, ßgal activity in cps obtained with pMOKßgal or pMVC1.4ßgal.

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

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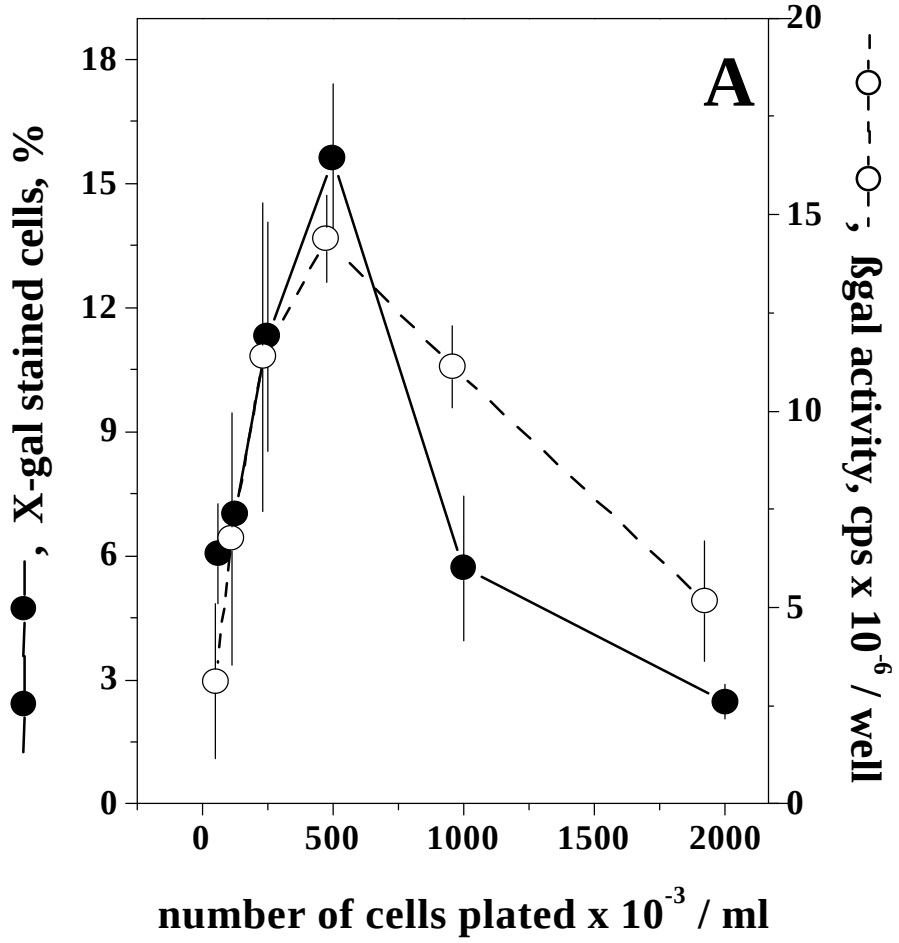
A
—●—, X-gal stained cells, %
- -○- - , βgal activity, cps x 10-6 / well
number of cells plated x 10-3 / ml
0
3
6
9
12
15
18
0
5
10
15
20
0
500
1000
1500
2000

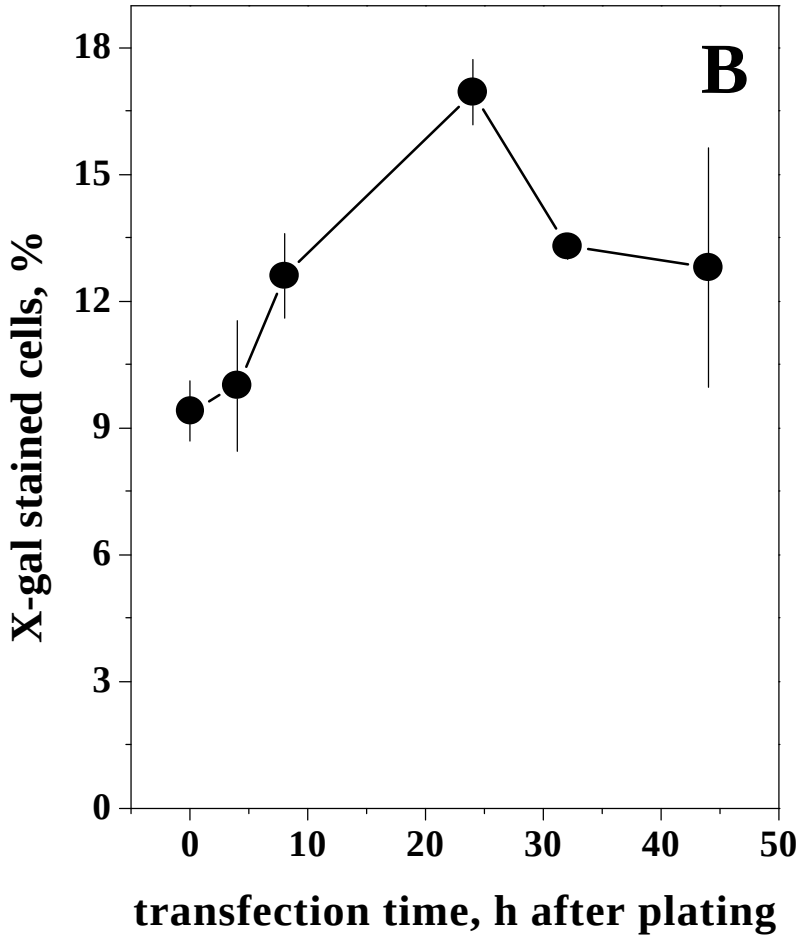
B
X-gal stained cells, %
transfection time, h after plating
0
3
6
9
12
15
18
0
10
20
30
40
50

Fig 1

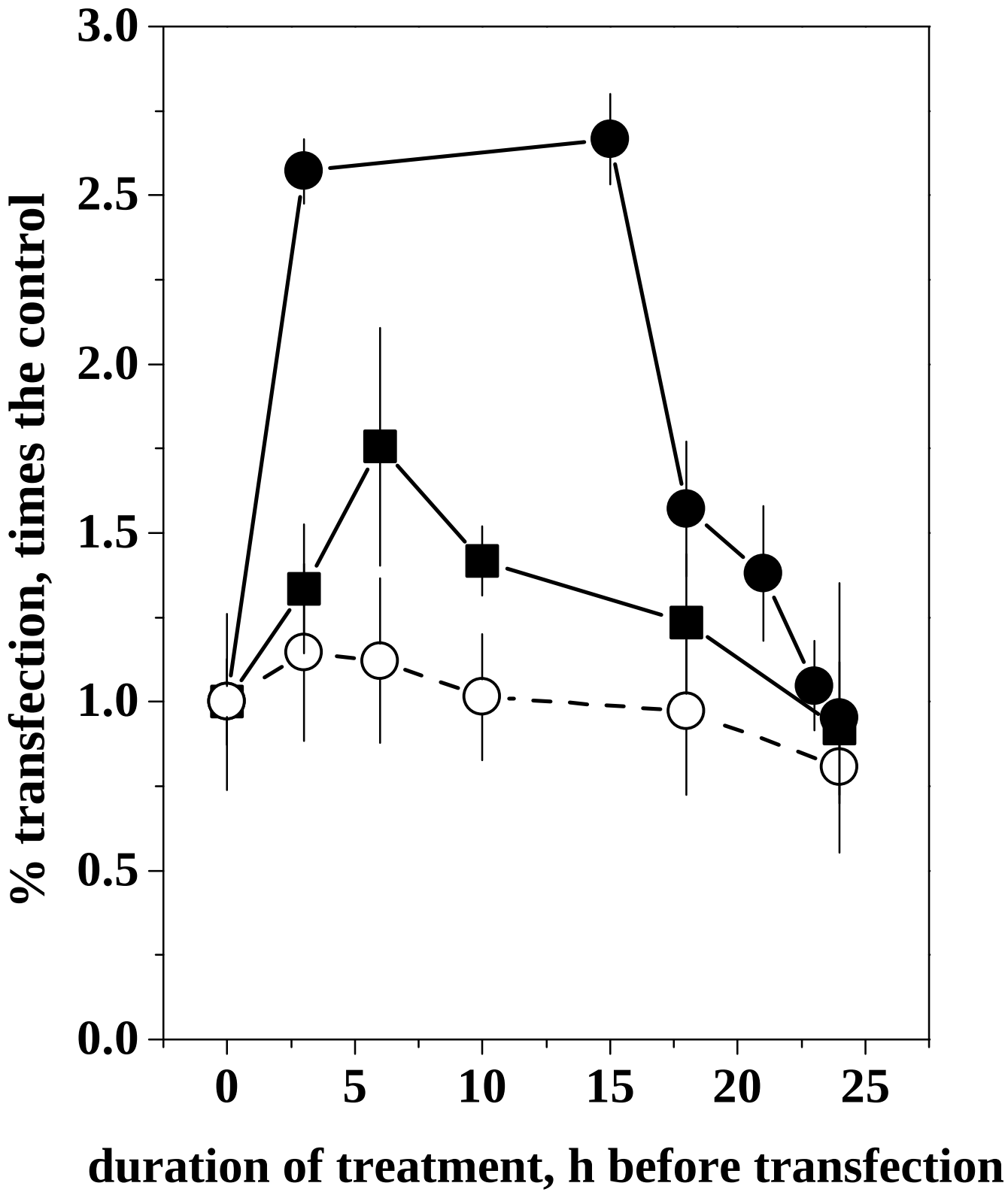
3.0
2.5
2.0
1.5
1.0
0.5
0.0
% transfection, times the control
0
5
10
15
20
25
duration of treatment, h before transfection

Fig 2

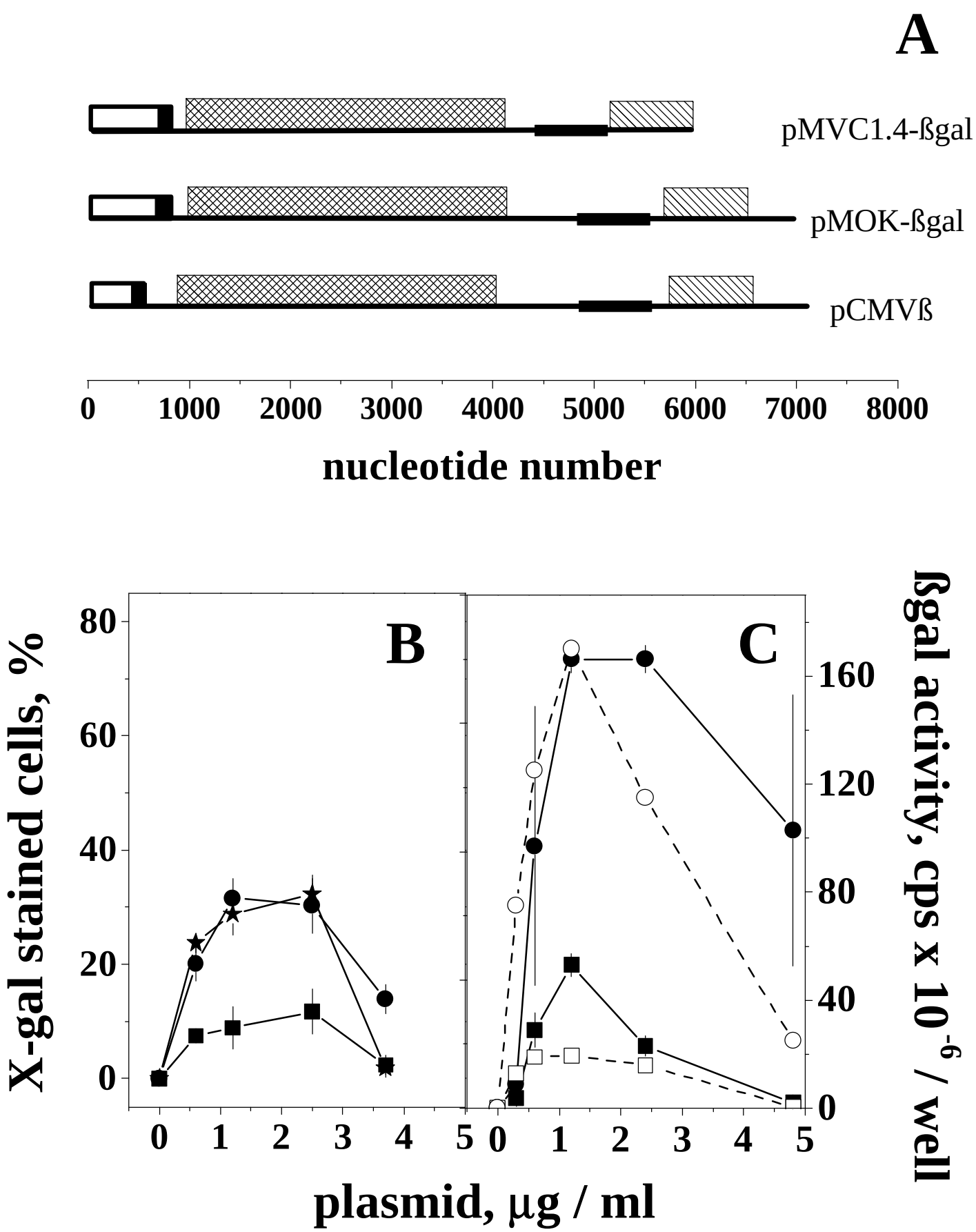
A
pMVC1.4-ßgal
pMOK-ßgal
pCMVß
0
1000
2000
3000
4000
5000
6000
7000
8000
nucleotide number
B
C
X-gal stained cells, %
ßgal activity, cps x 10-6 / well
plasmid, µg / ml

Fig 3