Spontaneous Transfection of Mammalian Cells with Plasmid DNA

Vania L. Tsoncheva, Kristina A. Todorova, Ivan G. Ivanov,
and Vera A. Maximova*

Institute of Molecular Biology, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria.
Fax: (+3592) 736237. E-mail: vmaximova@obzor.bio21.bas.bg

* Author for correspondence and reprint requests

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A simple method for spontaneous transfection into mammalian cells (both adherent and suspension in culture) with plasmid DNA is described. This method does not require any specific DNA carrier or technical device and can be applied for obtaining both transient and stably transfected cells. The efficiency of spontaneous transfection is slightly lower in comparison with that of the conventional calcium phosphate and lipofectin transfection methods and does not depend on the type of cell culture used.

Key words: Spontaneous Transfection, Mammalian Cells

Introduction

DNA-mediated gene transfer into cultured mammalian cells is a routine approach for studying gene function in eukaryotes. Following the report by Szybalska and Szybalski (1962) on the ability of mammalian cells to take up exogenous DNA, a great number of sophisticated methods for introducing DNA into mammalian cells have been developed. The most popular of which are: the DNA-calcium phosphate precipitation method and its various modifications (Chen and Okayama, 1987, 1988; Orrantia *et al*., 1990); the DEAE-dextran mediated method (Milman and Herzberg, 1981); polybrene (Chaney *et al*., 1986) and polycationic liposome transfection (Schaefer-Ridder *et al*., 1982); the electric field transfection method (electroporation) (Potter *et al*., 1984) or bombardment with DNA carrying colloidal (gold) particles (Matsumo *et al*., 2003); transfection by viral vectors (Robbins *et al*., 1998). All these methods have advantages and disadvantages and their improvement as well as the development of new approaches for gene transfer is still appreciated. In recent years the promising reports (Weiss, 2003; Perletti *et al*., 2003) and investigations from several laboratories have shown that naked plasmid DNA can be delivered efficiently to cells *in vivo* either via electroporation or by intravascular injection for gene immunization and therapy (Herweijer and Wolff, 2003). The uptake and expression of naked DNA is a general property of animal cells within a tissue, which is lost when the cells are maintained in culture. That tempted us to study in more detail the possibility for spontaneous transfection of mammalian cells in cultures with naked plasmid DNA.

Materials and Methods

Cell cultures and media

The adherent cell cultures WISH (amnion epithelial cells), HeLa (human cervix carcinoma cells), MCF-7 (epithelial human breast cancer cells) and PLC (human liver tumor cells) were maintained in Dulbecco MEM/Nut Mix F-12 (HAM) (Gibco, Paisley, Scotland) supplemented with 10% fetal bovine serum (Biochrom KG). The cells were cultivated in flasks (Corning) at 37 °C in humidified air containing 5% CO_2. Every 3–4 d the cells were subcultivated by trypsinization with 0.25% trypsin (Gibco) in versen [0.02% EDTA in PBS (phosphate buffer saline: 0.14 M NaCl, 10 mM phosphate buffer, pH 7.2)] at 37 °C for about 5 min.

The suspension cell culture Namalva (human Burket lymphoma cell line) was grown in RPMI 1640 (Gibco) medium with 10% fetal bovine serum.

Expression plasmids

Two plasmid vectors, pEGFP-N1 and pDsRed2-N1 (Clontech, Palo Alto, CA, USA), have been used to express the green and red fluorescence protein, respectively, in a cell line as transfection marker. Plasmid DNA was obtained from *Escherichia coli* strain HB101 as described earlier

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(Todorova *et al.*, 2002). DNA was dissolved in sterile Tris [(hydroxymethyl)amino-ethane]-EDTA, pH 7.4, and quantified spectrophotometrically.

Transfection methods

DNA-calcium phosphate precipitation method

Calcium phosphate-mediated DNA transfection of adherent mammalian cells was performed in accordance with Chen and Okayama (1987) with some modifications. Cells (5×10^5) were grown in 50 mm petri dishes to about 60% confluence. After washing with PBS, 0.5 ml fresh DNA-precipitate solution was layered on the top of the cell monolayer and incubated for 30 min at 37 °C. Then 3 ml medium (without serum) were added and the dishes were incubated about 4–6 h at 37 °C. The cells were washed with PBS to remove precipitates and left for 24–48 h in fresh medium supplemented with serum.

Lipofection method

The liposome-based procedure was carried out with lipofectin (Gibco), according to the manufacturer's protocol. Briefly, 20 μl lipofectin and 20 μg plasmid DNA were combined in 0.5 ml medium (without serum) and left for 30 min at room temperature. The transfection mixture was diluted twice with medium and added to the pre-washed cells in a ratio of 1 ml per 50 mm petri dish. The cells were incubated at 37 °C for 5 h and 2 ml medium supplemented with fetal serum was added.

Spontaneous transfection method

1 d before transfection, the cell cultures were grown in 50 mm dishes. Adherent mammalian cells were transfected as follows: Cells cultivated in dishes (to 80% confluence) were trypsinized with 0.25% trypsin at 37 °C for 5 min and enzyme activity was neutralized with 0.5 ml medium containing 10% bovine fetal serum (normal or heat-inactivated). Plasmid DNA (5 μg/ml) was added and the cells in this small volume were incubated at 37 °C for 30 min. Finally, the cells were diluted to 3 ml medium including 10% fetal bovine serum.

In case of suspension cell cultures, the plasmid DNA (5 μg/ml) was added straight to the suspension of 1 ml (8×10^5) cells without any preliminary treatment. Following a 30 min incubation at 37 °C, fresh medium with 10% heat inactivated serum was added.

The efficiency of transient transfection for both adherent and suspension cell lines was estimated after 24 h.

Stable transfection

For this purpose, the cells were transfected in 6-well plates according to the protocol for the spontaneous method (as described above). 1 d after treatment, the medium was exchanged by the same medium supplemented with 1 mg/ml geneticin (G-418) (Gibco BRL) in 100 mM Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] (pH 7.4). The cells were grown as above, changing the selective medium every 3 d. Two weeks later the colonies of living transfected cells attached to the bottom of the plates were harvested and the expression of the reporter genes were evaluated by monitoring and measurement of the fluorescence marker protein.

Transfection monitoring and measurement

The efficiency of spontaneous transfection was evaluated by counting the number of fluorescence cells by a fluorescence microscope. The cells were grown and transfected straight on microscope glass slides in dishes. After 24–48 h the slides were flushed in PBS, fixed with 4% paraformaldehyde in PBS at room temperature for 10 min and washed twice with PBS. The cells from suspension culture were collected, washed with PBS and fixed by adding 3 volumes of paraformaldehyde solution. These fixed cell suspensions were spotted onto thoroughly cleaned glass slides and air-dried. The slides were scrutinized by a microscope fitted for epifluorescence.

For quantitative fluorescence measurements, adherent cells were washed twice with PBS, scraped off by a rubber rod and resuspended in 1 ml bidistilled water. The cell suspension was sonicated on ice with 20 short bursts for disruption of cells to release intracellular components. The cell debris was removed by centrifugation at $250 \times g$ for 15 min at 4 °C. The fluorescence of the clear cell lysates was measured on a fluorescence spectrofluorometer (Shimadzu RF-5000) under the following conditions: $\lambda_{ex}/\lambda_{em}$ = 488 nm/507 nm and $\lambda_{ex}/\lambda_{em}$ = 558 nm/583 nm for the green and red fluorescence protein, respectively. In the case of suspension cultures, the cells were harvested by centrifugation, washed with PBS and disrupted as above. The intensity of fluorescence maximum was

represented in arbitrary units (a.u.) and was referred to 1 mg/ml protein (quantified spectrophotometrically at 280 nm). Through the autofluorescence of the cells, the value of fluorescence intensity of the non-transfected cells (background) was substracted from that of the transfected cells. The fluorescence intensity of transfected cells increased about 5-fold compared to background.

Results and Discussion

To evaluate the efficiency of spontaneous transfection in comparison with that of the conventional methods, four adherent cell lines (WISH, HeLa, MCF-7, and PLC) were tested. The cells were transfected by both spontaneous and calcium phosphate transfection methods using the plasmid pEGFP-N1. The results presented in Fig. 1A show that the efficiency of spontaneous transfection was almost constant and independent of the cell line used. The same four cell lines, however, responded differently to the calcium phosphate-mediated DNA transfection (see Fig. 1B). Comparing the efficiency of transfection of the two methods, one can see that except for HeLa, the rest of the cell lines responded better to the calcium phosphate-mediated transfection. The data indicate that the efficiency of spontaneous transfection of HeLa cells was about twice lower than that obtained by both lipofectin and calcium phosphate methods.

The overall efficiency of spontaneous transfection (transfected versus non-transfected cells) as determined by counting the fluorescence of WISH and HeLa cells was about 30%. The fluorescence protein (EGFP) was visualized by fluorescence microscopy into the cytosol of the transiently transfected cells.

Besides with adherent cells, the spontaneous transfection method was applied successfully also for transfection of suspension cell lines such as Namalva.

In this study the efficiency of transient transfection was measured 24 h after cells treatment with plasmid DNA. The spontaneous transfection method was also applied for obtaining stably transfected cells. Studying the expression of fluorescent protein genes in the latter cells, we found that the fluorescent intensity gradually increased up to the 21st day, after which it remained constant (see Fig. 2).

To standardize the conditions for spontaneous transfection, we examined the effect of some factors such as DNA concentration, temperature, growth medium composition on the efficiency of transfection. As shown in Fig. 3, no detectable correlation was found between DNA concentration and the yield of green fluorescence protein in

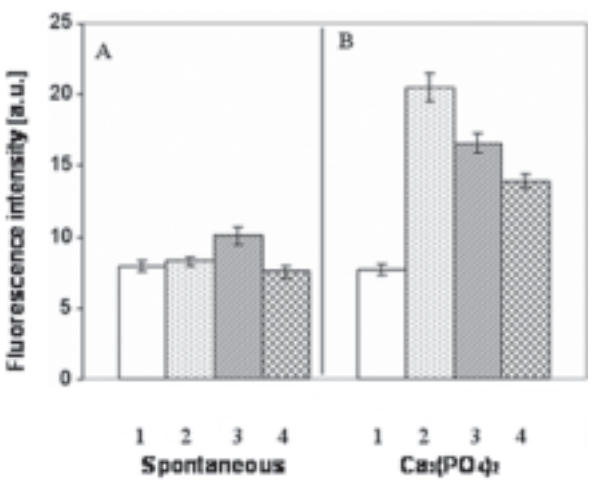

Fig. 1. Transfection with pEGFP-N1 of adherent cell lines by the (A) spontaneous and (B) calcium phosphate transfection methods (cell lines: 1, HeLa; 2, MCF-7; 3, PLC; 4, WISH). The fluorescence intensity of expressed EGFP in clear cell lysates (see Materials and Methods) (λ_{ex} = 488 nm/λ_{em} = 507 nm) is presented in arbitrary units (a.u.) and refers to 1 mg/ml protein.

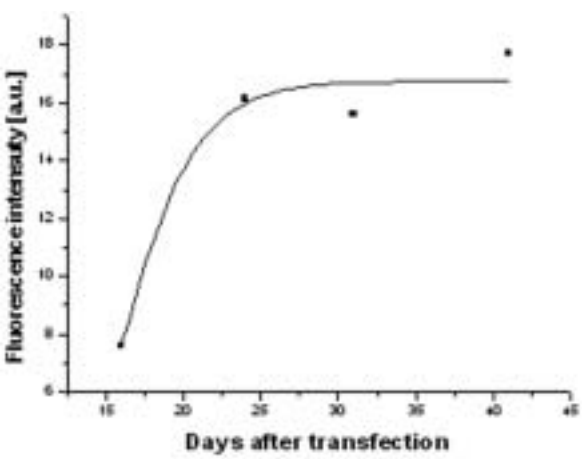

Fig. 2. Stable transfection of HeLa cells by the spontaneous transfection method. HeLa cells were transfected with plasmid DNA (pDsRed2-N1) and cultivated in selective medium (with G-418, 1 mg/ml). The fluorescence intensity of expressed DsRed2 protein in clear cell lysates (λ_{ex} = 558 nm/λ_{em} = 583 nm) is presented in arbitrary units (a.u.) and refers to 1 mg/ml protein.

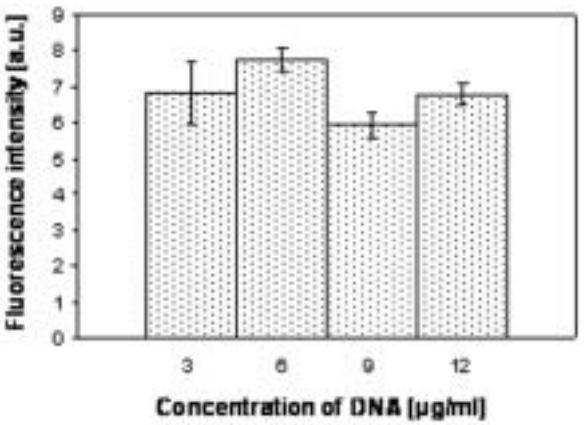

Fig. 3. Effect of plasmid DNA concentration on the efficiency of spontaneous transfection of HeLa cells with pEGFP-N1. The fluorescence intensity of clear cell lysates (λ_{ex} = 488 nm/λ_{em} = 507 nm) is presented in arbitrary units (a.u.) and refers to 1 mg/ml protein.

transfected cells. That is why we chose to use 5 μg/ml plasmid DNA in all further experiments. To investigate the effect of temperature on spontaneous transfection, two adherent (HeLa and WISH) and one suspension (Namalva) cell lines were incubated with plasmid DNA (pDsRed2-N1) for 1 h at either 37 °C or 4 °C. As seen in Table I, the efficiency of transfection, determined by fluorescence at λ_{ex} = 558 nm/λ_{em} = 583 nm, was higher at 4 °C for all cell lines used. Table I shows also that the three cell lines responded differently at lower (4 °C) temperature, where a maximal increase (42%) was registered with the HeLa cells. The effect of low temperature on efficiency of transfection might be explained by the low activity of the nucleases in the serum.

All above experiments are carried out in medium containing non-inactivated bovine serum. Varying the composition of growth medium, we found that the supplementation with heat-inactivated serum had a positive effect on transfection increasing its efficiency over 30% for all cell lines used. That is why we recommend to use heat- inactivated serum for spontaneous transfection.

Table I. Effect of temperature on spontaneous transfection.

Cell line	Fluorescence intensity [a.u.][a]	
	Transfection at 37 °C	Transfection at 4 °C
Namalva	4.9 ± 0.4	5.4 ± 0.5
HeLa	5.9 ± 0.3	10.2 ± 0.3
WISH	4.6 ± 0.25	5.3 ± 0.3

[a] The fluorescence intensity of clear cell lysates (λ_{ex} = 558 nm/λ_{em} = 583 nm) is presented in arbitrary units (a.u.) referred to 1 mg/ml protein.

Besides the criticism of the calcium phosphate method [where over 50% of the transfected DNA is immediately degraded (Orrantia and Chang, 1990], it is still the most commonly used approach for DNA mediated gene transfer (Basolo *et al*., 1990). This method, as well as the lipofectin transfection, has another disadvantage. In both cases the uptake of DNA is different for the different cell lines (Malek and Khaledi, 2003). This shortcoming is overcome with the spontaneous DNA transfection method described in this paper, where the efficiency of transfection is independent of the cell culture type. The only disadvantage of our method is its slightly lower efficiency compared to the conventional transfection techniques. In return it is simple, reproducible and does not require expensive chemicals or technical devices.

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Basolo F., Elliott J., and Russo J. (1990), Transfection of human breast epithelial cells with foreign DNA using different transfecting techniques. Tumori **76**, 455–460.

Chaney W. G., Howard D. R., Pollard J. W., Sullustio S., and Stanley, P. (1986), High frequency transfection of CHO cells using polybrene. Somat. Cell Mol. Genet. **12**, 237–244.

Chen C. and Okayama H. (1987), High efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. **7**, 2745–2751.

Chen C. and Okayama H. (1988), Calcium phosphate-mediated gene transfer: a highly efficient transfection system for stably transforming cells with plasmid DNA. Biotechniques **6**, 632–638.

Herweijer H. and Wolff J. A. (2003), Progress and prospects: naked DNA gene transfer and therapy. Gene Therapy **6**, 453–458.

Malek A. H. and Khaledi M. G. (2003), Monitoring liposome-mediated delivery and fate of an antisense drug in cell extracts and in single cells by capillary electrophoresis with laser-induced fluorescence. Electrophoresis **24**, 1054–1062.

Matsumo Y., Iwata H., Umeda Y., Takagi H., Mory Y., Miyazaki J., Kosugi A., and Hirose H. (2003), Nonviral gene gun mediated transfer into the beating heart. ASAIO J. **49**, 641–644.

Milman G. and Herzberg M. (1981), Efficient DNA transfection and rapid assay for thymidine kinase activity and viral antigenic determinants. Somatic Cell Genet. **7**, 161–170.

Orrantia E. and Chang P. L. (1990), Intracellular distribution of DNA internalized through calcium phosphate precipitation. Exp. Cell Res. **190**, 170–174.

Orrantia E., Li Z. G., and Chang P. L. (1990), Energy dependence of DNA-mediated gene transfer and expression. Somat. Cell Mol. Genet. **16**, 305–310.

Perletti G., Marras E., Dondi D., Grimaldi A., Tettamanti G., Valvassori R., and DeEguileor M. (2003), Assessment of the biological activity of an improved naked-DNA vector for angiogenesis gene therapy on a novel non-mammalian model. Int. J. Mol. Med. **11**, 691–696.

Potter H., Weir L., and Leder P. (1984), Enhancer-dependent expression of human kappa immunoglobulin genes introduced into mouse pre-B lymphocytes by electroporation. Proc. Natl. Acad. Sci. USA **81**, 7161–7165.

Robbins P. D., Tahara H., and Ghivizzani S. C. (1998), Viral vectors for gene therapy. Trends Biotechnol. **16**, 35–40.

Schaefer-Ridder M., Wang Y., and Hofschneider P. H. (1982), Liposomes as gene carriers – efficient transformation of mouse L-cells by thymidine kinase gene. Science **215**, 166–168.

Szybalska E. H. and Szybalski W. (1962), Genetics of human cell lines IV. DNA-mediated heritable transformation of a biochemical trait. Proc. Natl. Acad. Sci. USA **48**, 2026–2034.

Todorova K., Kolev V., Nacheva G., and Ivanov I. G. (2002), Isolation of plasmid DNA by absorption on glass fibers. Biotechnol. Biotec. Eq. **16**, 145–147.

Weiss S. (2003), Transfer of eukaryotic expression plasmids to mammalian hosts by attenuated *Salmonella* spp. Int. J. Med. Microbiol. **293**, 95–106.