Original Article

Optimization of PTS2-EGFP Expression in CHO and Vero Cells

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Abstract

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Objective: Reporter gene transfer to mammalian cells receives a great deal of attention due to its importance for molecular biology, embryology and developmental biology studies. Among DNA transfer technologies to eukaryotic cells, lipofection is known as the most widely used because of its easy handling procedure, low cell mortality and the natural pathway it undertakes.

Materials and Methods: In this study we have examined the transfectability of two cell types: CHO and Vero cells via Lipofection in four different treatments, with combination of exposure duration, 3 and 6 hrs, and different plasmid DNA concentration, 0.5 and 1µgs. A fusion protein expression vector, pUcD2. PTS2-EGFP was used to direct the EGFP protein to peroxisomes after expression of related cDNA. An SPSS analysis was preformed after counting the positive cells.

Results: optimum gene expression was found when using 1 µg DNA treated for three hrs for CHO cells, and 1 µg DNA treated for six hrs for Vero cells.

Conclusion: The result suggests that CHO lipofection efficiency is significantly increased by both the DNA concentration and exposure time increment; however, an increase in exposure time has less significant effect on low DNA concentration conditions. The same results have been observed for Vero cells. Optimum expression was obtained with highest DNA concentration.

Keywords: Expression Vector, Targeting Signal, Lipofection, Lipoplex

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Introduction

Under appropriate conditions, eukaryotic cells can take up exogenous DNA, and the up-taken DNA can become localized in the nucleus. The phenomenon has been exploited to obtain both transient and stable expression of various genes (1). However, due in part to the size and charge of DNA and to the multitude of enzymatic and membrane barriers imposed by the cell, the spontaneous entry of intact DNA into the cell and its subsequent expression in the nucleus are very inefficient (2). Thus, a wide variety of methods have been developed to facilitate this process. These methods include the use of poly-cations, calcium phosphate, liposome fusion, retroviruses, microinjection, electroporation, and protoplast fusion (3). All Gene delivery systems can be categorized in three distinct groups: physical, viral, and chemical (4, 5). Physical methods require a high number of cell density and DNA concentration, and has high cell mortality. Viral technology undertakes infection pathway which may end in cytotoxicity (6, 7). Chemical methods include DEAE-dextran, calcium phosphate and cationic lipid-mediated transfection (8).

Lipid-mediated transfection is known as lipofection. The use of various lipids for mediating gene delivery was studied as early as 1980 by Flenger *et al* (9). Researchers found that mixing lipids with DNA in water leads to the formation of hollow spheres in lipids called liposomes, with when these liposomes were added to cells growing in vitro, some of the liposomes fused with cellular plasma membranes and were taken up into the cells via endocytosis (10).

However, the effectiveness of these early liposomes was very poor since they did not bind to target cell membranes efficiently. Also, the endocytotic pathway by which the entrapped DNA entered the cells led to fusion with lysosomes, and subsequently to degradation by the digestive enzymes therein (10).

Today, Lipofection is probably the most commonly used gene transfer method (11). Cationic transfection lipids are typically composed of a positively charged head group, such as an amine, a flexible linker group such as an ester or ether, and two or more hydrophobic tail groups. When combined with DNA, cationic lipids spontaneously act to form structures known as lipoplexes, which are much more complex than simple liposomes (12). Under appropriate conditions, lipoplexes maintain an overall positive charge, enabling them to efficiently bind to negatively charged cell surfaces. Subsequently, the lipoplexes enter cells via the endocytotic pathway. This pathway would normally result in fusion with lysosomes and degradation of the DNA.

However, neutral "helper" lipids, such as dioleoylethanolamine (DOPE), are typically included with the cationic lipid, allowing entrapped DNA to escape the endosomes. From there, the DNA can make its way to the nucleus and gain access to the transcriptional machinery of the cell (13).

Lipofection may be used for transient and stable expression of interested gene and is shown that lipofection has a higher efficiency comparing to other chemical methods aforementioned above (14). Even though lipofection method is rapid and applicable and it does not need expensive machinery, its efficiency generally varies between cell lines. Though, DNA concentration and lipoplex exposure duration need to be optimized for different cell types and expression of interested genes e.g. marker genes (15). Chinese Hamster Overy (CHO) and Vero cells are useful to investigate molecular and cellular mechanism involved in gene expression experiments (16-18).

In this study, we have optimized lipofection efficiency for CHO and Vero cell types using duration of exposure and DNA concentration as variables by means of the expression vector pUcD2. Hygro. PTS2-EGFP (19) which carry Enhanced Green fluorescent protein, EGFP, and peroxisome targeting signal 2, PTS2. Due to the fact that PTS2 directs the EGFP into the proxisome (20) and facilitate gene expression detection, this vector has been used. EGFP has the advantage of being detected directly under fluorescence microscopy without further staining procedure (21).

Materials and Methods

Plasmid Preparation

The expression vector, pUcD2. Hygro. PTS2-EGFP obtained from the Department of Biology, Kyushu University, Fukuoka, Japan (19) transformed to *E. coli* DH5α chemically competent cells (Invitrogen, Spain) by heat shock transformation (15). Plasmid DNA was prepared according to QIAprep Miniprep plasmid extraction kit (Cat. No. 27104, Qiagen, Belgium) and diluted in 0.05 mL TE (0.5 mg/mL).

Cell Culture

CHO-K1 (Chinese Hamster Ovary) and Vero (African Green Monkey Epithelial) cells were obtained from Royan Institute (Tehran, Iran) and cultured (22, 23). CHO-K1 cell line was cultured in a 75 cm^2 flask (TPP, Sweden) to reach 90% confluency, incubated at 37°C, 5% CO_2, 90% humidity for 48 hrs in Ham's F12 (D8900-1L, Sigma), supplemented with 10% FBS (10270-106, Gibco, EU), 100UmL^{-1} penicillin (15070-063 Gibco, EU), and 100 μgmL^{-1} streptomycin (15070-063 Gibco, EU). Vero cell line was cultured to reach 90% confluency incubated at 37°C, 5% CO_2, 90% humidity for 48 hrs in DMEM-F12, Dulbecco's Modified Eagle Medium – F12, (Gibco, 21331-020), 10% FBS (10270-106, Gibco, EU), 100UmL^{-1} penicillin, and 100μgmL^{-1} streptomycin (15070-063 Gibco, EU) in a 75 cm^2 flask.

CHO and Vero cells were seeded on two cover-glassed six-well tissue culture plates (TPP, Trasadingen, Switzerland) at the concentration of 1x 10^5 cells per well and incubated at 37°C, 5% CO_2, 90% humidity for 18 hrs prior to transfection.

Medium was substituted by serum-free media for 30 min before transfection (24).

DNA Transfection

Plasmid DNA was transfected using lipofectamine Reagent (Invitrogen, Spain). In order to optimize the expression of the EGFP marker gene, a modified procedure of manufacturer instruction was performed using four different treatments. Both cell types exposed for 3 and 6 hrs to a lipoplex, containing plasmid DNA concentrations of 0.5µg and 1µg with 12µg of lipofectamine. The exposure was repeated for three times. For each treatment, control cells were cultured with lipoplex without plasmid DNA. In order do the transfection, appropriate amount of DNA was mixed with 0.2 mL Opti-MEM I (Gibco, USA) and 12 µg of lipofectamine, followed by incubation at room temperature for 30 minutes. Finally 0.8 mL Opti-MEM I (Gibco, USA) was added to the mixture. Cells were incubated with lipoplex in place of serum free media for 3 and 6 hrs. Treatments included 0.5µg for 3 hrs, 1µg for 3 hrs, 0.5µg for 6 hrs, and 1µg for 6 hrs are referred as treatment 1, 2, 3, and 4 respectively.

EGFP expression detection

Two days post-transfection, both cell types were washed with Phosphate Buffer Saline and fixed by 4% Para-formaldehyde for 45 min. The EGFP expression was visualized by fluorescent microscopy (Olympus BX51, Japan). The percentage of EGFP positive cells out of the total number of cell per 10 fields were counted using Olysia software (Olympus, Japan) for all three repeats. Statistical analyses were performed using SPSS and Excel Software and compared with control treatments.

Results

Kinetics of PTS2-EGFP expression in CHO-K1 cells

Transient expression assay was used to search for the optimum condition of EGFP expression via lipofection. Figure 1 shows punctate fluorescent pattern of PTS2-EGFP in CHO cells after each treatment. These cells were considered as positive or transformed cells. Percentage of positive fluorescent cells out of the total cells per each field was counted in each group and results are presented in figure 2.

DNA showed expression rates of 15.12% and 15.37% positive cell for 3 and 6 hrs exposures of the cells to lipoplex containing 0.5 µg plasmid, respectively. We found 24.03% and 33.54% positive cells after transfection of CHO cells with 1µg plasmid DNA for 3 and 6 hrs, respectively.

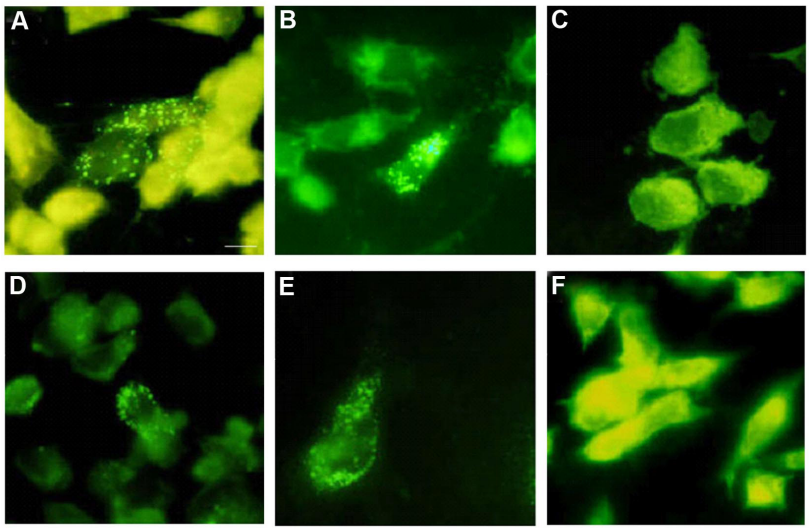

Fig 1: CHO cells expressing PTS2-EGFP.

Green punctate particles, presumably peroxisomes, were visualized by fluorescence microscopy. 10^5 CHO cells were transfected with (A) 0.5µg and (B) 1µg plasmid DNA (C) control cells incubated for 3 hours; (D) 0.5µg and (E) 1µg plasmid DNA (F) control cells incubated for 6 hours are shown as control. Bar=20µM.

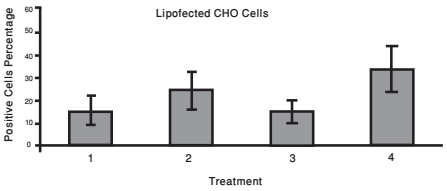

Fig 2: Quantitative analysis of CHO cells showed positive PTS2-EGFP particles.
Mean percentage EGFP - positive cells out of 10 different fields of total cells transfected. Treatments include 0.5µg for 3 hrs, 1µg for 3 hrs, 0.5µg for 6 hrs, and 1µg for 6 hrs treatments are referred to as 1, 2, 3, and 4, respectively ($p<0.05$).

SPSS software was used to perform *chi* square test between treatments 1 and 2; 1 and 3; 2 and 4; and 3 and 4. There were significant differences among all the treatments except the treatments 2 and 4. The highest yield observed when cells were exposed to 1µg plasmid DNA for 6 hrs but the differences were not significant compared with the results of 1µg plasmid DNA for 3 hrs treatment.

Kinetics of PTS2-EGFP expression in Vero cells

The same evaluation methodology was used to monitor the transient expression and the transfection efficiency of the same four different treatments preformed on Vero cells. Vero cells transfected with 0.5 µg plasmid DNA showed 14.6% and 18.48% positive cells when exposed to the lipoplex for 3 and 6 hrs, respectively. Transfection of Vero cells with 1µg plasmid DNA exhibit 17.23% and 41.96% positive cells after exposure to lipoplex for 3 and 6 hrs, respectively (Fig 3).

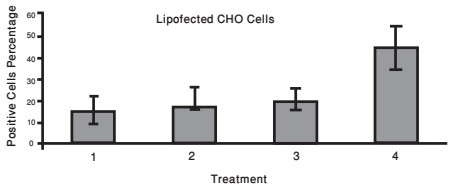

Fig. 3: Vero cells expressing PTS2-EGFP
Green punctate particles, presumably peroxisomes, were visualized by fluorescence microscopy. 10^5 Vero cells were transfected with (A) 0.5µg and (B) 1µg plasmid DNA (C) untreated cells incubated for 3 hours and (D) 0.5µg and (E) 1µg plasmid DNA (F) untreated cells incubated for 6 hours are shown as control. Bar=20µM

SPSS analysis of these data showed that all four treated groups were significantly different. The comparison was done between treatments 1 and 2, 1 and 3, 2 and 4, and 3 and 4. The highest yield was observed when Vero cells were exposed to 1µg plasmid DNA for 6 hrs (Fig 4).

Discussion

The aim of this study was to optimize the transient expression efficiency of pUcD2. PTS2-EGFP in CHO and Vero cell lines. Several methods are currently used for gene transfer to mammalian cells such as lipofection, DEAE-dextran, and calcium phosphate. These methods are categorized as chemical methods of transfection (9, 25). Major drawbacks of DEAE-dextran method are the limited range of cell types with which it works effectively, its lack of efficiency in creating stable cell lines, and its toxicity, especially when DMSO or glycerol is used as a supplemental chemical shock to increase gene transfer efficiency (26).

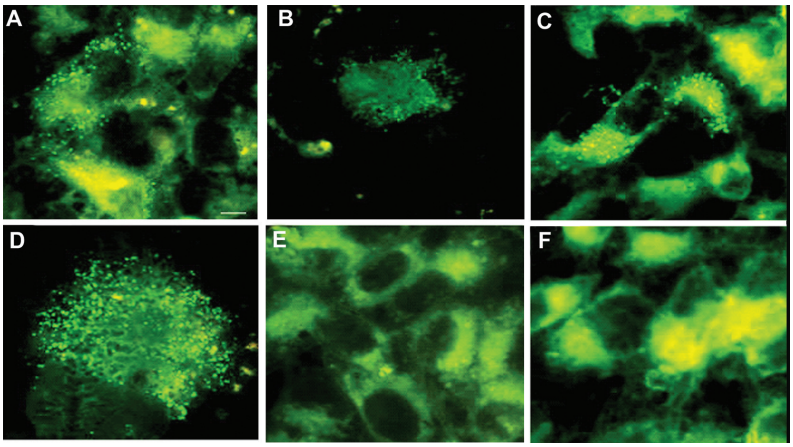

Fig 4: Quantitative analysis of Vero cells showed positive PTS2-EGFP particles.
EGFP - positive cells mean percentage out of 10 different fields of total cells transfected. Treatments include 0.5µg for 3 hrs, 1µg for 3 hrs, 0.5µg for 6 hrs, and 1µg for 6 hrs are referred as 1, 2, 3, and 4 respectively ($p<0.05$).

DEAE-dextran is, therefore, appropriate when transfecting cell types that have already been proven to efficiently uptake DNA administered via this method, or when a low cost transfection reagent with moderate efficiency is preferred over a higher cost reagent with higher transfection efficiency (27). The other one, calcium phosphate method, is advantageous because of its simplicity, low cost, and its applicability to a wide variety of cell types. Moreover, unlike DEAE-dextran, it can be used to generate stably transfected cell lines (28). The disadvantages of the calcium phosphate method are sensitivity to slight changes in buffer salt concentrations, temperature, and pH, as well as, its relatively poor transfection efficiency compared to newer transfection methods, especially in suspension cells such as lymphocytes. Calcium phosphate co-precipitation has a broader range of effectiveness than DEAE-dextran; however, it typically does not achieve transfection efficiencies as high as cationic lipids (29). Cytotoxicity and high cell mortality of these gene transfer mehods have been made cationic lipid-mediated transfection a common method (29).

The main advantages of cationic lipid transfection reagents are their ability to transfect a wide range of cell types with higher efficiencies than previously developed transfection methods (25). Also, cationic lipids are valued for their ease of use, their reproducibility, and relatively low cost and toxicity (30). Despite these advantages, cationic lipids have some limitations that render them less than optimal for certain gene delivery applications (31). For example, several types of primary cultured cells, such as primary neurons, primary dendritic cells, and primary endothelial cells remain recalcitrant to non-viral mediated transfection methods, including cationic lipids (32). Other reasons include the hampered ability of cationic lipids to deliver genes efficiently in the presence of high serum concentrations typically found *in vivo* and that the plasmid DNA concentration and exposure duration should be optimized for every cell types(1).

Therefore, CHO and Vero cells transfected via lipofection to transiently express EGFP in peroxisomes. The data showed that the CHO cells expression efficiency is significantly different when exposed to 1 µg DNA for 3 and 6 hrs and no significant difference observed when exposed to 0.5 µg DNA for 3 and 6 hrs. Significant difference was observed when cells were exposed to 1 and 0.5 µg DNA for 3 and 6 hrs, showing that increase in exposure duration alone did not elevate transfection efficiency when low DNA concentration was used, while promoting DNA concentration did increase transfection efficiency even in lower exposure durations. These results are consistent with the experiments of Ghaedi *et al* (21, 33) and Plisek *et al* (34) who took 4 hrs to efficiently transfect CHO cell using 1µg DNA. The same results were observed for Vero cells; briefly, significant difference was observed when cells exposed to 1 µg DNA for 3 and 6 hrs, and when cells were exposed to 1 and 0.5 µg DNA for 6 hrs. Non-significant differences were obtained when cells exposed to 0.5 µg DNA for 3 and 6 hrs, and when cells were exposed to 1 and 0.5 µg DNA for 3 hrs. The expression efficiency of 1 µg DNA and 6 hrs was significantly higher in Vero cells. Nogal *et al* exposed the Vero cells to lipoplex containing 1µg for five hours to reach highest transient expression (35) and Pertel et al incubated Vero cell for 6 hrs and 1µg DNA (36).

Conclusion

Our recommendation is to use more than 1.0 - 2.0 µg plasmids DNA for 3-4 hrs for lipofection of CHO cells, and 5-6 hrs for Vero cells. Further studies, may be needed to investigate the lipofection efficiency of typical cell lines with higher and lower concentration of lipofectamine.

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