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GFP gene expression in transfected rainbow trout fibroblast cell line RTG-2 using a polycationic reagent (Superfect)

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In this study, GFP reporter gene was transfected into a fibroblast cell line RTG-2 using a polycationic transfection reagent (Superfect) and showed a successful expression of GFP. The transfection efficiency by Superfect was compared to the commonly used transfection method, i.e. DNA-calcium phosphate coprecipitation. Transfection by Superfect was more effective than calcium phosphate coprecipitation method (frequency of cell expressing GFP was 11.3% and 3.5%, respectively). The optimal expression of GFP and β-galactosidase was observed when 5-6 μℓ of Superfect per μg DNA was used for transfection, 1:5-6 ratio between DNA (μg) and Superfect (μℓ).

Key words : Transfection, Gene expression, GFP, RTG-2, Superfect

Introduction

The ability to introduce exogenous genes into cells provides a powerful tool to investigate gene regulation and protein function as well as to develop DNA vaccine. Reporter genes, such as luciferase, β-galactosidase, and chloramphenicol acetyltransferase (CAT) genes, have been used to test the introduction of exogenous gene and to assess the expression of transfected genes. Green fluorescent protein (GFP) is a relatively recent reporter gene that provides the unique ability to collect *in vivo* data, as well as *in vitro*, on gene expression patterns in a non-destructive manner. For example, when coupled to a stress inducible promoter, such as that of *Arabidopsis* Alcohol dehydrogenase (Adh) gene, GFP can be used to monitor a plant responsible to stress in real time (Manak *et al.*, 2000). GFP system has also been used in fish to study the invasion pathway of *Edwardsiella tarda in vitro* and *in vivo* (Ling *et al.*, 2000), and the secretory pathway of carp retinol-binding protein (Bellovino *et al.*, 2002).

Here we report a successful expression of GFP reporter gene in a fibroblast cell line rainbow trout gonad-2 (RTG-2) when transfected using a highly branched polycationic transfection reagent (Superfect, Qiagen).

Materials and Methods

Plasmids

A modified version of the green fluorescent protein (GFP) plasmid driven by cytomegalovirus (CMV) promoter (pEGFP-N3, Clontech) and a β-galctosidase vector fused with SV40 promoter /enhancer (pSV-β-gal, Promega) were purchased for transfection experiment. The plasmid maps are available at web sites (http://www.clontech.com for pEGFP-N3; http://www.promega.com for pSV-β-gal).

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RTG-2 Cell line

RTG-2 cells were kindly gifted by Prof. Secombes (University of Aberdeen, U.K.). The cells were cultured at 18℃ in L15 medium containing 10% fetal bovine serum (FBS) and P/S (100 U penicillin/㎖, 100 ㎍ streptomycin/㎖) and transferred into a 25 cm^2 flask at the density 10^5-10^6 cells/ml one day prior to transfection.

Dose response of transfected GFP plasmid and Superfect reagent

DNA-Superfect complexes were prepared using 10 or 20 ㎍ pEGFP-1 and 30, 50, 100 ㎕ Superfect reagent. Complex formation was induced by placing the reaction at room temperature for 10 min. After incubation of cells with the DNA-Superfect complexes for 6 h, the chemical complexes were removed and 5 ml of fresh growth medium was added. GFP expression in transfected cells was observed *in situ* under a fluorescent microscope (Carl Zeiss) and took the pictures of GFP expressing cells under fluorescent.

Comparison with Ca-phosphate precipitation method

The transfection efficiency of Superfect reagent was compared with a common transfection technique using calcium phosphate-DNA precipitates (Higgins *et al.*, 1999). For a transfection using calcium phosphate-DNA precipitates, RTG-2 cells were transfected with 12.5 ㎍ of pEGFP plasmid using 30 ㎕ of Superfect reagent as mentioned above or transfected with a calcium chloride method optimised for transient transfection of RTG cells (Collet and Secombes, 2001). Cells were plated in 25 cm^2 flasks a day before transfection. DNA 12.5 ㎍ was diluted in 135 ㎕ of 1 X TE (10mM Tris 1mM EDTA, pH 8.0) buffer and mixed with 15 ㎕ of 2.5 M $CaCl_2$ and 150 ㎕ of 2 X Hepes buffer (280 mM NaCl, 1.5 mM Na_2HPO_4, 50 mM Hepes, pH 7.05). The mixture was incubated at room temperature until the calcium phosphate-DNA coprecipitated. This mixture was diluted in 3 ml of complete culture medium (L15 medium plus 10 % FBS and P/S), immediately added to the RTG cells and mixed gently. After 24 h incubation at 18℃, the cells were shocked for 1 min using 20% glycerol solution in NaCl/Pi (Gibco-BRL). The cells were then washed twice with culture medium and further incubated for 48 h at 18℃.

β-galactosidase gene expression and assay

RTG-2 cells were placed in a 24 well cell culture plate and transfected using a β-galactosidase plasmid (pSV-β-gal, Promega) for 6 h before replacing the growth media. DNA-Superfect complexes were formed using 0.1, 0.5, or 1 ㎍ pSV-β-gal and 3 or 6 times of Superfect. After incubation with the DNA-Superfect complexes, the complexes were removed and 350 ㎕ of fresh growth medium was added. Cells were cultured for 48 h and subjected to a β-galactosidase assay using a commercial kit (Promega). Briefly, transfected cells were lysed and incubated with equal volume of 2X assay buffer for 30 min at 37℃. The β-galactosidase activity was measured at 420 nm using a spectrophotometer.

Results and Discussion

Transfection of heterologous genes into cells has been used as an important tool for studying DNA vaccine and the function of newly identified gene or promoter. Transfection methods can be broadly divided into two main categories: first, viral systems based on retrovirus, adenovirus, or adeno-associated virus derived vectors and second, non-viral systems such as chemical treatment, particle bombardment, and electroporation.

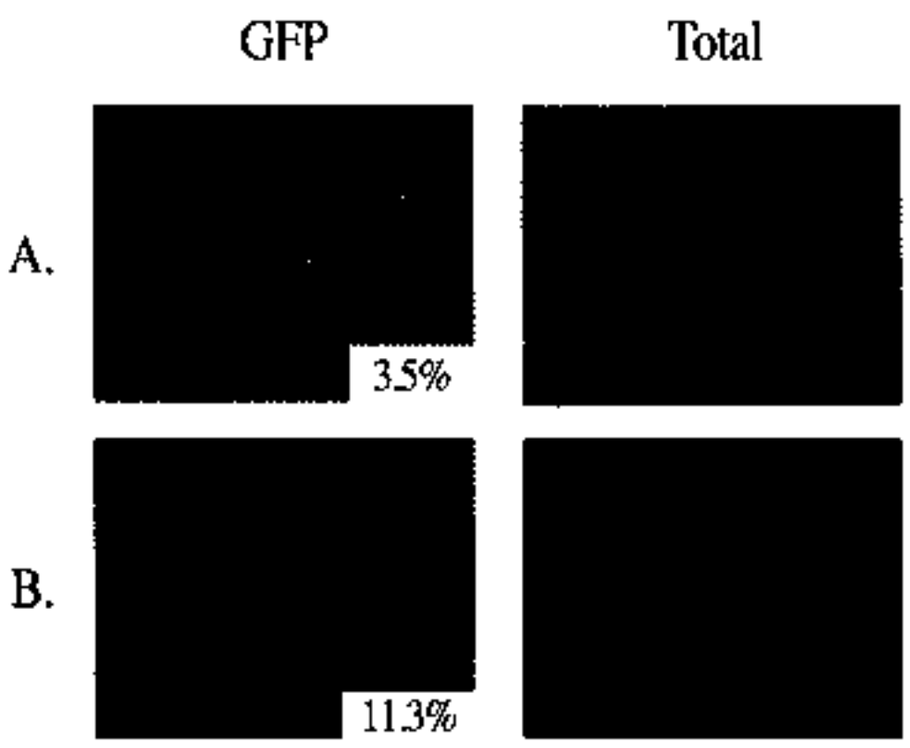

Fig. 1. Comparison of transfection efficiency of Ca-phosphate and Superfect. RTG-2 cells were transfected with 12.5 μg of pEGFP-N3 plasmid using Ca-phosphate precipitation method (a) or with 8 μg of pEGFP-N3 plasmid using 30 $\mu\ell$ of superfect reagent (b). The left column shows transfected cells expressing GFP under a fluorescent microscope *in situ* while the right column represents total cells. The frequency of cell expressing GFP is represented as % in the left column.

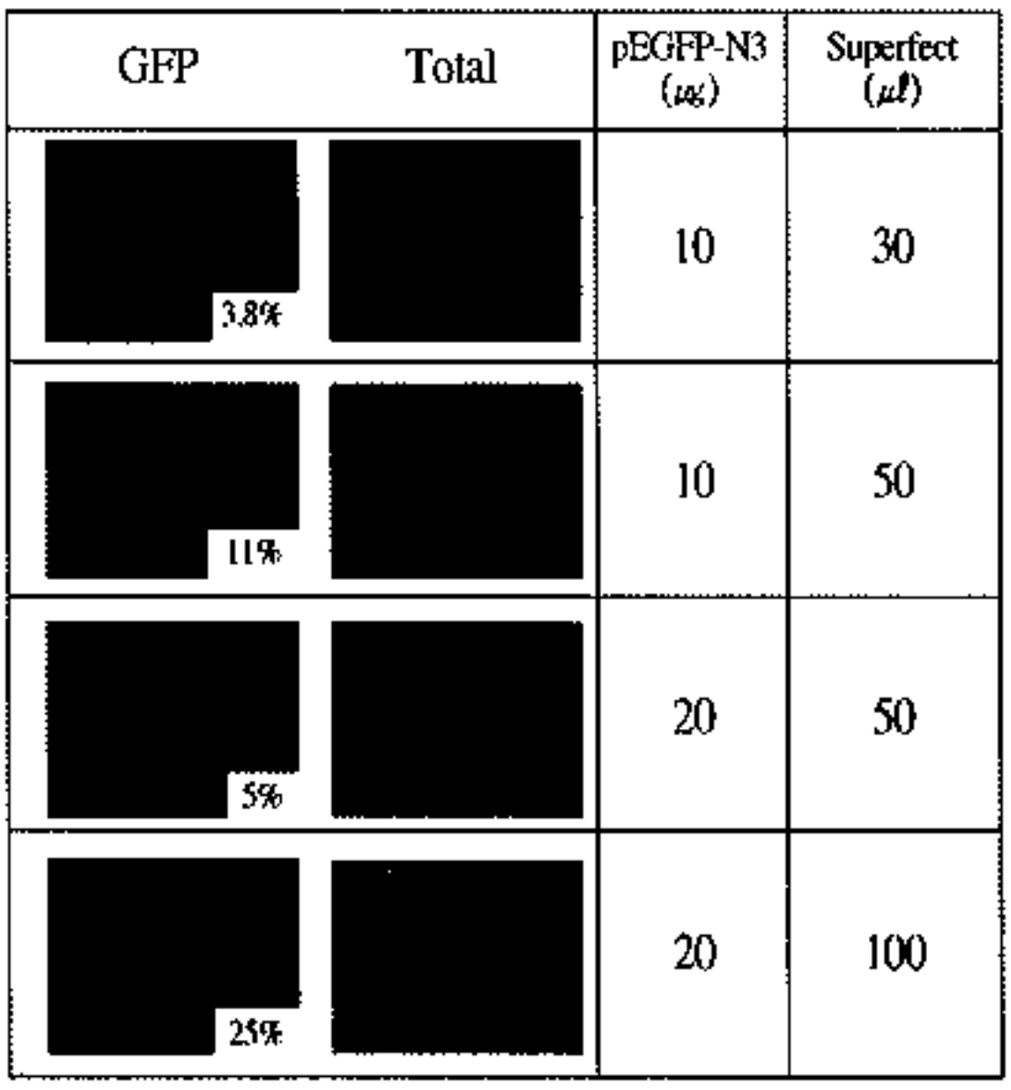

Fig. 2. Expression of GFP in RTG-2 cells transfected with different amount of DNA and Superfect. Various doses of pEGFP-N3 vector (10-20 μg) were conjugated with Superfect at different ratios (1:3 or 1:5) for 10 min at room temperature and transfected into RTG-2 cells. The left column shows transfected cells expressing GFP under a fluorescent microscope *in situ* while the right column represents total cells 5 days after transfection. The frequency of cell expressing GFP is represented as % in the left column.

Non-viral methods including electroporation (Schegel *et al.*, 1988; Rorth *et al.*, 1990), liposomes (Fenjves, 1994), calcium phosphate coprecipitation (Jiang *et al.*, 1991), DEAE dextran (Jiang *et al.*, 1991), and polybrene (Staedel *et al.*, 1994) are either not very efficient or have toxic side effects. RTG-2 cells have been commonly used for promoter assays using a luciferase reporter system (Collet and Secombes, 2001; Collet *et al.*, 2003). RTG-2 cell line is a fibroblast cell line that is essentially ubiquitous in tissue and an attractive target for transfection (Byrnes *et al.*, 2002). Transfection efficiency is often dependent on transfection reagents. In this study, two different transfection systems such as Superfect and calcium phosphate coprecipitation were compared for their ability to transfect plasmid DNA into RTG-2 cell line. Transfection by Superfect seems to be more effective than calcium phosphate coprecipitation method (Fig. 1). The frequencies of cell expressing GFP were 11.3% and 3.5%, respectively

The mass ratio between Superfect and DNA was optimized using a reporter construct, pEGFP-N3 (Fig. 2). Various amounts of pEGFP-N3 vector (10-20 μg) were conjugated with Superfect at different ratio (1:3 or 1:5) and transfected into RTG-2 cells. GFP expression was observed after 5 days under a fluorescent microscope *in situ* since the GFP expression started to appear from 4 days after transfection. This is quite later than in a mammalian cell line, hepatocellular carcinoma (HCC) HepG2 cells which the GFP expression appeared in 24 h after transfection with adenovirus expressing GFP (AdCMV/GFP) (Liu *et al.*, 2002). Kelly-Spratt (1998) has also reported that the first few green fluorescent cells were faintly visible within 12 h, 25-30% peak after 48-72 h and the GFP expression was seen through out the entire cells after 7 days in rat pheochromocytoma PC-12 cells. The differences

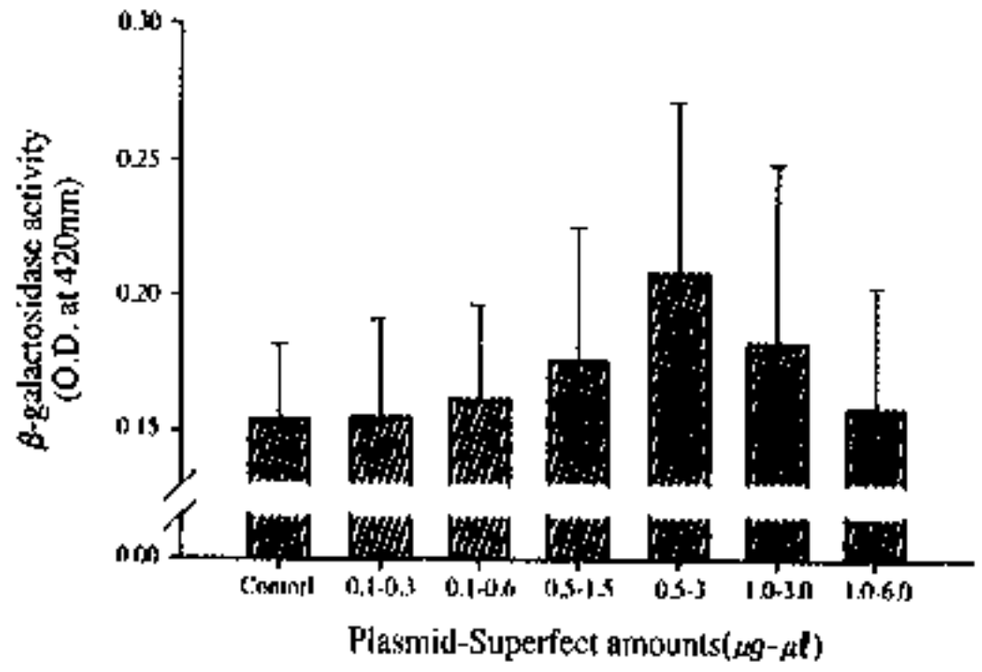

Fig. 3. β-galactosidase expression in RTG-2 cells transfected with different amount pSV-β-gal vector and Superfect reagent. Various doses of pSV-β-gal vector (0.1-6 μg) were conjugated with Superfect at different ratios (1:3 or 1:6) for 10 min at room temperature and transfected into RTG-2 cells. Transfected cells were lysed after 2 days and assayed for β-galactosidase expression. β-galactosidase activity was measured at O.D. 420nm using a spectrophotometer.
* Significantly higher than untransfected control analysed by ANOVA oneway Fisherman's test ($p<0.05$).

between RTG-2 cells and other mammalian cells could be due to the different culture temperature because RTG-2 cells were cultured at lower temperature (18℃) than mammalian cells (37℃).

The highest GFP appearance was obtained using 5 μl of Superfect per μg pEGFP-N3 (Fig. 2). Although the mixture of 20 μg of pEGFP-N3 vector and 100 μl of Superfect gave the best transfection efficiency (25%), 100 μl of Superfect had a strong cytotoxic effect on RTG-2 cells as shown in Fig 3. Thus 10 μg of DNA and 50 μl of Superfect (11%) seems to be the best combination to transfect 10^5-10^6 cells in 25 cm^2 flask.

Gene transfer and expression by Superfect reagent was also analyzed by assessing β-galactosidase expression in RTG-2 cells transfected with pSV-β-gal vector. Various doses of pSV-β-gal vector (0.1-1 μg) were conjugated with Superfect at different ratios (1:3 or 1:6). The highest β-galactosidase activity was also obtained at 1:6 ratio between DNA and Superfect using 3 μl of Superfect and 0.5 μg pSV-β-gal (Fig. 3) in 24 well culture plate.

In conclusion, GFP reporter gene was well expressed in RTG-2 cell line when transfected using Superfect. Superfect transfection system was found to be more efficient to transfect RTG-2 cells than DNA-calcium phosphate coprecipitation method. The optimal expression of GFP and β-galactosidase gene was obtained using 5-6 μl of Superfect per μg of pEGFP-N3 and pSV-β-gal vectors for transfection.

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