

# STABLE TRANSFORMATION OF CULTURED CHICKEN CELLS

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## Summary

A plasmid vector, RSVLTR/ $\beta$ G2, containing lacZ gene under the control of the RSVLTR promoter were transfected into chicken embryo fibroblasts by three different transfection methods. Calcium phosphate, liposome and DEAE-dextran techniques were applied for transfection of chicken cells. A histochemical assay with X-gal was used as a simple method for screening transfected cells. Plasmid RSVLTR/ $\beta$ G2 was expressed proficiently in the chicken embryo fibroblast. Calcium phosphate-DNA precipitate transfection resulted in the highest efficiency for transient expression of RSVLTR/ $\beta$ G2. Transfected cells formed colonies on the 9th day of incubation indicating stable transformation of the inserted plasmid.

(Key Words: Plasmid Vector, Transfection, Calcium Phosphate, Liposome, DEAE-Dextran, Chicken Fibroblast)

## Introduction

Transfection of foreign DNA by retroviruses into chicken cells is well established (Bosselman et al., 1989; Shuman and Shoffner, 1986; Salter and Crittenden, 1989). On the other hand, linear or circular plasmid vectors have not successfully been used to introduce foreign DNA into chicken germ line cells. In this study, the lacZ reporter gene in plasmid RSVLTR/ $\beta$ G2 was used under a variety of conditions to determine the efficiency of transfection in chick embryo fibroblasts *in vitro*.

Methods for introducing cloned plasmid DNA into cultured cells has made it possible to express foreign DNA in eukaryotic cells. Three conditions must be fulfilled to achieve efficient stable transformation of chicken cells by DNA transfection. First, the DNA must be delivered efficiently into the cell nucleus. Second, integration has to be promoted into the host chromosome during

replication. Third, the transduced gene should be expressed sufficiently to be recognized. These factors were examined by three different methods of transfection of plasmid DNA. The methods used were CaPO<sub>4</sub>-DNA co-precipitation, liposome-mediated DNA transfection, and transfection mediated by DEAE-dextran. In addition, factors affecting optimal transfection efficiency were examined. The objectives of this study were to: 1) identify the transfected marker plasmid, 2) compare transfection methods which could be used for application to primordial germ cells (PGCs) transfection, 3) evaluate factors affecting optimal transfection efficiency, and 4) examine the stability of transfected plasmid DNA.

## Materials and Methods

### Vector construction

The RSVLTR/ $\beta$ G2 plasmid was provided by Hua-Ming Wang, University of Utah. This plasmid contains the lacZ gene under the control of Rous sarcoma virus (RSV) long terminal repeat (LTR) promoter. The lacZ gene for  $\beta$ -galactosidase originated from plasmid pGA307 (An et al., 1982). This plasmid contains the tufB-lacZ fusion in which the distal part of tufB, a gene for elongation factor EF-Tu, was replaced with the bacterial lacZ structural gene. The promoter RSVLTR originated from the LTR/tk plasmid

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and is a strong promoter, as well as an enhancer, when introduced into a variety of eukaryotic cells (Gorman et al., 1982). The pGA307 was completely digested with Hind III and Bam HI to produce the DNA fragment containing tufB-lacZ gene. To remove DNA fragments containing tk genes, LTR/tk was also thoroughly digested with Hind III and Bam HI. These two fragments were ligated to produce RSVLTR/ $\beta$ G2 plasmid. Figure 1 illustrates the plasmid structure.

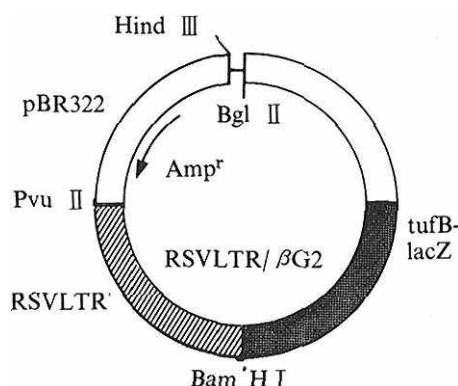


Figure 1. Structure of plasmid RSVLTR/ $\beta$ G2. The plasmid was kindly provided by Hua-Ming Wang, Univ. of Utah. Size of plasmid is approximately 13 kb. Drawing not scaled or proportioned.

#### Isolation of plasmid DNA

Plasmid RSVLTR/ $\beta$ G2 DNA was transformed and maintained into *E. coli* strain 7118. Bacterial transformation followed the standard protocol of Maniatis et al. (1982). Isolation of plasmid DNA and restriction enzyme digestion were performed as described by Maniatis et al. (1982). Supercoiled plasmid DNA was purified by two cycles of CsCl density gradient centrifugation in a VTi65 rotor (50K rpm, 20-22 hr). A spectrophotometer was implemented for quantifying the amount of DNA. Purified plasmid DNA ( $OD_{260}/OD_{280} > 1.8$ ) was used for DNA transfection into chicken embryo fibroblasts (CEF). Linearized DNA was obtained by complete digestion with the restriction enzyme Hind III.

#### Chicken embryo fibroblasts (CEF)

Chicken embryo fibroblasts (CEF) were prepared from the skin of 4 to 6 day incubated

embryos. Cell dissociation was accomplished with  $1 \times$  trypsin-EDTA in phosphate-buffered saline ( $Ca^{2+}$  and  $Mg^{2+}$  free) for 5 min. Cells were mechanically dispersed by repeated aspiration with a Pasteur pipet. CEF cells were grown in a medium consisting of 50% Dulbecco's modified Eagle's medium (DME) and 50% Ham's nutrient mixture F-12 (Sigma), supplemented with 10% calf serum (or chicken serum), 1% antibiotic-antimycotic (Sigma), and 1% L-glutamine (Sigma). The sodium bicarbonate level of this medium was 1.2 g/liter. Cultures were maintained at 41°C in a humidified incubator with 5%  $CO_2$ . Early passage CEF cells were used in the transfection experiments. Primary and secondary passage cells were not used because of possible contamination from other cell types. Other routine procedures were followed according to the cytogenetics laboratory manual, University of Minnesota. In all experiments 1-3 control flasks were grown alongside treated flasks.

#### Calcium phosphate mediated transfection

The following procedure for plasmid DNA transfection is a modification of the method used by Chen and Okayama (1987, 1988). The modification consisted of introducing  $CaPO_4$  in a very gradual way. Three to four Corning 25  $cm^2$  tissue culture flasks containing approximately  $1-3 \times 10^6$  cells per flask were plated 15 to 20 hr before transfection. Cells were grown overnight in 5 ml of a medium with serum to a confluency of approximately 10-50%. DNA was diluted with 225  $\mu$ l distilled, deionized water, and 25  $\mu$ l of 2.5 M  $CaCl_2$  and 250  $\mu$ l of 2XBBBS (pH 6.95, 50 mM N, N-bis (2-hydroxy ethyl)-2-aminosulfonic acid, 280 mM NaCl, 1.5 mM  $Na_2HPO_4$ ) (Stratagene) were added. After mixing gently, this mixture was allowed to incubate 10-20 min at room temperature. The calcium phosphate-DNA mixture was added to 5 ml of medium in a 25  $cm^2$  tissue culture flask and swirled gently to distribute evenly. The flask was then incubated for 4 to 16 hr at 37°C in 2%-3%  $CO_2$  atmosphere. Following a 4-16 hr incubation, the medium was removed, and the cells were washed twice with PBS (8 g of NaCl, 0.2 g of KCl, 1.44 g of  $Na_2HPO_4$ , and 0.24 g of  $KH_2PO_4$  in 1 liter of distilled  $H_2O$ , pH 7.0). The cells were refed and incubated in a 5%  $CO_2$  atmosphere at 41°C for 2 days. The transfection efficiency comparisons

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were evaluated using various amounts of circular plasmid from 1-40  $\mu\text{g}$  or linearized DNA from 1-30  $\mu\text{g}$ , levels of cell confluency at transfection (10-50%), and incubation times with  $\text{CaPO}_4$ -DNA mixture (4-16 hr).

### Liposome-mediated transfection

The procedure for liposome-mediated transfection was followed from the methods of Felgner and Holm (1989). Lipofectin is a preformed cationic liposome which is thought to facilitate DNA transfection by forming a complex with nucleic acid. Subsequently, the complex fuses with the cell membrane and delivers DNA to the cell. CEFs were plated in 25  $\text{cm}^2$  tissue culture flask at a density of  $2.5 \times 10^5$  cells per flask in 2.5 ml DME/Ham's F-12 supplemented with 10% calf serum (or chicken serum). Cells were incubated at 41°C in a humidified 5%  $\text{CO}_2$  environment overnight (15-20 hr). At this time, cell confluency was approximately 40-50%. DNA and 40  $\mu\text{g}$  of Lipofectin™ Reagent (Bethesda Research Laboratories) each were diluted with distilled, deionized water to form 50  $\mu\text{l}$  mixtures. Each of the 50  $\mu\text{l}$ , DNA and Lipofectin™ Reagent dilutions were combined in a polystyrene snap cap tube to obtain 100  $\mu\text{l}$  of mixture solution per 25  $\text{cm}^2$  flask and mixed gently. This DNA-lipofectin complex was incubated 15 min at room temperature. The cells were washed twice with serum free medium and 3 ml Opti-MEM® Reduced Serum Medium (Gibco) was added per flask. In addition, 100  $\mu\text{l}$  of DNA-Lipofectin™ Reagent per flask was applied dropwise, gently swirling the flasks, and incubated for 8-10 hr at 37°C. After incubation with the DNA-liposome complex, the medium was removed, and DME/Ham's F-12 with 10% serum was added. The flask was incubated for 48 hr at 41°C in a humidified 5%  $\text{CO}_2$  level atmosphere. Transfection efficiency was compared according to DNA content (circular: 1-40  $\mu\text{g}$ ; linear plasmid DNA: 1-20  $\mu\text{g}$ ) and amount of Lipofectin™ Reagent (10-40  $\mu\text{g}$ ).

### Transfection mediated by DEAE-dextran

The method modified from McCutchan and Pagano (1968) and the procedure prepared from Holter et al. (1989) (without chloroquine) were used for transfection mediated by DEAE-dextran. Cells were washed with DME/Ham's F-12 to remove serum. DNA was introduced into 2 ml

DME/Ham's F-12 and 25  $\mu\text{g}/\text{ml}$  DEAE-dextran was added. In the sequential method (Holter et al., 1989), cells were washed with DMF/Ham's F-12 and incubated 30 sec with a medium containing 500  $\mu\text{g}/\text{ml}$  DEAE-dextran. After washing the cells with medium, DNA was applied in a volume of 2 ml. At the end of the incubation period with DNA, cells were shocked for 30 sec in prewarmed 5% glycerol, washed and cultured in DMF/Ham's F-12 containing 10% calf serum for 48 hr at 41°C with 5%  $\text{CO}_2$ . Transfection efficiencies were compared under varying incubation times and amounts of DNA.

### Transient expression

To secure an estimate of transfection stability, the calcium-phosphate-DNA and liposome mediated DNA transfection methods were screened every 3 days from the onset of transfection. The data were averaged for two trials with two replications using the circular RSVLTR/ $\beta\text{G}2$ . In the  $\text{CaPO}_4$  transfection method, 10  $\mu\text{g}$  of DNA per 25  $\text{cm}^2$  flask was applied to 30% confluent cells for an 8 hr transfection. In the liposome mediated DNA transfection, 10  $\mu\text{g}$  of DNA and 40  $\mu\text{g}$  of Lipofectin™ Reagent were used to transfect 50% confluent cells for 8 hr. Following 8 hr of incubation at 37°C, the cells were refed with DME/Ham's F-12 media supplemented with 10% serum and incubated 24 hr at 41°C. Flasks were subdivided into 3 flasks and incubated at 41°C for another 24 hr. Two flasks were screened for  $\beta$ -galactosidase activity and one flask was subdivided into 3 flasks and incubated another 3 days. After transfection of CEF, the transfection efficiency was examined every 3 days for a 2 wk duration.

### Histochemical staining for $\beta$ -galactosidase activity

Histochemical staining for  $\beta$ -galactosidase activity was used to determine transfection efficiency in all trials. The method of A.M. Gibbins (1990, personal communication), modified from Sanes et al. (1986) was followed. Cells were fixed for 5 min in 50 mM phosphate buffer (pH 7.4) containing 22% (V/V) glutaraldehyde, 2% (V/V) formaldehyde and 2 mM  $\text{MgCl}_2$ . Cells were then rinsed three times with 2 mM  $\text{MgCl}_2$  and 0.02% (V/V) NP-40 in 50 mM phosphate buffer, pH 7.4 for 5 min each. The cells were then overlaid with a histochemical reaction mixture containing

0.5 mg/ml 5-bromo-4-chloro-3-indolyl- $\beta$ -galactosidase (X-gal), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 2 mM  $MgCl_2$  in a 50 mM phosphate buffer (pH 7.4). The endogenous  $\beta$ -galactosidase activity was detected by using 0.1 M citrate buffer (pH 4.3) instead of 50 mM phosphate buffer (pH 7.4). X-gal was dissolved in dimethyl sulfoxide (DMSO) at 20 mg/ml, then diluted into the reaction mixture. Incubation time was 12-16 hr at 37°C. Subsequent to incubation, blue cells as indicators of transfection were counted in the inverted phase contrast microscope. When counting blue cells, 25 cm<sup>2</sup> tissue culture flasks were divided into 36 small sections, and 9 sections were counted then multiplied by 4 to calculate the total number of blue cells. The transfection efficiency was calculated by adding the total number of blue cells then dividing by the total number of cells per flask. All trials were replicated 3 to 4 times and averaged for estimates of transfection efficiency.

## Results

### Transfection efficiency with $CaPO_4$ -DNA precipitation method

Controls not containing RSVLTR/ $\beta$ G2 did not result in blue cells by 24 hr of incubation with X-gal at 0.05 M phosphate buffer solution, pH 7.4. Transfected cells with RSVLTR/ $\beta$ G2 were detected after 5 min of X-gal treatment. Following 3 hr of X-gal treatment, approximately 50% of the transfected cells expressed  $\beta$ -galactosidase activity. Almost all of the transfected cells were detected after 12-16 hr incubation with X-gal treatment, and exceeding 16 hr of incubation did not increase the blue cell count significantly. The expression of endogenous chicken  $\beta$ -galactosidase gene was not detected after 1 hr of incubation but after 3 hr of incubation with X-gal at 0.1 M citrate buffer, pH 4.3. Following 13 hr of incubation with X-gal at pH 4.3, approximately 60% of the total cells (not transfected) changed to a blue color, and approximately 90% turned blue after 30 hr of incubation indicating that chicken chromosomes express  $\beta$ -galactosidase genes. Expression of exogenous plasmid DNA was successful only at pH 7.4 *in vitro*.

The transfection efficiency was compared by levels of circular or linear plasmid DNA RSVLTR/ $\beta$ G2 introduced and integrated in cells as

shown by the appearance of blue cells. Figure 2 illustrates the transfection efficiency indicated by the percent of blue cells in chicken embryo fibroblasts culture with circular plasmids. The conditions of transfection were 8-12 hr incubation with  $CaPO_4$ -DNA precipitate, 2.5%  $CO_2$ , and 50-60% confluency of cell at the time of transfection application. Ten  $\mu$ g of DNA per 5 ml of medium in a 25 cm<sup>2</sup> flask resulted in the highest transfection efficiency, 1.24% (figure 2). Linear DNA resulted in a lower transfection than circular DNA. Cultures incubated with 1  $\mu$ g and 10  $\mu$ g of linear DNA per 5 ml of medium showed higher transfection efficiencies. Ten percent confluency of the cell at the time of plasmid application was more efficient than 50% confluency for linear DNA (figure 3). Therefore, cell confluency is a factor affecting transfection efficiency. Figure 4 illustrates the effect of confluency of the cell monolayer at time of transfection on transfection efficiency using circular DNA. Ten percent confluency of cells at the time of transfection increased efficiency more than 2-fold. Transfection efficiency was also examined for various time durations (figure 5) at 10% confluency of the cells and application of 10  $\mu$ g of circular DNA. Eight hours of incubation with  $CaPO_4$ -DNA precipitates transfected DNA into the cells with a higher efficiency. After 16 hr of incubation using this method, cytotoxicity was observed.

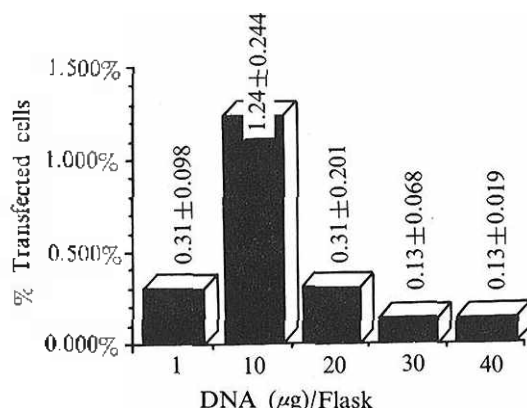


Figure 2. Effect of the amount of the circular plasmid DNA, RSVLTR /  $\beta$ G2, on transfection efficiency in CEF at a confluency of 50-60% after an 8-12 hr incubation. The  $CaPO_4$ -DNA co-precipitation method was used.

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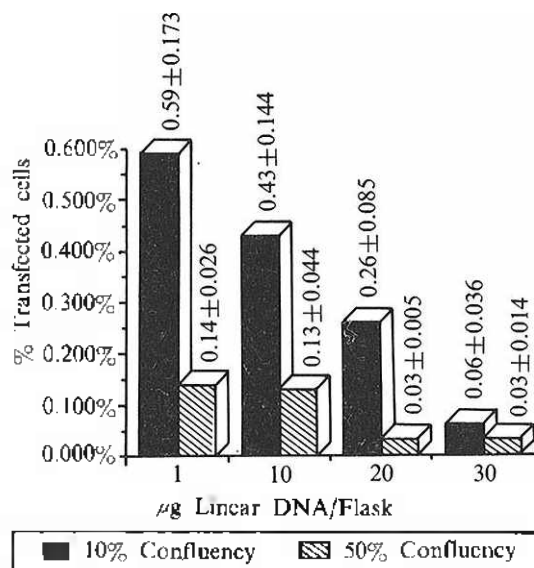


Figure 3. Effect of linear DNA content and confluency of the cells with 8 hr incubation on transfection efficiency. The  $\text{CaPO}_4$ -DNA co-precipitation method was used.

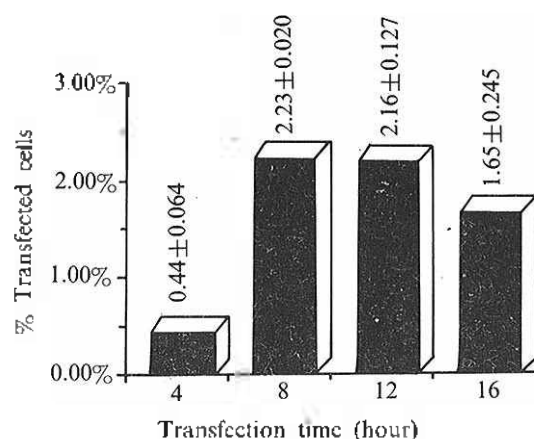


Figure 5. Effect of incubation time with calcium phosphate-DNA precipitates on the transfection efficiency. 10 µg of circular DNA and 10% confluency of the cells were used.

## Liposome mediated transfection

Figure 6 shows the results of lipofection mediated transfection by amount and form of DNA. A high transfection efficiency was achieved with 10 µg of DNA per 25 cm<sup>2</sup> flask for both circular and linear plasmid (0.41% and 0.35% respectively). There is little difference between

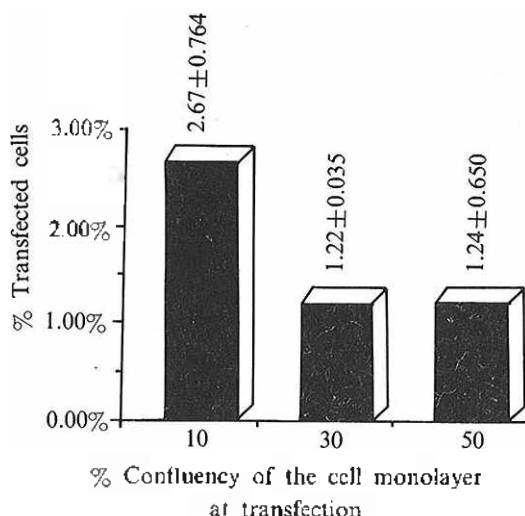


Figure 4. Effect of the confluency of the cell on the transfection efficiency in the  $\text{CaPO}_4$  transfection method. Ten µg of circular DNA was used and cells were incubated for 8 hr.

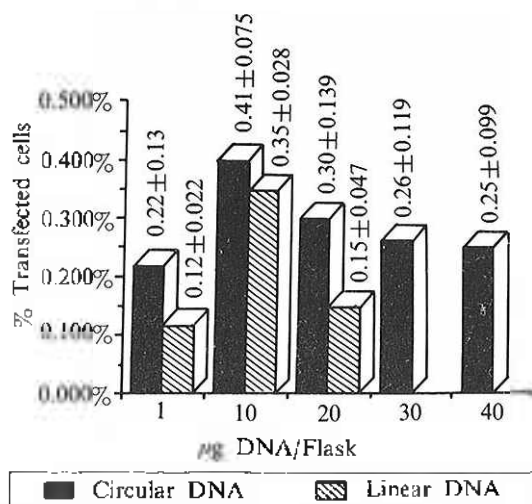


Figure 6. Effect of various concentrations of circular and linear DNA with 40 µg of lipofectin on transfection efficiency. Linear DNA was applied only in 1 µg, 10 µg, and 20 µg amounts.

linear and circular DNA efficiencies for liposome protected plasmids, as compared to the calcium-phosphate transfection method, in which linear DNA was considerably lower. While efficiency of liposome mediated transfection was lower than that of the calcium phosphate-DNA precipitate method, liposome mediated transfection did not

result in cytotoxicity with 10  $\mu\text{g}$ , 20  $\mu\text{g}$ , and 30  $\mu\text{g}$  of DNA, whereas  $\text{CaPO}_4$ -DNA mediated transfection showed some cytotoxicity at 8 hr of transfection and much cytotoxicity at 16 hr of transfection. Increasing concentrations of liposome improved transfection of CEF with 10  $\mu\text{g}$  of circular DNA (figure 7). Forty  $\mu\text{g}$  of lipofectin did not result in cytotoxicity.

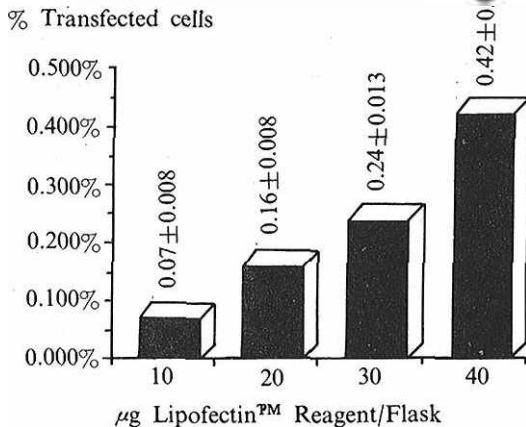


Figure 7. Effect of liposome concentrations on transfection efficiency. Ten  $\mu\text{g}$  of circular DNA was applied for 5-8 hr in 50-60% confluent cells.

#### Transfection mediated by DEAE-dextran

Figure 8 illustrates transfection efficiencies resulting from different amounts of DNA applied and compares the standard and sequential DEAE-dextran procedures without chloroquine. Sequential treatment with DEAE-dextran and DNA resulted in an improved transfection efficiency over the standard method. Maximal efficiency occurred with 1  $\mu\text{g}$  of DNA treatment in the sequential method and with 0.5  $\mu\text{g}$ -1  $\mu\text{g}$  of DNA treatment in the standard method. The percentage of transfection of chicken embryo fibroblast was 0.53 % and 0.25% respectively. Incubation periods exceeding 4 hr did not enhance transfection (figure 9). DEAE-dextran mediated DNA transfection had some toxicity to the cells. The sequential method was more toxic than the standard method possibly due to high concentration of DEAE-dextran treatment (500  $\mu\text{g}/\text{ml}$ ) or glycerol shock of the cells.

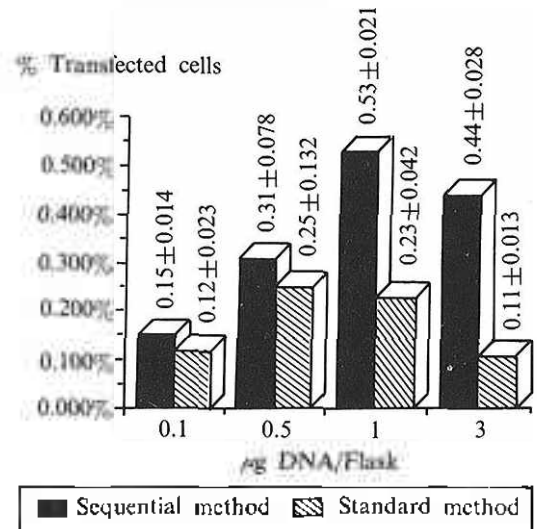


Figure 8. Effect of DNA amount on transfection efficiency of CEF when treated with DEAE-dextran and circular DNA. Cells were incubated for 12 hours at 50% confluency.

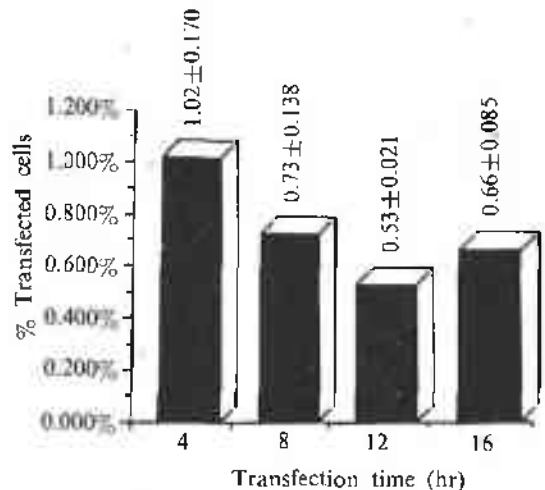


Figure 9. Effect of transfection time on efficiency in sequential treatment of DEAE-dextran and circular DNA.

#### Transient expression

As shown in figure 10, almost all the DNA degraded between 3 days and 6 days of transfection. At the 6th day of transfection, only 10-20% of transfected DNA was left indicating that almost all plasmid DNA expresses  $\beta$ -galactosidase without integration into chromosomes and DNA was degraded during the replication

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of the cell. Transfected blue cells were colonized from the 9th day of transfection (figure 11), suggesting that transfected plasmid DNA was stably transformed. The number of colonized cells per colony was 4-20, indicating that the rapidly growing cells divided 2-5 times in the 3 day interval. The efficiency of stable transformation was  $7 \times 10^{-4}$  in  $\text{CaPO}_4$  transfection method and  $5 \times 10^{-4}$  in liposome mediated DNA transfection.

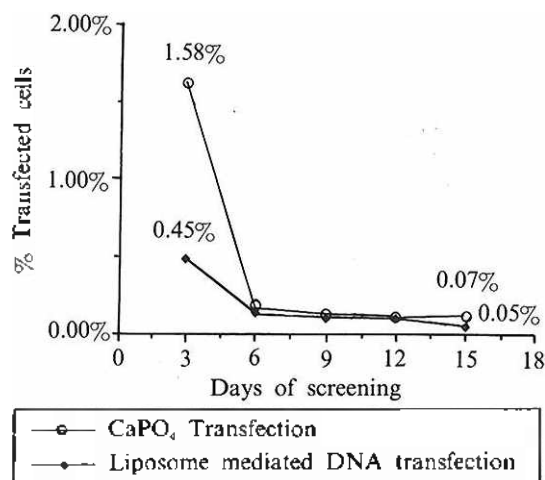


Figure 10. The efficiency of stable transfection.  $\text{CaPO}_4$  transfection and liposome mediated DNA transfection, were examined for transfection efficiency every 3 days. Ten  $\mu\text{g}$  of DNA per 25  $\text{cm}^2$  flask was applied for 8 hr in both methods.

### Discussion

The calcium phosphate precipitation technique produced higher efficiencies (2.67%) than liposome mediated DNA transfection (0.42%) or DEAE-dextran method (1.02%). The efficiency of calcium phosphate precipitation is affected by the pH level, amount of DNA, level of  $\text{CO}_2$ , form of the DNA, different cell types, and cell confluency (Corsaro and Pearson, 1981; Chen and Okayama, 1987). The level of  $\text{CO}_2$  at transfection (2-3%) and pH level of the buffer (pH 6.95) was used as suggested by Chen and Okayama (1987) for high transfection efficiency. By keeping the amount of DNA to approximately 10  $\mu\text{g}$  per dish, the efficiency of the calcium phosphate-DNA precipitation technique was improved. Linear

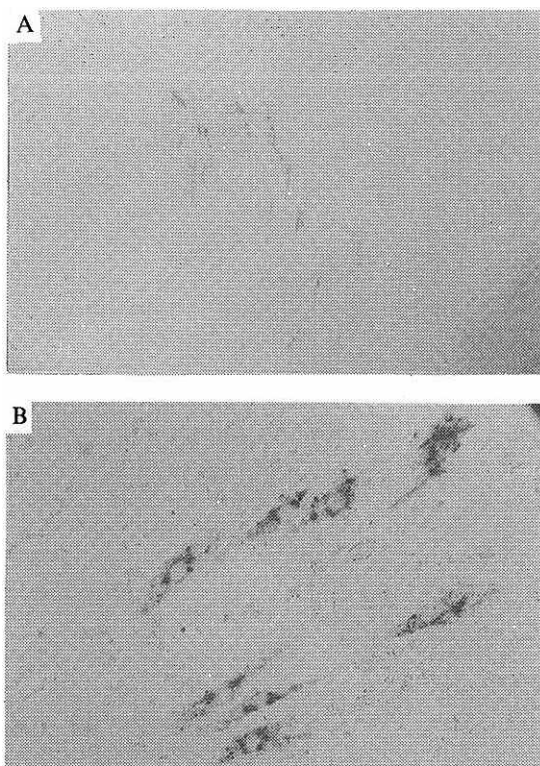


Figure 11. Colonization of chicken embryonic fibroblast transfected by RSVLTR /  $\beta\text{G2}$ . (A: 100X, B: 400X).

DNA produced poor transfection corresponding to the reports by Chen and Okayama (1987). The linear DNA may be susceptible to nuclease degradation during transport of the DNA to the nucleus following endocytosis and it may degrade more rapidly than circular DNA in the cytoplasm. Chen and Okayama (1987) reported 10-50% stable transfection by using the pcDneo plasmid in various mammalian cell lines. The approximately 3% transient transfection efficiency in CEF, is significantly lower than the results of Chen and Okayama (1987). The different efficiencies resulting from the same method could be due to different recipient cell types (Corsaro and Pearson, 1981; Van Pel et al., 1985) or the nature of the introduced gene and transcriptional control region. The reasons why low density of the cell increases transfection efficiency is unclear. Possibly more replication can be plated at 10% than 50%.

Lipofection resulted in a very low 0.4-0.5% transfection efficiency. The efficiency of lipofectin mediated transfection was dependent on the

amount of liposome and DNA added. The optimal amount of DNA was 10  $\mu$ g per 25 cm<sup>2</sup> flask. Increasing the concentration of liposomes improved transfection in CEF linearly. These results correspond to those of Felgner et al. (1987) and Burrin and Jameson (1989). In CV-1 cells, maximal expression of pSV2 cat was achieved with 10  $\mu$ g-20  $\mu$ g of DNA per 100 mm tissue culture plates (Felgner et al., 1987), and in rat primary pituitary cells, maximal expression of RSVcat or  $\alpha$ CAT plasmid was evident in 20  $\mu$ g of DNA per 60 mm dish (Burrin and Jameson, 1989). Time of incubation with lipofection-DNA complexes did not significantly affect transfection efficiency after 3-5 hr of treatment. Liposomes are relatively less sensitive to a broad range of DNA concentrations, form of DNA, cell confluency, CO<sub>2</sub> level and pH level when compared to the calcium phosphate-DNA precipitation transfection.

Optimal condition for DEAE-dextran mediated transfection were a 1  $\mu$ g of DNA with 4 hr of incubation. Holter et al. (1989) reported that sequential treatment of DEAE-dextran and DNA yielded up to 60% in HeLa cells positive for a surface protein encoded by the transfected sequence (pRSVcat DNA). They also found this method to be nontoxic. In contrast, approximately 1% efficiency was obtained in CEF using this method, but chloroquine was not applied in this study. It is unclear whether the different transfection efficiencies are the result of chloroquine treatment or the different cell types and plasmid DNA. In this experiment, the sequential method was more toxic to CEF than the standard method, which did not agree with the results of Holter et al. (1989). The small amount of DNA (1  $\mu$ g) was sufficient to promote transfection. Compared to calcium phosphate DNA precipitation transfection and liposome mediated transfection, only a small amount of DNA is needed for sufficient transfection by DEAE-dextran. This result corresponds to other reports (Sompayrac and Danna, 1981; Holter et al., 1989). A transfection time of 4 hr resulted in optimal transfection efficiencies but others report 8 hr as the optimal transfection time (Holter et al., 1989; Lopata et al., 1984), possibly due to different cell types or no treatment with chloroquine. The sequential treatment of DEAE-dextran and DNA resulted in an approximately 2-fold higher effi-

ciency over the standard method.

In conclusion, the lacZ gene under the control of the RSVLTR promoter was expressed in cultures of chicken embryo fibroblasts, and the histochemical assay with X-gal was a simple method for screening transfected cells. By 24 hours of X-gal treatment, the expressed exogenous RSVLTR/ $\beta$ gal was clearly differentiated by the blue color. In these experiments, the CaPO<sub>4</sub>-DNA precipitate transfection resulted in the highest efficiency for transient expression with the RSVLTR/ $\beta$ gal plasmid. Cytotoxicity was evident after 16 hr of incubation with CaPO<sub>4</sub>-DNA precipitates. The optimal conditions for this method were more exacting than liposome mediated DNA transfection. The liposome-mediated transfection method did not show cytotoxicity at 10  $\mu$ g-20  $\mu$ g of DNA and is less sensitive to a broad range of conditions. In both the CaPO<sub>4</sub>-DNA precipitates and liposome mediated transfection methods, transfected cells formed colonies by the 9th day of incubation, suggesting stable transformation of the inserted plasmid. DEAE-dextran mediated transfection method displayed cytotoxicity with low expression of transfected RSVLTR/ $\beta$ gal. Furthermore, it is practically impossible to apply the 30 sec DEAE treatment and 30 sec glycerol shock to embryos *in vivo*.

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