Efficient transfection of DNA by mixing cells in suspension with calcium phosphate

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The most commonly used method for transfecting DNA into recipient cells is the co-precipitation of the DNA of interest with calcium phosphate (CaPO_4). The standard method of transfection requires the preparation (and eventually application) of the precipitate of DNA- CaPO_4 individually for each plate containing a monolayer of cells (1). When a number of plates are used, the standard method results in a variation of transfection efficiency among the plates and difficulty in performing the experiment. Here we have successfully introduced the β -amyloid precursor protein (β APP) promoter DNA into the N1E-115 cells by incubating the cell suspension with the DNA- CaPO_4 precipitate, and investigated the effects of time and temperature on the efficiency of transfection.

Plasmids. The plasmid pAmylCAT was constructed by inserting a 1.2 kb fragment of the β APP promoter into the plasmid pBLCAT3 upstream of the chloramphenicol acetyltransferase (CAT) reporter gene (2). The pAmylCAT was co-transfected with the plasmid pSV β GAL. The SV40 early gene promoter drives transcription of the *lacZ* gene which encodes the β -galactosidase enzyme (β GAL) (3). The amount of DNA used for transfection was 6 $\mu\text{g}/10^6$ cells.

Cell culture. The N1E-115 cells (4) were cultured in a DMEM medium containing 10% fetal bovine serum (FBS). Rat PC12 cells were cultured in a RPMI 1640 medium containing 5% FBS and 10% horse sera. The average number of cells at transfection was $1-3 \times 10^6$ cells/5 ml of complete medium.

'Standard method' of transfection by adding the DNA- CaPO_4 precipitates in adherent cells. In this method, a precipitate containing CaPO_4 and DNA was allowed to form by slowly mixing a HEPES-buffered saline with a solution of CaCl_2 and DNA. The suspension was incubated at room temperature (RT) for 20 min and the precipitate was added to the cells which were cultured at 37°C. The cells were harvested 48 h later.

'Modified method' of transfection by mixing the DNA- CaPO_4 precipitates with the suspension of cells. Briefly, a precipitate containing CaPO_4 and DNA was allowed to form like in the 'standard method' and then the precipitate was added directly to the cell suspension which was prepared from several plates depending on the number of transfections to be performed. For example, if we had planned to perform DNA transfection in eight plates containing growing cells of 80% confluence, we carried out the

following steps. First we prepared a combined 8 ml CaPO_4 -DNA precipitate in a tube by incubating at RT for 20 min. During this period, we harvested the cells from eight plates, centrifuged down and resuspended the cells in 8 ml medium. We mixed the CaPO_4 -DNA precipitate with the pooled cells in a tube, incubated them for 30 min at RT and divided the whole mixture equally onto eight plates. The cells were cultured in 5 ml of medium at 37°C, harvested 48 h later and analyzed.

Electroporation. Briefly, the cells were harvested, resuspended in serum-free medium and incubated for 10 min with the plasmid DNA at 4°C. The cell-DNA mixture was pulsed using the Gene Pulser (Bio-Rad, CA). Electroporation was performed under different parameters of voltage (250–350 V) and capacitance (125–960 μF) in either PBS or regular medium as per the manufacturer's instructions. The pulsed cells were returned to ice for 10 min before seeding them onto different plates. The cells were harvested 48 h later and assayed for CAT and β GAL activity.

CAT assay. The transient expression of the reporter gene was monitored by measuring the CAT activity followed by thin-layer chromatography (TLC) (2,5). Briefly, 40–75 μl of extract (50 μg of the cellular proteins preheated at 60°C for 10 min) was made up to 100 μl with 0.20 M Tris-HCl (pH 7.8), 1.76 mM acetyl coenzyme A and 0.05 μCi of [^{14}C]chloramphenicol (50 mCi/mmol, NEN). The reaction was performed at 37°C for 2 h and the chloramphenicol was extracted with ethylacetate. The solvent was dried, the pellet resuspended in ethylacetate and spotted on a TLC plate to separate the native chloramphenicol from its acetylated derivatives. The radioactive spots were cut out and counted. The CAT activity was expressed as the percentage conversion of chloramphenicol into its total acetylated forms.

β -GAL assay. Briefly, 5 μg of the proteins from the cell extract were incubated with 1 mM MgCl_2 , 5 mM chlorophenol red- β -D-galactopyranoside (CPRG) and 0.1 M HEPES in a 100 μl reaction at 37°C for 1.5 h. The reaction was stopped by adding Na_2CO_3 and the optical density was measured at 574 nm. The β -GAL activity was calculated from the standard curve of the enzyme and expressed as U/10 mg of protein.

For comparison, we transfected the N1E-115 cells with DNA by the methods of electroporation and CaPO_4 . We were unable to introduce DNA into the N1E-115 cells by electroporation (Fig. 1,

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Figure 1. Autoradiogram showing the CAT activity in cell extracts transfected by the electroporation method. N1E-115 cells were either not transfected with any plasmid (lane 1) or transfected with the plasmid pAmylCAT (lanes 2–5). Electroporation was carried out using the Gene Pulser apparatus at either 960 μ F/350 V (lane 2), 960 μ F/250 V (lane 3), 500 μ F/350 V (lane 4) or 500 μ F/250 V (lane 5). The PC12 cells were either transfected with pAmylCAT at 960 μ F/350 V (lanes 6 and 8) or not transfected with any plasmid (lane 7). The cells were harvested and the CAT assay was performed as described in the text.

lanes 1–5). Only 0.54% of conversion was observed in the cell extracts (lane 5). However, we successfully used the electroporation method to transfect pAmylCAT into PC12 cells. Over 90% of [14 C]chloramphenicol was converted into its acetylated derivatives using 50 μ g of the PC12 cell extracts at 960 μ Fd and 350 V (lanes 6 and 7).

For the N1E-115 cells, we performed the original steps of the CaPO₄ procedure in four different ways. First, we followed strictly the 'standard method' to transfect DNA in two plates. This was achieved by preparing 1 ml of DNA–CaPO₄ suspension in each of two tubes and the precipitate from each tube was added to its corresponding plate (Fig. 2, lanes 2 and 3). The percent conversion of chloramphenicol to its total acetylated derivatives was 26 ± 11 and 34 ± 14 , respectively, from the untransfected cell extracts (lanes 1–3). In the second method, we prepared 1 ml of DNA–CaPO₄ suspension in each of two tubes. The precipitates from the two tubes were pooled and then divided equally into two plates (lanes 4 and 5). The percent conversion was 24 ± 5 and 26 ± 4.7 , respectively. In the third method, we prepared a combined 2 ml of DNA–CaPO₄ suspension in a tube and then equally divided it onto two plates (lanes 6 and 7). The percent conversion was 26 ± 4.7 and 24.5 ± 6.5 , respectively. Preparing the DNA–CaPO₄ precipitate separately for each cell dish had little effect on transfection efficiency ($P > 0.96$, lanes 2–7). Thus pooling the contents from the tubes either before or after the formation of precipitates did not significantly change the results.

In the fourth method, we added the DNA–CaPO₄ precipitate directly to the cell suspension using the 'modified method'. To determine the best conditions for transfection, we incubated the DNA–CaPO₄ precipitate and cell suspension at different temperatures for different periods of time (Fig. 2). When we incubated the DNA–CaPO₄ precipitate with the cell suspension we obtained a higher CAT activity than when the precipitate was directly added to the adherent cells. The degree of increase in promoter activity was dependent on the temperature of incubation of the DNA–CaPO₄ precipitate with the cell suspension. For example, CAT activities of 49 ± 18 and 55 ± 15 were observed when the incubation was for 10 and 30 min at RT, respectively

Figure 2. Assay of CAT and β GAL activities in cell extracts transfected by the 'standard' (lanes 2–7) and 'modified' (lanes 8–15) CaPO₄ methods. Lane 1 shows the results from the untransfected cell extracts. The pAmylCAT and pSV β GAL plasmids were either transfected into N1E-115 cells without (lanes 2–7) or with (lanes 8–15) the suspension of cells. One ml of the DNA–CaPO₄ suspension was incubated at RT for 20 min and the precipitate was added to a plate of cells (lanes 2). The same experiment was repeated for another plate (lane 3). One ml each of the DNA–CaPO₄ suspension was prepared separately in two tubes and incubated at RT for 20 min. The precipitates from the two tubes were pooled and then divided equally onto two plates (lanes 4 and 5). Two ml of a combined DNA–CaPO₄ suspension was prepared in a tube and incubated at RT for 20 min to form the precipitate which was then added in equal amounts to two plates (lanes 6 and 7). A combined 8 ml of DNA–CaPO₄ suspension was prepared in one tube and incubated at RT for 20 min. One ml of the DNA–CaPO₄ precipitate was added to each of eight tubes containing 1 ml of cell suspension. After mixing the DNA–CaPO₄ precipitate and cell suspension, they were incubated at either RT for 5 min (lane 8), 10 min (lane 9), 20 min (lane 10), 30 min (lane 11); 4°C for 10 min (lane 12), 30 min (lane 13) or 37°C for 10 min (lane 14), 30 min (lane 15). The rest is as described in the text. The results of CAT and β GAL activities shown here is average data from four different experiments with indicated S.E.M. ($n = 4$).

(lanes 9 and 11). On the contrary, lower CAT activities of 14 ± 1.3 and 14 ± 1.2 were observed when the incubation was for 10 and 30 min at 37°C, respectively (lanes 14 and 15). Thus the time of incubating the cell suspension with the precipitate had no significant effect on the transfection efficiency of pAmylCAT ($P > 0.15$, lanes 8–11) but the temperature of incubation of the cell suspension with the precipitate did have a significant effect e.g., incubation at RT resulted in a higher promoter activity than incubation either at 4 or 37°C ($P < 0.0046$, lanes 8–15). To rule out the possibility of any bias of the modified procedure to a particular plasmid, we successfully reproduced the whole procedure with another unrelated plasmid, pSV β GAL. The result obtained with pAmylCAT was consistent with the pSV β GAL cotransfection (Fig. 2).

We have observed that two kinds of situations may arise during the transfection of DNA in cells. (i) Cells that can be transfected by both the electroporation and CaPO₄ methods (e.g., PC12 cells). Here one can perform DNA transfection by the electroporation method but the apparatus itself is very expensive and so is the cuvette for each transfection. In this situation, the 'modified method' reported here is another option available to study the transcriptional control of the promoter in adherent cell types. (ii) Cells that can be transfected by the CaPO₄ method only. As shown in Figure 1, we could not transfect DNA into N1E-115

neuroblastoma cells by the method of electroporation under the conditions used. We therefore tried the 'standard method' of transfection by CaPO_4 . Although it is an easy and simple method of transfection for many cell types, the 'standard method' has limitations. For adherent cell lines such as PC12 and N1E-115, the 'standard method' requires the preparation (and eventually the application) of the coprecipitates of DNA- CaPO_4 separately for each plate. When a number of plates are used, this method results in variation in transfection efficiency among plates. To normalize the results from different plates we transfect the cells with pAmylCAT and cotransfect the same cells with a control plasmid, pSV β GAL. Following transfection of and reculturing the cells, we assay for both CAT and β GAL activities in cell extracts. We calculate the CAT activity which is then normalized for equal transfection efficiency within a given cell type, by considering the CAT activity per 500 U β -GAL activity. Therefore, in the 'standard method' (a) one requires another control plasmid for co-transfection and assaying for another reporter gene and (b) depending upon the concentration and the molar ratio of the plasmids, the expression of the co-transfected control plasmid is likely to be modulated by a transacting enhancer or silencer DNA element. This would, in turn, overestimate the enhancer/promoter activity of the co-transfected plasmid (6).

In our cell-suspending procedure, one can eliminate this co-transfection step. Since the DNA precipitate was incubated with the cell suspension which was then divided into a number of plates, each plate should be identical in terms of transfection

efficiency. This would not have been possible using the standard procedure where the DNA precipitates were added individually onto each plate of adherent cells. On comparing the percent conversion between the modified method (55 ± 14) and the standard method (26 ± 11), we conclude that transfection by the calcium phosphate method worked best when the precipitate of DNA- CaPO_4 were incubated with the cell suspension. Our results have shown that suspending the adherent cell prior to transfection did not interfere with the cell's ability to be transfected by using the calcium phosphate method. Thus our results indicated that the 'modified method' works better than the standard CaPO_4 method both in terms of promoter activity as well as eliminating the variability of transfection efficiency among different plates of cells.

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