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Transfecting mammalian cells: optimization of critical parameters affecting calcium-phosphate precipitate formation

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ABSTRACT

DNA–calcium phosphate co-precipitates arise spontaneously in supersaturated solutions. Highly effective precipitates for transfection purposes, however, can be generated only in a very narrow range of physico-chemical conditions that control the initiation and growth of precipitate complexes. The concentrations of calcium and phosphate are the main factors influencing characteristics of the precipitate complex, but other parameters, such as temperature, DNA concentration and reaction time are important as well. An example for this is the finding that almost all of the soluble DNA in the reaction mix can be bound into an insoluble complex with calcium phosphate in <1 min. Extending the reaction time to 20 min results in aggregation and/or growth of particles and reduces the level of expression. With improved protocols we gained better reproducibility and higher efficiencies both for transient and for stable transfections. Up to 60% of cells stained positive for β-gal and transient production of secreted proteins was improved 5- to 10-fold over results seen with transfections using standard procedures. Similar improvements in efficiency (number of recombinant cell colonies) were observed with stable transfections, using co-transfected marker plasmids for selection. Transient expression levels 2 days after DNA transfer and titers obtained from stable cell lines, emerging weeks later, showed strong correlation.

INTRODUCTION

Co-precipitates composed of ‘calcium phosphate’ (hydroxyapatite) and purified DNA have been used for >20 years for the transfer to and expression of genetic information in mammalian cells in culture (1). This technique has become one of the major methods for DNA transfer to mammalian cells. A number of papers (2–6) have addressed observed variability of DNA transfer and low efficiency and they usually contain specific recommendations for ‘optimal’ procedures. However, these communications did not report on the complex relationships between different components responsible for the creation of a supersaturated status. We present here data that provide the basis for a better understanding, at the physico-chemical level, of some of the most crucial aspects concerning the formation of DNA containing precipitate complexes. We have applied gained insights in sets of transfections both for transient and stable expression of recombinant proteins. We used two popular immortalized cell lines for these studies; human embryo kidney 293 cells (HEK-293) (7–9) and dihydrofolate reductase-minus (DHFR–) Chinese hamster ovary cells (CHO) (10–13). We were able to improve the efficiency of DNA transfer and expression in both cell lines.

This paper also illustrates that a simple centrifugation–OD assay can be useful in studying kinetics and reproducibility of precipitate formation. This test provides information on the reliability of precipitate formation and gives greater assurance for successful DNA transfer to cells.

MATERIALS AND METHODS

Plasmid isolation

Propagation of AmpR plasmids were done in the bacterial strain DH5a using 50 μg/ml carbenicillin in LB medium. DNA was isolated using the alkaline lysis method and was purified by double equilibrium centrifugation in CsCl–ethidium bromide gradients as described by Sambrook *et al.* (23).

Calcium phosphate–DNA co-precipitation

A solution of 100 μl of 2.5 M $CaCl_2$ and the desired amount of plasmid DNA was diluted with 1/10 TE buffer (1 mM Tris–HCl, 0.1 mM EDTA, pH 7.6) to a final volume of 1 ml. One volume of this 2× Ca/DNA solution was added quickly to an equal volume of 2× HEPES solution: 140 mM NaCl, 1.5 mM Na_2HPO_4, 50 mM HEPES, pH 7.05 at 23°C, or alternatively phosphate-free 2× HEPES buffer was prepared and supplemented with phosphate from a 300 mM stock solution of pH 7.05 containing 195 mM Na_2HPO_4 and 105 mM NaH_2PO_4. The two

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solutions were mixed quickly once, and added to the cell culture medium after the time frame indicated.

Centrifugation assay

Precipitate formation was confirmed and quantified by absorption at 320 nm against a blank lacking the phosphate or alternatively lacking both, DNA and phosphate. The association (binding) of DNA with precipitate was determined by OD measurement at 260 and 320 nm of a 250 µl aliquot of the supernatant of the precipitation mixture after 30 s centrifugation (16 000 *g*) in an Eppendorf centrifuge. The supernatant was analyzed immediately after the centrifugation step. Precautions were undertaken to prevent heating up of the rotor when multiple samples were analyzed. For precipitation experiments at 0°C the rotor was cooled down on ice before it was used.

Cell culture

Cell culture was performed according to Doyle *et al.* (21). Both CHO cells and 293 cells were grown in a DMEM/F12 1:1 based medium, supplemented with 2% fetal calf serum. CHO cells were maintained attached in 75 cm^2 flasks. Cells (293 HEK) were adapted to growth in suspension and grown in 250 ml spinner flasks. Both cell lines were subcultivated once or twice a week at ratios between 1:10 and 1:100.

Transient transfections

Cells from the exponential growth phase were seeded (1–4× 10^5 cells/ml) into 12-well or 60 mm plates the day before the transfection was done. One hour before the precipitate was added, the medium was replaced with fresh medium (pH 7.4). For each ml of medium, 100 µl of precipitate was added. With a calcium concentration of 125 mM in the precipitation mixture, the resulting final concentration of calcium in the cell culture medium was ~12.5 mM. The cells were exposed to the precipitate for 2–6 h at 37°C at a pH of 7.3–7.6. For CHO cells, a glycerol shock was applied at this point. The cells were exposed to 20% glycerol in PBS. After 1 min the glycerol was removed by adding fresh medium, aspiration of the mixture and replacement with fresh medium. For 293 cells the medium was replaced by fresh medium without applying a shock. The cells were then incubated for 1–6 days before the supernatant was harvested and analyzed by ELISA. Transfection efficiency was determined by staining β-galactosidase expressing cells with X-Gal after 24 h (22).

Stable transfection for CHO cells

A 1:1 (w/w) mixture of linearized and purified plasmid containing DHFR as a selective marker or the TNK–tPA (16) expression cassette was precipitated as described above. The cells were grown for 2 days after transfection in non-selective medium and the supernatant was harvested to measure transient expression. The cells were trypsinized and seeded in 100 mm plates under different selective pressure. Of these cells, 2% were seeded in 100 mm plates in medium lacking glycine, hypoxanthine and thymidine (GHT– medium), 20% in GHT– medium containing 30 nM methotrexate (MTX) and 40% at 100 nM MTX. After 10 days, the product titers from pools of cells were assayed by ELISA. For each level of selective pressure, titers seen from emerging pools of stable cells were normalized against each other.

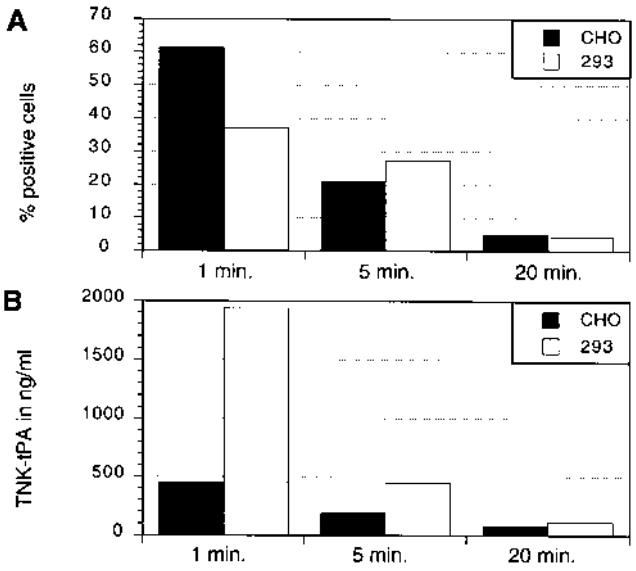

Figure 1. Transient expression of β-gal or TNK–tPA in HEK-293 and in CHO cells transfected with a calcium phosphate–DNA precipitate complex. The precipitate was allowed to form for 1, 5 or 20 min after mixing and then added to the cells. (**A**) Cells were fixed and stained with X-gal 24 h after transfection. (**B**) Supernatant was harvested and analyzed after 6 days.

RESULTS

Complete DNA binding can be achieved within seconds upon initiation of crystal formation

Subsequent to the mixing of the calcium/DNA solution with the HEPES/phosphate solution a slight opacity appears within a few minutes, indicating that a precipitate has been formed. With the intention of having the reaction 'complete', standard protocols suggest an incubation period of up to 20 (4) or even 30 min (3) at room temperature. To measure the rate at which the DNA is being incorporated into or associating with the forming precipitate an assay was developed. This test was based on a centrifugation step. The DNA concentration remaining in the clarified solutions was assessed by determining the optical density at 260 nm. Surprisingly, at a pH of 7.05 and a DNA concentration of 25µg/ml, adsorption of DNA occurred within 30 s. In fact 30 s was the shortest period for completion of this test. When either calcium or phosphate was missing in the mixture, no DNA was found to be adsorbed (data not shown).

Time-dependent changes in the precipitation complex that affect the efficiency for transfections

To determine the optimal incubation time for the formation of an efficient precipitate for transfections, the reaction mix was transferred to cells at different time points upon initiation of the reaction. A β-galactosidase (β-gal) plasmid (14) was used to estimate the number of transfected cells (transfection efficiency). An expression vector for human tissue plasminogen activator (TNK-tPA) (15,16) was used in a second transfection experiment.

Highest transfection efficiencies were observed with precipitation mixtures that had been incubated for short periods of time. In a representative experiment ~60% of CHO cells and 38% of

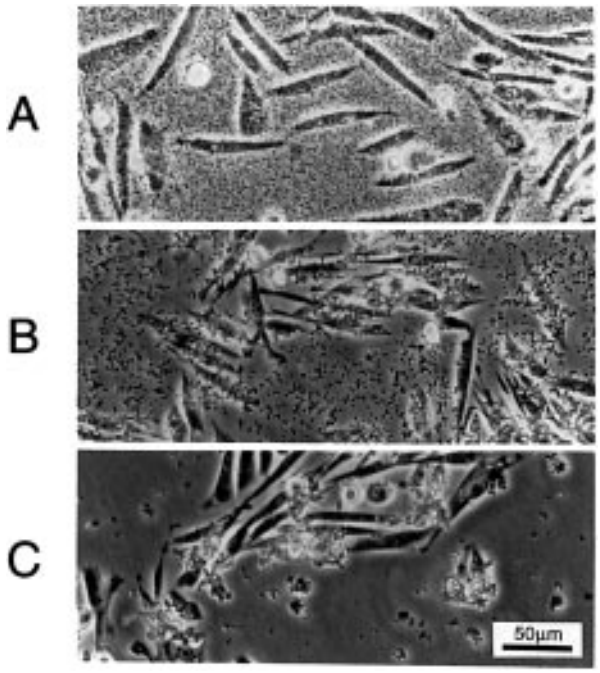

Figure 2. CHO cells with precipitates, visualized by phase-contrast microscopy (400× magnification) 4 h after adding the transfection cocktail to the cells. The precipitate mix was incubated for (**A**) 1, (**B**) 5 or (**C**) 40 min before transfer to the cells.

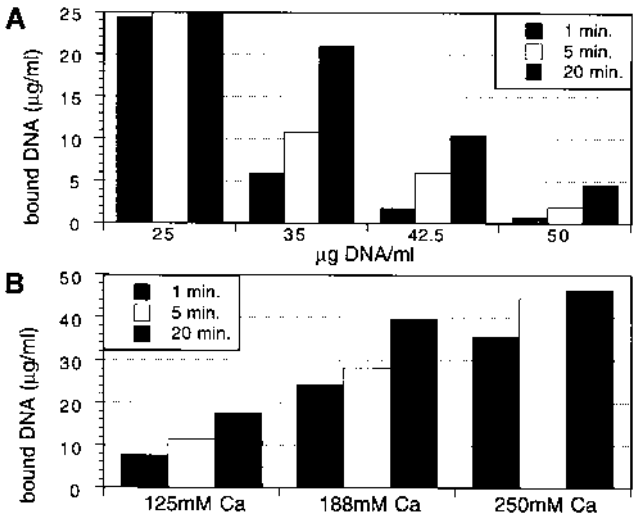

Figure 3. DNA binding capacity of a forming calcium phosphate precipitate at 1, 5 and 20 min. (**A**) Increasing DNA concentrations from 25 to 50 µg/ml at 125 mM calcium. (**B**) Precipitation of 50 µg/ml DNA at different calcium concentrations.

HEK-293 cells stained positive for β-galactosidase in plates which had been exposed to 1 min precipitate complexes (Fig. 1A). The transfection efficiency decreased to 3–5% when precipitates were used after an incubation period of 20 min. Using a 5 min reaction time gave intermediate results. Similar differences, here in yield of a secreted protein, were observed with a transfected TNK–tPA vector (Fig. 1B). Almost 2 µg/ml for HEK-293 cells and 0.5 µg/ml for CHO cells of TNK–tPA were detected by ELISA in the supernatant of cells exposed to an 'early' precipitate-complex (1 min). 'Late' precipitates (20 min) gave only 10% of these titers, confirming observations made by O'Mahoney *et al.* (6).

These differences in transfection efficiency or in the levels of secreted recombinant protein could be correlated with the nature of the calcium phosphate precipitate in plates (Fig. 2). Precipitates added 1 min after mixing, consisted of a large number of very small particles covering the surface of individual cells almost completely. Many particles seemed to adhere to the cells, however many were floating in the medium and exhibited Brownian motion.The large number of particles and, possibly, Brownian motion may be responsible for the image being of poor quality. Precipitate formed within a 5 min reaction time consisted of fewer but larger particles. None of these particles were floating. After 40 min the precipitate consisted of even fewer particles, some of them as big as the cells themselves. The highest transfection efficiency correlated with the generation of many very small particles.

Factors affecting the kinetics of precipitate formation

Multiple transfections, done on the same day with the same solutions usually give reproducible results but the transfection efficiency can vary dramatically if experiments are performed on different days (own observations and personal communications). This provided the motivation to systematically search for parameters which affect the precipitate formation and would change the efficacy in transfections. Chen and co-authors (5) reported that the plasmid concentration needs to be optimized to achieve high transfection efficiencies. We verified this observation and found that the amount of DNA can have a major effect on the precipitation reaction (Fig. 3A). At 25 µg/ml all the DNA was bound to the forming precipitate within 1 min. Higher concentrations of DNA partially inhibited the formation of precipitates, resulting in reduced amounts of DNA being associated with an insoluble precipitate complex. A DNA concentration of 50 µg/ml DNA almost completely blocked the formation of precipitates. Even after a 20 min incubation time, <20% of the DNA was associated with a precipitate.

In an independent experiment (no temperature control) we show that increasing the calcium concentration could reverse this phenomenon. 125 mM calcium (standard) was compared with calcium concentrations of up to 250 mM (Fig. 3B). At a concentration of 250 mM calcium twice the amount of DNA was transferred into an insoluble precipitate, almost as fast as seen under the standard calcium concentration for 25 µg/ml DNA.

These data indicate that DNA participates early in the process of precipitate complex formation. The interplay of concentrations of calcium and DNA points to the possibility that DNA molecules may affect early events during nucleation and 'crystal' growth.

Another important parameter is the temperature. DNA binding experiments were performed with a HEPES–phosphate buffer in which the phosphate concentration was reduced from 0.75 to 0.6 mM. Results of one of these experiments are presented here (Fig. 4). Under reduced phosphate concentration the rate of precipitate formation was significantly slower. The individual reactions were performed at temperatures ranging from 0 to 37°C. At 20°C, a 1 min reaction time was no longer sufficient to completely bind the DNA in solution. Even after 20 min only ~80% of the DNA was associated with a precipitate. At a temperature of 37°C, however, fast and complete removal of DNA from the solution occurred during the centrifugation step. At 0°C

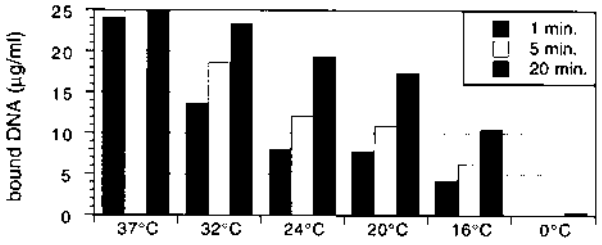

Figure 4. Effect of temperature on formation of DNA–calcium phosphate precipitate complexes under a reduced phosphate concentration of 0.6 mM.

on the other hand, all the DNA remained in the supernatant and almost no precipitate could be detected after 20 min. This indicates a reduced solubility of calcium phosphate at elevated temperature, and it appears that relatively small variations in temperature are able to affect the kinetics of precipitate complex formation.

Developing an assay to quantify the precipitation step

Spontaneous precipitation occurs only if concentrations of calcium and phosphate are high enough to ensure supersaturation. All the data presented above suggest that conditions which affect the solubility of 'calcium-phosphate' would directly affect the nature of the precipitate complexes. To demonstrate this we tested five different calcium concentrations between 12.5 and 250 mM in combination with 10 different phosphate concentrations between 0.15 and 6 mM and determined the quantity of DNA remaining in solution after a centrifugation step (Fig. 5A). With a calcium concentration of 250 mM, the DNA was co-precipitated at a phosphate concentration of 0.5 mM or higher. When the calcium concentration was decreased, higher phosphate concentrations were needed to co-precipitate DNA. Precipitate-complex formation and binding of DNA could be initiated with each of the calcium concentrations used, yet at 12.5 mM calcium, the phosphate concentration had to be ≥4 mM.

To assess precipitate complex formation directly, the mixture (suspension) was transferred into a spectrophotometer cuvette. The non-precipitated solutions show no absorption at 320 nm (Fig. 5B). An increase of absorption at 320 nm indicates the appearance of a precipitate, which could be confirmed visually: the higher the phosphate concentration, the more cloudy the suspension. Increasing concentrations of phosphate resulted for each calcium–DNA mixture in a typical curve, showing increasing optical densities at higher phosphate concentrations. This assay is very reliable and gave reproducible results when performed with the same solutions repeatedly. It allows to distinguish precipitates created with different phosphate concentrations together with the same calcium and DNA solutions. It should be noted, that absorption at 320 nm is influenced by various parameters such as crystal size, particle number and structural characteristics of the precipitate complex and that an individual value does not give indications about the ratio of effects mediated by these different parameters.

Complete binding of DNA correlates with the appearance of a visible precipitate. Incomplete binding of DNA occurs in a very narrow concentration range of phosphate. While at a phosphate concentration of 0.77 mM all DNA was associated with the

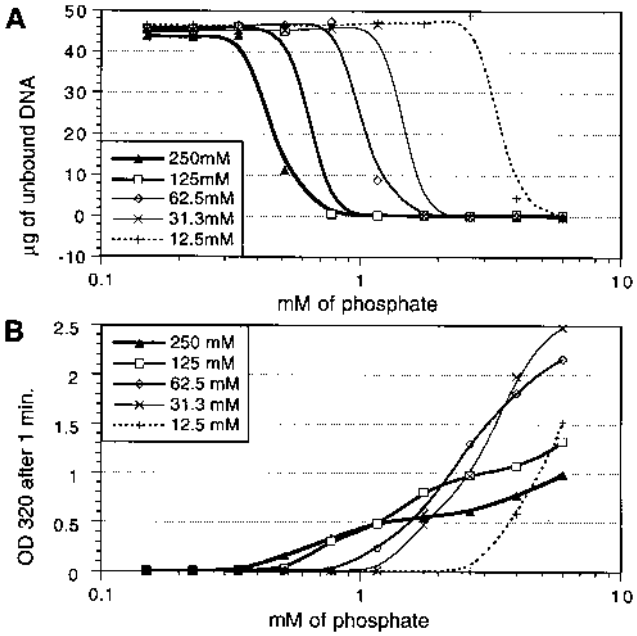

Figure 5. Effect of calcium and phosphate concentration on the precipitation of DNA (50 µg DNA/ml, 23°C, pH 7.05, reaction time = 1 min). Five calcium concentrations, ranging from 12.5 to 250 mM were tested, as indicated in the figure inserts. (**A**) Soluble DNA in the supernatant as a function of the phosphate concentration. (**B**) Turbidity of the precipitation mixture (suspension) measured at 320 nm.

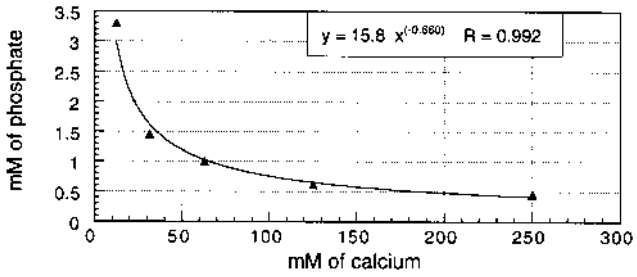

Figure 6. Relationship between calcium and phosphate concentrations sufficient to precipitate 50% of the DNA within 1 min at 23°C in a solution with 50 µg/ml DNA at pH 7.05.

precipitate (at 125 mM calcium); a reduction of phosphate by 35% to 0.51 mM completely prevented binding of DNA.

In Figure 6 conditions are summarized which address incomplete binding of DNA. Concentrations of calcium and phosphate that will precipitate and bind 50% of the DNA provided were calculated from the data of Figure 5A. These data show that the association of DNA with an emerging calcium phosphate precipitate is a function of both calcium and phosphate concentration.

Correlation between precipitated DNA and levels of expression

Using the phosphate concentration as a tool to change the nature of the precipitate, we designed an experiment to correlate the optical characteristics with the transfection efficiency. Sufficient volumes of solutions were mixed to quantify the precipitation step and also to

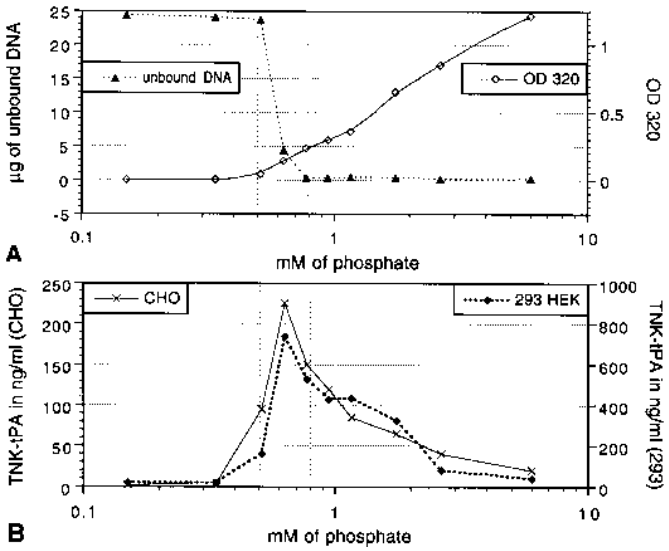

Figure 7. Assessment of various phosphate concentrations during the formation of a precipitate (25 µg DNA/ml, 23°C, 125 mM calcium, 1 min and pH 7.05). (**A**) Quantity of soluble DNA in the supernatant after centrifugation of the precipitation mix and optical density of the precipitation mixture at 320 nm. (**B**) Transient expression levels of tPA in the supernatant of CHO or 293 HEK 42 h after transfer of the precipitation mixture to cells. The dotted vertical lines indicate concentrations of 0.5 (left) and 0.8 mM phosphate (right).

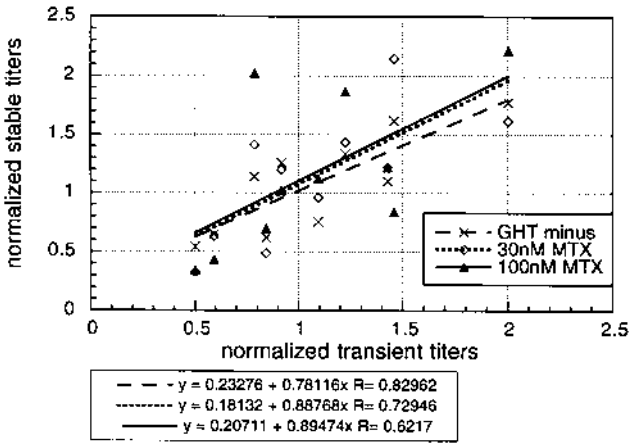

Figure 8. Correlation between transient and stable expression of tPA in a set of 10 independent transfections. From each plate cells were seeded in selective medium with increasing selective pressure (GHT–, 30 nM MTX and 100 nM MTX) 2 days after transfection. Transient expression levels were assessed 2 days after transfection, stable expression levels were assessed from pools of cells after 2 weeks.

transfect two different cell lines with the same solutions on the same day in order to assess a possible correlation. While standard pH, calcium and DNA concentration were chosen for these experiments (at 23°C), the incubation time was reduced to 1 min. Shown in Figure 7A are DNA-binding and precipitate formation as measured by absorption at 320 nm, over a range of phosphate concentrations from 0.15 to 6 mM. Corresponding transient expression levels for TNK–tPA in CHO and HEK-293 cells in relationship to the phosphate concentrations are shown in Figure 7B.

Phosphate concentrations of <0.51 mM did not result in the formation of precipitates within 1 min and no transfection occurred. At a phosphate concentration of 0.63 mM, >80% of the DNA was bound to the precipitate which consisted of many very small particles. This condition also gave best transfection efficiencies for both lines. Correlating with coarser precipitates seen under the microscope, higher phosphate concentrations resulted in lower transient titers. No precipitate was detected in precipitation mixtures with 0.15 or 0.34 mM phosphate. However, in plates precipitates became visible after ~4 h. Similar precipitates were seen in plates in which the calcium concentration was raised by 12.5 mM by addition of a calcium stock solution.

Stable transfections: early 'transient' titers predict number of stable clones

A series of stable transfections was performed in CHO cells. In order to facilitate the integration of plasmid DNA into the cellular genome, all plasmids were linearized by restriction enzyme digestion. A DHFR vector as a selectable marker was co-transfected together with the expression vector for TNK–tPA (16). A single plasmid mixture and cells prepared and set up on a single day were used. Transfection protocols were deliberately altered to reflect various precipitation reactions. When stable clones were selected, the number of emerging clones after the selection period of 2–3 weeks was found to be different for each type of transfection used. Most importantly, conditions that generated efficient precipitate complexes for transient expression, also resulted in the largest number of stable clones (data not shown, 17).

Figure 8 shows that expression levels generated transiently (2 days after exposure of DNA to the cells) correlate to the protein levels observed in pools of stable cells selected after 2 weeks. Normalized values for transient expression were plotted against normalized values representing stable expression of pools of recombinant cells, selected under three different stringency conditions: medium free of glycine, hypoxanthine and thymidine (GHT–), or GHT– media containing either 30 nM methotrexate (MTX) or 100 nM MTX. Stable titers were normalized within each selection group. The stringency of selection, as expected, affected the stable transfection efficiency (numbers of clones observed): higher selective pressure resulted in fewer clones as well as in slightly smaller colony size. Linear regression of the plotted data gave correlation coefficients of 0.83 for the values seen in the GHT– case (99% chance of correlation), 0.73 in the 30 nM MTX case (95% chance of correlation) and 0.62 in the 100 nM MTX case (90% chance of correlation). It is remarkable that the correlation for the 100 nm MTX case remained good, in spite of the fact that the individual pools of stable clones consisted of only six clones (in the one case which gave lowest transient titers) to 35 clones (in one of the transfections which gave better transient titers). Therefore, early assessment of a transfection, using the supernatant just 2 days after DNA exposure, indicates whether few or many good clones are likely to emerge weeks later.

DISCUSSION

The principle of the calcium-phosphate–DNA precipitation method, originally developed by Graham and Van der Eb (1), is simple. Nevertheless the formation of an optimal DNA–calcium phosphate co-precipitate is difficult to reproduce (18) because multiple

parameters affect the solubility of calcium and phosphate and the appropriate window of conditions seems to be narrow.

Two interesting issues concerning the generation of a calcium phosphate–DNA precipitate complex have been discerned. An excess of soluble DNA in a supersaturated solution of calcium and phosphate may prolong the period during which a precipitate forms. If high enough in concentration, DNA may even prevent precipitate formation entirely. Therefore an error in the determination of the concentration of DNA, e.g. due to contamination with impurities which can influence the OD_{260} reading might substantially affect the formation of the precipitate. The second issue is temperature: the solubility of hydroxyapatite (i.e. 'calcium-phosphate') is higher at lower temperatures. Kjer *et al.* (5) showed that this influences transfections in mosquito cells. Although calcium phosphate is considered 'insoluble' in water (19), no precipitate at all may be formed in standard mixtures at temperatures close to 0°C. Most protocols recommend 'room temperature' for the preparation of the transfection mixture. Our results indicate that stricter temperature controls are appropriate for optimal results.

In order to overcome problems with the precipitation step we suggest use of simple tools for the early testing of solutions for transfections. A separate phosphate stock solution can be used for adjustment purposes. Multiple precipitations can be very consistent in terms of binding the DNA (one plasmid batch) or in terms of precipitate formation as assessed by absorption at 320 nm. Once new solutions are characterized and optimized, the precipitation procedure can be performed in a reproducible way and does not need to be re-optimized for each new transfection. New DNA preparations and new compositions (20) of supersaturated solutions can be tested quickly. The absorption at 320 nm, assessed with an aliquot of the preparation, can be used as an approach to study the kinetics and efficacy of precipitation.

The formation of the precipitate is a dynamic process which is terminated by transferring the precipitation mix into the culture medium and diluting the precipitate 10-fold. The time period allowed for generation of a precipitate is an important parameter. Subsequent to binding most of the soluble DNA into precipitate complexes, particles tend to grow further and transfection efficiency will be reduced. Theoretically, the standing time can be optimized for each set of parameters, as has been pointed out by Mahoney and Adams (6). In most cases a 1 min standing time appears to be a practical reaction time and the phosphate concentration should be used as a parameter to optimize and to control the precipitation step.

We show that concentrations of calcium, phosphate and DNA as well as temperature and reaction time affect the formation of DNA–hydroxyapatite particles in a profound way. Most importantly, transfection efficiency and expression levels in both transient and stable transfections are influenced by these parameters.

The work presented here led to research evaluating the use of calcium-phosphate for DNA transfer to cells grown in stirred laboratory bioreactors [Jordan, Köhne and Wurm (1995), submitted].

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