S-phase synchronized CHO cells show elevated transfection efficiency and expression using CaPi

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Abstract

Various methods exist to transfect mammalian cells in culture. It is generally accepted that individual methods have to be optimized for each of the cell lines or cell types used. Despite the use of optimized protocols, significant day-to-day variations in transfection efficiency regularly occur. We postulate that the 'status' of cell populations prior to transfection is involved in such variability. This study evaluates standardized transfections done at different phases of the cell cycle. Cell synchronization was achieved using mimosine. Transfection efficiency was monitored by fluorescence quantification of GFP (Green Fluorescent Protein). We show that transfection using the calcium-phosphate-DNA co-precipitation method, at different phases of the cell cycle, yields variable expression levels of GFP. Highest GFP expression levels were seen when transfecting cell populations with a dominant representation of S-phase-cells.

Introduction

Calcium-phosphate-DNA co-precipitation has been used as an efficient transfection method since almost 30 years (Graham and Van der Eb, 1973), and still remains very popular today. In spite of its widespread use, many questions remain unaddressed. DNA transfer and DNA processing within the cells are important research topics (Batard et al., 2001). When studying transfectability over a 24-hour period after plating, we have observed variable efficiency with calcium-phosphate (CaPi) transfection. This observation on non-synchronous cell population led us to speculate that cell cycle could play an important role in determining susceptibility to transfection by the CaPi method.

Studies addressing cell cycle and transfectability have been reported for other non-viral and viral transfection methods, such as Lipofectamine, adenovirus-enhanced transferrinfection, recombinant adenovirus

(Brunner et al., 2000) and stabilized plasmid-lipid particles (Mortimer et al., 1999). We show here, using synchronized cells, that high transfectability, using CaPi, is correlated with a high percentage of cells in S-phase.

For synchronization of our cell populations, we used mimosine. Mimosine inhibits ribonucleotide reductase, a class of enzymes that are involved in the synthesis of deoxyribonucleotides (dNTPs) and require the presence of $\text{Fe}^{2+}/\text{Fe}^{3+}$ undergoing redox cycles in the active center. Mimosine, an iron salt chelator, lowers dNTPs levels available intra-cellularly, thus preventing entry of the cell into synthesis phase (Dai et al., 1994; Dijkwel and Hamlin, 1992; Krude, 1999; Reichard, 1993; Tsai and Ling, 1971). After mimosine treatment, the majority of cells are arrested at the border between G1 and S-phase. Through optimised addition and removal of mimosine from the culture (a simple wash step with fresh medium), we generated cultures whose cells remain highly syn-

Figure 1. Experimental approach for analysis of GFP expression and cell cycle status upon transfections at various time-points during cell cycle.

chronized, at least for one, if not two, complete cycles. These synchronized cell populations were transfected at various time points throughout the cell cycle. Through measurement of expression of the fluorescent reporter gene (GFP), we determined the transfection efficiency at these various time points.

Materials and methods

Experimental approach

After release from mimosine arrest, batches of synchronized CHO cells were divided into two groups (Figure 1). One part of the cell population was transfected at given times after cycle recovery, while the other half were fixed, each time a transfection was performed, for cell cycle analysis.

Cell culture

CHO (DG44) cell line was maintained in culture in DMEM/F12 medium (GIBCO, Scotland) supplemented with 2% FCS (SeraTech, Germany) in T-75 flasks

(TPP, Switzerland) at 37 °C in a 5% CO₂ atmosphere. Cells were passaged every two or three days.

Cell synchronization

CHO cells were arrested with mimosine at the border between G0/G1 and S phases prior to transfection. Prior to mimosine treatment, we executed a pre-synchronization step, by incubating cells in low serum-containing medium at 0.2% FCS (Sera-Tech, Germany) for 48 h. The medium was then changed to DMEM/F12 + 10% FCS + 100 μM mimosine, cells were subsequently incubated for an additional 14 h. To reinitiate the cycle, the medium was changed to mimosine-free medium + 2% FCS.

We used synchronization by contact inhibition as a mimosine-free synchronized control (Davis et al., 2001). Cells were left to grow in 12-well plates until they reached total confluence. The cells were checked by flow cytometry until a high G1 phase synchrony was reached. Replating contact-inhibited cells at lower density resulted in continued cell cycle progression from early G1. Starvation-synchronization was also tested, but cells were poorly transfectable afterwards, at least for one sub-cultivation cycle. For starvation synchronization, cells were grown for 5 to 7 days in medium with a low serum content (~0.2%). Both methods block cells in the G0/G1 phase. Contact inhibition synchronization was preferred as cells were still transfectable after being released from contact inhibition.

Cell transfection

CHO cells were transfected using an optimised CaPi method (Jordan et al., 1996) with pEGFP-N1 plasmid (Clontech, Palo Alto, CA) as reporter gene. Briefly, we transfected approximately 1.5×10^5 cells in 1 ml DMEM/F12 medium (Gibco BRL) per well, using 12-well plates. Hundred μl of transfection solution (125 mM Ca²⁺, 0.7 mM PO₄³⁻ and 2.5 μg DNA per 100 μl of solution) were added to each well; cells were then incubated for 3 h at 37 °C before a glycerol shock was applied for one minute by changing transfection medium with PBS containing 10% glycerol at 37 °C. The glycerol-PBS solution was quickly removed and fresh medium containing 2% serum was added.

Reporter gene quantification

Reporter gene (GFP) expression was assessed for a period of five days after transfection, corresponding

Figure 2. Progression through the cycle. Cells were pre-synchronized for 48 h in 0.2% FCS containing medium and then synchronized with mimosine for 14 h. Samples were taken for cycle analysis from 2 to 16 h after mimosine release (left to right, top to bottom), stained with DAPI and analyzed by flow cytometry (PAS III, Partec GmbH).

to the stationary phase of the cell culture used within these conditions. GFP expression was quantified with a fluorescent plate reader (Cytofluor, multi-well plate reader, Series 4000 by Perseptive Biosystems), excitation = 485 nm, emission = 530 nm.

Cell cycle analysis

Trypsinized cells were centrifuged (5 min at 700 rpm) and resuspended in 70% ethanol for 1 min for fixation. Ethanol was then removed and cells were resuspended in 1 × PBS and stored at 4 °C until further treatment.

Fixed cells samples were centrifuged to remove PBS, this was followed by the addition of 2 ml DAPI (FLUKA, Buchs, Switzerland) solution (1:1000 in MeOH) to each sample ($\sim 1.5 \times 10^6$ cells per sample). Cells were incubated for 15 min at 37 °C, centrifuged, resuspended in 1 × PBS and stored at 4 °C until analysis.

Non-transfected cell samples were fixed at the same time that CaPi-DNA complexes were added to the cell samples being transfected. Once all samples were collected, chromosomal DNA was stained with DAPI and analysis by flow cytometry was performed

Figure 3. GFP expressing cells transfected in the different parts of the cells cycle, 24 h after transfection. S phase cells clearly show the highest GFP expression.

Figure 4. Levels of GFP expression per 1×10^5 cells correlate with percentage of cells in the S-phase. Transfections were done every hour after release from synchronization and cell cycle distribution was measured for every cell population being transfected.

within 6 h after staining. At least 30 000 events were acquired for each sample.

Results

For synchronization of our cell populations, we used mimosine, which gives good synchronization rates and was reported to have minimal long-term effects once removed (Orren et al., 1995). Through removal of mimosine from the culture (a single wash step with fresh medium), we generated cultures in which cells grow in a synchronized mode, at least for one complete cycle.

Flow cytometric analysis (PAS III, Partec GmbH, Münster, Germany) of the cycle distribution showed

that progression through the cycle restarted after mimosine had been washed away from the cells (Figure 2).

We transfected synchronized cell populations at different time points throughout the cell cycle, approximately once every hour. Transfection efficiency, as judged by the number of GFP-positive cells that are visible under a fluorescence microscope, was found to be strongly dependent from the cell cycle (Figure 3).

For more precise quantification, the fluorescence of GFP was measured directly within the 12-well plates. When this signal was corrected for the variable number of cells that were actually present at the time of transfection (during the period of 14 h, the synchronized cells did at least one doubling, thus increasing the number of cells that could be potentially

Figure 5. Cells were synchronized by contact inhibition. Transfections were performed after passaging, in order to reinitiate the cell cycle. The same relationship between GFP expression and percentage of cells in the S-phase at the time of transfection was obtained.

transfected), it correlated perfectly with cell cycle distribution: the evolution of GFP expression was always proportional to the fraction of S-phase cells at the time of transfection (Figure 4). The experiment was repeated three times and results were reproducible.

The reporter gene expression had to be corrected according to the number of cells present at the time of transfection (as cells cycle in a synchronous way, when they enter G1 phase the second time, the number of cells being transfected has doubled). In order to take into account changes due to cell growth, relative fluorescence units have been normalized to 1×10^5 cells.

To exclude a direct effect of mimosine treatment on the observed correlation, synchronization was induced differently by allowing cells to grow to confluence. Due to contact inhibition, CHO cells cultured in T-flasks are arrested at the G0/G1 phase and stop growing. Upon resuspension in fresh culture medium, cell cycle was reinitiated. The same correlation between percentage of cells in S-phase and GFP expression was observed again (Figure 5).

Conclusion

For CaPi transfections, complexes enter cells using a cellular import system which is thought to be endocytosis (Orrantia and Chang, 1990). These authors pro-

posed a model for transport of CaPi-DNA involving the endocytotic pathway, but the mechanism of transfer from endosomes and/or lysosomes to the nucleus still remains largely unknown.

A relatively small fraction of CHO cells in an asynchronous population express GFP after transfection by CaPi. This could be a reflection of the various 'states' of cells at the time of transfection. It is reasonable to assume that endocytosis activity varies through the cell cycle. If import activity would be low at a certain stage in the cell cycle, then all those cells would not be accessible for transfection, resulting in low or no expression. Thus working with synchronized cells populations and transfecting them at the right time points of the cell cycle improves the efficiency for DNA uptake and expression.

The observation that S-phase cells obtain a higher efficiency than mitotic cells indicates that a certain time is needed for the CaPi-DNA complexes in endosomes/lysosomes to become distributed within cells so that at least a few will be localized in the proximity of the nucleus. At mitosis, when the nuclear membrane disintegrates, and before it is rebuilt again, plasmid DNA may become enclosed into the nuclear environment. If transfected during G1, the nuclear membrane will not disrupt early enough and plasmid DNA will get mostly degraded in the lysosomes or cytoplasm (plasmid degradation in cytoplasm has been

shown to be important (Tsai and Ling, 1971). If transfected during mitosis, the nuclear membrane will be rebuilt before plasmid DNA complexes are close to the nucleus.

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