SHORT TECHNICAL REPORTS

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Transient Transfection of Primary Cultured Hepatocytes Using $CaPO_4$/DNA Precipitation

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ABSTRACT

We present a detailed protocol for the transient transfection of non-proliferating primary cultured hepatocytes that is easily reproducible. Using a modification of the classical $CaPO_4$/DNA precipitation method, this protocol is an inexpensive alternative to other methods that are often cumbersome, expensive, difficult to reproduce or harmful to primary hepatocytes. Because only 0.5 ´ 10^6 cells are needed for a single transfection experiment, several reporter genes can be introduced into hepatocytes of a single liver preparation. With our protocol, different plasmids can be introduced into one cell. In this way, cis-trans *interactions can be examined and reporter gene expression can be normalized for transfection efficiency. Furthermore, we describe details of a transfection experiment with two different reporter gene vectors using a luciferase gene and a* lacZ *gene. The results presented may be helpful to other groups concerned with improved timing of transfection experiments.*

INTRODUCTION

A successful gene therapy for genetic deficiencies manifested in the liver depends, among others, on the characteristics of the regulatory elements controlling the genes that should be expressed in the target organ. It will be important to construct vectors that express the genes in a hepatocyte-specific manner and with a controllable efficiency. To determine which elements do control hepatocyte-specific expression, there is urgent need for a test system that allows large-scale transfection of reporter gene constructs. For these experiments, non-proliferating, primary cultured hepatocytes should be used that represent the in vivo differentiation state as closely as possible in cell culture. This approach has a clear advantage over introducing reporter genes into immortalized or permanent cell lines of questionable biological significance, as is frequently done.

Although a number of protocols for the transfection of primary cultured hepatocytes have been published, many of these protocols are not suitable for large-scale transfection experiments for several reasons. For example, electroporation is difficult because it has to take place immediately after perfusion when the cells are still in suspension. At this time the cells are very fragile, and electroporation causes cell death to a high extent. Lipofection on the other hand is expensive if several experiments with different kinds of reporter gene constructs need to be done. The second disadvantage of lipofection is its severe cytotoxic effect on primary cultured hepatocytes. Retroviral approaches are usually well suited to get high efficiency transfections but are cumbersome in terms of the construction of appropriate vectors. As an alternative, $CaPO_4$/DNA transfections are inexpensive and render it possible to introduce different vectors into one cell. This is especially useful because transcription of reporter gene constructs can be corrected for transfection efficiency. Furthermore, co-transfections with transcription-factor-expressing plasmids can be done to address questions of *cis-trans* interactions.

We present a new protocol for the $CaPO_4$/DNA transfection (3) of non-proliferating, primary cultured hepatocytes that has several advantages over protocols previously published (e.g., Reference 9). First of all, it allows the transfection of hepatocytes within 24 h after isolation without substantial cell death, and cells can be harvested 10–20 h after transfection. This is of great necessity if one wants to get as close as possible to the in vivo situation, since hepatocytes in culture may undergo steady changes regarding their biochemical functions. The protocol presented also allows the transfection of hepatocytes plated on collagen instead of on plastic. Therefore the loss of liver-specific functions because of culture conditions is further minimized. The protocol can be easily performed and, if care is taken to use identical materials, it always provides reproducible results. Aside from being highly reproducible, the protocol has the great advantage

that only 0.5×10^6 hepatocytes in a 35-mm dish are needed for a single transfection experiment.

MATERIALS AND METHODS

As a preliminary remark, it should be emphasized, that the protocol should be followed strictly. It is strongly recommended to use the same disposable materials as the ones cited. Disposables from other producers, even if they look the same and are made from identical material, may not work.

Isolation of Hepatocytes

Male Sprague-Dawley rats weighting 220–280 g were purchased from the Süddeutsche Versuchstierfarm (Tuttlingen, Germany). They were kept on a standardized diet of Alma® H1003 (Botzenhardt, Kempten, Germany) and water ad libitum. Total hepatocytes were isolated by in vitro perfusion technique with collagenase (2). Periportal liver parenchymal cells (from zone 1 of the acinus) and pericentral hepatocytes (from zone 3 of the acinus) were isolated by in vitro perfusion with digitonin/collagenase (2). Viability of the isolated hepatocytes, ranging from 85% to 95%, and the efficiency of the separation between periportal and pericentral cells was determined as described (2).

Reporter Genes

The plasmid pSV-β-galactosidase that encodes a β-galactosidase (β-gal) was purchased from Promega (Serva, Heidelberg, Germany). The plasmid pT109, which carries a luciferase gene under the control of a Herpes simplex thymidine kinase promoter, was constructed and described by Nordeen (8).

Preparation of DNA for Transfection

Plasmid DNA for transfection experiments can be isolated from overnight grown cultures of XL1-Blue or DH5α and purified by affinity chromatography on QIAGEN® columns (Qiagen, Hilden, Germany) according to manufacturer's description. The quality of the DNA is determined by spectrophotometry and agarose gel electrophoresis (0.8% agarose) with 0.4 μg/mL ethidium bromide in the gel. Usually, plasmid DNA obtained from QIAGEN columns can be used directly for transfection, as already shown by others (4). If DNA nicking is a problem (i.e., more than 5% of the DNA is in the relaxed form, as estimated by gel electrophoresis) a CsCl dye-buoyant density gradient (1) is strongly recommended. DNA with a spectrophotometrically determined optical density (OD) ratio (OD_{260}/OD_{280}) of more than 1.85 should not be used. If the ratio is below 1.6, a 30-min float dialysis on a VSWP0.025-μm filter (Millipore, Eschborn, Germany) against TE (pH 7.4) (1) is

strongly recommended. With dialysis, it is usually possible to enhance the quality of the DNA; otherwise, DNA should not be used for transfection.

Preparation of $CaPO_4$/DNA Precipitate for Transfection

The $CaPO_4$/DNA mixture is prepared 25–30 min before it is added to the cells. Five hundred microliters of a solution of 250 mM $CaCl_2$ ($CaCl_2 \cdot 4H_2O$ Suprapur®; Merck, Darmstadt, Germany) containing 12 µg of supercoiled plasmid DNA are added dropwise with a glass Pasteur pipet to 500 µL of 2× HeBS (0.28 M NaCl, 10 mM KCl, 1.5 mM Na_2HPO_4, 42 mM HEPES, 2% glucose, pH 7.10) in a polystyrene conical tube (Falcon® 2095, 17 × 120 mm; Becton Dickinson, Heidelberg, Germany). During addition, the mixture is bubbled from the bottom of the tube with air using a mechanical pipettor and a second glass Pasteur pipet. The addition of the DNA/$CaCl_2$ mixture should take at least 1 min. After addition, the mixture is vortex mixed at maximum speed for 2–3 s, incubated for 25–30 min at room temperature and added to the cells as described below.

Transfection

Hepatocytes used for transfection experiments are plated at a density of 0.5×10^6 cells per 35-mm dish of a 6-well plate (Macroplate, TC; Greiner, Nuertingen, Germany), pre-coated with collagen as described (5) and cultured in William's medium E (Sigma Chemical, Muenchen, Germany) supplemented with 10% of fetal calf serum (C.C. Pro, Karlsruhe, Germany), 2 mM glutamine, 10^{-9} M insulin, 10^{-7} M dexamethasone, 500 U/mL penicillin and 40 U/mL streptomycin (all purchased from Sigma Chemical). The cells are incubated in 1.5 mL medium per well and at 37°C and 90% humidity in an atmosphere containing 5% CO_2 and 95% air. Two and twenty hours after seeding of the cells, medium is removed, and fresh medium is added. Twenty-four hours after seeding of the cells, 200 µL of the $CaPO_4$/DNA-mixture are added to the cells in 1.5 mL of medium. Before addition to the cells, the $CaPO_4$/DNA-solution is mixed by pipetting through a 1000-µL pipet tip (Mµlti®; Roth, Karlsruhe, Germany) that is also used to add the material. During addition, the dish is swirled gently. It is emphasized that the cells should not be kept at room temperature and normal atmosphere for more than 6 min during addition of the DNA. The cells are exposed to the precipitate for 8 h followed by a 2-min glycerol shock (15% glycerol in 1× HeBS). First, the cells are washed two times with Hank's solution (137 mM NaCl, 5.4 mM KCl, 0.4 mM $MgSO_4 \cdot 7H_2O$, 0.5 mM $MgCl_2 \cdot 6H_2O$, 0.35 mM $Na_2HPO_4 \cdot 2H_2O$, 0.44 mM KH_2PO_4, 2 mM HEPES, pH 7.4 with NaOH), then 0.5 mL of glycerol solution is added to each well at room temperature for exactly 2 min. After removal of the glycerol solution, the cells are washed with Hank's solution. After a wash with fresh medium, 1.5 mL of fresh medium is added, and the cells are incubated until lysis of the cells.

Enzyme Assays and Determination of the Level of Expression from Reporter Genes Carrying Luciferase or *LacZ* Genes

Transfection efficiencies were determined by transfection of hepatocytes with the pSV-β-galactosidase vector and counting of transfected cells after in situ staining with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) (6).

For the determination of expression of luciferase or β-gal, cells are lysed in 100 µL of lysis buffer (77 mM K_2HPO_4, 23 mM KH_2PO_4, 0.2% Triton® X-100, 1 mM dithiothreitol [DTT], pH 7.8), and cell debris is removed by centrifugation (2 min, 12000× *g*). Enzyme activity can be determined directly after cell lysis, but extracts can also be stored at -20°C without loss of activity.

In the experiments presented, each enzyme activity was measured using 10 µL of cell extract. β-gal activity was determined by a fluorometric assay using 4-methylumbelliferyl-β-D-galactoside as described (7). Fluorescence was measured in a Model 1000 Fluorescence Spectrophotometer (Perkin-Elmer, Ueberlingen, Germany). Firefly luciferase activity was determined by a protocol of Promega (Technical Bulletin No. 161) using a Lumat® LB 9501 (Berthold, Wildbad, Germany) in the 10-s integration mode.

RESULTS AND DISCUSSION

One important factor in achieving good transfection efficiencies is the quality of DNA. If care is taken to have

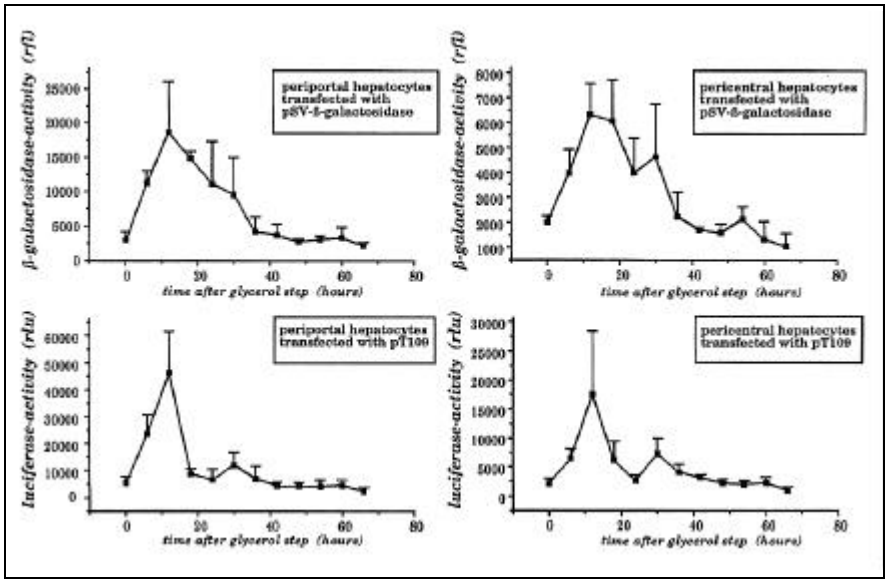

Figure 1. Level of expression from the reporter genes pSV-β-gal and pT109 at different times after the glycerol step. Cells were lysed at the times indicated, and enzymatic assays were performed. Each value of β-gal activity is the average of four single transfections. Values for luciferase activity are the mean of eight single transfections. Every enzymatic assay was performed in duplicate. Luciferase activity is given in relative light units (rlu), β-gal activity in fluorescence units (rfl).

more than 95% supercoiled DNA with an OD_{260}/OD_{280} between 1.6 and 1.8, different DNA-preparations always yield comparable transfection efficiencies. Another important parameter is the pH of the HeBS solution. It is recommended to prepare batches of HeBS with pH between 7.0 and 7.2 in steps of 0.05 and test them in a transfection experiment. Best results are usually obtained at pH 7.10. Often solutions with a pH within the range from 7.05–7.15 can also be used. The third important parameter is cell viability and density. Viability should be higher than 80%, and a cell density of more than 0.5×10^6 cells in a 35-mm dish is not advisable.

To demonstrate the suitability of the method described, we transfected the vectors pT109 and pSV-β-galactosidase into primary cultured subpopulations of hepatocytes and lysed the cells at different times after the glycerol step.

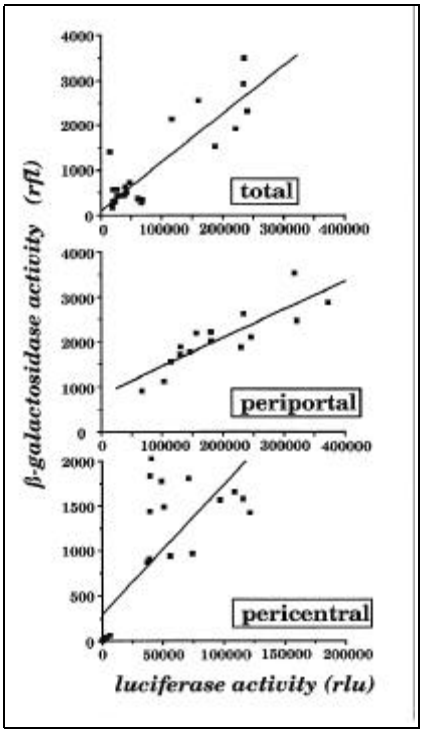

Figure 2. Comparison of β-gal and luciferase activities in co-transfection experiments. Primary cultured hepatocytes from different liver preparations were transfected with reporter genes from different DNA preparations. For preparation of the $CaPO_4$-precipitate, pT109 and pSV-β-galactosidase were mixed in the ratio 5:1. All cells were lysed 36 h after the glycerol shock and the rfl and rlu determined. The straight line represents a linear regression of the data.

The luciferase activity and the β-gal activity determined by enzymatic assays at different times are shown in Figure 1. The results indicate that highest expression is observed around 10 h after the glycerol step. The luciferase activity is more transient than the β-gal activity. This may be the result of a more stable β-gal mRNA or protein, which cannot be discriminated in these experiments. In addition, it is obvious that there is a difference in the enzymatic activities obtained with pericentral and periportal hepatocytes. Because β-gal and luciferase both show this difference, it can be interpreted as the result of different transfection efficiencies, which was confirmed by in situ staining with X-gal. In fact, transfection efficiencies usually show a variability within one order of magnitude, as indicated by the error bars in Figure 1. Such a difference in transfection efficiency may lead to a misinterpretation of the level of expression from a transfected reporter gene. To overcome this problem, a second reporter gene with a different enzyme activity can be co-transfected with the constructs to be tested. As shown in Figure 2, there is generally a direct relationship between β-gal activity and luciferase activity in each individual transfection, although the correlation is not as good in pericentral cells as in periportal or total hepatocytes. Thus, the luciferase expression plasmid, pT109, can be used to correct transfection efficiencies when using reporter genes with *lacZ*, while the pSV-β-galactosidase vector can be used in combination with luciferase-encoding reporter plasmids. If the endogenous β-gal activity of hepatocytes causes problems with the pSV-β-galactosidase vector as control, it is possible to use a vector with a stronger promoter, e.g., the cytomegalovirus (CMV) promoter (10).

The transfection efficiencies obtained by transfection of primary cultured hepatocytes with our method, as determined by X-Gal stain of cells transfected with pSV-β-galactosidase, is usually in the range between 1% and 5% for total hepatocytes and slightly less in subpopulations. This is less than the 5%–10% we and others obtained by lipofection, but in our hands, high transfection efficiencies obtained by lipofection were always correlated with

a substantial cytotoxic effect in primary cultured hepatocytes. This cytotoxic effect, as measured by determining cellular protein per dish and by microscopic examination of the cultures, was present even if antibiotics or other supplements were omitted from the medium during transfection. Considering this toxicity and the high prices for reagents used for lipofection, our method is an inexpensive and gentle alternative that is particularly useful if many transfections will be performed with a single liver cell preparation. Furthermore, with the enzymatic assays described, even weak promoters can be investigated using as few as 0.5×10^6 cells in a single transfection experiment. Another advantage is that different plasmids can be introduced into the same cell. This should be a valuable tool if protein-DNA interactions of a transacting factor with the transfected DNA need to be examined.

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