Histone H1-Mediated Transfection: Serum Inhibition Can Be Overcome by Ca^{2+} Ions

Annekathrin Haberland,[1] Thomas Knaus,[1] Sergei V. Zaitsev,[1,2] Bernd Buchberger,[3] Andreas Lun,[4] Hermann Haller,[1] and Michael Böttger[1,4]

Received July 23, 1999; accepted October 6, 1999

Purpose. One of the drawbacks of polycationic and cationic liposomal gene transfer is its sensitivity to serum. Gene therapy requires the transfectant-DNA complex to be resistant to serum as well as blood. Since Ca^{2+} has proved to be an efficient cofactor of polycationic gene transfer, we decided to investigate its effects on transfection in the presence of serum.

Methods. We studied transgene expression of luciferase gene (pCMV Luc) on ECV 304 human endothelial cells using H1 histone and DOSPER as transfectants in the presence of 0–100% fetal calf serum.

Results. H1-and DOSPER-mediated transfection was found to be inhibited by serum above the concentration of 10%. If 2 mM Ca^{2+} or 2 mM Ca^{2+}/0.1 mM chloroquine was included in the culture medium which replace the transfection mixture and was left on the cells for 24 hours postincubation, the inhibiting effect of even 100% serum was overcome.

Conclusions. A high serum level does not interfere with binding and uptake of H1- and DOSPER-DNA complexes, but inhibits subsequent steps such as endosomal escape. Ca^{2+} in the form of nascent calcium phosphate microprecipitates and other lysosomolytical agents facilitate endosomal/lysosomal release by their fusigenic and membranolytic activity.

KEY WORDS: transfection; serum inhibition; H1 histone; DOSPER; calcium.

INTRODUCTION

Non-viral gene therapy is a promising approach for the treatment of acquired and genetic disorders (1). Cationic lipid based systems and DNA vectors exploiting receptor-specific endocytosis as a target-specific mechanism have been used for gene delivery. However, the efficiency of gene vectors in vivo still needs to be improved. Although in many cases the efficiency in vitro is high, the in vivo efficiency cannot be predicted (2,3). One of the reasons is the inactivation of cationic DNA vector systems by serum or blood components (4–6). Many cationic lipid based transfection systems are inactivated in as low as 5–10% serum (7–10).

We have focussed on the use of nuclear proteins as DNA carrier systems for gene delivery because they are constituents of chromatin and natural interaction partners of DNA. We prepared acid nuclear extracts and subsequent protein fractions by fractionated precipitation. Single transfection-active proteins were obtained by FPLC. In the presence of Ca^{2+} ions in the transfection medium, H1 histone was identified as a very transfection efficient protein. The high mobility group proteins HMG1 and HMG2 were less efficient (11,12). Synthetic polycations also showed a high transfection efficiency in the presence of Ca^{2+}. We suggested that Ca^{2+} in the form of nascent $Ca(PO_4)_i$ microprecipitates formed in the presence of phosphates has lysosomolytic activity and supports the release of the H1-DNA complexes from endosomes/lysosomes into the cytoplasm (13).

In this paper, we would like to report on transfection results using H1 histone as a DNA packaging protein in the presence of serum. We demonstrate that the presence of Ca^{2+} ions in the cell culture medium can overcome the transfection-inhibiting effects of serum. Similar results were obtained using the cationic transfection reagent DOSPER. The aim of these investigations is to find experimental conditions supporting gene delivery in vivo.

MATERIAL AND METHODS

Histone H1 and DNA

Nuclear proteins were extracted from calf thymus nuclei with 5% perchloric acid and fractionated by acetone precipitation as described previously. Histone H1 was obtained by FPLC using anionic and cationic ion exchanger columns Mono Q and Mono S (Pharmacia). The fractions 2VAS1 and 2.5VAS1/2 are FPLC-pure H1 (11,12).

The reporter plasmid pCMV Luc was grown in E.coli DH5 and isolated and purified using standard methods. It encodes the American firefly luciferase gene under the control of the cytomegalovirus promoter.

Commercial Transfectants

DOSPER, a cationic lipid with a spermine head group (4 positive charges), was obtained from Roche Diagnostics GmbH Germany.

Experimental Design

In order to increase the transfection efficiency, Ca^{2+} can be added to the transfection or/and postincubation medium. If Ca^{2+} is added to the postincubation medium, the transfection process can be divided into two steps, complex uptake and translocation to the nucleus. Both steps can be studied and affected separately. In the absence of Ca^{2+} H1-DNA complexes are taken up by the cells in the transfection phase and stored in endosomal/lysosomal vesicles (13). In this paper, we studied

[1] Franz Volhard Clinic at the Max Delbrück Center for Molecular Medicine, Berlin-Buch, Germany.
[2] Institute of Cytology of the Russian Academy of Sciences, St. Petersburg, Russia.
[3] Roche Diagnostics GmbH, Penzberg, Germany.
[4] Institute of Laboratory Medicine and Pathobiochemistry, Charité, Humboldt University, Berlin, Germany.
[5] To whom correspondence should be addressed. (e-mail: mboettg@mdc-berlin.de)

ABBREVIATIONS: $Ca(PO_4)_i$, calcium phosphate microprecipitate; cq, chloroquine; DOSPER, 1,3-Di-oleoyloxy-2-(6-carboxy-spermyl)-propylamid; FCS, fetal calf serum; FPLC, fast protein liquid chromatography; RLU, relative light unit; RPMI, cell culture medium; XTT, sodium 3′-[1-(phenyl-amino-carbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro)-benzene sulfonic acid hydrate.

the effects of increasing serum concentration on the cellular uptake by adding serum to the transfection medium as well as on translocation or transfection by adding serum to the postincubation medium in the presence of Ca^{2+} as outlined above. In order to facilitate the understanding of the figures, the following schematic description of the graphs was used: transfection medium/postincubation medium with the experimental conditions before or after the dash as given. Transfection medium describes buffer or cell culture medium, here RPMI, with or without serum including transfectant and DNA which is added to the cells for transfection. The postincubation medium is cell culture medium containing Ca^{2+} and serum which is added to the cells after removing the transfection medium and washing the cells.

Cell Culture and Transfection Protocol

ECV 304 cells, a spontaneously transformed, human umbilical vein endothelial cell line, were cultured in endothelial cell medium M199 with Earle's salt and L-glutamine (Gibco BRL) supplemented with 10% fetal calf serum (FCS). Transfection was performed as published previously (11,12). Plasmid DNA and H1 histone as specified in the text were mixed in 0.15 M NaCl, 10 mM Tris-HCl, pH 7.6 at room temperature and shaken for 15 min. 2 μg of plasmid DNA pCMV Luc were used as a reporter gene. Weight input ratios r_i (protein/DNA) of 1 and 5 were used. From these values molar charge ratios +/− of 0.8 (here approximated as neutral) and 4 on the basis of lysines + arginines/DNA phosphate can be calculated. The final complex volume was 100 μl. It was mixed with 0.9 ml culture medium (RPMI 1640) containing varying amounts (0–100%) of fetal calf serum FCS (Gibco BRL) and 2–6 mM $CaCl_2$. At the highest FCS concentration (100%) the RPMI was fully replaced by serum. $2 \cdot 10^5$ cells per well were transfected with these transfection mixtures at 5% CO_2 and 37°C for 4 hours. Then the transfection mixture was removed and replaced by 1 ml RPMI/10% FCS/2 mM $CaCl_2$ and/or 0.1 mM chloroquine. After incubation for about 30 hours, the cells were harvested and the luciferase activity measured by the Promega assay system using a Lumat LB 9501 luminometer (Berthold). The relative light units RLU refer to 20 μl cell extract from a total of 150 μl containing $2 \cdot 10^5$ cells.

Measurement of the Calcium Concentration

The calcium concentration in the presence of RPMI and FCS was determined by photometry in a Hitachi 747 by means of the o-cresolphtalein-complexon method supplied as kit by Roche Diagnostics GmbH Germany. Some measurements were performed by flame photometry in an ELEX 6361 flame photometer (Eppendorf Hamburg, Germany).

Free Ca^{2+} in the presence of RPMI and FCS was determined potentiometrically by means of the calcium electrode using the blood gas analyzer OMNI, AVL, Graz Austria.

Viability Measurements

Viability measurements were performed in 96-well plates using the cell proliferation kit (XTT) from Boehringer Mannheim, now Roche diagnostics, Germany, as described previously (12). The measurements were performed similarly to the transfection experiments in order to check the influence of Ca^{2+} and higher serum concentrations on the viability of the ECV 304 cells. Since H1-DNA complexes per se are not toxic (12), they were not included in the viability experiments.

RESULTS

Ca^{2+} Ions Stabilize the H1-Mediated Transfection at High Serum Concentrations

We checked the transfection activity of histone H1 and of commercial transfectants in complex with plasmid DNA in the presence of serum. Transfection experiments performed with H1-pCMV Luc complexes of adjusted molar charge ratios +/− of about 0.8 (here taken as "uncharged") and +4 (cationic) are shown in Fig. 1. ECV 304 cells were transfected with neutral H1-pCMV Luc complexes (molar charge ratio +/− = 0.8) in the presence of 0–100% serum and 6 mM Ca^{2+} in the transfection medium. The postincubation medium contained 10% serum as usual (13). If there was no Ca^{2+}/chloroquine in the postincubation medium, the transfection efficiency given in relative light units (RLU) was inhibited at serum concentrations higher than 10–20%. However, as further demonstrated in Fig. 1, this serum inhibition can be overcome completely if 2 mM Ca^{2+}/0.1 mM chloroquine are added to the postincubation medium. High transfection efficiencies were observed at serum concentrations between 10 and 100% (in the transfection medium) which greatly exceeded the control values. Similar effects, however with a slightly lower transfection efficiency, were obtained

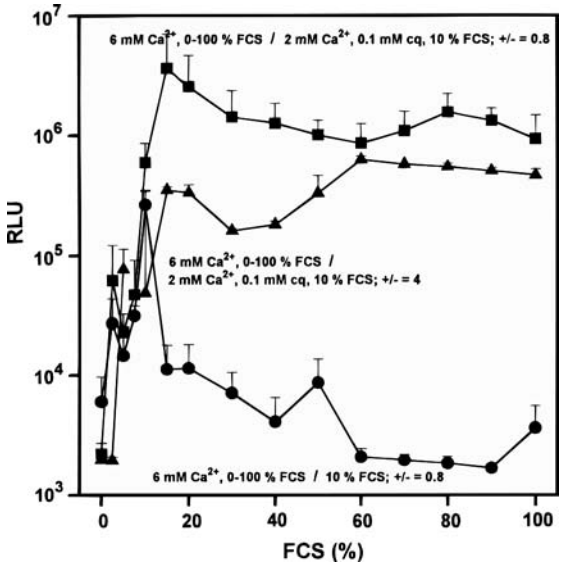

Fig. 1. Transfection efficiency (RLU) of histone H1-DNA complexes as a function of the concentration of fetal calf serum (FCS) in %. Transfection experiments under conditions of no or low serum inhibition with the transfection mixture containing 6 mM Ca^{2+}, 0–100% FCS and the postincubation medium containing 2 mM Ca^{2+}/0.1 mM chloroquine, 10% FCS. "Uncharged" complexes with a molar +/− charge ratio of 0.8 or input ratio r_i (H1/DNA by weight) of 1 (■), positive charged complexes of a molar charge ratio +/− = 4, r_i = 5 (▲). Transfection under conditions of high serum inhibition with the transfection medium as above and the postincubation medium with 10% FCS without Ca^{2+}, uncharged comlpexes with +/− = 0.8 or r_i = 1 (●). 2 μg pCMV Luc, $2 \cdot 10^5$ ECV 304 cells; with more than 2 experiments mean values and standard error are given.

with H1-DNA complexes of a molar charge ratio +/− = 4. We also have observed that the stabilization of the transfection effect against higher serum concentrations does not require chloroquine. Transfection on a lower level can be achieved by Ca^{2+} alone (not shown). As further shown in Fig. 1, decreasing RLU values were observed below 10% serum in the transfection medium under all transfection conditions used here. As shown in Fig. 2 in viability measurements by means of the XTT test, this decrease in RLU is due to the toxicity of Ca^{2+} on cells not sufficiently protected by serum (the blank value without DNA is about $2 \cdot 10^3$ RLU). H1-DNA complexes were also included in the viability experiments. They do not influence the viability (not shown).

The question if Ca^{2+} fulfills a cell-specific role in overcoming the serum inhibition or acts merely indirectly by neutralizing negative electrostatic charges on the extracellular level is of importance. In the latter case Mg^{2+} also should exhibit similar effects. In Fig. 3 experiments are shown in which the influence of the divalent cations Ca^{2+} and Mg^{2+} on the transfection efficiency in the presence of serum was compared. In addition to Ca^{2+} and Mg^{2+}, increasing serum concentrations up to 100% were present in the transfection medium. The postincubation medium contained 2 mM Ca^{2+}, 0.1 mM chloroquine and 10% serum. Whereas in the presence of 6 mM Ca^{2+} in the transfection medium the highest RLU values were observed, Mg^{2+} in the transfection medium resulted in a slightly lower transfection efficiency that remained nearly constant over the full range of serum concentrations. Therefore, the complexes were taken up in the presence of Mg^{2+}. Complexes of a charge ratio of +/− = 4 exhibited a similar behavior (not shown). Furthermore, Mg^{2+} was not toxic at low serum concentrations (no decrease in RLU) unlike Ca^{2+} which proved to be toxic under these conditions. However, if Mg^{2+} was present in the transfection and postincubation media, no transfection was observed (in the absence of Mg^{2+} or Ca^{2+} in the postincubation medium as well). Therefore, Mg^{2+} seems not to be involved in cellular processes as in the endosomal release of the complexes in the presence of serum because transfection is suppressed if Mg^{2+} instead of Ca^{2+} is present in the postincubation medium.

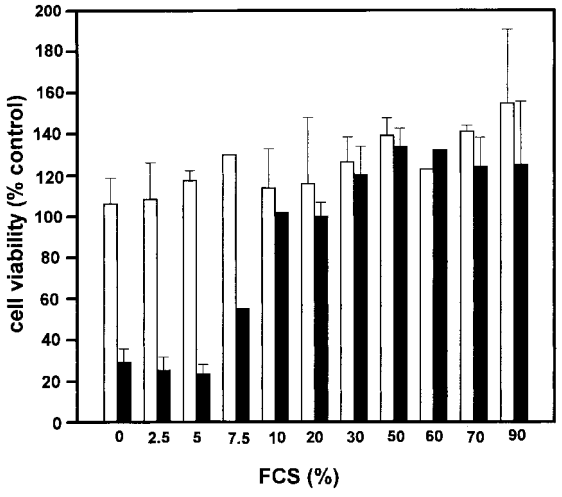

Fig. 2. Cell viability of ECV 304 cells in the presence of 6 mM Ca^{2+} (black columns) as a function of the fetal calf serum (FCS) concentration in %. The percentages of the controls in the absence of Ca^{2+} (white columns) are given. H1-DNA complexes not added to the cells (see text); XTT assay performed in 96-well plates with 4×10^4 cells, mean ± s.e., three experiments.

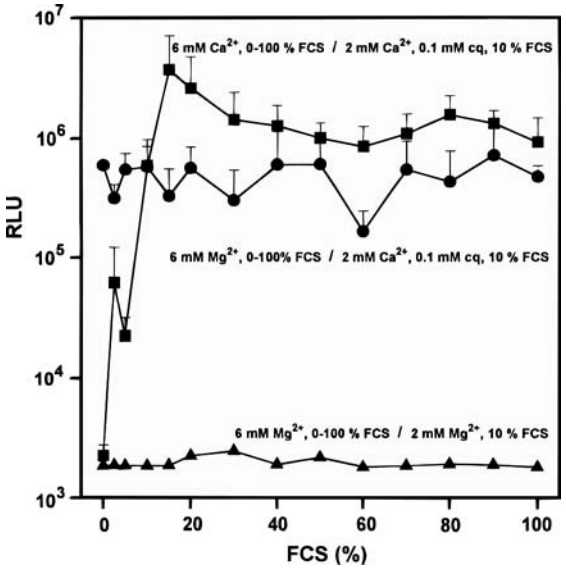

Fig. 3. Comparison of the effects of Ca^{2+} and Mg^{2+} ions on the transfection efficience of H1-DNA complexes in the presence of serum. Composition of the media: Transfection medium 6 mM Ca^{2+}, 0–100% FCS, postincubation medium 2 mM Ca^{2+}/0.1 mM chloroquine, 10% FCS (■); transfection medium 6 mM Mg^{2+}, 0–100% FCS and postincubation medium 2 mM Ca^{2+}/0.1 mM chloroquine, 10% FCS (●), transfection medium 6 mM Mg^{2+}, 0–100% FCS and postincubation medium 2 mM Mg^{2+}, 10% FCS (▲), +/− = 0.8 or r_i = 1; 2 μg pCMV Luc, $2 \cdot 10^5$ ECV 304 cells, when more than 2 experiments mean values and standard error are given.

Ca^{2+} Is Not Needed for the Complex Uptake

In order to see if the complex uptake by the cells is inhibited by higher concentrations of serum, we varied this basic experiment by omitting Ca^{2+} in the transfection medium. Previously, we had shown that complexes were taken up by cells in the absence of Ca^{2+} (13). Fig. 4 shows the transfection efficiencies of neutral H1-DNA complexes without Ca^{2+} in the transfection medium at varying serum concentration under different conditions. When increased serum concentrations were present in the transfection medium and 2 mM Ca^{2+}/0.1 mM chloroquine together with 10% serum in the postincubation medium, high transfection efficiencies in the total range of serum concentrations were observed. If under the same conditions increasing serum concentrations were also present in the postincubation medium or in both, in transfection and postincubation medium, an inhibition of the transfection was observed. This inhibition was more pronounced in the latter case. Thus, it seems that the uptake of the complexes is either not inhibited or only slightly inhibited in the presence of even high serum concentrations. On the other hand, intracellular events such as the endosomal release of the complexes or the nuclear entry and/or the expression of the DNA could be inhibited by serum. Since we have suggested that $Ca(PO_4)_i$ microprecipitates rather than the free Ca^{2+} support the endosomal/lysosomal release of the complexes (13, 14), it is very likely that at higher serum concentrations

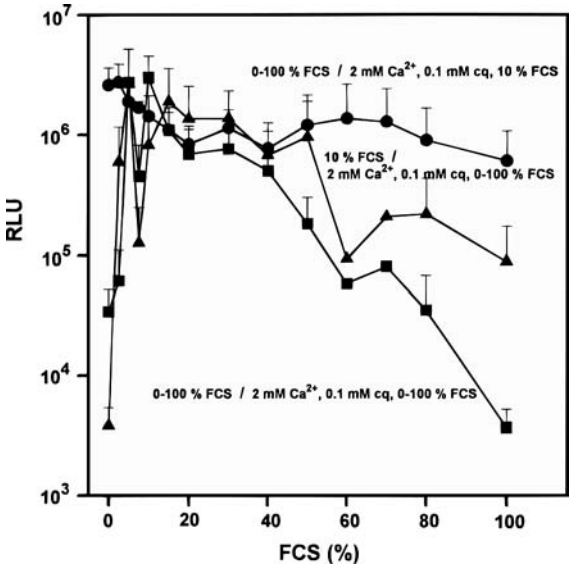

Fig. 4. Transfection measurements providing evidence for the cellular uptake of H1-DNA complexes (see text) as a function of the serum concentration. The media had the following composition: Transfection medium contained 0–100% FCS without Ca^{2+} and postincubation medium 2 mM Ca^{2+}/0.1 mM chloroquine, 10% FCS (●); transfection medium with 10% FCS and without Ca^{2+} and postincubation medium 2 mM Ca^{2+}/0.1 mM chloroquine, 0–100% FCS (▲); transfection medium without Ca^{2+} and with 0–100% serum and postincubation medium 2 mM Ca^{2+}/0.1 mM chloroquine and 0–100% FCS (■). +/− = 0.8 or r_i = 1 μg pCMV Luc, 2 · 10^5 ECV 304 cells; with more than 2 experiments mean values and standard error are given.

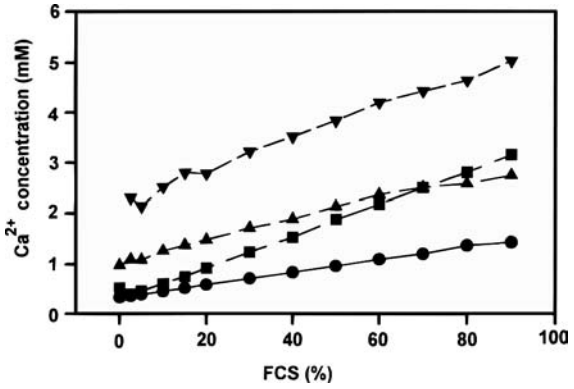

Fig. 5. Concentration of total Ca^{2+} and ionic Ca^{2+} in RPMI containing 0–90% FCS. The total (■) Ca^{2+} concentration and the ionic (free) Ca^{2+} concentration (●) were determined in RPMI containing 0–90% serum and after adding 2 mM exogenously Ca^{2+} to RPMI and serum, (▼) total and (▲) ionic Ca^{2+}. The measurements were performed twice, mean values are given.

the PO_4 concentration is not sufficient to generate $Ca(PO_4)_i$ microprecipitates (the phosphate concentrations in RPMI and concentrated FCS were 5.6 mM and 2.4 mM, resp.). In a series of experiments, we therefore added 12 mM PO_4 to the medium at high serum concentrations in order to regenerate the high transfection efficiencies observed at 10% serum. An improvement could actually be achieved. At 50% serum the transfection efficiency is increased 3.5-fold, at 90% about 50-fold, however, without reaching the initial values at 10% serum (not shown here). The low RLU values in the complete absence of serum are due to cell damage (see Fig. 2).

Free Ca^{2+} Concentration in Serum-Containing Media

To elucidate the mechanism of Ca^{2+}'s ability to overcome the transfection inhibition by serum, we performed measurements of the total and free Ca^{2+} concentration in RPMI containing serum concentrations of 0–90% FCS (Fig. 5). Since without addition of exogeneous Ca^{2+} there was no transgene expression, we were confronted with a problem of the availability of free Ca^{2+} for cellular processes related to transfection. Binding of Ca^{2+} to serum proteins could reduce free Ca^{2+} concentration from the media which should be measurable. The free Ca^{2+} concentration in serum reaches a value of about 1.44 mM at 90% FCS. At 0% FCS, i.e. in RPMI, we find 0.33 mM Ca^{2+}. The total Ca^{2+} concentration in this range increases from 0.45 mM to 3.15 mM Ca^{2+}. When no exogeneous Ca^{2+} is added, transgene expression was not detected in the full serum range. Only after addition of 2 mM exogeneous Ca^{2+} to the postincubation medium, transgene expression was obtained at all serum concentrations in the transfection medium (Fig. 1). Fig. 5 also shows the measured free and total Ca^{2+} concentrations after the addition of 2 mM exogeneous Ca^{2+} to the RPMI/serum solutions, i.e. under conditions which would result in transgene expression. Free Ca^{2+} concentrations between 0.98 mM and 2.74 mM Ca^{2+} were measured in the full concentration range of FCS. In contrast to this, 2.22 mM at 2.5% FCS to 5.0 mM Ca^{2+} were measured for the total Ca^{2+} concentration. At 0% FCS the total Ca^{2+} concentration after addition of 2 mM Ca^{2+} was not measurable because of binding of free Ca^{2+} to cuvette walls. These data prove that a considerable lack of free Ca^{2+} exists in RPMI and that this lack increases as the serum concentration increases. We suggest that the major part of the added Ca^{2+} is bound to free phosphates contained in RPMI (5.6 mM) which results in the formation of calcium phosphate microprecipitates $Ca(PO_4)_i$. With increasing serum concentrations further amounts of free Ca^{2+} also could be bound to negative serum proteins. This could be derived from the graph for the free Ca^{2+} concentration after the addition of 2 mM exogeneous Ca^{2+} that increased less compared with that of the total Ca^{2+} concentration (Fig. 5). Therefore, it is likely that $Ca(PO_4)_i$ microprecipitates play a role in overcoming the inhibitory effect of serum on transfection. However, substituting the free Ca^{2+} by preformed $Ca(PO_4)_i$ precipitates (total concentration of 2 mM Ca^{2+}) in the postincubation phase did not result in the full transfection efficiency. We obtained about 20% of the efficiency in the presence of ionic Ca^{2+} at 10 and 50% serum and 80% of the efficiency at 90% serum. Therefore, other unknown factors are also involved in the effect. Fig. 6a shows the relationship between transfection efficiency and input Ca^{2+} concentration in RPMI for Ca^{2+} concentrations <2 mM. The transfection medium contained 10% FCS and no Ca^{2+}. The postincubation medium contained increasing Ca^{2+} concentration and 10% FCS. In Fig. 6b the measured total and the free Ca^{2+} concentration is given as a function of the Ca^{2+} input concentration. The figure shows that even at 1 mM Ca^{2+} added in the postincubation phase the full transfection activity was reached. The difference of total and free Ca^{2+} measured at

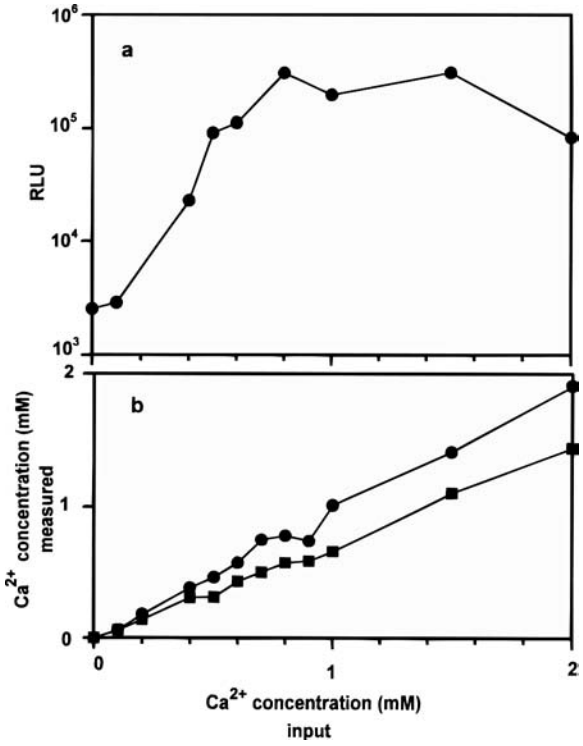

Fig. 6. Transfection efficiency (RLU) of H1-DNA complexes (a) and of the measured Ca^{2+} concentration as a function of the Ca^{2+} input concentration (b), total Ca^{2+} concentration (●), ionic Ca^{2+} concentration (■). 2 μg pCMV Luc, $r_i = 1$; $2 \cdot 10^5$ ECV 304 cells. The measurements were performed twice, mean values are given.

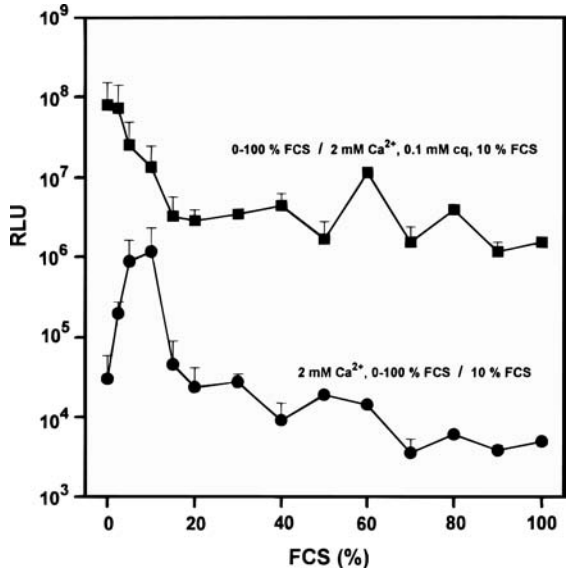

Fig. 7. Transfection efficiency (RLU) of the cationic liposomal reagent DOSPER as a function of the serum concentration. Transfection medium 2 mM Ca^{2+}, 0–100% FCS, postincubation medium 10% FCS (●), transfection medium without Ca^{2+}, 0–100% FCS, postincubation medium 2 mM Ca^{2+}/0.1 mM chloroquine, 10% FCS (■). 5 μg DOSPER, 2μg pCMV Luc, $2 \cdot 10^5$ ECV 304 cells; with more than 2 experiments mean values and standard error are given.

these low Ca^{2+} concentrations could indicate the existence of nascent microprecipitates.

Ca^{2+} Increases the Serum Stability of DOSPER

Finally, we studied the sensitivity to higher serum concentrations of the commercial transfectant, DOSPER. A question of great interest for the use of the cationic liposome DOSPER was, whether DOSPER which like most of the lipoplexes is inhibited by serum during transfection, could be stabilized in a similar way as H1 against serum inhibition by Ca^{2+}. Fig. 8 shows that DOSPER has a maximum transfection around 10% FCS when 2 mM Ca^{2+} is present in the transfection medium. The serum concentration ranged from 0–100% in the transfection medium. The postincubation medium contained 10% FCS. The decrease at 0% is due to Ca^{2+} toxicity. If there was 2 mM Ca^{2+}/0.1 mM chloroquine in the postincubation medium, transfection efficiency at first decreased 20–30 fold from the highest levels of transfection at low serum (0–5%) and then remained on this level in the full serum range. This means stabilization against serum on a lower transfection level.

DISCUSSION

The ability of serum to inhibit transfection is a well-known phenomenom. It represents one of the most important drawbacks of the nonviral gene transfer in vivo. The processes which result in the inhibition of the gene delivery are described for lipofection in some detail. Yang and Huang (5) demonstrated that this inhibition is due to negative serum proteins which bind to lipoplexes resulting in an inhibition of their binding to cells. They worked with 20% fetal bovine serum. It is possible to circumvent these effects by increasing the +/− charge ratio of the complexes resulting in an overcompensation of the negative charges of the serum proteins. Ogris et al. (6) were able to identify negative serum proteins which specifically bind to DNA/Transferrin-PEI complexes. It is expected that these proteins, IgM, fibrinogen, fibronectin, and complement C3, are transfection-inhibiting serum proteins. Using lipopolyamines Escriou et al. (16) found that in the presence of 10% serum the DNA uptake is only slightly affected, but the transgene expression greatly reduced. Lipoplexes were present in small vesicles, probably endosomes, in the presence of serum and were not translocated to the nucleus. In the absence of serum, the vesicles were larger and heterogeneous in size and shape. They contained large aggregates of lipoplexes which were active in transfection. The authors argue that the smaller vesicles in the presence of serum have difficulties in releasing the transgene DNA complex into the cytoplasm. This could be an additional explanation for the inhibition of the transfection by serum. Mizugichi et al. (10) suggested that the serum inhibition of transfection could be due to the presence of serum nucleases degrading the transgene DNA. Our results exclude this possibility. Another explanation of these authors concerned the binding of serum components to the cell surface interfering with the uptake of lipoplexes by the cells is also in contradiction to our results.

Transfection experiments in the presence of more than 20% serum have only been published rarely (10,17,18). However, such experiments are necessary in order to obtain full information on the effects of serum under in vivo conditions.

Using histone H1 as a DNA carrier protein, we have demonstrated that transfection is possible even in the presence of 100% serum if 2 mM Ca^{2+}/0.1 mM chloroquine is present in the postincubation medium. Although most of the experiments were performed in the presence of Ca^{2+} and chloroquine, the same conclusion was also obtained with Ca^{2+} alone. Here, we observed 20% smaller RLU values. Previously, we have shown that the addition of small concentrations of Ca^{2+} (2 mM) to the cells after the transfection, i.e. in the postincubation phase, resulted in high transfection rates. 10% serum was present in the postincubation medium, postincubation time was 24 hours (13). While the DNA uptake during the transfection period of 4 hours was not affected by Ca^{2+}, transgene expression was observed only if Ca^{2+} was present during postincubation. This was attributed to a facilitated endosomal/lysosomal release of the complexes in the presence of Ca^{2+}. According to these results, also a higher serum level (up to almost 100%) does not interfere with binding and uptake of complexes, but inhibits subsequent steps such as endosomal/lysosomal escape. Ca^{2+} as well as Ca^{2+}/ chloroquine, the latter with a higher efficiency, still functions as an lysosomolytic factor under these conditions. From our measurements of free and total Ca^{2+} concentration, we suggested that Ca^{2+} under our transfection conditions in the presence of serum is present in the form of calcium phosphate microprecipitates $Ca(PO_4)_i$ which have fusogenic and membranolytic activity (13,14). Several transfection experiments which were performed at Ca^{2+} concentrations <2 mM, in the absence of chloroquine and at 10% serum indicated that even at 0.8 mM Ca^{2+} the maximum in transfection was reached. There was evidence from our Ca^{2+} measurements that beginning with 0.4 mM Ca^{2+} nascent microprecipitates exist. This conclusion is further supported by the solubility product (on a molar basis), $Ca^{2+} \cdot (HPO_4)^{- -} = 2.4 \cdot 10^{-6}$, at pH 7.0, which indicates that $Ca(PO_4)_i$ microprecipitates will form whenever this product is exceeded (15). This condition was fulfilled in our experiments.

We studied complexes with approximately neutral +/− charge ratio which should not bind to negatively charged serum proteins and complexes of a charge ratio +/− of 4 which should bind to such proteins. The positively charged complexes showed a decrease in the transfection efficiency of 50%. This decrease could be due to an interaction of the complexes with acid serum proteins resulting in a diminished uptake by the cells (5). How is Ca^{2+} able to overcome the serum inhibition of transfection if it is not by binding serum proteins to the complexes or to the cell surface? The experiments in which Ca^{2+} was replaced by Mg^{2+} indicate the specific role of Ca^{2+} and exclude interactions between the extracellular components of the system which also would be influenced by Mg^{2+}. In the presence of serum, irrespective of the presence of Mg^{2+} in the transfection or postincubation medium, Ca^{2+} has to be present in the postincubation phase in order to overcome serum inhibition. The complexes are also taken up by cells in the absence of Ca^{2+}, otherwise we would not observe transfection if Ca^{2+} is present in the postincubation phase. An immunocytochemical study indicating that the uptake of the complexes takes place in the transfection phase and not after cell adsorbtion during the postincubation is in preparation (see also ref. 13). On the other hand, using neutral Hl-DNA complexes, the serum inhibition was restricted to the postincubation phase. The postincubation phase involves the translocation of the transgene DNA from the endosomes/lysosomes to the nucleus, the nuclear entry and the expression. As already mentioned, Ca^{2+} might be endocytosed in form of $Ca(PO_4)_i$ microprecipitates (19) and quickly distributed over the vesicular compartment of the cell. Due to their fusogenic and membranolytic activity, these microprecipitates are able to support transport and release of the transfecting Hl-DNA complexes from endosomes/lysosomes into the cytoplasm in the perinuclear region. Therefore, we suggest that the unknown serum effectors inhibit transfection on this level. It could be serum proteins which are taken up by the cells together with the transgene DNA complexes by endocytosis. They could inhibit the endosomal/lysosomal release of the transgene DNA complexes in the presence of serum. The endpoint would be their lysosomal digestion.

It should be mentioned that similar results concerning an overcoming of the serum inhibition of the transfection by addition of Ca^{2+}/chloroquine were also obtained in single experiments using CHO cells (not shown here). However, in this stage, a generalization of these results on other cell types is not yet possible. The results obtained with the cationic liposomal transfectant DOSPER concerning the stabilization against serum components using 2 mM Ca^{2+}/0.1 mM chloroquine compare well with H1. The only similarity between H1 and DOSPER is its cationic character. Therefore, our conclusions might be valid for polycations in general.

ACKNOWLEDGMENTS

This work was supported by a grant from the BMBF (01KV9548/1).

REFERENCES

1. F. D. Ledley. Nonviral gene therapy: the promise of genes as pharmaceutical products. *Hum. Gene Ther.* **6**:1129–1144 (1995).
2. E. R. Lee, J. Marshall, C. S. Siegel, C. Jiang, N. S. Yew, M. R. Nichols, J. P. Nietupski, R. J. Ziegler, M. B. Lane, K. X. Wang, N. C. Wan, R. K. Scheule, D. J. Harris, A. E. Smith, and S. H. Cheng. Detailed analysis of structure and formulations of cationic lipids for efficient gene transfer to the lung. *Hum. Gene Ther.* **7**:1701–1717 (1996).
3. Y. Saeki, N. Matsumoto, Y. Nakano, M. Mori, K. Awai, and Y. Kaneda. Development and characterization of cationic liposomes conjugated with HVJ (Sendai virus): reciprocal effect of cationic lipid for in vitro and in vivo gene transfer. *Hum. Gene Ther.* **8**:2133–2144 (1997).
4. C. Plank, K. F. Mechtler, F. C. Szoka Jr., and E. Wagner. Activation of the complement system by synthetic DNA complexes: a potential barrier for intraveneous gene delivery. *Hum. Gene Ther.* **7**:1437–1446 (1996).
5. J. P. Yang and L. Huang. Overcoming the inhibitory effect of serum on lipofection by increasing the charge ratio of cationic liposome to DNA. *Gene Ther.* **4**:950–960 (1997).
6. M. Ogris, S. Brunner, S. Schüller, R. Kircheis, and E. Wagner. PEGylated DNA/transferrin-PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. *Gene Ther.* **6**:595–605 (1999).
7. X. Zhou, A. L. Klibanov, and L. Huang. Lipophilic polylysines mediate efficient DNA transfection in mammalian cells. *Biochim. Biophys. Acta* **1065**:8–14 (1991)
8. J. G. Lewis, K. Y. Lin, A. Kothavale, W. M. Flanagan, M. D. Matteucci, R. S. DePrince, R. A. Mook Jr., R. W. Hendren, and R. W. Wagner. A serum-resistant cytofectin for cellular delivery of antisense oligodeoxynucleotides and plasmid DNA. *Proc. Natl. Acad. Sci. USA* **93**:3176–3181 (1996).
9. L. Vitiello, A. Chonn, J. D. Wasserman, C. Duff, and R. G. Worton. Condensation of plasmid DNA with polylysine improves liposome-mediated gene transfer into established and primary muscle cells. *Gene Ther.* **3**:396–404 (1996).

10. H. Mizuguchi, T. Nakagawa, M. Nakanishi, S. Imazu, S. Nakagawa, and T. Mayumi. Efficient gene transfer into mammalian cells using fusogenic liposomes. *Biochem. Biophys. Res. Commun.* **218**:402–407 (1996).
11. M. Böttger, S. V. Zaitsev, A. Otto, A. Haberland, and V I. Vorob'ev. Acid nuclear extracts as mediators of gene transfer and expression. *Biochim. Biophys. Acta* **1395**:78–87 (1998).
12. S. V. Zaitsev, A. Haberland, A. Otto, V. I. Vorob'ev, H. Haller, and M. Böttger. H1 and HMG17 extracted from calf thymus nuclei are efficient DNA carriers in gene transfer. *Gene Ther.* **4**:586–592 (1997).
13. A. Haberland, T. Knaus, S. V. Zaitsev, R. Stahn, A. J. Mistry, C. Coutelle, H,. Haller, and M. Böttger. Ca^{++} ions as efficient cofactor of polycation-mediated gene transfer. *Biochim. Biophys. Acta* **1445**:21–30 (1999).
14. N. Zakai, R. G. Kulka, and A. Loyter. Membrane ultrastructural changes during calcium phosphate-induced fusion of human erythrocyte ghosts. *Proc. Natl. Acad. Sci. USA* **74**:2417–2421 (1977).
15. W. Kübler and A. M. Katz. Mechanism of early pump failure of the ischemic heart: possible role of adenosine triphosphate depletion and inorganic phosphate accumulation. *Am. J. Cardiol.* **40**:467–471 (1977).
16. V. Escriou, C. Ciolina, F.Lacroix, G. Byk, D. Scherman, and P. Wils. Cationic lipid-mediated gene transfer: effect of serum on cellular uptake and intracellular fate of lipopolyamine/DNA complexes. *Biochim. Biophys. Acta* **1368**: 276–288 (1998).
17. J. Y. Legendre, A.Trzeciak, B.Bohrmann, U. Deuschle, E. Kitas, and A. Supersaxo. Dioleoylmelittin as a novel serum-insensitive reagent for efficient transfection of mammalian cells. *Bioconjug. Chem.* **8**:57–63 (1997).
18. L.Vitiello, K. Bockhold, P. B. Joshi, and R. G. Worton. Transfection of cultured myoblasts in high serum concentration with DODAC:DOPE liposomes. *Gene Ther* **5**:1306–1313 (1998).
19. E. Orrantia and P. L. Chang. Intracellular distribution of DNA internalized through calcium phosphate precipitation. *Exp. Cell Res.* **190**:170–174 (1990).