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Phosphate-enhanced transfection of cationic lipid-complexed mRNA and plasmid DNA

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Abstract

Cationic lipid-mediated gene transfer has been shown to be a competent albeit inefficient mechanism of promoting cellular gene transfer. One way to improve the efficacy of cationic lipid-mediated transgene expression is to optimize conditions for complex formation between the lipids and nucleic acids. In this report we describe the beneficial effects of using phosphate buffer to precondition lipofectin (a 1:1 (w/w) mixture of *N*-[1-(2,3-dioleyloxy)propyl]-*n*,*n*,*n*-trimethylammonium chloride (DOTMA), and dioleoyl phosphatidylethanolamine (DOPE)) prior to complexing with plasmid DNA or mRNA. Under such optimized conditions we studied the kinetics of DNA- and RNA-mediated transgene expression in a human osteosarcoma cell line (HOS). Preincubation of lipofectin in phosphate buffer resulted in up to 26- and 56-fold increases in luciferase expression from plasmid DNA and mRNA, respectively. Addition of chloroquine (50 μM), which enhanced plasmid-mediated gene delivery 3-fold, was synergistic with phosphate resulting in an additional 46-fold increase in luciferase expression. The preincubation with phosphate shortened both the time required for cellular uptake and the time to achieve maximal transgene expression. Optimal transfection was achieved in the presence of 30–80 mM phosphate, at pH 5.6–6.8 under which the phosphate anion is divalent. The effect of phosphate anion was specific in that monovalent Cl^- and acetate anions were not stimulatory. These results demonstrate that divalent phosphate anion plays a stimulatory role during complex formation and transfection when cationic lipids come in contact with negatively charged nucleic acids and cell membranes. These findings delineate specific conditions which dramatically enhance transfection efficiency for both DNA and mRNA, and provide an effective procedure for gene transfection studies. © 1998 Elsevier Science B.V.

Keywords: Cationic lipid; Gene transfer; Transfection; mRNA; Plasmid DNA; Kinetics; DOTMA

1. Introduction

The use of gene therapeutic approaches in the treatment of disease has received increasing interest. Viral based delivery has proved thus far to be the most efficacious form of gene delivery. Viral therapies however have a number of limitations including variable transfection efficiencies, activation of host immune response, concern over possible viral recom-

Abbreviations: DOTMA, *N*-[1-(2,3-dioleyloxy)propyl]-*n*,*n*,*n*-trimethylammonium chloride; DOPE, dioleoyl phosphatidylethanolamine; HOS, human osteosarcoma cell line; RLU, relative light unit

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bination and cellular toxicity [1,2]. Non-viral delivery systems have been developed with the hope of overcoming some of the problems associated with viral gene delivery (for review see [3]). In non-viral methods some type of lipid vehicle, usually cationic liposome, is employed to deliver plasmid DNA or in vitro transcribed mRNA to the target cells [4,5]. Evaluation of transfection efficiency usually involves comparison of different formulations using transgene expression as the endpoint. Cationic lipid transfer, however, results in relatively low transfection efficiency when compared to the viral systems, although progress in this process of so called ''lipofection'' [6] has been made throughout the years, which has resulted in improvements in gene transfer [7]. Potent vectors have been developed [8,9], novel lipids have been introduced [8,10,11] and complex formulations have been optimized [10,12–14]. Advancements have also been made in the physico-chemical characterization of the DNA/cationic lipid interaction during complex formation [15,16] and cell transfection [17]. The physical structure of the complex has been investigated by metal-shadowing, freeze-fracture and transmission electron microscopies [18–22], and more recently by combined in situ optical microscopy and x-ray diffraction [23].

Despite all of this progress and utilization of sophisticated tools, the new lipofection protocols are developed empirically by trial-and-error methods. This is largely due to the fact that the kinetics of complex formation between nucleic acid and lipid carrier, and the mechanisms of cellular uptake and intracellular trafficking of the complex remains poorly understood [22,24]. Little is known concerning the individual parameters that influence complex formation and the characteristics of the complex that are relevant to transfection efficiencies in gene therapy [10,13,22,25]. A better understanding of these molecular and cellular mechanisms would lead to a rational approach in the design of cationic lipid–nucleic acid complex, and subsequently to an improvement of gene transfer methods.

The aim of the present study was to evaluate the ability of phosphate anions to improve cationic lipid-mediated gene transfer. An established cell line was used to test the expression of luciferase reporter protein from template plasmid DNA or from in vitro mRNA transcripts. We demonstrate that when lipofectin is preincubated with sodium or potassium phosphate buffer the transfection efficiency of both RNA and DNA is dramatically improved. This beneficial effect of phosphate buffer was concentration and pH-dependent, specific, and resulted in accelerated protein expression. The relatively simple modifications described here have profound effects on the ability to efficiently transfect a wide variety of cell types with both mRNA as well as DNA. Finally, on the basis of these findings, we propose a mechanism to explain the effects of divalent phosphate anions upon the process of gene transfer.

2. Materials and methods

2.1. Cells, plasmid DNA and mRNA

A human osteosarcoma cell line (HOS) was obtained from American Type Culture Collection (ATCC, Rockville, MD) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Cell viability was assayed using Trypan blue exclusion test. For DNA transfection studies we utilized pCMVintLucΔ5′ gene expression plasmid vector which was a gift from Dr. Kris Fisher. This vector consisted of the full coding region of firefly *P. pyralis* luciferase cDNA driven by the human cytomegalovirus promoter. The 5530 bp plasmid also contained a polyadenylation signal and 5′ upstream intron sequences that were both SV40-related. For mRNA transfection we employed synthetic mRNA transcripts obtained by in vitro synthesis. The DNA template plasmid pT7-TEV-LUC-A50 was a gift from Dr. Daniel Gallie. Transcript derived from this plasmid contained the luciferase coding region, leader of the tobacco etch viral RNA, and polyA tail. The pT7-TEV-LUC-A50 plasmid was first linearized at a *Nde*I site 3′ to the incorporated stretch of 50 bp dA:dT. The linearized and purified DNA template was then used to synthesize the luciferase encoding 5′-capped transcript using MessageMachine kit (Ambion, Austin, TX) according to the manufacturer. Purification of the 1.8 kb transcript was performed by DNase I digestion followed by LiCl precipitation and washing with 75% ethanol. The quality of each batch of purified mRNA was tested by agarose gelelectrophoresis for degradation and for presence of con-

taminating DNA template. Plasmids were purified using the Qiagen kit (Qiagen, Santa Clarita, CA). The plasmid DNA and mRNA samples were quantitated by measuring O.D. at 260 nm. Samples were stored frozen at −20°C in 0.5–1.0 μg/μl final concentrations.

2.2. Preparation of the complex

Sodium and potassium phosphate buffers with different pH were prepared by combining different volumes of the appropriate 1 M solutions [26]. The stock solutions were prepared by making 0.1–1.0 M dilutions of the 1 M buffers (pH 4.0–9.2) and supplementing them with 1 μg/μl bovine serum albumin (BSA) (Sigma, St. Louis, MO). All stock solutions were kept in polypropylene Eppendorf tubes at −20°C. Lipofectin was obtained from Gibco BRL (Gaithesburg, MD).

First, dilutions were prepared using serum-free cold DMEM with high glucose content. The stock phosphate buffer was diluted 10-fold to 10–100 mM, and nucleic acids were diluted to 0.07 μg/μl concentration. Usually 0.23 μg nucleic acid was complexed with 0.8 μg lipofectin for transfecting cells in 1 well of a 96-well plate. Since experiments were performed in triplicate, 2.4 μl lipofectin (2.4 μg) was added to 21.3 μl phosphate buffer diluent containing 0.1 μg/μl BSA, and incubated for 10 min at room temperature in a 0.5 ml polypropylene tube. Then 9.9 μl nucleic acid (0.69 μg) was added and the mixture was further incubated for 10 min at room temperature. Finally 116.4 μl prewarmed (37°C) DMEM was added to bring up the final volume to 150 μl. The mixture was vortexed and 45 μl of it was added directly to 3 wells of a 96-well plate containing confluent culture of 2×10^4 cells per well each. Transfected cells were incubated for 1 h at 37°C in a 5% CO_2 incubator. The mixture was then replaced with fresh prewarmed culture medium containing 10% FCS, and further incubated. The incubations were terminated 5 h or 24 h after the initiation of transfection when mRNA or plasmid DNA was used, respectively. In some experiments freshly made 50 μM chloroquine (Sigma) was also present in the transfection mixture. In control experiments for complexing we followed the lipofectin manufacturer recommendations. Diluted lipofectin and diluted nucleic acid solutions were preincubated in separate tubes at room temperature for 30 min, then combined by adding the lipofectin solution to the nucleic acid, and incubated for an additional 15 min before placing on the cells. The calculated charge ratio of lipoplex prepared as described above is 1. In the time course experiments when cells were treated with lipoplex longer than 1 h, we added 45 μl DMEM supplemented with 20% FCS to the cells after the first hour of incubation.

2.3. Analysis of luciferase expression

Following incubation, the medium was removed from the cells and 50 μl luciferase cell culture lysis reagent (Promega, Madison, WI) was added to each well of the 96-well plate. The detection of luciferase activity was performed using Promega luciferase assay kit. 10 μl aliquots of the 50 μl cell lysates were analyzed for activity using LUMAT LB 950 (Berthold/EG&G Wallac, Gaithesburg, MD) luminometer with a 10 s measuring time. The background activity obtained with non-transfected cells was ~100 relative light units (RLU). Using recombinant luciferase (Promega), it was determined that the standard curve was linear, with 1.7×10^7 RLU activity corresponding to 1.4 ng luciferase. Given that the molecular weight of luciferase is 60 kDa, 1.4 ng luciferase contains $\sim 1.4 \times 10^{10}$ molecules. All measurements were performed in the linear range of the standard curve. The measured activity values were normalized for 10 μg cellular protein determined by the Bradford method (Bio-Rad, Hercules, CA). The protein content of $\sim 2 \times 10^4$ HOS cells that were present in a well of a 96-well plate during transfection was ~10 μg. Data represent RLU values obtained from cells present in 1 well.

2.4. Data analysis

Experiments were performed in triplicate, and each experiment was repeated independently several times. Data are shown as means of individual experiments. We detected some level of variation with different batches of lipofectin. Statistical analyses of the measured activities were performed using 2-tailed Student's *t*-test. A probability of less than 0.05 was considered to be statistically significant.

3. Results

3.1. The effect of potassium phosphate supplementation on the efficiency of lipofection

In an attempt to improve gene delivery we tested the stimulatory effect of chloroquine and viral protein isolates on the efficiency of lipofection of plasmid DNA and mRNA. After performing the appropriate control experiments, the stimulatory effect first attributed to the proteins was indeed elicited by the small amount of potassium phosphate buffer that was present in the isolates. To demonstrate the effect of the buffer, lipofectin was preincubated in DMEM supplemented with 20 mM potassium phosphate buffer pH 6.4 and BSA (100 ng/μl) prior to complexing with plasmid DNA. The complex was then further diluted with DMEM, delivered to human osteosarcoma cell line (HOS) and luciferase gene expression was evaluated. As shown in Fig. 1(A), supplementation with potassium phosphate buffer had a significant, 26-fold stimulatory effect on transfection efficiency. Under the same experimental conditions we also tested the effect of chloroquine. Chloroquine has been shown to enhance gene transfection, including cationic lipid-mediated gene transfer by its lysosomotropic activity which can enable escape of endocytosed materials from the endosome [10]. Chloroquine increased gene delivery to HOS by 3-fold, and also had a synergistic effect on the phosphate buffer-mediated enhanced plasmid transfection which resulted in a 46-fold increase in luciferase production (Fig. 1(A)). The effect of chloroquine and phosphate buffer was also tested on lipofectin-mediated mRNA transfection. Using this type of formulation the mRNA transfection was increased 56-fold. The treatment caused no obvious cytotoxicity, although the transfection complex was kept on the cells for only 1 h.

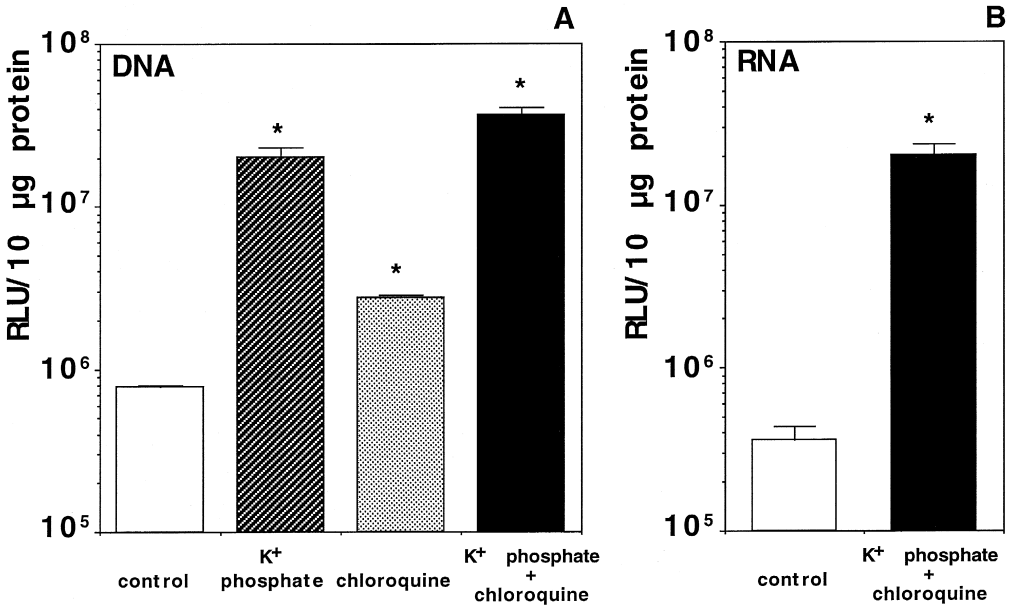

Fig. 1. Phosphate enhances cationic lipid-mediated DNA and RNA transfection. Transfection complex was prepared by mixing 0.23 μg luciferase-encoding plasmid DNA or mRNA with 0.8 μg lipofectin as described in Section 2. Complexes were added to a confluent monolayer of human osteosarcoma cell line (HOS) seeded in a 96-well microtiter plate. After a 1 h treatment, the transfection mix was replaced with culture medium and the cells were further incubated. (A) Expression of luciferase from a DNA template (pCMVintLucΔ5′) was assayed at 24 h post-transfection and from (B) an RNA template (mTEV-LUC-A50) 5 h after initiation of transfection. Lysates were normalized for protein content. The approximately 2×10^4 cells present in each well contained ~10 μg protein. Prior to complexing, the lipofectin was preincubated in DMEM containing 20 mM potassium phosphate buffer pH 6.4 and 100 ng/μl BSA (*K^+ phosphate*). Where it is labeled (*chloroquine*), 50 μM chloroquine was present during the 1 h transfection. Control cells were transfected with non-pretreated lipofectin and without chloroquine addition (*control*). Data represent the mean ± SD for triplicates after correction for background. Statistical differences from the controls are labeled * $P < 0.05$.

3.2. The effect of different counterion supplementation on cationic lipid-mediated transfection efficiency

To test if the increased gene transfection efficiency elicited by potassium phosphate was mediated by potassium, we compared the effect of KCl and NaCl to that of potassium phosphate. We found that KCl and NaCl were ineffective in enhancing lipofectin-mediated plasmid DNA delivery under the same experimental conditions where potassium phosphate had significant effect (Fig. 2(A)). We also tested whether the effect of potassium phosphate on gene transfection was phosphate specific. We subjected the lipofectin to 20 mM sodium phosphate, potassium phosphate and sodium acetate buffers with pH 5.8 treatments during the complexing process. As Fig. 2(B) demonstrates, the phosphate buffers were equally effective and increased the efficiency of lipofectin-mediated transfection about 10-fold. However sodium acetate supplementation during complexing inhibited the transfection efficiency. When sodium citrate buffer was tested we could detect modest level of stimulation, however it was not statistically significant and somewhat varied in different experiments (not shown). There was no obvious cytotoxicity related to any of the buffer treatments. These results suggest that the enhancement of transfection efficiency is phosphate specific.

3.3. Concentration-dependent effect of potassium phosphate

We tested whether stimulation of gene transfection by phosphate buffer supplementation was dependent on the concentration of the buffer. We used 0.01–0.10 M potassium phosphate buffer, pH 5.8 to pretreat the lipofectin prior to complexing with plasmid DNA. In this particular experiment the cells were exposed only for 30 min to the transfection mixture, and 50 μM chloroquine was also present for this time period. The results shown in Fig. 3 demonstrate that the maximum transfection efficiency can be achieved when the phosphate buffer concentration is between

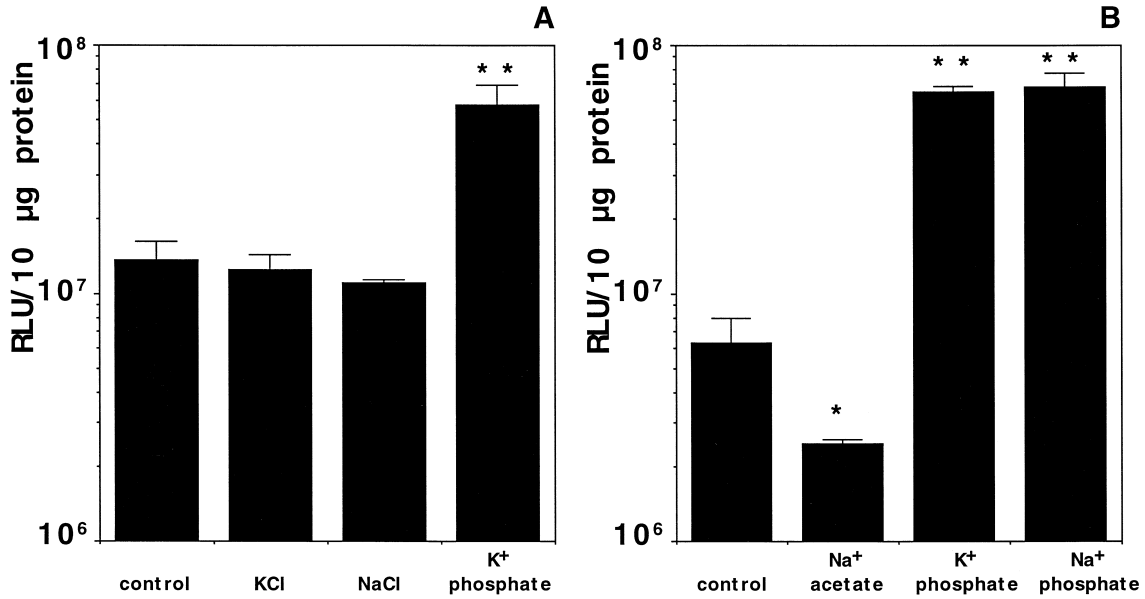

Fig. 2. The effect of different counterions on cationic lipid-mediated DNA transfection. Transfection and luciferase assaying were performed as described in Fig. 1 and in Section 2. Complex was prepared by mixing 0.23 μg luciferase-encoding plasmid DNA with 0.8 μg lipofectin that was preincubated with DMEM containing 100 ng/μl BSA and one of the following solutions: 20 mM KCl (*KCl*), 20 mM NaCl (*NaCl*), 20 mM potassium phosphate pH 5.8 (K^+ *phosphate*), 20 mM sodium phosphate pH 5.8 (Na^+*phosphate*), 20 mM sodium acetate pH 5.8 (Na^+*acetate*) or none (*control*) prior to complexing. After 1 h treatment the transfection mix was replaced with culture medium and the cells were further incubated for 23 h, when they were lysed and assayed. Lysates were normalized for protein content. Data represent the mean ± SD for triplicates after correction for background. Statistical differences from the controls are labeled $* P < 0.05$, $** P < 0.001$.

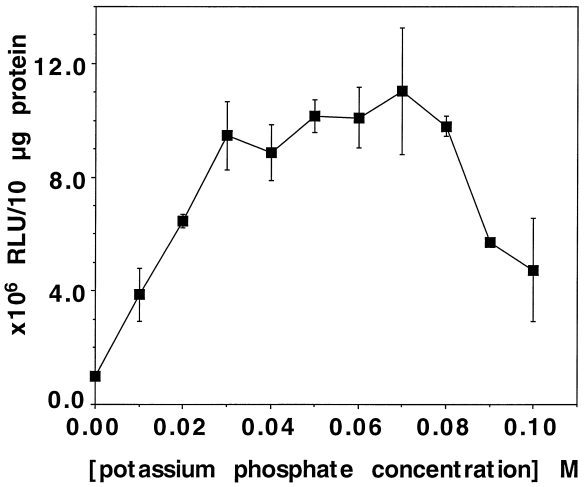

Fig. 3. Concentration-dependent effect of potassium phosphate on cationic lipid-mediated DNA transfection. Transfection and luciferase assaying were performed as described in Fig. 1 and in Section 2. The complexes were prepared by mixing 0.23 μg pCMVintLucΔ5′ with 0.8 μg lipofectin that was preincubated with DMEM containing 100 ng/μl BSA and different concentrations (10–100 mM) of potassium phosphate buffer pH 5.8. After 30 min treatment the transfection mix was replaced with culture medium and the cells were further incubated for 24 h. Cells were lysed and assayed for luciferase activity. Values are normalized for protein contents of the lysates. Data represent the mean ± SD for triplicates after correction for background. Determinations were made in 3 independent experiments.

0.03 and 0.08 M. Cells transfected with mixtures prepared with 0.09 and 0.1 M phosphate buffer supplementation appeared normal, so the decrease in transfection efficiency was unlikely due to cytotoxicity.

3.4. pH-dependent effect of potassium phosphate on transfection efficiency

During pilot studies we noticed that when pH 8.0 phosphate buffer was tested for lipofectin pretreatment, microscopically detectable tangled aggregates were formed instantly. These aggregates adhered to the cells and seemed to be toxic. To test if the pH of the phosphate buffer was a critical parameter in complex formation and in subsequent transfection, we screened the effect of potassium phosphate buffer between pH 4.0 and 9.2. The result of one of the representative experiments in which 0.05 M potassium phosphate buffer was used is shown in Fig. 4. Data suggest that maximal enhancement of gene delivery can be achieved using potassium phosphate buffer with pH 5.6–6.8. We obtained similar results when 0.08 M phosphate buffer was tested, or when mRNA was complexed instead of plasmid DNA. In all cases our finding was that if the buffer pH was 7.6 or more, there was a sharp 2–4-fold drop in the transfection efficiency. This decline is likely caused by cytotoxicity induced by lipofectin-DNA aggregates that formed during complexing. When complex was generated with potassium phosphate buffer at pH 7.4 or 8.0, transfection resulted in partial or complete aggregate coverage of the cells with differing degrees of cell death evident in these wells. No cell death was detected however in wells transfected with complexes generated with phosphate buffer at pH 4.0–5.2. We also tested whether the pH-dependent effect of potassium phosphate is related to degradation of plasmid

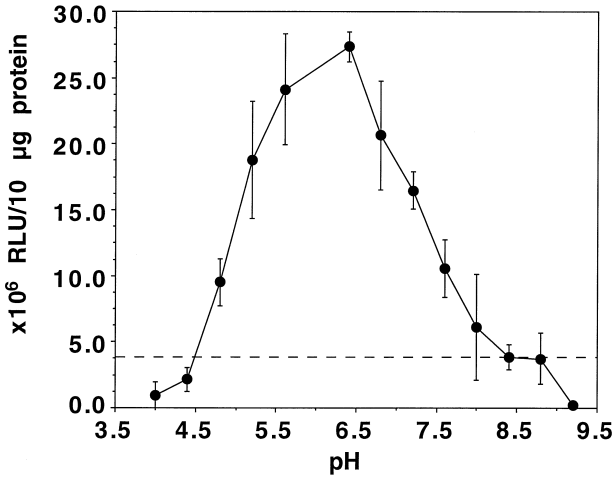

Fig. 4. pH-dependent effect of potassium phosphate on cationic lipid-mediated DNA transfection. Transfection and luciferase assaying were performed as described in Fig. 1 and in Section 2. The complexes were prepared by mixing 0.23 μg pCMVintLucΔ5′ with 0.8 μg lipofectin that was preincubated with DMEM containing 100 ng/μl BSA and 50 mM of potassium phosphate buffer with different pH (pH 4.0–9.2). After 1 h treatment the transfection mix was replaced with culture medium and the cells were further incubated for 24 h. Cells were lysed and assayed for luciferase activity. Values are normalized for protein content of the lysates. Data represent the mean ± SD for triplicates after correction for background. Determinations were made in 3 independent experiments. Dotted line indicates luciferase level obtained from cells transfected with complex that was made with non-preincubated lipofectin.

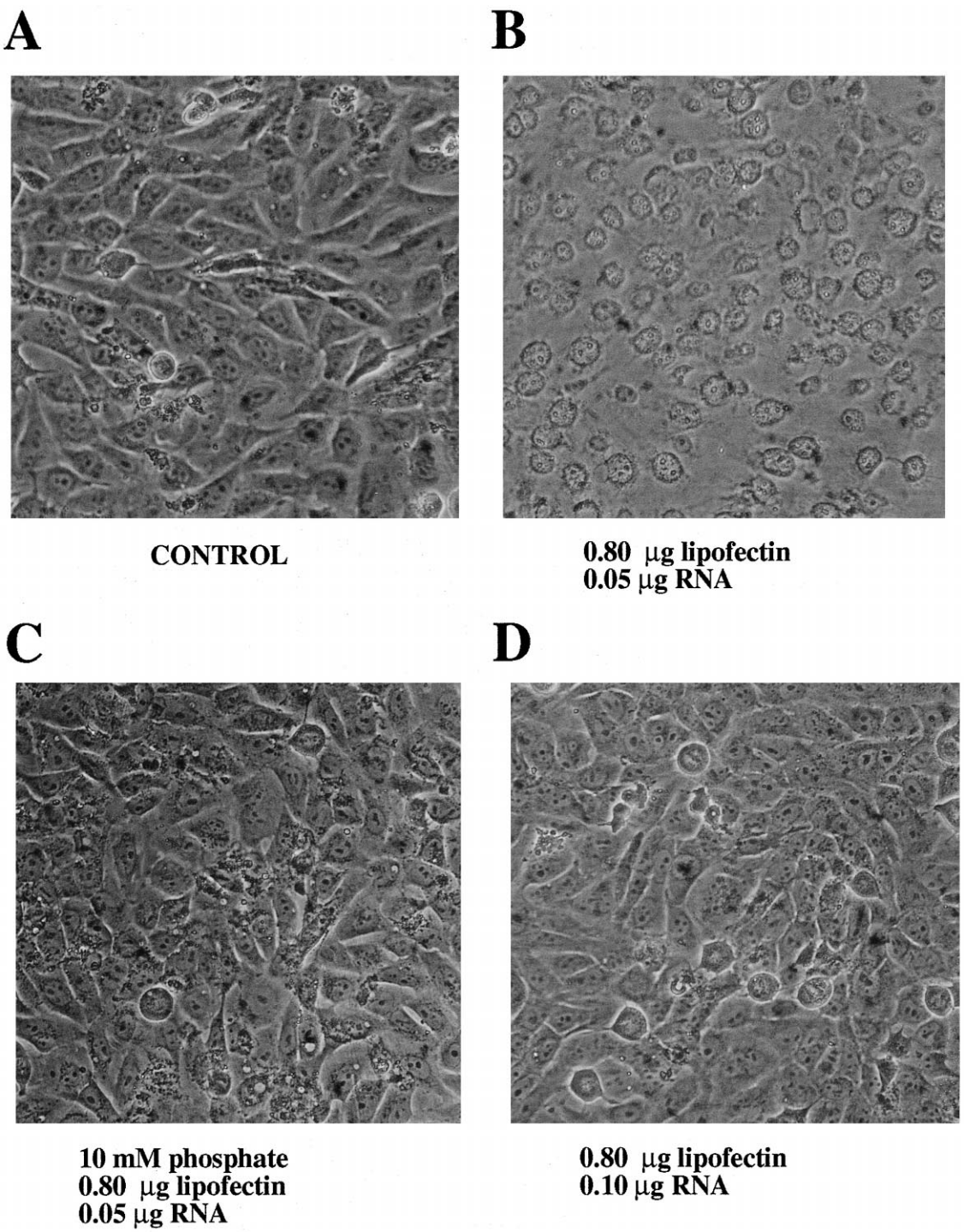

Fig. 5. Potassium phosphate inhibits cationic lipid-induced cytotoxicity. Transfection was performed as described in Section 2. The complexes were prepared by mixing 0.05 μg (B and C) or 0.1 μg (D) of mRNA (mTEV-LUC-A50) with 0.8 μg lipofectin that was preincubated in DMEM containing 100 ng/μl BSA and supplementation of 10 mM of potassium phosphate buffer pH 5.8 (C), or without supplementation (B and D). After 1 h treatment the transfection mixes were removed and pictures were taken. Untreated cells are shown in (A). Similar results were obtained in 3 independent experiments.

DNA which could occur during complexing at very low or very high pH. In some experiments we harvested the medium from the cells after the 1 h incubation period and tested for the presence of intact plasmid. All samples contained intact plasmid and no degradative products were detectable, suggesting not only that no pH-related degradation occurred, but also that significant amount of plasmid is still present in the medium at the time when the treatment with the transfection mix is terminated (not shown).

3.5. Phosphate diminishes cytoxicity of lipofectin

It has been long appreciated that cationic lipids can exhibit significant cellular toxicity. It has also been noted that cationic lipid dispersions without DNA are more toxic than those complexed with DNA [27]. We also noticed that under our experimental conditions non-complexed lipofectin was very toxic to HOS cells (not shown). Significant cell death was also observed following treatment with lipofectin complexed with a low amount of RNA (Fig. 5(B)). The charge ratio in the complex (+/−) in that particular experiment shown in Fig. 5(B) was 5. When the amount of nucleic acid was increased in the complex and the charge ratio became 2.5 no cytotoxicity was detectable (Fig. 5(D)). Importantly however phosphate buffer could eliminate the cytotoxicity of lipofectin when low concentration RNA was complexed to it (Fig. 5(C)). Apparently the phosphate anions act as a charge ''shield'' by substituting for the phosphate of mRNA and neutralizing excess positive charge present in the lipofectin.

3.6. Dose-response curves

One of the parameters that is most frequently tested and optimized in gene transfection protocols is the ratio of lipid and nucleic acid [10,12]. As we described in the methods section, usually 0.23 μg nucleic acid was complexed with 0.8 μg lipofectin for transfecting cells in 1 well of a 96-well plate. This ratio of nucleic acid lipofectin resulted in a +/− charge ratio of 1:1. This value seems suboptimal when compared to those reported by others for lipofectin [10,12]; however, it is possible that the potassium phosphate preincubation could impact upon true charge ''availability'' through the shielding effect. To determine the dependency of transfection efficiency on nucleic acid:lipid ratio, we transfected the cells with nucleic acid–lipofectin complex which was prepared by complexing variable amounts of mRNA or plasmid DNA with fixed amounts of lipofectin which was pretreated with phosphate buffer. The results shown in Fig. 6 suggest that ~0.1 μg mRNA or DNA is sufficient to obtain maximal transfection efficiency when complexed with 0.8 μg lipofectin pretreated with 0.01 M potassium phosphate, pH 6.8. The maximal luciferase activity elicited by the mRNA was ~8 million RLU/10 μg protein, and by DNA was ~32 million RLU/10 μg protein. These values correspond to 0.65 and 2.63 ng of luciferase production per 2×10^4 cells transfected with mRNA and DNA, respectively.

We hypothesized that there could be two reasons why the dose-curves reached saturation levels. One reason could be that excess nucleic acid and limited

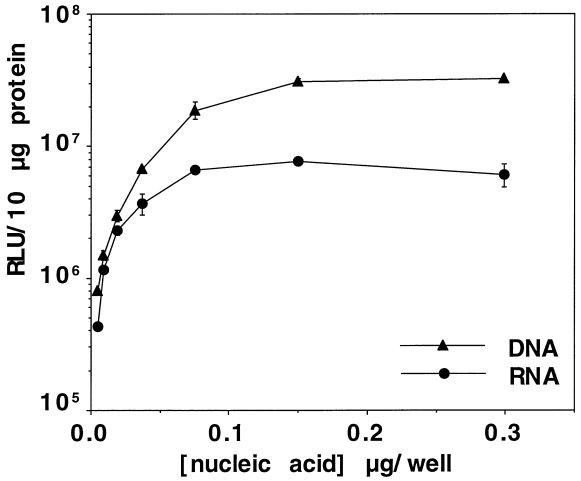

Fig. 6. Kinetics of phosphate-enhanced DNA and RNA transfection. Dose response curve. Transfection and luciferase assaying were performed as described in Fig. 1 and in Section 2. The complexes were prepared by mixing different amounts of plasmid DNA (pCMVintLucΔ5′) or mRNA (mTEV-LUC-A50) with fixed amounts of lipofectin (0.8 μg) that was preincubated with DMEM containing 100 ng/μl BSA and 10 mM potassium phosphate buffer pH 6.8. After 1 h treatment the transfection mix was replaced with culture medium and the cells were further incubated. Cells exposed to mRNA containing complexes were lysed 5 h after initiation of the transfection. Expression of luciferase from DNA template was assayed at 24 h post-transfection. Lysates were normalized for protein content. Values for DNA (*filled triangles*) and mRNA (*filled circles*) are shown. Data represent the mean ±SD for triplicates after correction for background.

amount of lipofectin is present during complexing, so the nucleic acid cannot be in its effective, lipid-complexed form during transfection. The other is that the cell has limited capacity for complex uptake or expression of protein. To determine which is the limiting factor, we generated two dose response curves. One with samples prepared by complexing variable amounts of mRNA with fixed amounts of lipofectin, the other by using aliquots of diluted preformed complexes. The stock complex for this latter curve was assembled using the usual 0.23 μg mRNA/0.8 μg pretreated lipofectin ratio. We reasoned that if the transfection efficiency decreased when using diluted aliquots of the most concentrated complex it would mean that the lipid is the limiting factor. The two dose-response curves were however almost identical (Fig. 7), suggesting that at high nucleic acid concentration (> 0.1 μg/well) excess transfective lipid-nucleic acid complexes are present, but cell-related parameters are limiting the transfection. This reasoning is further supported by results from several experiments where transfection solutions were reused. We could reach the same high level of transfection efficiency by reusing a complex solution of 0.23 μg nucleic acid and 0.8 μg lipofectin exposed to cells for 1 h (not shown). However the efficiency of transfection did not increase significantly when the incubation time was increased from 1 h. This observation not only means that there is excess transfective complex in the solution, but also suggests that the transfective particles are relatively stable and the cells are imposing a limitation to further increase the transfection efficiency.

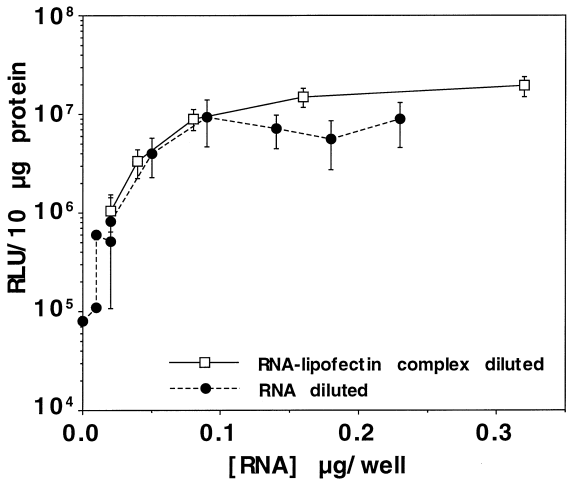

Fig. 7. Kinetics of phosphate-enhanced RNA transfection. Dose response curves. Transfection and luciferase assaying were performed as described in Fig. 1 and in Section 2 here using 2 different transfection mixes. For the first transfection mix (*filled circles*) the complexes were prepared by mixing the indicated amount of mRNA (mTEV-LUC-A50) with fixed amount of lipofectin (0.8 μg) that was preincubated with DMEM containing 100 ng/μl BSA and 20 mM potassium phosphate buffer pH 6.8. For the second transfection mix (*open squares*) the complex was prepared by mixing 0.96 μg mRNA with 2.4 μg lipofectin pretreated as described for the first mix. The preformed complexes were diluted in DMEM then added to the cells. After 1 h treatment both transfection mixes were replaced with culture medium and the cells were further incubated. Cells were lysed 5 h after initiation of the transfection and assayed for expression of luciferase. Values were normalized for cytoplasmic protein content. Data represent the mean ± SD for triplicates after correction for background. Similar results were obtained in at least 3 independent experiments.

3.7. Time course of gene expression

The time course of the transfected gene expression depends on two time components. One is the time required to transfer the gene to the cell, the other the time required to transcribe and translate the gene. The first time component is more dependent on the delivery method, while the second is dependent on the characteristics of the cell and gene type. To learn more about the phosphate buffer-stimulated delivery mechanism we tried to separate the two time components by performing different types of time course experiments. In these studies we compared the properties of two transfection mixes. One mix was made by complexing 0.23 μg plasmid DNA with 0.8 μg lipofectin which was preincubated with 80 mM potassium phosphate buffer, pH 6.4. The other control transfection mix was assembled according to the manufacturer of lipofectin using 0.23 μg DNA and 0.8 μg lipofectin. In the first time course study the cells were treated with the transfection mix for the indicated time period (20 min–5 hr), then the mix was removed and the cells were further incubated. The cells were lysed and assayed 24 h after initiation of the transfection. Results shown in Fig. 8(A) demonstrate that gene transfer to the cell is a very rapid process. In a short period of time, significant amounts of DNA became associated with the cells, entered or

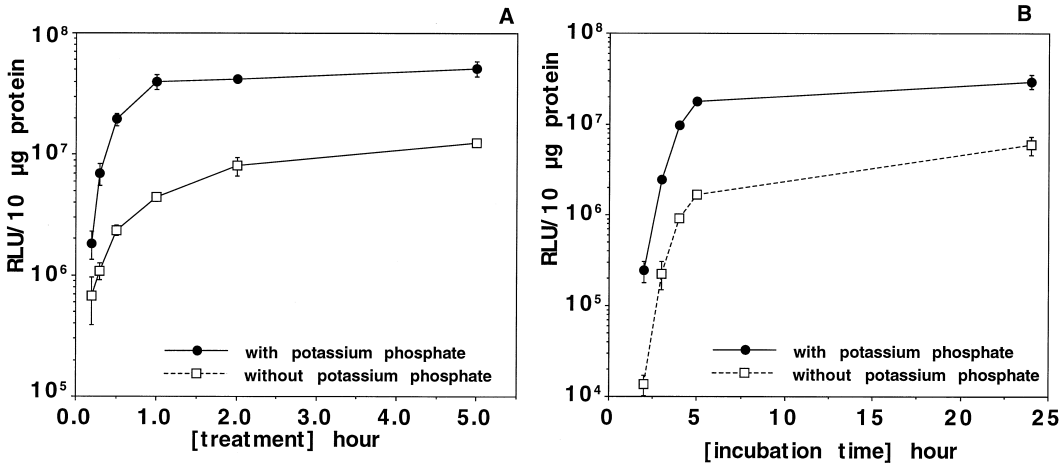

Fig. 8. Kinetics of phosphate-enhanced DNA transfection. Time course experiments. Transfection and luciferase assaying were performed as described in Fig. 1 and in Section 2. Complex was prepared by mixing 0.23 μg luciferase-encoding plasmid DNA with 0.8 μg lipofectin that was preincubated with DMEM containing 100 ng/μl BSA and supplemented with 80 mM potassium phosphate pH 6.4 (*filled circles*) or without supplementation (*open squares*) prior to complexing. (A) Cells were treated for the indicated time, then the transfection mix was replaced with culture medium and they were further incubated. Cells were lysed 24 h after initiation of the transfection and assayed for expression of luciferase. (B) Cells were incubated for 1 h with transfection mixes, then the transfection mix was replaced with culture medium and they were further incubated. Cells were lysed at the indicated time and assayed for expression of luciferase. Values were normalized for cytoplasmic protein content. Data represent the mean ±SD for triplicates after correction for background.

stuck to the surface in such a way that it could not be washed off. The results also suggest that phosphate buffer possibly accelerates the gene delivery process. One hour treatment was sufficient to elicit the maximal effect, and exposing the cells to the transfection mix for additional hours did not result in further increases in gene expression. In the control experiment maximal gene expression was significantly less than that which could be reached with phosphate buffer stimulation. In addition, more than 2 h were required to reach the maximal transfection efficiency.

In the second time course experiment all cells were treated for 1 h with the two types of transfection mixes described above. At the end of the 1 h treatment, the mix was removed, the cells were further incubated for 1–24 h, and lysed and assayed at the indicated time points. Luciferase activity was below detection level after 1 h of incubation, which coincided with the 1 h treatment period (not shown). Results presented in Fig. 8(B) demonstrate that at least 2 h incubation was required to detect luciferase activity. By that time the measured luciferase activity was 500-fold of the background level in cells lipofected with phosphate-treated samples while the same value was only 27-fold in cells lipofected with the conventional method. These results suggest that in HOS cells intracellular trafficking, transcription and translation of luciferase encoding plasmid DNA takes about 2 h to occur and about 5 h to reach its maximal capacity. Additional incubation up to 24 h resulted only in a marginal 1.6- and 3.5-fold further increase in gene expression elicited by the phosphate buffer-mediated and control transfections, respectively. These results also suggest that the DNA–lipid complex, made with phosphate buffer, requires less time to be expressed in higher quantities compared to the control complex made without the buffer. To insure that results of time courses are not skewed by the presence of limited amounts of transfective complex, we reused the transfection solution removed from the cells in a second set of time course experiments. With the reused transfection mix we obtained results similar to those shown in Fig. 8(B). Surprisingly, we could generate curves almost identical to Fig. 8(B), confirming that the original experiment was performed in the presence of excess effective complex. This result also demonstrates that both types of complexes are very stable.

3.8. Time course of mRNA transfection

To understand the mechanism by which the phosphate buffer affects efficiency of gene transfection we needed to determine more precisely the time frame of cellular uptake and processing of transgene to protein product. To achieve this we performed time course studies using mRNA and plasmid DNA. The nucleic acids were complexed with lipofectin that was pretreated with 30 mM potassium phosphate pH 5.8. Cells were treated with the transfection mixes for 1 h at which time the mix was replaced with prewarmed medium containing 10% FCS, unless the cells were lysed and assayed at an earlier time point. Cells were harvested at various time points after initiation of transfection. Results of the experiment are shown in Fig. 9. Much to our surprise at 15 min after exposing the cells to mRNA–lipid complex we could detect very significant amounts of luciferase activity in the cells. The luciferase expression elicited by the mRNA transfection was extremely rapid and the mRNA translation was almost at its full capacity in the first hour. Meanwhile, under the same experimental setting, luciferase expression from the plasmid DNA followed a kinetic similar to the one presented in Fig. 8(B). These experiments clearly demonstrate that the cellular uptake of the lipid–mRNA complex formed in the presence of phosphate buffer proceeds very quickly and that the majority of mRNA probably enters directly to the cytoplasm where translation occur almost instantly. This also suggests that the extended time needed for luciferase expression from the plasmid DNA vs. mRNA is probably required for nuclear transport of the DNA, transcription and cytoplasmic relocation of the in vivo made transcript.

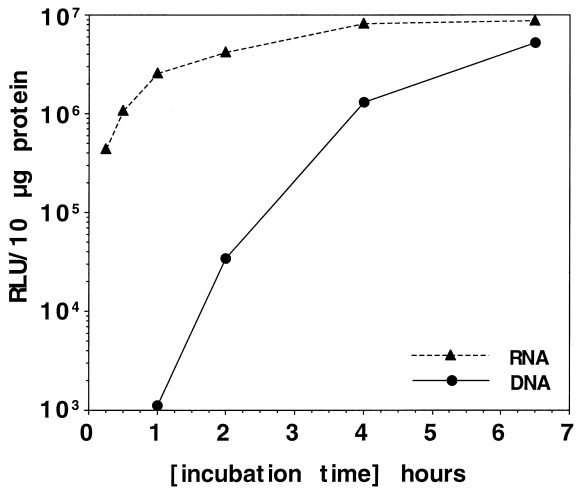

Fig. 9. Kinetics of phosphate-enhanced RNA and DNA transfection. Time course experiments. Transfection and luciferase assaying were performed as described in Fig. 1 and in Section 2. Complex was prepared by mixing 0.23 μg luciferase-encoding mRNA (mTEV-LUC-A50) (*filled triangles*) or plasmid DNA (pCMVintLucΔ5′) (*filled circles*) with 0.8 μg lipofectin that was preincubated with DMEM containing 100 ng/μl BSA and 30 mM potassium phosphate pH 5.8 prior to complexing. Cells were incubated for 1 h with the transfection mixes, then the transfection mix was replaced with culture medium and they were further incubated unless the cells were lysed and assayed at earlier time points. Cells were lysed at the indicated time following the initiation of transfection and assayed for expression of luciferase. Values were normalized for cytoplasmic protein content.

In some experiments the expression of luciferase was followed for an extended time period (not shown). Abundant luciferase expression was maintained for up to 8 h when mRNA was transfected to the cells. By 24 and 48 h, expression declined to about 4% and 1% of the maximal level, suggesting that both the luciferase mRNA and protein are labile in HOS cells. Following DNA transfection, we determined that by day 2 the expression had declined to 20% of the day 1 level. A further gradual decline was detected up to day 4, at which point 10% of day 1 level luciferase could be detected, suggesting that both transcription and translation have been sustained, though at a lower level, for 4 days.

4. Discussion

One of the goals of this study was to improve cationic lipid-mediated mRNA and plasmid DNA transfection by exploiting the stimulatory effect of phosphate anions on lipofection. The other goal was to use this improved transfection technique to gain insight into the mechanism of lipofection, and subsequently understand the processes by which phosphate exerts its effect.

We have demonstrated that the phosphate anion-evoked processes improved lipofection by expediting transgene expression and also by increasing the amount of synthesized transgene products. These effects of phosphate were specific in that other tested

compounds could not generate similar enhancement. It was also shown that the enhancement of gene expression depended upon the concentration and pH of the phosphate initiated effect.

A combination of electrostatic and hydrophobic interactions play crucial roles during complex formation and transfection when molecules such as the negatively charged DNA and cell membranes and the positively charged lipofectin are in contact within themselves and with each other. These interactions intrinsically are dependent on the ionic characteristics of the solution in which they are present. Therefore we can identify the following three important interactions in which the presence of phosphate anions in the solution can potentially exert their effect, leading to improved transfection. The first is the lipofectin–lipofectin interaction, the second is the lipofectin–nucleic acid interaction, and the third is the cell membrane interaction with the nucleic acid/lipofectin complex.

First consider the interaction between lipofectin–lipofectin in a lipofectin dispersion. It is possible that the effect of the negatively charged phosphate anion on the positively charged lipofectin is analogous to the long appreciated effect of divalent cations on the negatively charged phospholipid bilayers [28,29]. It has been demonstrated that divalent cations (i.e. Ca^{2+}, Ba^{2+}) but not monovalent cations (i.e. Na^{+}) induce membrane fusion when a critical amount of divalent cation is bound per phospholipid molecule [30,31], and indeed, Düzgünes et al. have shown that it is probable that divalent anions such as phosphate induce fusion of cationic lipids through the formation of a complex between positive charges present on the opposed lipid bilayers [15]. Phosphate anion was found to be fusogenic when it was present at 20 mM or higher concentration at pH 7.4. Monovalent anion such as acetate did not induce membrane fusion [15]. The conditions that we utilized for the lipofectin preincubation step was very similar to the above described condition in which phosphate anions induced fusion of cationic lipids. We achieved maximal transfection efficiency when the phosphate concentration was 30–80 mM during the lipofectin preincubation step. We determined a pH optimum of 5.6–6.8 under which the phosphate is present as divalent anion. We also identified that monovalent anions such as acetate and Cl^{-}, which have been shown to not induce membrane fusion [15], did not enhance the transfection efficiency (Fig. 2). These results suggest a potential correlation between cationic lipid fusion and increased transfection efficiency.

The second interaction that can potentially be influenced by the presence of phosphate anions occurs between the lipofectin and nucleic acid. Several models have been proposed to describe the morphologically distinct structures detected by analyzing complexes formed during nucleic acid and cationic liposome interactions. Depending primarily on the detection techniques employed, nucleic acid are described as being encapsulated within [18] or ensheated between lipid bilayers [19], or to be bound to the exterior surface of the lipid vesicles [20]. In addition, recent studies have identified the complexes as highly organized lamellar structures in which two-dimensionally condensed DNA molecules are sandwiched between cationic bilayers [23]. Despite the differences, all models fundamentally agrees, that the predominant interactions that lead to the formation and subsequently maintain the proposed structures are electrostatic in nature. It has been reported [23], that as a result of the electrostatic interactions the phosphate groups on the nucleic acids are likely to be neutralized by the cationic lipid, while the originally condensed counterions get released from both components. Thus, it is possible that phosphate-pretreatment of cationic lipid dispersion might result in formation of special lipid intermediates that are capable to promote nucleic acid condensation, and subsequently, to improve transfection efficiency. Such an explanation is also supported by a report concerning correlations between transfection activity and DNA condensation [21].

The third interaction which can potentially be influenced by phosphate anion is the cellular uptake of the nucleic acid/lipofectin complex. Goodarzi et. al. reported that in the presence of low pH phosphate buffer oligonucleotides bind to the cell membrane at much higher efficiency than at neutral pH [32]. The bound oligonucleotides could be competed off with plasmid DNA and tRNA or eluted from the membrane at pH 7.5. Under our experimental conditions it seemed that the phosphate was more effective when the pH was in the acidic range, therefore we tested whether increased nucleic acid binding to the cells under low pH phosphate condition could account for

the observed increased transfection efficiency. We tested whether washing the cells at pH 7.5 following transfection could diminish the phosphate-mediated increase of transgene expression. We found no difference in transfection efficiency when the cells were washed with high pH phosphate buffer following lipofection (not shown), suggesting that the phosphate-enhanced transgene expression is not mediated by increased nucleic acid binding to specific receptors such as described by Goodarzi [32].

Physico-chemical studies simulating the interactions between the complex and the cell membrane suggested the following potential entry mechanism. After contact, the complex initiates destabilization of the membrane by flip-flop of the anionic lipids from the membrane. The anionic lipids diffuse to the complex and displace the DNA from the complex permitting the DNA to enter the cytoplasm [17]. It has also been demonstrated that negatively charged water soluble molecules can also displace the DNA from the complex. Interaction with these water soluble molecules in the extracellular space could interfere with transfection resulting in premature release of the DNA to the medium rather than to the cytoplasm. Interestingly none of the tested phosphate containing molecules had such a DNA replacing activity [17].

It has long been appreciated in studies of lipofection that one of the many variables which affects the efficacy of gene expression is the medium used for lipid:DNA complex formation [13]. However, we are aware of only one study in which the effect of different counter anions of a cationic lipid on the transfection efficiency has been directly addressed. Performing a systematic study, Aberle et al. reported that bisulfate counter anion had the highest stimulatory effect on transfection [33]. The effects of phosphate and acetate anions were negligible. These results contradict our finding, but the sample preparation might explain the discrepancies. Aberle et al. added counterions to the lipids by ion exchange chromatography, rather than by preparing the lipid dispersion in a buffer containing excess of the anion, as we have done [33].

It has been reported [27], and we also found, that cationic lipid dispersions are more toxic without nucleic acid than with nucleic acid (Fig. 5(B) vs. (D)). In addition we have demonstrated that phosphate preconditioning can also inhibit lipofectin-induced cell toxicity (Fig. 5(B) vs. (C)). Other phosphate containing molecules such as tRNA were also reported to inhibit lipid-mediated cytotoxicity resulting in the use of tRNA routinely as a carrier during complex formation and transfection [5]. The phosphate-mediated reduction of lipofectin-evoked toxicity could be due to a shielding effect of phosphate anions on the excess charges of the lipofectin. A recent observation reported by Li and Hui [34] supports this explanation. While investigating the cationic liposome-induced cell toxicities, they found that phosphate buffered saline (PBS), which contains 5 mM polyvalent phosphate anions, reduced cationic lipid-induced cell–cell fusion and cell lysis [34].

Analyzing the kinetics of mRNA and DNA transfection, we determined that at higher nucleic acid concentration (0.1–0.3 μg nucleic acid/well) the transfection efficiency was not concentration dependent (Figs. 6 and 7). By diluting or repeatedly reusing the transfection mix we have demonstrated that the transfection mix contains a huge excess of transfectionally active complex. However the cellular uptake of those complexes are limited (Fig. 7). Using FACS analysis we have found that phosphate-enhanced transfection of the urokinase receptor-encoding mRNA resulted in transgene expression in 89% of the targeted cells (K. Karikó, A. Kuo, E.S. Barnathan, unpublished data). These data suggest that following phosphate-enhanced transfection most of the cells are expressing the transgene and higher levels of expression of transgene by individual cells is limited. The limitation is probably at the cellular uptake level. It has long been recognized that relative efficiency of naked polynucleotide uptake by the cells dramatically diminishes at high polynucleotide concentration [35]. However, using lipofectin-mediated gene delivery there was a linear relationship between the quantity of transfected mRNA or plasmid DNA and the quantity of synthesized proteins encoded by the nucleic acids [5,10]. We are not aware, however, of any studies where transfectability of already used transfection mix was tested. To understand why the cells cannot take up more complex needs further investigation.

Analysis of the time courses of DNA and RNA-mediated transgene expression demonstrated that phosphate preconditioning significantly accelerated both cellular uptake and transgene expression (Figs. 8

and 9) compared to those reported by conventional lipofection [4,5,12,13]. This acceleration was not limited to HOS cells, because almost identical time courses could be obtained using NIH 3T3 and K562 cell lines (not shown). In addition, our data also demonstrate a synergistic effect between lysosomotropic chloroquine and phosphate, suggesting that the classic endosomal escape is an unlikely mechanism for the phosphate-mediated effects (Fig. 1). Therefore it is possible that nucleic acids, in the presence of phosphate anion, are more efficiently encapsulated to a condensed structure, more easily cross the membranes of the cells and the endosomes, and become readily translatable resulting in superior non-viral gene transfection efficiency.

In summary, we have described here the superior characteristics of a non-viral, lipofectin-mediated mRNA- and plasmid DNA-based transfection. The highly transfective material was made by complexing nucleic acid with lipofectin that was pretreated with phosphate buffer. Although further study will be required to define the exact mechanisms, our findings support a hypothesis that could explain the phosphate-mediated enhancement. Divalent phosphate anions might induce fusion of the cationic lipid vesicles aggregated along the nucleic acid chains. Such a fusion could result in a more efficient nucleic acid encapsulation and subsequent formation of highly transfective particles.

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