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Enhancement of Transfection Efficiency by Protamine in DDAB Lipid Vesicle-Mediated Gene Transfer[1]

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We have previously developed a simple gene transfection procedure mediated by cationic lipid vesicles for animal cells, in which a commercially available cationic surfactant, dimethyldioctadecyl ammonium bromide (DDAB), was used for making lipid vesicles. In the present study, we examined enhancement of transfection efficiency for this method by adding protamine to plasmid DNA solution before the formation of DNA/lipid vesicle complexes. Both free-base protamine and protamine sulfate provided enhanced transfection efficiency and expression level, but the optimal amount of the two protamines was different. The enhancement in transfection efficiency and expression level by protamines was observed in all the cell lines (COS-7, Hela, NIH3T3, MDCK, and BHK-21C13) and all the plasmids (pCMVβ, pmiwZ, and pCH110) tested. The enhancement in both transfection efficiency and expression level was at most 20-fold compared with that using only DDAB lipid vesicles. Protamines seemed to protect DNA from degradation by DNase and promote DNA delivery into a nucleus.

Key words: cationic lipid vesicles, DDAB, gene transfection, protamine.

Gene transfection into eukaryotic cells has become an increasingly important technique for analysis of gene function, production of recombinant gene products, and gene therapy. The strategy for introducing genes includes viral and nonviral methods that possess unique characteristics. Viral-mediated methods can offer high efficiency and stable integration of the exogenous gene into host chromosomes at the risk of proto-oncogene activation. Nonviral methods such as calcium phosphate precipitation (*1*), DEAE-dextran (*2*), liposomes (*3-5*), reconstituted viral envelopes (*6-8*), electroporation (*9-11*), and particle gun (*12*) have relatively low efficiency and are not well suited for *in vivo* use. None of these methods works efficiently for all cell lines, and the transfection efficiency may be irreproducible and variable with different cell types.

As an alternative nonviral DNA delivery carrier for gene transfection, cationic liposomes have recently been frequently used (*13-18*). Cationic liposomes are generally prepared by evaporation of a lipid solution in chloroform, followed by dispersion in an aqueous solution and sonication with either a high energy probe immersed in the lipid dispersion or with a bath sonicator. The mechanism of effective DNA delivery by cationic liposomes into cells includes at least four fundamental steps: contact of DNA/cationic lipid vesicle complexes with cells, penetration of the complexes through the plasma membrane, intracellular processing of the complexes inside the cytoplasm, for example, by endosome degradation, and delivery of DNA into the nucleus. Cationic liposomes may deliver DNA either directly across the plasma membrane (*13*) or through the endosome compartment (*19, 20*). The latter pathway is dominant for liposome uptake, and much of the DNA accumulates in the endosome compartment. Several approaches have been investigated to prevent loss of the exogenous DNA in the endosomal compartment by protecting it from hydrolytic digestion. They include the use of weak amines such as chloroquine, which presumably prevent DNA degradation by inhibiting endosomal acidification (*21*). Viral coat proteins or whole viruses have been used to disrupt endosomes or promote fusion of liposomes with endosomes and facilitate release of DNA into the cytoplasm (*22, 23*). However, the efficient gene delivery from the cytoplasm into the nucleus may be the most critical step. For this process, the virus-mediated transfer is much more efficient than cationic liposome procedures (*24, 25*). Recently, the use of nuclear protein such as high mobility group-1, -2 mixtures to carry the plasmid DNA into the cell nucleus efficiently was reported to enhance the transfection efficiency (*26, 27*).

We have previously reported a surfactant-mediated gene transfection method (*28*), in which effective lipid vesicles were simply prepared by dissolving a cationic surfactant, dimethyldioctadecyl ammonium bromide (DDAB), and a nonionic surfactant, Tween 80, in double-distilled water at 60°C without the use of special devices such as an evapora-

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Abbreviations: BSA, bovine serum albumin; CMV, cytomegalovirus; DDAB, dimethyldioctadecyl ammonium bromide; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; MEM, minimum essential medium; NEAA, non-essential amino acids; ONPG, *o*-nitrophenyl β-D-galactopyranoside; PBS, phosphate-buffered saline.

tor and a sonicator. Although some commercially available transfection reagents exhibit cytotoxic effects at the concentrations used for gene transfection, the lipid vesicles used in this method have relatively low cytotoxicity for animal cells (*28*), and high viability and good transfection efficiency were obtained for gene transfection to quail embryos (*29*). We also modified the lipid vesicles with some ligands to enhance the transfection efficiency and/or cell-type specificity (*30*).

With this simple method, cell lines such as CHO and BHK cells showed high transfection efficiency of more than 80% under the optimal conditions (*28*). However, the efficiency still depended on the cell line, as observed with the other procedures. In the present study, we examined the addition of free-base protamine or protamine sulfate to plasmid DNA solution before the formation of DNA/lipid vesicle complexes to enhance the transfection efficiency.

MATERIALS AND METHODS

Cell Lines, Media, and Plasmid DNAs—Baby hamster kidney, BHK-21C13, African green monkey kidney, COS-7, human epithelial-like aneuploid, Hela, NIH Swiss mouse embryo, NIH3T3, and canine kidney, MDCK, cell lines were obtained from RIKEN cell bank and used as host cells for gene transfer. COS-7, Hela, NIH3T3, and MDCK cells grew in an anchorage-dependent manner, and BHK-21C13 cells subcloned from anchorage-dependent BHK-21 cells grew in suspension.

COS-7, Hela, and MDCK cells were cultured in minimum essential medium (MEM; Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; JRH Sciences, Lenexa, KS, USA). BHK-21C13 cells were cultured in MEM medium supplemented with 10% FBS and 1% non-essential amino acids (NEAA). NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL) supplemented with 10% FBS. The cells were cultured at 37°C in a 5% CO_2 incubator. Media for all cultures routinely included 100 units/ml penicillin and 100 μg/ml streptomycin.

Recombinant plasmids pCMVβ (*31*), which encodes the *lacZ* gene under the control of cytomegalovirus (CMV) early promoter, pCH110 (*32*), which encodes the *lacZ* gene under the control of SV40 early promoter, and pmiwZ (*33*), which encodes the *lacZ* gene under the control of β-actin promoter, were used for transient expression. The recombinant plasmid pCMVgfp (pGREEN LANTERN-1; Gibco BRL) (*34*), which includes the green fluorescent protein (GFP) gene under the control of cytomegalovirus early promoter as a reporter, or pMC1neo (Stratagene, La Jolla, CA, USA), which encodes the G418 resistance gene, was used for evaluating stable expression.

Preparation of Cationic Lipid Vesicles—The preparation procedure of cationic lipid vesicles was reported previously (*28*). Briefly, a commercially available cationic surfactant, dimethyldioctadecyl ammonium bromide (DDAB; Sigma Chemical, St. Louis, MO, USA), and a nonionic surfactant, Tween 80 (Wako Pure Chemical Industries, Tokyo), were dissolved in double-distilled water in a 1:1 weight ratio by vortexing at 60°C. This lipid vesicle solution (1 mg/ml DDAB) was autoclaved at 120°C for 20 min, then preserved in a refrigerator. The solution was prewarmed and homogenized before use.

Transient Transfection Assay—Tissue cultures in plastic dishes (35 mm in diameter; Iwaki Glass Works, Tokyo) with 50 to 70% confluent cells were rinsed twice with serum-free opti-MEM medium (Gibco BRL) to remove serum from the culture. In the case of BHK-21C13 cells, the cells were collected and washed by centrifugation. The opti-MEM medium was removed just before the addition of the solution containing lipid vesicle/DNA complex. Typically, 3 μg of DNA and 36 μg of lipid vesicles containing DDAB and Tween 80 in a weight ratio of 1:1 were used for transfection. Each was diluted in 0.5 ml of opti-MEM medium. Free-base protamine (derived from salmon) and protamine sulfate (derived from salmon) were purchased from Sigma Chemical and Wako Pure Chemical Industries, respectively. A predetermined amount of free-base protamine or protamine sulfate dissolved in phosphate-buffered saline (PBS; 8 g NaCl, 0.2 g KCl, 2.9 g $Na_2HPO_4 \cdot 12H_2O$, 0.2 g KH_2PO_4 per liter, pH 7.4) was added to the plasmid DNA solution, and the resulting solution was gently mixed with the lipid vesicle solution. The mixture was allowed to stand at room temperature for about 30 min to form the complex. The solution containing the protamine/DNA/lipid vesicle complexes was added to the rinsed cells in culture dishes. After 6 h of incubation, the supernatant was carefully removed and growth medium was added. Transient expression of β-galactosidase was assayed 24 h post-transfection.

In situ staining was performed to identify β-galactosidase-expressing cells. Cells were rinsed twice with PBS, fixed with 1 ml of 2% (v/v) formaldehyde and 0.2% glutaraldehyde in PBS for 5 min at room temperature, rinsed twice with PBS, then stained for 2 h to overnight with 1 ml of X-gal solution: 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, and 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Wako Pure Chemical Industries) in PBS. Stained cells were counted under a microscope and the percentage of stained cells (transfection efficiency) was determined as the average count in five different fields.

For quantification of β-galactosidase activity, cells were rinsed twice with ice-cold PBS and harvested in 1 ml of ice-cold PBS. The samples were centrifuged at 15,000 rpm for 20 s. After removing the supernatant, 0.8 ml of reaction buffer (10 mM KCl, 1 mM $MgCl_2$, 0.1% Triton X-100, 5 mM 2-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, 0.1 M sodium phosphate buffer, pH 7.5) was added, and cell lysates were obtained by vortexing. After centrifugation, 0.6 ml of the supernatant was transferred to a new tube and incubated at 37°C for 10 min, then 0.1 ml of prewarmed 4 mg/ml *o*-nitrophenyl β-D-galactopyranoside (ONPG) in 0.1 M sodium phosphate buffer (pH 7.5) was added. After a suitable time of reaction, 0.3 ml of 1 M Na_2CO_3 was added, and the absorbance at 420 nm of the solution was measured with a spectrophotometer. The β-galactosidase activity was expressed as ONPG unit (1 unit: the activity producing 1 μmol *o*-nitrophenol per minute, normalized against the inoculated cell number). The β-galactosidase activity was determined as the average value from triplicate dishes under the same transfection conditions.

Stable Transfection Assay—Complexes of protamine/DNA/lipid vesicle were prepared as mentioned above. COS-7 cells were plated at the cell density of 1.5×10^5 cells

per 35-mm dish on the day before transfection, then transfected by incubation for 6 h with protamine/DNA/lipid vesicle solution containing 3 μg pCMVgfp or pMC1neo vector, 36 μg of lipid vesicles, and 5 μg protamine sulfate in 1 ml of opti-MEM serum free medium. The medium was then replaced with MEM medium supplemented with 10% FBS. Two days after the transfection, the cells were diluted and seeded at 10 cells/well in a 96-well tissue-culture plate for pCMVgfp or at 1,000 cells/well in a 24-well tissue-culture plate for pMC1neo. In the case of pMC1neo vector, G418 (Sigma Chemical) was added to the medium at the concentration of 800 μg/ml. After 2-3 weeks, cell colonies were fixed and counted under a microscope for pMC1 or under a fluorescence microscope using FITC filters for pCMVgfp.

Protection Assay for DNA Degradation—For the analysis of plasmid degradation, 3 μg of plasmid DNA (pCMVβ), in the presence or absence of 5 μg of protamine sulfate, was incubated at 25°C for various times in 30 μl of degradation buffer (5 mM $MgCl_2$, 100 mM sodium acetate, pH 5.0 at 25°C) after adding 0.5 μl of 10 μg/μl DNase I (Sigma Chemical) in storage buffer (5 mM $CaCl_2$, 0.1 mM phenylmethylsulfonyl fluoride, 50% glycerol, 20 mM sodium acetate, pH 6.5). After the incubation, an equal volume of 5 M NaCl was added to dissociate protamine from the complex, and 15-μl samples were mixed with 3 μl of sample loading buffer (40% sucrose, 0.25% bromophenol blue, 200 mM Tris-acetate buffer containing 5 mM EDTA, pH 7.8) and analyzed by electrophoresis on 1% agarose gel in 40 mM Tris-acetate buffer containing 1 mM EDTA.

FITC Labeling of Protamine—Fluorescein isothiocyanate (FITC; Sigma Chemical) was dissolved in dimethyl sulfoxide (best grade available, with no water) at 1 mg/ml, and 50 μl of this solution was added slowly in 5-μl aliquots to 1 ml of protamine or bovine serum albumin (BSA; Wako Pure Chemical Industries) solution (20 mg/ml in 0.1 M sodium carbonate, pH 9.0) with gentle, continuous stirring. After leaving the solution for 8 h in dark at 4°C, NH_4Cl was added to 50 mM, incubation was continued for 2 h at 4°C, then 0.1% xylene cylanol and 0.5% glycerol were added. The mixture was dialyzed against PBS at 4°C to remove the unbound dye from the conjugate.

RESULTS AND DISCUSSION

Enhancement of Transfection Efficiency in DDAB Lipid Vesicle-Mediated Gene Transfer by Protamine—Protamines, which are found in the sperm of most vertebrates, are small peptides (MW 4,000-5,000) with high arginine content and strongly basic (*35, 36*). The major role of protamine in sperm is thought to be to form a complex with DNA, which may have a compact structure, and then deliver DNA into the nucleus of the egg after fertilization. Therefore, protamine may also promote the efficient delivery of DNA from the cytoplasm into the nucleus in gene delivery by nonviral DNA carriers. Thus, we used protamine to enhance the transfection efficiency of a surfactant-mediated gene transfer method that we developed (*28*).

The effects of protamine amount added to DNA solution on transfection efficiency and expression level of exogenous gene were examined. Increasing amounts of free-base protamine or protamine sulfate were added to 3 μg of plasmid pCMVβ DNA. After mixing with 36 μg of cationic lipid vesicles, the transient transfection efficiency was measured with COS-7, Hela, and NIH3T3 cell lines. As shown in Figs. 1 and 2, the addition of either free-base protamine or protamine sulfate enhanced both the transfection efficiency and the expression level in all the cell lines tested. The percentage of β-galactosidase-expressing cells (transfection efficiency) increased 2-5-fold and the β-galactosidase expression level increased 5-20-fold over those of cells transfected with the same amount of DNA and DDAB lipid vesicles without protamines.

Transfection efficiency was influenced by the amount of free-base protamine or protamine sulfate added. The maximal transfection efficiency (over 50-60%) was obtained with 5 μg of protamine sulfate or 20 μg of free-base protamine in all the cell lines tested (Fig. 1). This difference in the optimal amounts of free-base protamine and protamine sulfate might be due to a difference in molecular weight and/or structure between free-base protamine and protamine sulfate that results in a difference in DNA-binding affinity or DNA packing as a biologically active complex. Despite this difference, the maximal transfection

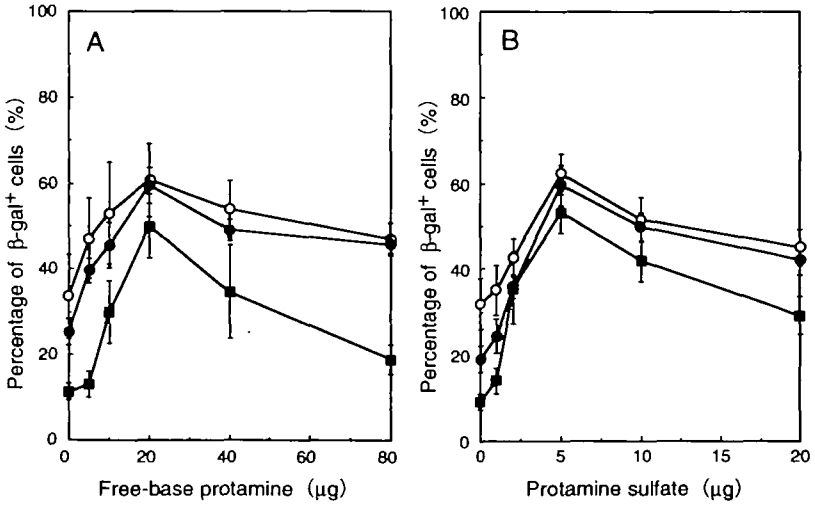

Fig. 1. **Effect of free-base protamine (A) and protamine sulfate (B) on transfection efficiency in DDAB lipid vesicle-mediated gene transfer.** A day before transfection, 1.5×10^5 COS-7 (○), Hela (●), and NIH3T3 (■) cells were plated in 35-mm dishes. For transfection, 3 μg of pCMVβ and 36 μg of lipid vesicles containing DDAB and Tween 80 in a weight ratio of 1:1 were used. *In situ* staining was performed 24 h post-transfection to identify β-galactosidase expressing cells.

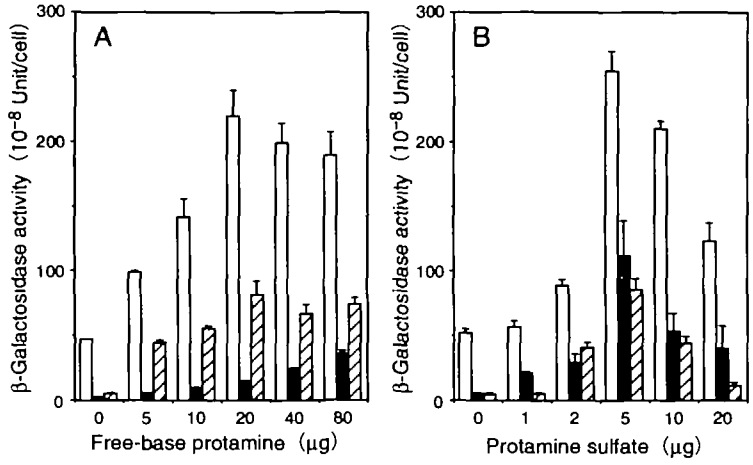

Fig. 2. **Effect of free-base protamine (A) and protamine sulfate (B) on expression level in DDAB lipid vesicle-mediated gene transfer.** A day before transfection, 1.5×10^5 COS-7 (□), Hela (▨), and NIH3T3 (▨) cells were plated in 35-mm dishes. Cells and the conditions for transfection were the same as in Fig. 1. β-Galactosidase activity was determined 24 h post-transfection.

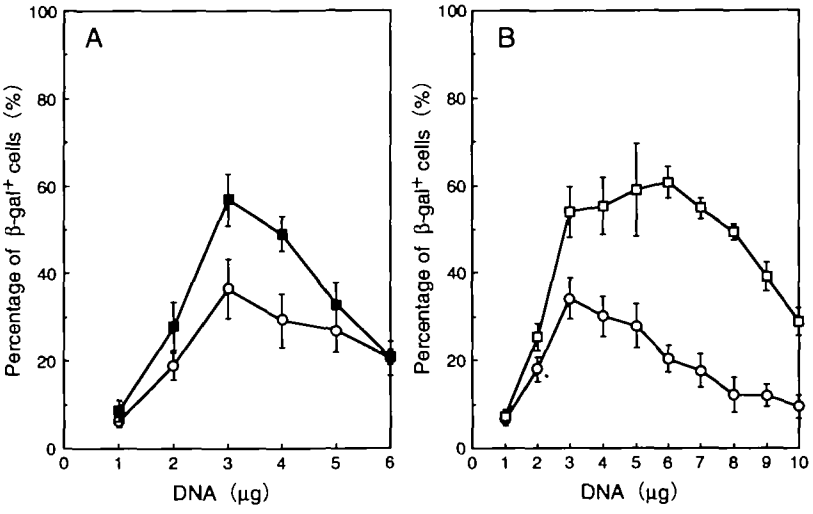

Fig. 3. **Effect of DNA amounts on transfection efficiency in the presence and absence of protamines.** A day before transfection, 1.5×10^5 COS-7 cells were plated in a 35-mm dish. For transfection, a variable amount of pCMVβ and 36 μg of lipid vesicles were used. *In situ* staining was the same as in Fig. 1. (○) DDAB lipid vesicles alone or with (■) 20 μg free-base protamine (20 μg/ml-medium), (□) 5 μg protamine sulfate (5 μg/ml-medium). A, free-base protamine; B, protamine sulfate.

efficiencies obtained with free-base protamine and protamine sulfate were similar. The maximal β-galactosidase expression level and the optimal transfection efficiency were obtained with the same amount of protamine sulfate (5 μg) in all the cell lines tested, but the maximal β-galactosidase expression level depended on the cell line in the case of free-base protamine (Fig. 2).

Optimization of Transfection Efficiency—As reported earlier (*13, 14, 28*), gene transfection using cationic liposomes as DNA carrier was influenced by many conditions, such as concentrations of lipid vesicles and DNA, medium, cell density and cell type. For instance, a serum-free medium (opti-MEM) should be used for incubation of the cells with protamine/DNA/DDAB lipid vesicle complexes to obtain high transfection efficiency. As well as medium, cell density was also important. For optimal transfection efficiency in this transfection procedure, 3×10^5 MDCK or 2×10^5 BHK-21C13 cells per 35-mm dish were plated 2 h before transfection, and 1.5×10^5 COS-7, Hela, or NIH3T3 cells per 35-mm dish were plated a day before transfection.

As shown in Figs. 1 and 2, transfection efficiency was highly influenced by the amounts of free-base protamine or protamine sulfate added. To investigate the effects of other conditions on transfection efficiency, the amounts of DNA and lipid vesicles added to the medium were examined

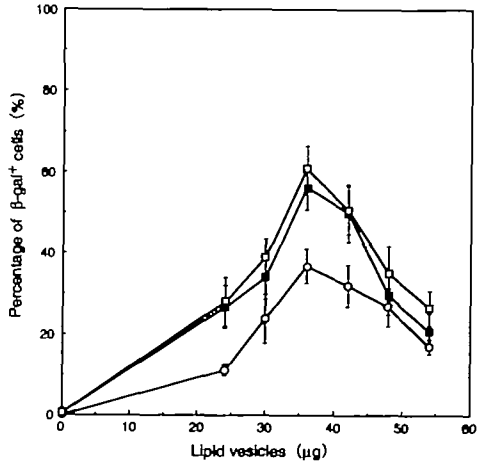

Fig. 4. **Effect of lipid vesicle on transfection efficiency in the presence and absence of protamines.** A day before transfection, 1.5×10^5 COS-7 cells were plated in a 35-mm dish. For transfection, 3 μg of pCMVβ and a variable amount of lipid vesicles were used. *In situ* staining was performed as shown in Fig. 3. (○) DDAB lipid vesicles alone, (■) 20 μg free-base protamine (20 μg/ml-medium), (□) 5 μg protamine sulfate (5 μg/ml-medium).

using COS-7 cells as host cells and pCMVβ as an exogenous DNA (Figs. 3 and 4). In these experiments, 5 μg of protamine sulfate or 20 μg of free-base protamine, the amount that gave the optimal efficiency (Figs. 1 and 2), was used. As shown in Fig. 3, the transfection efficiency strongly depended on the amount of DNA with free-base protamine, and excess DNA lowered the transfection efficiency. With protamine sulfate, on the other hand, excess DNA within a limited range did not decrease transfection efficiency. The maximal transfection efficiencies were obtained with 3 μg (3 μg/ml-medium) and 6 μg (6 μg/ml-medium) of DNA for free-base protamine and protamine sulfate, respectively. In the case of protamine sulfate, enhanced transfection efficiencies were observed in the range of 3–8 μg DNA. Thus, to obtain the maximal transfection efficiency, choice of appropriate amount of DNA is necessary.

The amount of DDAB lipid vesicles also influenced the transfection efficiency in both the presence and absence of protamines. In our previous results, the optimal amount of lipid vesicles depended on the amount of DNA (*28*). As shown in Fig. 4, the optimal amount of lipid vesicles was 36 μg (36 μg/ml-medium) with 3 μg of pCMVβ, regardless of protamine addition. This showed that the ratio of DNA and lipid vesicles was also important in the presence of protamines, as well as in transfection without protamines (*28*). It should be noted that both free-base protamine and protamine sulfate caused near background level of transfection efficiency in the absence of DDAB lipid vesicles (0 μg lipid vesicle amount in Fig. 4). This indicated that the DNA incorporation into the cells was mediated by DDAB lipid vesicles. Protamine may serve in a subsequent intracellular step, such as nuclear transfer or DNA stabilization from DNase digestion.

Generalities of the Protamine Effects—To clarify the generality of the enhanced transfection efficiency of protamine, the other cell lines, MDCK and BHK-21C13, in addition to COS-7, Hela, and NIH3T3, were transfected with the same plasmid pCMVβ. COS-7 cells were also transfected with different plasmids such as pCMVβ, pmiwZ, and pCH110, which contain different promoters. In these experiments, only protamine sulfate was checked, due to its good potency and availability. The results are summarized in Tables I and II. Although enhancement factors of transfection efficiency varied with the cell line or plasmid, enhanced transfection efficiency by protamine was observed in all cell lines and plasmids tested. In particular, both transfection efficiency and expression level were enhanced 23-fold with MDCK cells. The cell lines with relatively low transfection efficiency without protamine (Hela, NIH3T3, and MDCK cells) tended to show a great increase in expression level by protamine addition. In the case of BHK-21C13 cells, high transfection efficiency of more than 90% was observed even without protamine. The high transfection efficiency was not significantly changed by protamine addition, but the expression level was enhanced 1.5-fold. The enhancement factors of expression level were greater than those of transfection efficiency in almost all the cell lines and plasmids tested. The transfection efficiency and expression level provided by this method using protamine were comparable to those of the commercial transfection reagents (data not shown).

Figure 5 shows photographs of β-galactosidase-expressing cells stained with X-gal. High transfection efficiency and expression level by using protamine/DNA/DDAB lipid vesicle complexes could be clearly observed from the number and color density of the stained cells.

Stable transfection efficiency was measured by using COS-7 cells as hosts and pCMVgfp and pMC1neo as plasmids. Cell colonies were counted 2–3 weeks after transfection under the same conditions as in transient transfection. Green fluorescent colonies were counted under a fluorescence microscope for pCMVgfp and total colonies were counted after culturing with G418 for pMC1neo. Stably transfected cells accounted for 7.6% of the total in the case of pCMVgfp and 2.1% in the case of pMC1neo in the presence of protamine, and 5.7 and 1.8% respectively in the absence of protamine. Thus, high

TABLE I. **Effect of protamine sulfate on transfection efficiency and expression level under optimal conditions in DDAB lipid vesicle-mediated gene transfer using COS-7, Hela, NIH3T3, MDCK, and BHK-21C13 cells as host cells.**

Cell Line	Percentage of β-gal$^+$ cells (%)		Enhancement factor	β-Galactosidase activity (10^{-6} Unit/cell)		Enhancement factor
	Protamine			Protamine		
	(−)	(+)		(−)	(+)	
COS-7	35.0±8.4	64.3±7.2	1.8	52.4±2.9	255±16	4.9
Hela	19.2±3.1	59.6±4.7	3.1	5.6±0.8	112±27	20
NIH3T3	9.2±1.9	53.4±5.0	5.8	4.8±1.0	85.8±8.7	18
MDCK	1.0±0.4	22.6±7.1	23	1.8±0.3	42.7±0.6	23
BHK-21C13	93.7±2.3	95.1±3.2	1.0	74.2±8.0	114±42	1.5

The data are shown as means±SD for triplicate experiments.

TABLE II. **Effect of protamine sulfate on transfection efficiency and expression level under optimal conditions in DDAB lipid vesicle-mediated gene transfer using pCMVβ, pmiwZ, and pCH110 as plasmids, and COS-7 cells as host cells.**

Plasmid	Percentage of β-gal$^+$ cells (%)		Enhancement factor	β-Galactosidase activity (10^{-6} Unit/cell)		Enhancement factor
	Protamine			Protamine		
	(−)	(+)		(−)	(+)	
pCMVβ	35.0±8.4	64.3±7.2	1.8	52.4±2.9	255±16	4.9
pmiwZ	12.9±4.0	47.8±2.9	3.7	4.2±0.2	88.9±3.9	21
pCH110	35.2±6.1	79.6±4.4	2.3	18.9±0.7	131±15	7

The data are shown as means±SD for triplicate experiments.

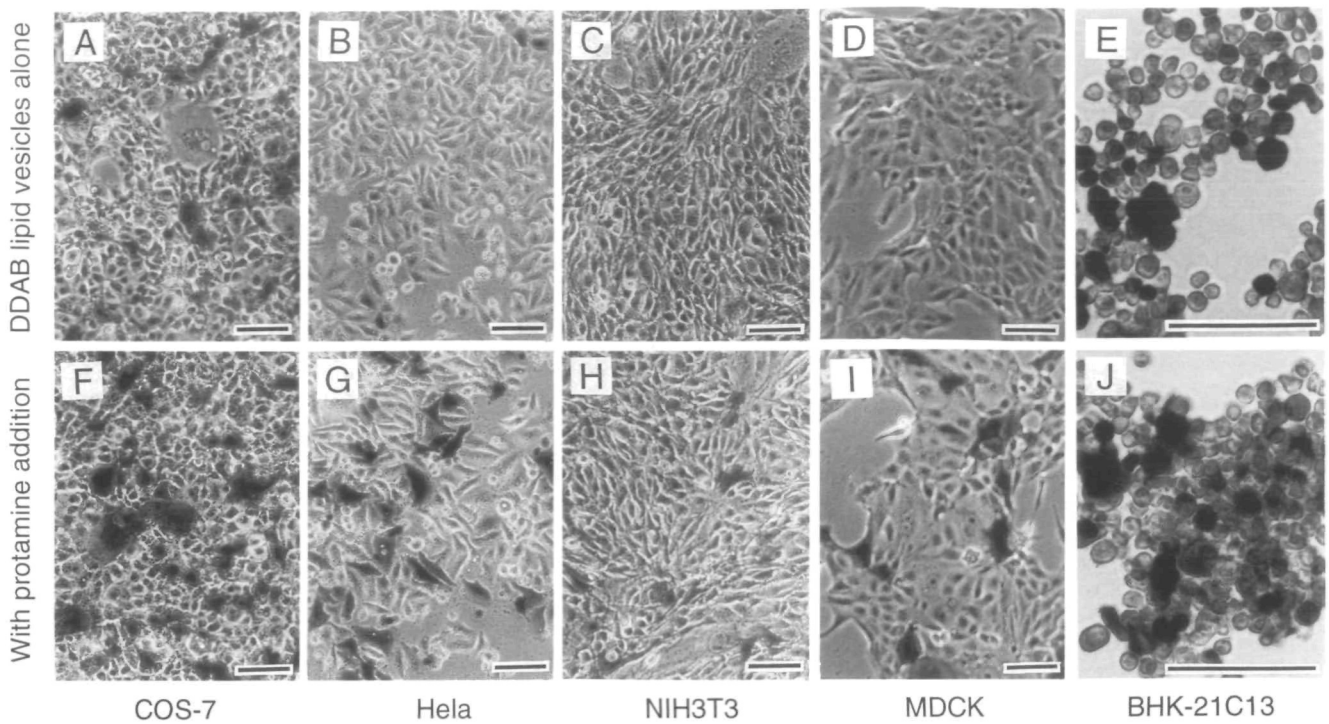

Fig. 5. **Photographs of β-galactosidase expressing cells after transfection.** A day before transfection, 1.5×10^5 COS-7 (A, F), Hela (B, G), and NIH3T3 (C, H) cells were plated in a 35-mm dish, and 3×10^5 of MDCK (D, I) and 2×10^6 of BHK-21C13 (E, J) cells were plated 2 h before transfection in a 35-mm dish. For transfection, 3 μg of pCMVβ and 36 μg of lipid vesicles in the absence (A-E) or presence (F-J) of 5 μg of protamine sulfate were used. Cells were stained with X-gal. Scale bar = 100 μm.

transfection efficiencies in long-term expression were obtained by this DDAB lipid vesicle-mediated procedure, as described previously (*28*). However, protamine did not show a marked enhancing effect in stable expression.

Mechanism of Protamine-Mediated Enhancement—Several possible mechanisms can be advanced to explain the enhancement by protamines of DDAB lipid vesicle-mediated gene transfection. Cationic polymers such as poly-L-lysine, histone, and protamine are known to condense DNA from an extended conformation to highly compact structures of about 30-100 nm in diameter (*37*). Recently, Sorgi *et al.* reported that protamines enhanced expression level in commercially available lipofection reagents-mediated gene transfection (*38*). They concluded that the electrostatic interactions between protamine and DNA may result in a charge neutralization of the complex and the formation of a condensed structure. This condensed structure, due to its diminished size, may be more favorable to enter the cells *via* an endocytosis pathway (*39, 40*). We also found that transfection efficiency in the DDAB lipid vesicle-mediated gene transfer was enhanced by the addition of a nonionic surfactant that can reduce the average size of the lipid vesicles (*28*). Thus, total size of the DNA complex seems to be a very important factor to obtain maximal transfection efficiency.

Previous investigators have demonstrated that naked DNA exposed to cell lysates and serum nucleases is readily degraded (*41, 42*). Therefore, assays were performed to examine whether protamine in protamine/DNA complexes protected DNA from DNase digestion (Fig. 6). Free DNA and protamine/DNA complex containing the same amount of DNA were incubated with DNase I for various times. As

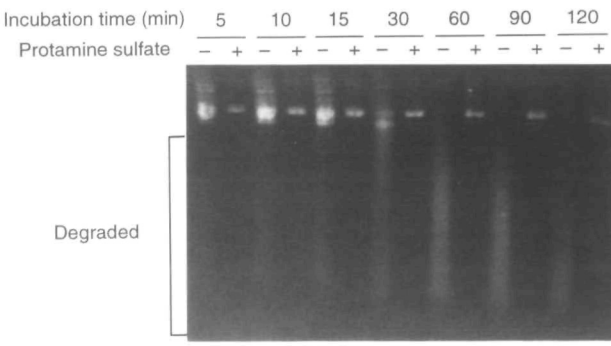

Fig. 6. **DNA degradation protection assay.** For analysis of plasmid degradation, 3 μg of pCMVβ DNA, either free or complexed to 5 μg of protamine sulfate, was incubated at 25°C with 5 μg of DNase I in 30 μl of reaction mixture for various times and then analyzed by electrophoresis on 1% agarose gel, in the presence (+) or absence (−) of protamine sulfate.

shown in Fig. 6, free DNA was rapidly degraded. On the other hand, DNA complexed with protamine sulfate was obviously resistant to the nuclease. The protection of DNA by protamine may increase the chance of delivery of undamaged, functional DNA into the cells and finally into the nucleus. Moreover, the availability of DNA in cytoplasm and endosomes might be enhanced, which should result in delivery of more DNA into the nucleus and an increase in its expression level. This may explain why the enhancement factors of β-galactosidase expression were greater than those of transfection efficiency (Figs. 1-3, Tables I and II).

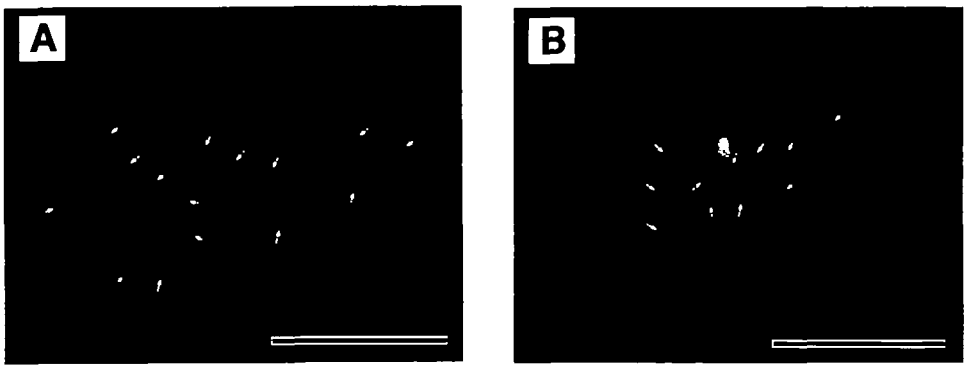

Fig. 7. **Nuclear localization of protamine in COS-7 cells.** A day before micro-injection, 1.5×10^5 COS-7 cells were plated in a 35-mm dish, and 0.1-0.5 μl of 1 μg/μl FITC-protamine (A) or FITC-BSA (B) was injected into the cytosol of the cells under a microscope using an autoinjector with a micromanipulator. The arrows indicate nuclei of the cells. Scale bar = 100 μm.

For the expression of an exogenous gene in host cells, a DNA molecule must be delivered into the nucleus and released from the carrier. Earlier study indicated that nuclear localization is a major rate-limiting step in microinjection of naked DNA into a cytoplasm (*43*). A plasmid may be relocated into the nucleus passively during the process of cell division, or alternatively DNA may be bound with newly synthesized peptides containing a host nuclear localization signal, which facilitates the entry of DNA into the nucleus through an active process. Since protamine contains a nuclear localization signal (*36, 44, 45*), it is possible that the active nuclear uptake of the protamine/DNA complexes increased. This effect was confirmed by using FITC-labeled protamine (Fig. 7). FITC-labeled protamine injected into the cytoplasm of cells was effectively transferred into the nucleus (Fig. 7A), whereas FITC-labeled BSA remained in the cytoplasm (Fig. 7B).

In addition to reduction of DNA complex size as reported by earlier, protamine could stabilize DNA against degradation and enhance nuclear localization. As shown in the present paper, these features of protamine may be general to cationic lipid vesicle-mediated gene transfection, and similarly enhanced transfection efficiency can be expected for the other transfection procedures.

In conclusion, the transfection efficiency of DDAB lipid vesicle-mediated gene transfection method was improved by addition of protamine. The transfection method developed in this study has the advantages of ease of preparation of effective lipid vesicles, low cytotoxicity, and economy in comparison with commercial transfection reagents. It can be used widely for gene transfection into animal cells both for laboratory and large-scale works.

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