J. Biochem. 129, 125–132 (2001)

Protamine-Modified DDAB Lipid Vesicles Promote Gene Transfer in the Presence of Serum[1]

Shinji Mizuarai, Ken-ichiro Ono, Jun You, Masamichi Kamihira,[2] and Shinji Iijima

Department of Biotechnology, Graduate School of Engineering, Nagoya University, Chikusa-ku, Nagoya 464-8603

Received August 2, 2000; accepted November 2, 2000

Cationic lipid vesicle–mediated gene transfer has become common for *in vitro* gene delivery. However, the transfection efficiency is often impaired by serum. DDAB (dimethyldioctadecyl ammonium bromide) lipid vesicle-mediated gene transfer, which we previously reported, has the same problem. To overcome this obstacle, we here report a novel transfection vehicle using protamine-modified DDAB lipid vesicles. While free protamine was simply added to the DNA/lipid complex in the previous study, in the present method the protamine is chemically conjugated to stearic acid and incorporated into DDAB lipid vesicles. Gene transfer was not significantly inhibited in 10% serum-containing medium by this method for the transfection of cultured cells. Protamine-modified DDAB lipid vesicles also enhanced virus transduction efficiency in the presence of serum using a replication-defective retroviral vector. Furthermore, the vesicles allowed efficient gene transfer for avian embryos *in vivo*. These results indicate that the method is useful for the production of transgenic animals and gene therapy.

Key words: cationic lipid, DDAB lipid vesicle, gene transfer, protamine, retrovirus.

Since the first development in the late 1980s, cationic lipids have been widely used as transfection reagents because of their several advantages (*1–5*). For example, it is safer and simpler than virus-mediated transfection (*6–8*), less toxic and with a higher transfection efficiency than other non-viral techniques such as electroporation (*9*) or calcium phosphate precipitation (*10*). However, serum-containing medium significantly impairs the transfection efficiency in cationic lipid–mediated transfection (*11–14*). This makes it difficult to apply the method to *in vivo* gene transfer. To overcome this problem, some lipids or non-lipid chemical substances have been developed (*15–21*). A cationic lipid reagent composed of 1,2-dioleoyl-*sn*-glycero-3-trimethylammonium-propane (DOTAP) mixed with cholesterol allows a high level of *in vitro* transfection efficiency in the presence of serum (*15*). The addition of polybrene to the medium also abrogates the decrease in transfection efficiency caused by serum using lipofection (*16*). A polyamine polymer with a glucose backbone, as an example of a non-lipid reagent, was found to give a higher level of reporter gene expression *in vitro* and *in vivo* than a 2,3-dioleyloxy-*N*-[2(sperminecarboxamido)ethyl]-*N,N*-dimethyl-1-propanaminium trifluoroacetate (DOSPA)–based cationic lipid (*17*).

There are many obstructive critical steps in exogenous gene expression: entry into cytoplasm, degradation in endosome compartment and nuclear transfer. Most of transfection reagents, including the ones described above, were only designed to work effectively at the entry into cytoplasm. However, it is necessary to treat all the obstructive steps in developing a non–viral gene vehicle.

Viruses can efficiently deliver their genes from the entry into the cell membrane to the nucleus and sometimes integrate themselves into the host genome. A capsid-coated viral genome escapes endosome degradation (*22*, *23*). A viral matrix attachment protein that includes a nuclear localization signal enables lentivirus to pass through the nuclear membrane (*24*, *25*). The retroviral genome can easily be integrated into the host chromosome by viral integrase (*26*, *27*). Viruses have evolved a specialized system for each obstructive step of gene transfer. Thus, it is interesting to incorporate a virus-like mechanism into non–viral gene transfer.

Based on this concept, we previously developed a lipid-based transfection method (*28–30*). In this method, lipid vesicles composed of dimethyldioctadecyl ammonium bromide (DDAB) and Tween 80 can efficiently introduce foreign DNA into cultured cell lines (*28*). To escape degradation inside the cytoplasm and to make it easier to pass through the nuclear membrane, a sperm protein protamine was added to the lipid/DNA complex (*29*). Protamine is highly basic and binds strongly to negatively charged DNA resulting in the protection of the delivered DNA from deg-

[1] This work was supported in part by Grants-in-Aid for Scientific Research from Ministry of Education, Science, Sports and Culture of Japan (Nos. 10145104 and 12555227).

[2] To whom correspondence should be addressed. Tel: +81-52-789-4277, Fax: +81-52-789-3221, E-mail: kamihira@proc.nubio.nagoya-u.ac.jp

Abbreviations: CMV, cytomegalovirus; DDAB, dimethyldioctadecyl ammonium bromide; DMEM, Dulbecco's modified Eagle's medium; DMRIE, 1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide; DOPE, dioleoyl phosphatidylethanolamine; DOSPA; 2,3-dioleyloxy-*N*-[2(sperminecarboxamido)ethyl]-*N,N*-dimethyl-1-propanaminium trifluoroacetate; DOTAP, 1,2-dioleoyl-*sn*-glycero-3-trimethylammonium-propane; DOTMA, *N*-[1-(2, 3-dioleyloxy) propyl]-*N,N,N*-trimethylammonium chloride; FBS, fetal bovine serum; MEM, minimum essential medium; MoMLV, Moloney murine leukemia virus; ONPG, *o*-nitrophenyl β-D-galactopyranoside; PBS, phosphate buffered saline; RSV, Rous sarcoma virus; TMTPS, *N,N′,N″,N‴*-tetramethyl-*N,N′,N″,N‴*-tetrapalmitylspermine; VSV-G, vesicular stomatitis virus G protein; X-gal,5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

radation by DNase. Furthermore, a protamine nuclear localization signal makes it possible for the foreign DNA to pass through the nuclear membrane (*29*). Recently, we reported the incorporation of a retroviral integrase in DDAB lipid vesicle-mediated gene transfer to enhance the integration of the transferred DNA into the host chromosome (*30*). By this method, the stable transfection efficiency was increased up to 15 times compared with DDAB lipid vesicles alone.

Although these DDAB-based transfection methods mimic a virus in some points and are superior to the first generation of cationic lipid–mediated gene transfer methods, the transfection efficiency decreases drastically in the presence of serum in the culture medium. In the present study, protamine was covalently bound to stearic acid, and the stearyl protamine was incorporated into lipid vesicles to make stable complexes between DNA and DDAB lipid vesicles. The protamine-modified DDAB lipid vesicles were applied to the transfection of cultured cells in the presence of serum, and also quail embryos as an *in vivo* transfection study.

MATERIALS AND METHODS

Cell Lines, Media, and Plasmid DNAs—African green monkey kidney, COS-7, NIH Swiss mouse embryo, NIH3T3, and Chinese hamster ovary, CHO-K1, cell lines were obtained from the RIKEN cell bank and used as host cells for gene transfer. COS-7 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). NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL) supplemented with 10% FBS. CHO-K1 cells were cultured in Ham's F12 medium (Gibco BRL) supplemented with 10% FBS. Media for all cultures routinely included 100 units/ml penicillin and 100 μg/ml streptomycin.

Recombinant plasmids pCMVβ (*31*) and pCMV-GFP (pGREEN LANTERN-1; Gibco BRL), which encode β-galactosidase and green fluorescent protein, respectively, under the control of the cytomegalovirus (CMV) early promoter, were used for the transient expression of cultured cells. A recombinant plasmid, pmiwZ (*32*), which encodes the β-galactosidase gene under the control of chicken β-actin and Rous sarcoma virus (RSV) hybrid promoter, was used for gene transfer of quail embryos.

Preparation of Cationic Lipid Vesicles—The procedure for the preparation of DDAB lipid vesicles was reported previously (*28, 29*). Briefly, a cationic surfactant, 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 at 4°C. The solution was prewarmed and homogenized before use. For the preparation of protamine-modified vesicles, stearic acid was first activated by *N,N'*-dicyclohexylcarbodiimide and mixed with protamine sulfate (Wako Pure Chemical Industries) solution at pH 9.5 to produce covalent binding between stearic acid and protamine. The molar ratio of stearic acid to protamine was 1.0. After dialyzing with phosphate buffered saline (PBS; 150 mM NaCl, 16 mM Na_2HPO_4, and 4 mM NaH_2PO_4, pH 7.4), the stearic acid–protamine conjugate was incorporated into the DDAB lipid vesicles by sonication (Fig. 1).

Transient Transfection Assay—Growth media in plastic dishes (35 mm in diameter; Iwaki Glass Works, Tokyo) with 50 to 70% confluent cells were replaced with 1.5 ml of fresh growth media containing the respective concentration of serum. Three micrograms of DNA and 36 μg (calculated as DDAB+Tween 80 weight) of protamine-modified vesicles, composed of DDAB and Tween 80 in a weight ratio of 1:1, were used for transfection. DNA and the lipid vesicle solutions were diluted separately in 250 μl of opti-MEM (Gibco BRL) and incubated for 10 min at room temperature. The solutions were then mixed and incubated at room temperature for 30 min to form complexes. The solution containing protamine-modified DDAB lipid vesicles/DNA complexes was added to the cells in culture dishes. The transient expression of β-galactosidase was assayed 24 h post-transfection. For transfections using commercially available transfection reagents, Lipofectin® (DOTMA/DOPE); 1:1 liposome formulation of *N*-[1-(2,3-dioleyloxy)propyl]-*N,N,N*-trimetylammonium chloride (DOTMA) and dioleoyl phosphatidylethanolamine (DOPE), Lipofectamine® (DOSPA/DOPE); 3:1 liposome formulation of DOSPA and DOPE, Cellfectin® (TMTPS/DOPE); 1:1.5 liposome formulation of *N,N',N'',N'''*-tetramethyl-*N,N',N'',N'''*-tetrapalmitylspermine (TMTPS) and DOPE, and DMRIE-C® (DMRIE/cholesterol); 1:1 liposome formulation of 1,2-dimyristyloxypropyl-3-dimethylhydroxy ethyl ammonium bromide (DMRIE) and cholesterol were purchased from Gibco BRL. The optimal volume

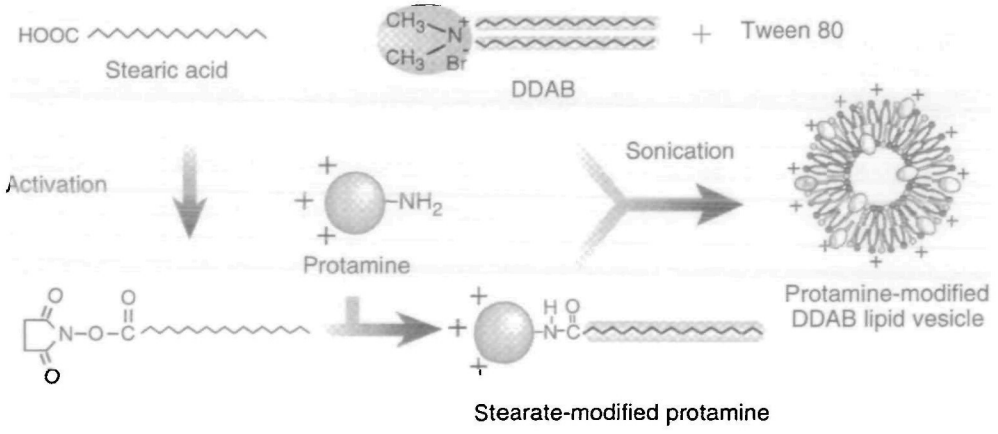

Fig. 1. **Schematic diagram of the modification of DDAB lipid vesicles by protamine.** Protamine sulfate and stearic acid were covalently bound by the carbodiimide method. The stearate-modified protamine was incorporated into DDAB lipid vesicles by sonication.

of each reagent and 3 μg of DNA were used as described in the manufacturer's instructions. Transfection methods with DDAB lipid vesicles alone or protamine-added lipid vesicles in the absence of serum were described previously (*29*).

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 3 times with PBS, and then stained for 10 h with 1 ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, and 1 mg/ml X-gal) 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, 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 to the harvested cells, and then 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, and then 0.1ml 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 reaction time, 0.3 ml of 1 M Na_2CO_3 was added, and the absorbance of the solution at 420 nm was measured with a spectrophotometer. β-Galactosidase activity is expressed as ONPG units (1 unit: the activity producing 1 μmol *o*-nitrophenol per minute, normalized against the inoculated cell number).

Viral Transduction—Viruses were produced using a pantropic retroviral expression system obtained from Clontech (Palo Alto, CA, USA). A green fluorescent protein gene was inserted into the multicloning site of pLXRN plasmid (pLGRN). A retrovirus packaging cell line GP293 expressing *gag* and *pol* genes derived from Moloney murine leukemia virus (MoMLV) was transfected with pLGRN to establish stable expression clones. After screening for a good virus producer clone, the cells were transfected with pVSV-G plasmid encoding the vesicular stomatitis virus G protein (VSV-G) gene to produce pantropic retroviruses in the medium. Then 0.25 ml of filtered and centrifuged viral supernatant was mixed at a volume ratio of 1:1 with opti-MEM containing 8 μg/ml of protamine-modified DDAB lipid vesicles, 8 μg/ml of DDAB lipid vesicles alone or 2 μg/ml of DOSPA/DOPE. After 20 min incubation, 0.5 ml of lipid/virus mixture was added to NIH3T3 cells that were cultured in 24-well tissue culture plates with 0.5 ml of the medium containing 20% serum. For infection with polybrene, 0.25 ml of polybrene (Sigma Chemical) dissolved in DMEM (8 μg/ml) was mixed with 0.25 ml of viral solution, and then added to NIH3T3 cells in the same manner. Viral titers were determined by counting GFP-expressing cells 48 h post-transduction under a fluorescence microscope. Without any additive, the viral titer was about 10^9 cfu/ml.

Transfection to Quail Embryos—Protamine-modified lipid vesicles and plasmid solutions were diluted separately to final concentration of 0.2 μg/ml DNA and 1 μg/ml DDAB in the same volume of opti-MEM. The mixtures were gently mixed and then left to stand at room temperature for about 30 min to form complexes. Fertilized quail eggs were obtained from Nisseiken (Yamanashi). The egg shells were wiped with 70% ethanol, and then cut horizontally with a drill to make an opening 10 mm in diameter at the sharp end. Then 2 μl of the protamine-modified lipid vesicle/DNA complex solution was injected into the heart of 48-h-stage embryos using a glass micropipette. For injection using commercially available reagents, the optimal ratios of reagent to DNA were chosen as described in the manufacturer's instructions. After the injection, the opening of the egg shell was sealed with a gas-permeable porous PTFE membrane (Milliwrap™; Millipore, Tokyo) and plastic film (Saran Wrap™; Asahi Kasei Kogyo, Tokyo) using quail thin albumen as glue. The eggs were incubated at 37.7°C and a relative humidity of 65% with rocking through an angle of 90°C at 30 min intervals for 2 days, and then the embryos were evaluated for gene expression.

Evaluation of Transfection Efficiency of Quail Embryos—*In situ* staining of quail embryos was performed to detect β-galactosidase expression 2 days post-transfection. Embryos washed with PBS were fixed in 0.4 ml of 2% glutaraldehyde in PBS for 40 min at 4°C, rinsed 3 times with PBS, and then stained with 0.4 ml of X-gal solution for 3 h at 37°C. The embryos were observed under a microscope.

For quantification of β-galactosidase activity, whole body of embryos were collected 2 days after transfection, washed with PBS, and then the heads, limbs and membranes were removed. The remaining body was cut in pieces and sonicated in 0.8 ml of reaction buffer to obtain cell lysates. β-Galactosidase activity of the lysates was measured using ONPG as a substrate, as described above.

RESULTS

In Vitro Gene Transfection Using Protamine-Modified DDAB Lipid Vesicles in the Presence of Serum—Figure 2A shows the transfection efficiency using protamine-modified DDAB lipid vesicles to COS-7 cells in serum-containing medium. To optimize the ratio of DDAB to protamine, various amounts of stearate-conjugated protamine were incorporated into DDAB lipid vesicles, and then COS-7 cells were transfected with pCMVβ using protamine-modified lipid vesicles in the presence of 10% serum. Maximal transfection efficiency was achieved when 3.6 μg (protein basis) of protamine conjugated with stearic acid was incorporated into 18 μg of DDAB lipid vesicles. The optimal ratio of DDAB to protamine (5:1) gave about 50% transfection efficiency, whereas 18:1 of DDAB/protamine ratio showed an efficiency below 5%. Figure 2B shows the effects of increasing concentrations of serum on transfection efficiency, and also shows comparative efficiency data with our previous methods, DDAB lipid vesicles alone and protamine-added (not modified) DDAB lipid vesicle–mediated gene transfer. When transfection was performed under serum-free conditions, protamine-modified and -added lipid vesicles achieved more than 50% transfection efficiency. About 30% of cells expressed β-galactosidase when DDAB lipid vesicles alone were used. While the transfection efficiencies of the two previous methods decreased drastically with increasing serum concentrations, the efficiency of protamine-modified vesicles was not significantly inhibited up to 10% serum concentration. In the presence of 10% serum, the transfection efficiencies in the previous methods were less than 3%. On the other hand, the efficiency of the protamine-modified method was 50%. Even with 20% serum, about 20% of the

cells still expressed β-galactosidase when protamine-modified lipid vesicles were used.

We then examined whether protamine-modified lipid vesicles are useful for various cell lines such as COS-7, NIH3T3, and CHO-K1 cells, by measuring the β-galactosidase activity as an index of transfection efficiency (Fig. 3). As shown in our previous study, DDAB lipid vesicle–mediated transfection introduces DNA into CHO-K1 cells at a high efficiency in the absence of serum (*28*), although relatively low efficiencies are achieved in COS-7 and NIH3T3 cell lines. The addition of protamine to the DDAB/lipid vesicle complex promotes the efficiency in the absence of serum (*29*). However, transfection is inhibited in 10% serum-containing medium. In the case of protamine-modified lipid vesicles, no significant decrease in β-galactosidase activity was observed regardless of the cell line. These data indicate that protamine-modified lipid vesicles are useful for transfection to various cell lines.

Comparative Study of Protamine-Modified DDAB Lipid Vesicles with Other Cationic Lipid Reagents—The transfection efficiency of protamine-modified lipid vesicles were compared to those of other cationic lipid reagents (DOTMA/DOPE, DOSPA/DOPE, TMTPS/DOPE, and DMRIE/cholesterol) under optimal conditions using pCMVβ as a reporter plasmid (Fig. 4A). When transfection was performed without serum, all reagents, including our previous methods, showed relatively high transfection efficiency (around 50%). In the presence of 10% serum, however, the transfection efficiencies of other cationic lipids decreased drastically. The efficiency of DMRIE/cholesterol, which showed the highest transfection efficiency among the four cationic reagents, was about 10% in the presence of serum. Another reporter plasmid, pCMV-GFP, allowing the visual evaluation of transfection efficiency, also gave similar results showing that protamine-modified lipid vesicles are superior to DOSPA/DOPE (Fig. 4B) and the other cationic lipid reagents (data not shown).

Protamine-Modified DDAB Lipid Vesicles Also Enhance Viral Transduction—Generally, retroviral transduction into cultured cells is performed in serum-containing medium. It is known that the addition of a polycation molecule, polybrene, enhances retroviral transduction (*33*, *34*). Recently, it was reported that cationic lipids such as DOSPA/DOPE promote retroviral transduction (*35*). Thus, we examined whether protamine-modified DDAB lipid vesicles enhance the viral transduction titer in the presence of 10% serum

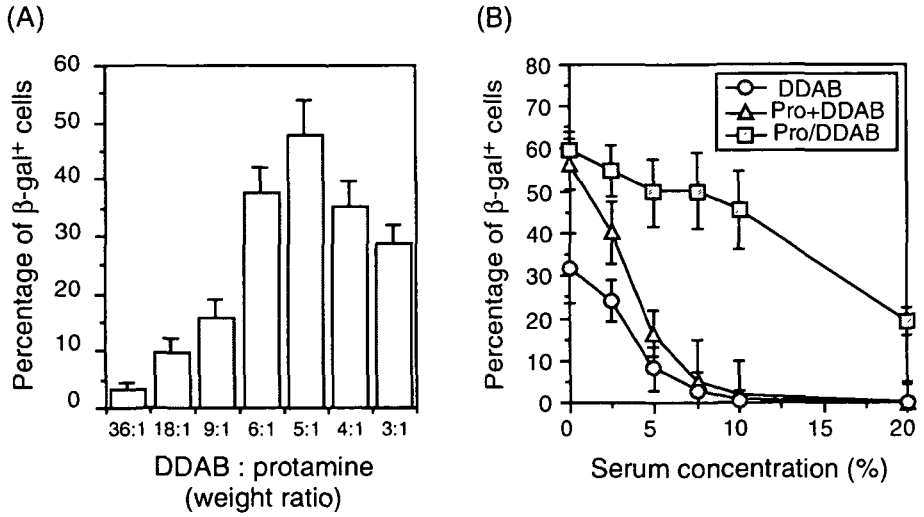

Fig. 2. **Enhancement of *in vitro* gene transfer into COS-7 cells using protamine-modified DDAB lipid vesicles in the presence of serum.** (A) Dose-response of transfection efficiency in the presence of 10% serum by DDAB lipid vesicles incorporating various amount of protamine. At each ratio of protamine to DDAB lipid vesicles, the weight of lipid was fixed at 36 μg. (B) Effect of serum concentration on transfection efficiency. Increasing concentrations of serum up to 20% were added to the culture medium at the transfection with protamine-modified lipid vesicles (Pro/DDAB). DDAB lipid vesicles alone (DDAB) and protamine-added lipid vesicles (Pro+DDAB) were used as controls. The experiments were performed in triplicate and the data represent means ± SD.

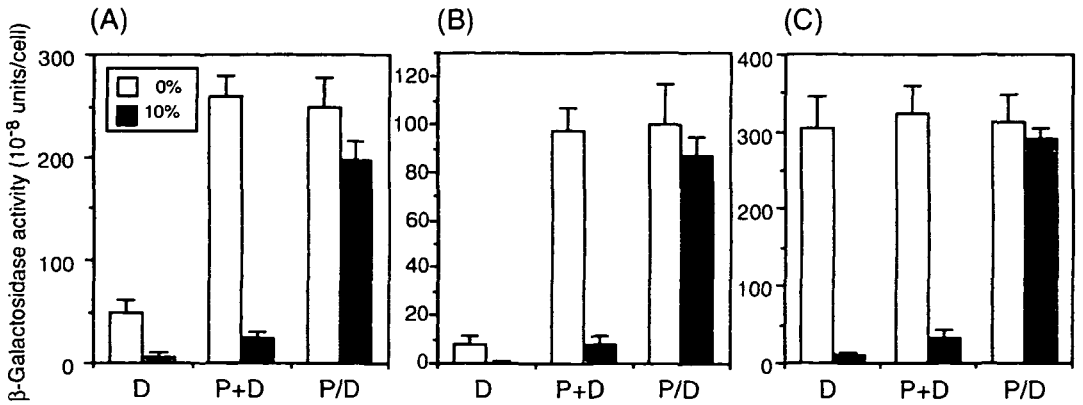

Fig. 3. **Expression levels of β-galactosidase in various cell lines.** Three cell lines, COS-7 (A), NIH3T3 (B), and CHO-K1 (C) were transfected with pCMVβ using DDAB lipid vesicles alone (D), protamine-added lipid vesicles (P+D), or protamine-modified lipid vesicles (P/D) in the absence (open bar) and presence (closed bar) of 10% serum. β-Galactosidase activities were measured 2 days post-transfection. The experiments were performed in triplicate and the data represent the mean ± SD.

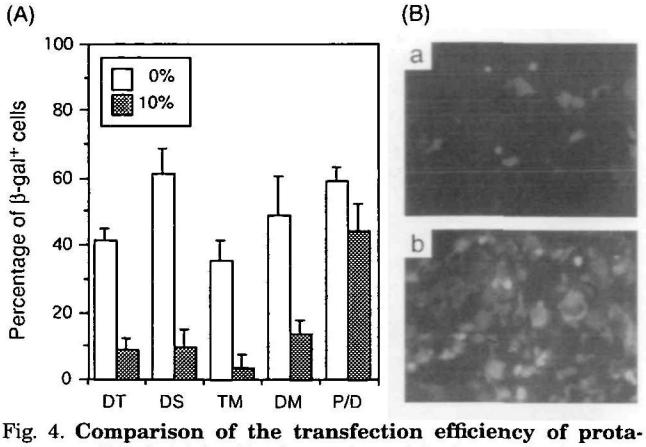

Fig. 4. **Comparison of the transfection efficiency of protamine-modified DDAB lipid vesicles with those of other cationic lipid reagents.** (A) COS-7 cells were transfected with pCMVβ using DOTMA/DOPE (DT), DOSPA/DOPE (DS), TMTPS/DOPE (TM), and DMRIE/cholesterol (DM) and protamine-modified DDAB lipid vesicles (P/D) in the absence (open bars) or presence (closed bars) of 10% serum. The experiments were performed in triplicate and the data represent the mean ± SD. (B) Photographs of cells expressing green fluorescent protein after transfection. A reporter plasmid, pCMV-GFP, was used to transfect COS-7 cells using DOSPA/DOPE (a) and protamine-modified DDAB lipid vesicles (b).

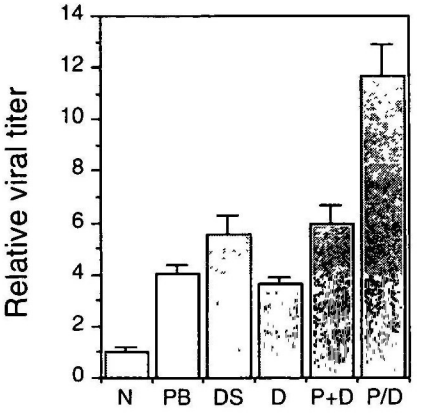

Fig. 5. **Improvement of retroviral transduction efficiency using protamine-modified DDAB lipid vesicles.** A pantropic retroviral vector pseudotyped by VSV-G protein was transduced to NIH3T3 cells without any reagent (N), with polybrene (PB), DOSPA/DOPE (DS), DDAB lipid vesicles (D), protamine-added lipid vesicles (P+D), or protamine-modified vesicles (P/D). The experiments were performed in triplicate and the data represent the mean ± SD.

(Fig. 5). A retroviral vector, in which the viral envelope was changed to VSV-G protein, was used. Pantropic retroviral vectors that express VSV-G as an envelope protein are superior to usual retroviral vectors such as MoMLV based vector in terms of host specifity and ease of obtaining concentrated viral solutions by ultracentrifugation (*36*, *37*). Compared with the virus alone, DDAB lipid vesicles, protamine-added lipid vesicles and DOSPA/DOPE increased the transduction efficiency by 4- to 6-fold. The degree of enhancement was almost the same as that of polybrene. On the other hand, protamine-modified lipid vesicles showed a 12-fold increase in transduction efficiency, indicating that the vesicles are not only useful as a nonviral gene vehicle but also as an enhancing reagent for retroviral transduction.

Enhancement of Gene Transfer to Quail Embryos—As described above, efficient gene transfer of cultured cells was achieved with protamine-modified DDAB lipid vesicles. Therefore, we applied the method to gene delivery for quail embryos as an example of *in vivo* transfection to an animal organ. A solution of protamine-modified lipid vesicles was administered into hearts of 48-h-stage embryos by direct injection. Among all organs, heart contains the largest amount of blood, thus it should reflect well the serum-effect on transfection. The reporter plasmid used in this experiment was pmiwZ, which encodes β-galactosidase and has been shown to be well expressed in quail embryos (*32*, *38*). For comparison, other cationic lipids were also utilized in these assays. Figure 6A shows the β-galactosidase activity per transfected embryo. Protamine-modified lipid vesicles achieved higher β-galactosidase activity than those of other cationic reagents. Whole mount-staining of the transfected embryos performed 2 days post-transfection is shown in Fig. 6B. Embryos transfected with DOSPA/DOPE showed faint and mosaic-like staining at the hearts [Fig. 6B (b)], and no staining was detected visually with other cationic reagents. On the other hand, gross staining of the hearts was observed in most of the transfected embryos when protamine-modified lipid vesicles were used. Unexpectedly some embryos showed mosaic-like staining around the heads [Fig. 6B (e, f)], although the vesicle/DNA solution was injected into the hearts. Furthermore, the chorioallantoic membrane was often stained only in embryos transfected using protamine-modified lipid vesicles (data not shown). In addition to the effectiveness of transfection to cultured cells in serum-containing medium, protamine-modified lipid vesicles might be useful for *in vivo* gene transfer.

DISCUSSION

A novel lipid-based gene vehicle that can be used for efficient gene transfer in the presence of serum was developed. Protamine, a DNA binding nuclear protein in sperm, was conjugated to stearic acid, and then incorporated into DDAB lipid vesicles. Our previously reported methods (DDAB lipid vesicles and the vesicles with protamine addition) undergo a significant loss of their transfection ability in serum-containing medium, whereas protamine-modified DDAB lipid vesicles enable efficient gene delivery in medium containing up to 10% serum. Comparative studies using other cationic lipid reagents also revealed the effectiveness of protamine-modified lipid vesicles. In addition, *in vivo* gene transfer to the hearts of quail embryos using protamine-modified lipid vesicles was successfully achieved.

The reason protamine-modified vesicles work effectively in the presence of serum is not clear. A possible mechanism is that a stable association of lipid vesicles with DNA is achieved by protamine-modification of the lipid vesicles. It is known that protamine is a highly basic protein and binds strongly to DNA (*39*). Thus, the protamine-modification of lipid vesicles may cause an increased +/− charge ratio and lead to the stabilization of DNA/lipid vesicle complexes, which cannot be achieved by the simple addition of protamine in the presence of serum. Several studies using other

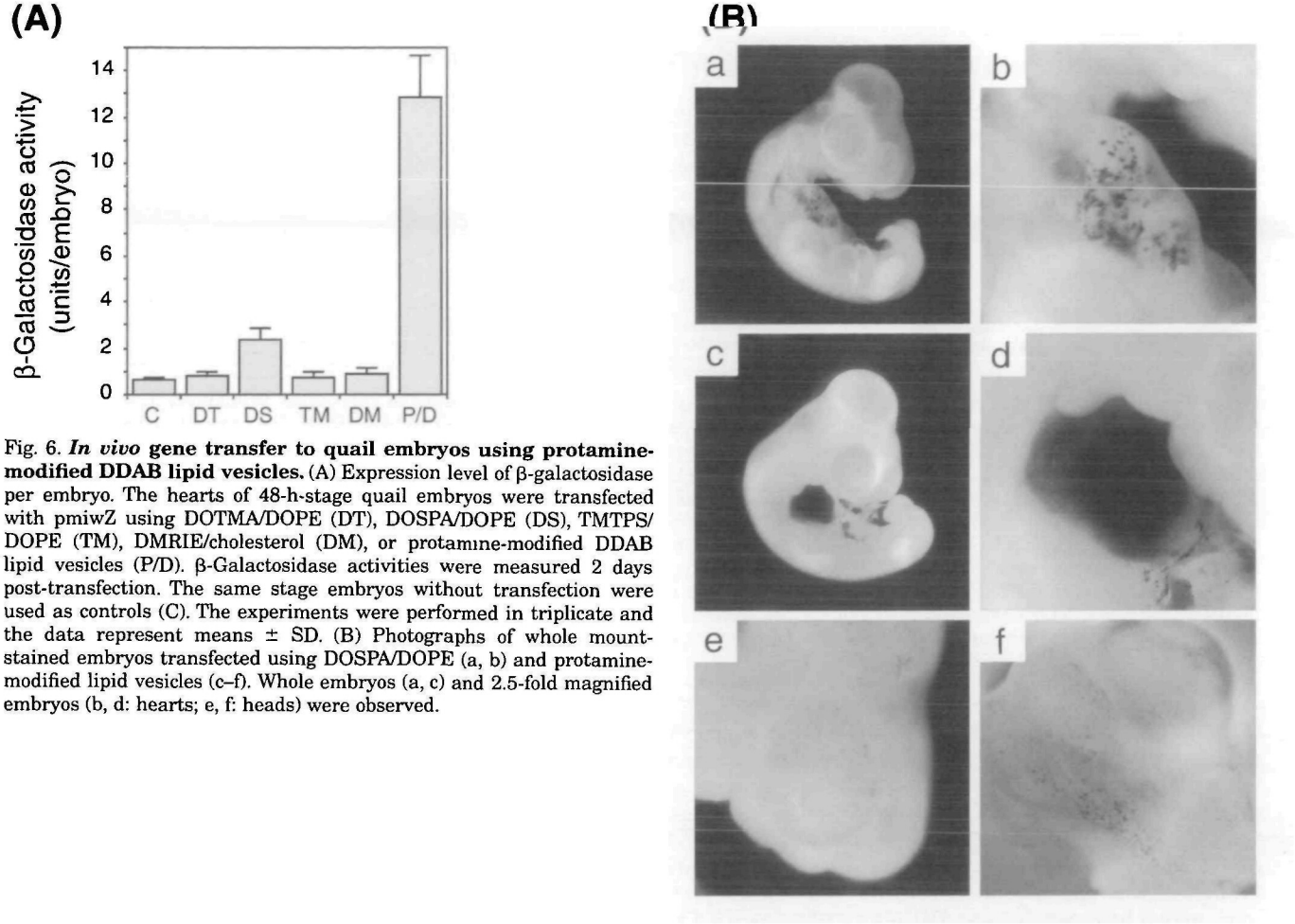

Fig. 6. ***In vivo* gene transfer to quail embryos using protamine-modified DDAB lipid vesicles.** (A) Expression level of β-galactosidase per embryo. The hearts of 48-h-stage quail embryos were transfected with pmiwZ using DOTMA/DOPE (DT), DOSPA/DOPE (DS), TMTPS/DOPE (TM), DMRIE/cholesterol (DM), or protamine-modified DDAB lipid vesicles (P/D). β-Galactosidase activities were measured 2 days post-transfection. The same stage embryos without transfection were used as controls (C). The experiments were performed in triplicate and the data represent means ± SD. (B) Photographs of whole mount-stained embryos transfected using DOSPA/DOPE (a, b) and protamine-modified lipid vesicles (c–f). Whole embryos (a, c) and 2.5-fold magnified embryos (b, d: hearts; e, f: heads) were observed.

cationic lipids support this hypothesis (*16*, *20*). In case of DC-chol liposome, constituted with 3β-(*N*-((*N'*,*N'*-dimethylamino)ethane)carbamoyl)-cholesterol and DOPE, the maximal transfection efficiency was achieved at a charge ratio of 2.0 in the absence of serum, whereas a ratio of 4.0 gave maximal efficiency in the presence of serum (*20*). The addition of a detergent (1% octylglucoside) at the formation of the (DOSPA/DOPE)/DNA complex to stabilize the complex improves transfection in the presence of serum (*21*). Moreover, several serum components that have inhibitory effects on transfection have recently been identified (*40*). For instance, the oleic acid and heparin contained in serum displaces DNA from lipid/DNA complexes, and interferes with gene delivery. These studies suggest again that the serum-resistant properties of protamine-modified lipid vesicles are attributable to the stabilization of lipid/DNA complexes due to a high affinity of protamine for DNA.

Protamine-modified lipid vesicles also enhance the viral transduction efficiency, as shown in Fig. 5. The addition of protamine-modified lipid vesicles to pantropic retroviral particles increased the viral titer 12-fold. One of important step in viral infection is the attachment of the viral particle to the cellular membrane. It is known that polybrene mediates the association between viral particle and cellular membrane, which results in the enhancement of infection efficiency (*33*, *34*). Previous research showed that DOSPA/DOPE also increase viral transduction in an amphotropic retroviral system (*35*). The reduction of the negative charge repulsion between the cell and viral membrane phospholipids, which facilitates interaction of the envelope glycoprotein and cell surface, was a possible mechanism for the enhancement of virus transduction by DOSPA/DOPE. In the case of protamine-modified lipid vesicles, a similar mechanism can be assumed, although the enhancement of pantropic retroviral transduction was 2-fold higher than that achieved by DOSPA/DOPE. Retroviral infection of cultured cells is usually performed in serum-containing medium. Since protamine-modified lipid vesicles are highly positively charged and stable in the presence of serum, as indicated in the DNA transfection study, it can effectively mediate the virus–cell interaction in the presence of serum and enhance retroviral transduction.

A lipid that mediates gene transfer in the presence of serum *in vitro* is not always effective in *in vivo* gene transfer (*19*, *41*). This makes the development of gene transfer more complicated. In a gene transfection study using a series of amphiphilic imidazolinium compounds, which differ only in the structure of the hydrophobic acyl-chains, the myristoyl-form was the most effective *in vitro*, whereas the oleoyl-form was the most effective *in vivo* (*19*). In the present study, protamine-modified lipid vesicles showed excellent transfection ability for *in vivo* gene delivery to quail embryos. In a study of transgenic birds, replication competent retroviruses have been often used, although they pos-

sess some problems in terms of safety and reproducibility (*42*, *43*). To avoid these problems, several researchers attempted the transfection of avian embryos using cationic lipid reagents (*44–47*). However, faint or mosaic-like expression of reporter gene was observed in the previous methods. In this study, gross staining of the reporter gene expression was observed at hearts by the injection of protamine-modified lipid vesicle/DNA complexes to hearts of quail embryos. Thus, this method may be used for site specific exogenous gene expression *in vivo* by direct injection.

Gene transfer using the protamine-modified lipid vesicles developed in this study effectively utilizes the function of protamine. As described in previous studies, protamine condenses DNA (*39*), prevents DNA from degradation by DNase, and enforces nuclear transport (*29*, *48*). By the addition of protamine, transfection efficiency is improved in cationic lipid vesicle-mediated gene transfer (*29*, *49*). In the present study, protamine might have similar effects since protamine-modified lipid vesicles gave almost same transfection efficiency as protamine-added lipid vesicles under serum-free conditions (Fig. 2B). Furthermore, the gene vehicle acquired serum durability by protamine incorporation. In our previous studies, DDAB lipid vesicles were conjugated to cell specific ligands to improve specifity (*49*), and combined with viral integrase to enhance genome integration of the transferred gene (*30*). In the future, incorporating these functions, gene transfer using protamine-modified lipid vesicles might be established as a safe and efficient method for gene delivery.

REFERENCES

1. Felgner, P.L., Gadek, T.R., Holm, M., Roman, R., Chan, H.W., Wenz, M., Northrop, J.P., Ringold, G.M., and Danielsen, M. (1987) Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci. USA* **84**, 7413–7417
2. Malone, R.W., Felgner, P.L., and Verma, I.M. (1989) Cationic liposome-mediated RNA transfection. *Proc. Natl. Acad. Sci. USA* **86**, 6077–6081
3. Gao, X. and Huang, L. (1995) Cationic liposome-mediated gene transfer. *Gene Ther.* **2**, 710–722
4. Caplen, N.J., Alton, E.W., Middleton, P.G., Dorin, J.R., Stevenson, B.J., Gao, X., Durham, S.R., Jeffery, P.K., Hodson, M.E., Coutelle, C., Huang, L., Porteous, D.J., Williamson, R., and Geddes, D.M. (1995) Liposome-mediated CFTR gene transfer to the nasal epithelium of patients with cystic fibrosis. *Nat. Med.* **1**, 39–46
5. Oudrhiri, N., Vigneron, J.P., Peuchmaur, M., Leclerc, T., Lehn, J.M., and Lehn, P. (1997) Gene transfer by guanidinium-cholesterol cationic lipids into airway epithelial cells *in vitro* and *in vivo*. *Proc. Natl. Acad. Sci. USA* **94**, 1651–1656
6. Kielian, T. and Hickey, W.F. (1999) Inflammatory thoughts about glioma gene therapy. *Nat. Med.* **5**, 1237–1238
7. Nabel, E.G., Gordon, D., Yang, Z.Y., Xu, L., San, H., Plautz, G.E., Wu B.Y., Gao, X., Huang, L., and Nabel, G.J. (1992) Gene transfer *in vivo* with DNA-liposome complexes: lack of autoimmunity and gonadal localization. *Hum. Gene. Ther.* **3**, 649–656
8. Thierry, A.R., Lunardi-Iskandar, Y., Bryant, J.L., Rabinovich, P., Gallo, R.C., and Mahan, L.C. (1995) Systemic gene therapy: biodistribution and long-term expression of a transgene in mice. *Proc. Natl. Acad. Sci. USA* **92**, 9742–9746
9. Chu, G., Hayakawa, H., and Berg, P. (1987) Electroporation for the efficient transfection of mammalian cells with DNA. *Nucleic Acids Res.* **15**, 1311–1326
10. Graham, F.L. and van der Eb, A.J. (1973) A new technique for the assay of infectivity of human adenovirus 5. *Virology* **52**, 456–467
11. Zabner, J., Fasbender, A.J., Moninger, T., Poellinger, K.A., and Welsh, M.J. (1995) Cellular and molecular barriers to gene transfer by a cationic lipid. *J. Biol. Chem.* **270**, 18997–19007
12. Senior, J.H., Trimble, K.R., and Maskiewicz, R. (1991) Interaction of positively-charged liposomes with blood: implications for their application *in vivo*. *Biochim. Biophys. Acta* **1070**, 173–179
13. Zelphati, O. and Szoka, F.C. Jr. (1996) Mechanism of oligonucleotide release from cationic liposomes. *Proc. Natl. Acad. Sci. USA* **93**, 11493–11498
14. Leventis, R. and Silvius, J.R. (1990) Interactions of mammalian cells with lipid dispersions containing novel metabolizable cationic amphiphiles. *Biochim. Biophys. Acta* **1023**, 124–132
15. Crook, K., Stevenson, B.J., Dubouchet, M., and Porteous, D.J. (1998) Inclusion of cholesterol in DOTAP transfection complexes increases the delivery of DNA to cells *in vitro* in the presence of serum. *Gene Ther.* **5**, 137–143
16. Abe, A., Miyanohara, A., and Friedmann, T. (1998) Polybrene increases the efficiency of gene transfer by lipofection. *Gene Ther.* **5**, 708–711
17. Goldman, C.K., Soroceanu, L., Smith, N., Gillespie, G.Y., Shaw, W., Burgess, S., Bilbao, G., and Curiel, D.T. (1997) *In vitro* and *in vivo* gene delivery mediated by a synthetic polycationic amino polymer. *Nat. Biotechnol.* **15**, 462–466
18. Lewis, J.G., Lin, K.Y., Kothavale, A., Flanagan, W.M., Matteucci, M.D., DePrince, R.B., Mook, R.A. Jr., Hendren, R.W., and Wagner, R.W. (1996) A serum-resistant cytofectin for cellular delivery of antisense oligodeoxynucleotides and plasmid DNA. *Proc. Natl. Acad. Sci. USA* **93**, 3176–3181
19. Solodin, I., Brown, C.S., Bruno, M.S., Chow, C-Y., Jnag, E-H., Debs, R.J., and Heath, T.D. (1995) A novel series of amphiphilic imidazolinium compounds for *in vitro* and *in vivo* gene delivery. *Biochemistry* **34**, 13537–13544
20. Yang, J-P. and Huang, L. (1997) Overcoming the inhibitory effect of serum on lipofection by increasing the charge ratio of cationic liposome to DNA. *Gene Ther.* **4**, 950–960
21. Hofland, H.E.J., Shephard, L., and Sullivan, S.M. (1996) Formation of stable cationic lipid/DNA complexes for gene transfer. *Proc. Natl. Acad. Sci. USA* **93**, 7305–7309
22. Seth, P. (1994) Mechanism of adenovirus-mediated endosome lysis: role of the intact adenovirus capsid structure. *Biochem. Biophys. Res. Commun.* **205**, 1318–1324
23. Greber, U.F., Willetts, M., Webster, P., and Helenius, A. (1993) Stepwise dismantling of adenovirus 2 during entry into cells. *Cell* **75**, 477–486
24. Gallay, P., Swingler, S., Song, J., Bushmann, F., and Trono, D. (1995) HIV nuclear import is governed by the phosphotyrosine-mediated binding of matrix to the core domain of integrase. *Cell* **83**, 569–576
25. Stevenson, M. (1996) Portals of entry: uncovering HIV nuclear transport pathways. *Trend Cell Biol.* **6**, 9–15
26. Craigie, R., Fujiwara, T., and Bushman, F. (1990) The IN protein of moloney murine leukemia virus processes the viral DNA ends and accomplishes their integration *in vitro*. *Cell* **62**, 829–837
27. Hansen, M.S.T., Smith III, G.J., Kafri, T., Molteni, V., Siegel, J.S., and Bushman, F.D. (1999) Integration complexes derived from HIV vectors for rapid assays *in vitro*. *Nat. Biotechnol.* **17**, 578–582
28. You, J., Kamihira, M., and Iijima, S. (1997) Surfactant-mediated gene transfer for animal cells. *Cytotechnology* **25**, 45–52
29. You, J., Kamihira, M., and Iijima, S. (1999) Enhancement of transfection efficiency by protamine in DDAB lipid vesicle-mediated gene transfer. *J. Biochem.* **125**, 1160–1167
30. Mizuarai, S., Kamihira, M., Nishijima, K., and Iijima, S. (1999) Integrase-mediated nonviral gene transfection with enhanced integration efficiency. *J. Biosci. Bioeng.* **88**, 461–467
31. MacGregor, G.R. and Caskey, C.T. (1989) Construction of plasmids that express *E. coli* β-galactosidase in mammalian cells. *Nucleic Acids Res.* **17**, 2365
32. Kadokawa, Y., Suemori, H., and Nakatsuji, N. (1990) Cell lineage analyses of epithelia and blood vessels in chimeric mouse

embryos by use of an embryonic stem cell line expressing the β-galactosidase gene. *Cell Differ. Dev.* **29**, 187-194
33. Andreadis, S. and Palsson, B.O. (1997) Coupled effects of polybrene and calf serum on the efficiency of retroviral transduction and the stability of retroviral vectors. *Hum. Gene. Ther.* **8**, 285–291
34. Sorokin, L.M., Morgan, E.H., Yeoh, G.C., and Coelen, R. (1989) A method for infection of cultured myogenic cells with Rous sarcoma virus using polybrene. *In Vitro Cell Dev. Biol.* **25**, 63–68
35. Hodgson, C.P. and Solaiman, F. (1996) Virosomes: Cationic liposomes enhance retroviral transduction. *Nat. Biotechnol.* **14**, 339–342
36. Burns, J.C., Friedmann, T., Driever, W., Burrascano, M., and Yee, J-K. (1993) Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: Concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. *Proc. Natl. Acad. Sci. USA* **90**, 8033–8037
37. Yee, J-K., Miyanohara, A., LaPorte, P., Bouic, K., Burns, J.C., and Friedmann, T. (1994) A general method for the generation of high-titer, pantropic retroviral vectors: Highly efficient infection of primary hepatocytes. *Proc. Natl. Acad. Sci. USA* **91**, 9564–9568
38. Oguchi, S., Kamihira, M., You, J., Tachibana, A., and Iijima, S. (1998) Exogenous gene transfection into quail embryo using cationic lipid vesicles. *J. Ferment. Bioeng.* **86**, 118–120
39. Ando, T., Yamasaki, M., and Suzuki, K. (1973) Protamines. Isolation, characterization, structure and function. *Mol. Biol. Biochem. Biophys.* **12**, 1–114
40. Zelphati, O., Uyechi, L.S., Barron, L.G., and Szoka, F.C. Jr. (1998) Effect of serum components on the physico-chemical properties of cationic lipid/oligonucleotide complexes and on their interactions with cells. *Biochim. Biophys. Acta* **1390**, 119–133
41. Aksentijevich, I., Pastan, I., Lunardi-Iskandar, Y., Gallo, R.C., Gottesman, M.M., and Thierry, A.R. (1996) *In vitro* and *in vivo* liposome-mediated gene transfer leads to human MDR1 expression in mouse bone marrow progenitor cells. *Hum. Gene. Ther.* **7**, 1111–1122
42. Takeuchi, J.K., Koshiba-Takeuchi, K., Matsumoto, K., Vogel-Hopker, A., Naitoh-Matsuo, M., Ogura, K., Takahashi, N., Yasuda, K., and Ogura, T. (1999) Tbx5 and Tbx4 genes determine the wing/leg identity of limb buds. *Nature* **398**, 810–814
43. Petropoulos, C.J. and Hughes, S.H. (1991) Replication-competent retrovirus vectors for the transfer and expression of gene cassettes in avian cells. *J. Virol.* **65**, 3728–3737
44. Watanabe, M., Naito, M., Sasaki, E., Sakurai, M., Kuwana, T., and Oishi, T. (1994) Liposome-mediated DNA transfer into chicken primordial germ cells *in vivo*. *Mol. Reprod. Dev.* **38**, 268–274
45. Rosenblum, C.I. and Chen, H.Y. (1995) *In ovo* transfection of chicken embryos using cationic liposomes. *Transgen. Res.* **4**, 192–198
46. Inada, S., Hattori, M.A., Fujihara, N., and Morohashi, K. (1997) *In vivo* gene transfer into the blastoderm of early developmental stage of chicken. *Reprod. Nutr. Dev.* **37**, 13–20
47. Muramatsu, T., Mizutani, Y., Ohmori, Y., and Okumura, J. (1997) Comparison of three nonviral transfection methods for foreign gene expression in early chicken embryos *in ovo*. *Biochem. Biophys. Res. Commun.* **230**, 376–380
48. Sorgi, F.L., Bhattacharya, S., and Huang, L. (1997) Protamine sulfate enhances lipid-mediated gene transfer. *Gene Ther.* **4**, 961-968
49. You, J., Kamihira, M., and Iijima, S. (1998) Enhancement of transfection efficiency using ligand-modified lipid vesicles. *J. Ferment. Bioeng.* **85**, 525–528