Follow-up of Exogenous DNA by Sperm-mediated Gene Transfer via Liposome

Hwang-Yun Cho, Ki-Hwa Chung[1] and Jin-Hoi Kim*

Major of Dairy Science. Division of Applied Life Science. College of Agriculture. Gyeongsang National University
Chinju. GyeongNam 660-701. Korea

ABSTRACT : To examine the feasibility of using a sperm vector system for gene transfer, we have investigated the binding and the uptaking of foreign DNA into the sperm nucleus by PCR, in situ hybridization and LSC. We have also examined the transportation of exogenous DNA into oocytes by immunofluorescene via PCR. Sperm cells were incubated with DNA/liposome complexes (1:4 ratio) in fertilization medium with BSA or without BSA. *In situ* hybridization demonstrated that the transfection rate of sperm cells with and without BSA was 41 and 68% respectively, when the cells were treated with liposome/DNA complexes and 13% for DNA alone. LSC analysis showed that the binding of exogenous DNA was greatly reduced by DNase I treatment which digests DNA bound onto spermatozoa, suggesting that some of the DNA was internalized into the sperm membrane. To find out whether transfected DNA was internalized into sperm intracytomembrane, sperm DNA was amplified by inverse PCR. No PCR products were detected from sperm cells, indicating that the foreign DNA was simply bound onto the sperm membrane. To investigate transfer rates of exogenous DNA into oocytes via sperm cells, we used immunofluorescene method to follow the distribution of foreign DNA via spermatozoa: a few exogenous DNA was located in the cytoplasm of early embryos (13/60, 21.7% for DNA+/liposome+/BSA) and was not located in the pronucleus and/or nucleus. These results suggest that most of the transfected sperm cells could carry the foreign DNA into the egg by in vitro fertilization, but that the transferred DNA is degraded in the developing embryos without stable integration into the zygote genome. Therefore, we have directly injected with transfected sperm cell into oocyte cytoplasm and observed that some of the exogenous DNA was detected in preimplantation embryonic cytoplasm and expressed at preimplantation stages, suggesting that exogenous DNA in early zygote has their integrity. In this study, we have not identified a noble mechanism that interfering transportation of foreign DNA into zygote genome via spermatozoa. Our data, however, demonstrated that inverse PCR and immunofluorescene methods would be used as a new tool for follow-up of gene distribution in oocyte via sperm cells. *(Asian-Aust. J. Anim. Sci. 2002. Vol 15, No. 10 : 1412-1421)*

Key Words : Gene Transfer, Sperm Vector, Intracytoplasmic Sperm Injection, Mouse, Bacterial Lacz

INTRODUCTION

A fundamental problem with microinjection in the production of transgenic livestock is the low efficiency of integration of exogenous DNA (Eyestone et al., 1994). Furthermore, it is very expensive and laborious to obtain zygote by superovulation and surgical collection: production costs range from $25,000 for a founder transgenic pig to over $500,000 for a founder transgenic calf (Wall et al., 1992). Another problem is mosaicism and variable germline transmission. Therefore, several transgenic lines for each application will be generated, because we are confident of having at least one with the desirable trait (Wilmut et al., 1997). By contrast, if it is possible to establish a procedure for gene transfer using sperm cells, thereby reducing the number of animals required in the production of a transgenic founder. Several groups have reported that sperm cells from a variety of different species were permeable to exogenous DNA (Brackett et al., 1971; Arezzo et al., 1983; Atkinson et al.,

1991; Castro et al., Clausen et al., 1991; Camaioni et al., 1992; Lavitrano et al., 1992a, 1992b and 1992c; Horan et al., 1992; Francolini et al., 1993; Zani et al., 1995) and that transgenic embryos or fetus were produced from oocytes fertilized by spermatozoa that had been exposed to exogenous DNA (Lavitrano et al., 1989; Arezzo et al., 1983; Milne et al., 1989; Hochi et al., 1990; Perez et al., 1991; Gruenbaum et al., 1991; Rottmann et al., 1992; Squires and Drake, 1994; Sperandio et al., 1996). However, it is clear that the original experiments from Lavitrano et al. (1989) have not been repeatable (Brinster et al., 1989; Maddox, 1989; Al-Shawi et al., 1990; Gandolfi et al., 1996; Kim et al., 1995, 1997).

Liposome is small bodies consisting of membrane-like lipid layers surrounding hydrous compartments. Furthermore, liposome can be used to protect a foreign DNA from digestion of proteases or DNase I present in the cytoplasm of egg (Schaefer-Ridder et al., 1982). Commercial available cationic liposome can spontaneously interact with RNA/DNA and fuse with the plasma membrane in a wide variety of cell types in culture to produce transfections (Schaefer-Ridder et al., 1982; Felgner et al., 1987; Stewart, 1991; Francolini et al., 1993). The cationic outer surface of complexes can allow internalization of nucleic acids into negatively charged sperm cells, but the production of transgenic animals by this

method has as yet not been reported (Bachiller et al., 1991). Our recent studies have demonstrated that some mature sperm cells could indeed transfer foreign DNA into oocytes at fertilization, but transferred DNA would ultimately be diluted and disappear from the developing embryos (Kim et al., 1997). In this study, we investigated the fate of foreign DNA into/onto sperm cells and followed the fate of exogenous DNA in embryos during fertilization. Although cationic liposome/DNA complexes were efficiently bound onto spermatozoa, we report that under an experimental conditions sperm-mediated gene transfer via liposome is an impractical technique for producing transgenic animals.

MATERIALS AND METHODS

Animals

ICR female (3-4 weeks) and male (12 weeks) mice were used for this experiment. All mice were kept under controlled conditions of light (light on: AM 8:00-PM 10:00) and temperature ($22\pm0.5^\circ$C). Animals were maintained and experiments were conducted in accordance with the GyeongSang National University guide for the Care and Use of Laboratory Animals.

DNA preparation

pZIP(X)hEPOSVneo plasmid, which contains human erythropoietin gene under the control of LTR promoter (Goto et al., 1988) or pCH110 (Takeda and Toyoda, 1991) which contains bacterial *lacZ* under the control of simian virus 40 promoter, were purified according to the method described by Sambrook et al. (1988). The pZIP(X)hEPOSVneo plasmid were digested with HindIII/EcoRI to isolate a 5.86 kb of fragment and the pCH110 plasmid were digested with Pst I to isolate 5.84 kb of fragment. The linear DNAs used for transfection were treated with calf intestinal alkaline phosphatase (CIP, TAKARA) to avoid concatemers and then tested by ligation reaction.

In situ hybridization

Incorporation or binding of exogenous DNA in sperm cells was examined by fluorescent *in situ* hybridization according to our previous method (Kim et al., 1997). Briefly, the transfected sperm samples were smeared on gelatin-coated glass slide, fixed with 4% paraformaldehyde for 15 min, treated with 0.2N HCl and 1 mg/ml of proteinase K at 37°C for 30 min, then re-fixed with 4% paraformaldehyde according to a slightly modified method described previously (Bachiller et al., 1991; Camaioni et al., 1992; Francolini et al., 1993). The probe (for hEPO and for lacZ gene) was labeled with digoxigenin dUTP-FITC using the method recommended by the manufacturer (Boehringer-Mannheim). After washing in PBS for 15 min at room temperature (RT), hybridization buffer containing 20 ng of probe DNA, 6×SSC, 5×Denhardt's solution, 45% formamide, and 10% dextran sulfate was placed on each slide and heated for 5 min at 95-100°C. Samples were briefly chilled on ice and incubated for 4 h at 42°C. After incubation, slides were rinsed twice in 4% formamide and 6×SSC for 15 min at 42°C, then washed twice in 2×SSC for 5 min at RT and once in 0.2×SSC for 15 min at 50°C. The signal of the exogenous DNA in spermatozoa was detected as a green fluorescence of the FLUOS-conjugated anti-digoxigenin antibody by epifluorescence microscopy. Sperm nuclei were counterstained with propidium iodide at a concentration of 0.5 mg/ml.

Immunofluorescene

To follow the transportation of foreign DNA into zygotes, the DNA for transfection was prepared by PCR for incorporation of fluorescein dUTP into their DNA according to method recommended by manufacturer (Boehringer-Mannheim). Sperm cells were then incubated with liposome/fluorescein labeled DNA for 2 h at 37°C 5% CO_2 in air and then, oocytes were fertilized by transfected sperm cells. The fertilized embryos were mounted onto slide glass, fixed in 4% paraformaldehyde for 10 min and washed using the method recommended by the manufacturer (Boehringer-Mannheim). The samples were incubated with anti-digoxigenin-fluorescein conjugate at a final concentration of 20μg/ml for 30 min. Excess antibody was removed by twice washing for 5 min in TBS (0.05 M Tris-HCl, pH 7.4 and 0.85% NaCl) at room temperature. For double staining, embryos were stained with propidium iodide at a final concentration of 0.01mg/ml for a few seconds and the signal of the introduced DNA in the embryos was evaluated by fluorescein microscopy. In some places, the yellow color indicates the colocalization of a green fluorescence and propium iodide emission.

Flow cytometry

The binding and uptake of exogenous DNA in spermatozoa were examined by Laser Scaning Cytometry analysis system (LSC^{TM}, CompuCyte). Spermatozoa with high mobility were selected by swimming-up. In situ hybridization was performed as described above and then co-stained with propidium iodide. In order to determine the percentage of FITC-positive cells, FITC and PI fluorescence emission were excited at 488 nm with an argon laser and the fluorescence signals were measured using band pass 530 nm and 570 nm filters. In all experiments, a minimum of 2,000 cells per slide were scanned and analyzed by LSC analysis. All experiments were duplicated at least three times.

Experimental designs

Exp. I. The aim of this experiment was to investigate

whether liposome/DNA complexes could be bound into sperm cells. Epididymal spermatozoa were obtained from caudal epididymis of ICR mouse as described by Lavitrano et al. (1989). Transfections of sperm cells were performed by using DNA alone (Lavitrano et al., 1989) and/or DNA/lipofectin complexes. For DNA/lipofectin complexes, each solution of DNA (600 ng) and lipofectin (2 μg, GIBCO) were diluted to 5 μl in sterile water, then placed for 30 min at room temperature after gentle mixing. DNA/lipofectin complexes were transferred into 200 μl drops of modified Whittens' medium and covered with paraffin oil. Mice spermatozoa (6×10^6/droplet) were incubated with 200 μl drops containing DNA/lipofectin complexes for 3 h. The transfected sperm cells were used for *in vitro* fertilization (IVF) according to the Lavitrano's method (1989) or in situ hybridization as described above.

Exp. II. The aim of this experiment was to examine whether interaction of DNA with sperm cells could be affected by the presence of BSA, an inhibitor of cationic liposome. The transfection was performed according to the method described above except that bovine serum albumin (BSA) in modified Whittens' medium was replaced with polyvinylalcohol (PVA). Afterwards the mouse spermatozoa were incubated with cationic liposome/DNA complexes for 2 h. BSA (10 mg/ml) was then added for a further 1 h to obtain capacitation, and used for *in situ* hybridization or IVF: oocytes were recovered from ampullae at 12 to 15 h post hCG in PBS containing 10% FBS. IVF was performed as follows: approximately 30 oocytes were placed in 50 μl droplets of IVF medium and 2 μl of the transfected sperm suspension (final concentration in droplet, 1×10^6 spermatozoa) were added (Lavitrano et al., 1989).

Exp. III. To find out whether exogenous DNA was simply bound to and/or taken up into sperm membrane, internalization of exogenous DNA into the sperm nucleus was identified by the polymerase chain reaction (PCR). It is generally known that transgenes were integrated into genome as a tandem array having head and tail arrangement (Burdon and Wall, 1992). We investigated whether exogenous gene is integrated in sperm chromatin and preimplantation embryos by PCR or not. If foreign DNA is incorporated into their genome, PCR products will be 340 bp, meaning that the incorporation was head to tail arrangement. The primers used were as follows: 5'-CAGGAGTGGGGAGGCACGAT-3' (1), anti5'-CGTAAACTGAAC CAAGATACG-3' (2). If the PCR products were 867 bp, it means that exogenous DNA was bound onto sperm membrane: 5'-GAGTTGGGAAGC-TAGACACTG-3' (3), anti 5'-CTCTCCCTCCTGCC-CCTCAGCAGC-3' (4). Spermatozoa were transfected with 5.897 kb of linear fragment derived from pZIP(X)hEPOSVneo plasmid. The PCR for spermatozoa was performed by using the same method described by Li et

al. (1988) except following conditions: the spermatozoa were treated with DNase I to remove the DNA bound onto sperm membrane, then completely washed, boiled at 95°C for 10 min to extract DNA, and cooled rapidly for 3 min.

Exp. IV. The ability of DNA transfer into oocytes was investigated by immunofluorescene via PCR. Female mice were superovulated by sequential injection of 5 IU of pregnant mare serum gonadotropin (PMSG, Sigma) followed by 5 IU of human chorionic gonadotropin (hCG, Sigma). Oocytes were recovered from ampullae at 12 to 15 h post hCG in PBS containing 10% fetal bovine serum (FBS). To examine the intracellular distribution of fluorescent labeled DNA in fertilized eggs, the DNA was prepared by PCR using a fluorescein labeling mixture. The epididymal sperm cells were diluted to 6×10^6/droplet and incubated with fluorescent labeled DNA/liposome complexes for 3 h. then IVF was performed by placing approximately 30 oocytes into 50 μl droplets of IVF medium containing BSA. After IVF, the embryos were analyzed by PCR and/or immunofluorescene as described above.

Exp. V. Finally, we have examined whether oocytes could adopt foreign DNA into their genome during early developmental stages, as it is intact without digestion by endonuclease enzyme. If foreign DNA has not been digested by endonuclease enzyme during fertilization, penetration of foreign DNA into the sperm membrane could increase the integration of the DNA into the embryonic chromosome. To prove this hypothesis, the transfected sperm cell was direct injected into oocyte by intracytoplasmic sperm injection (ICSI), fixed in 4% formalin solution after formation of male pronuclei and then the exogenous DNA was located by immunofluorescene.

RESULTS

Binding patterns of exogenous DNA in sperm cells

Table 1 shows the labeled proportions of sperm cells incubated with DNA alone and transfected with DNA/liposome complexes in the presence or in the absence of BSA. In DNA alone, of the 3,785 sperm cells examined, only 13% of spermatozoa were labeled the exogenous DNA and among them, 57.3% of sperm cells showed whole labeling, but 42.7% were restricted to the anterior or posterior of the sperm head. This contrasts with the labeling of sperm cells transfected by cationic liposome/DNA complexes (41% and 68%) in which the labeling was located to the whole sperm cell including posterior and anterior heads of spermatozoa. Furthermore, the transfection efficiency of cationic liposome DNA

Table 1. Labeling patterns of foreign DNA in the transfected mouse sperm cells

Treatment	DNase I	No. of sperm counted	No. of sperm labeled	Binding patterns			
				Acrosome	Anterior head	Poster head	Whole
Simple DNA	+	3,785	492 (13%)	19	58	133	282 (57.3%)
Lipofectin DNA withBSA	+	4,652	1,907 (41%)	57	143	187	1,520 (79.7%)
Lipofectin DNA without BSA	+	4,897	3,329 (68%)	0	16	47	3,266 (98.1%)

Table 2. Location of exogenous DNA transferred into oocytes fertilized by sperm cell transfected with fluorescein labeled DNA

Methods	Location of foreign DNA		
	Zona pellucida	Cytoplasm	Pronucleus/nucleus
DNA alone BSA+	28/60	0/60	0/60
Liposome+ DNA+ BSA-	39/60	5/60	0/60
Liposome+ DNA+	47/60	13/60	0/60

BSA(-) is added shortly after tranfection with exogenous DNA.

complexes with and without BSA was significantly higher (41 and 68%) than that of DNA alone (13%). In photography, the signal of exogenous DNA binding in sperm cells was slightly reduced by DNase I treatment, but the number of transfected sperm cells was not changed, indicating that some DNA was incorporated into the sperm membrane (Figure 3A and B).

BSA inhibits the activity of cationic liposome

In the presence of BSA, the number of labeled sperm cells (1907/4652, 41%) was significantly reduced and labeling patterns of foreign DNA were restrained to the posterior and anterior head of spermatozoa (Table 1). Conversely, the absence of BSA increased the numbers of labeled sperm cells (3329/4897, 68%) and the patterns of labeled sperm cells were extended to the connecting piece containing the whole head of spermatozoa (Table 1). These results indicate that BSA reduced the activity of cationic liposome and consequently inhibited the interaction of DNA with sperm cells. To elevate transfection efficiency, sperm cells were incubated with DNA/lipofectin complexes into BSA-free IVF medium and BSA was added shortly after transfection to induce the capacitation. Fertility of sperm cells was not affected by addition of BSA (10 mg/ml) after the sperm cells were left in transfection medium for up to 3 h following transfection.

DNA internalization in sperm nucleus

Figure 1 shows representative labeling patterns of sperm cells transfected with DNA/lipofectin complexes. The association of foreign DNA with the sperm membrane was easily detected, but the internalization of exogenous DNA into the membrane was not confirmed. The effects of DNase I treatment in the transfected sperm cells were
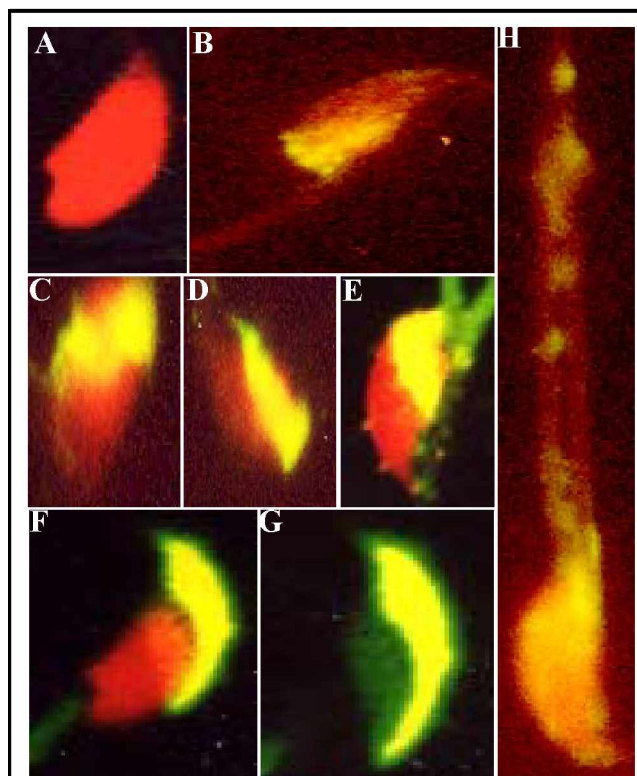

Figure 1. A representative patterns of DNA labeling in mouse sperm cells. For the detail, see the Materials and Methods. Red color indicates staining of sperm nuclei by Hoechst 33258, while green and yellow means binding or uptake by association with DNA. A; control (red), B; neck and post-acrosomal region (weak, yellow), C; post-acrosomal region (strong, yellow), D; post-acrosomal region and equatorial segment (yellow), E; post-acrosomal and tail (yellow and green), F; plasma membrane (yellow), G; whole staining, but plasma membrane was strongly stained, H, whole head and dot staining in tail.

investigated: in both DNase I treated and untreated groups, there is no microscopic difference in the number of the labeled sperm cells (Figure 3A and B). However, LSC analysis demonstrated that the total DNA concentration in the transfected sperm cells which were treated with DNase I, was significantly lower than that of the DNase I-untreated groups (Figure 3C). This indicates that most of the exogenous DNA was bound into the sperm membrane. To explain the LSC analysis, internalization of introduced DNA in sperm cells was investigated by PCR. As shown in Figure 4, only single bands were detected, which indicates simple binding of foreign DNA onto sperm membranes. Taken together, these results suggest that exogenous DNA
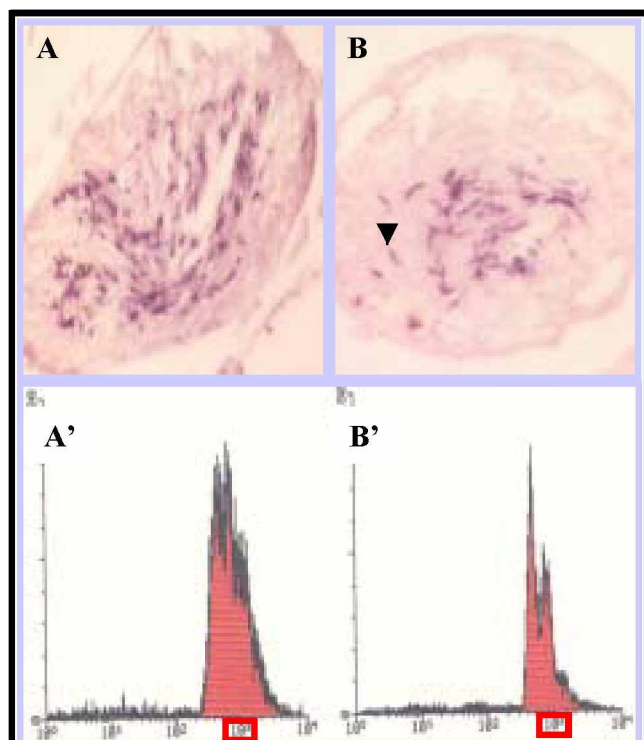
Figure 2. Effect of BSA for the transfection of the sperm cells. Sperm cells were transfected with lipofectin/DNA complexes in the presence and absence of BSA. In A and B, brown color indicates the number of sperm cells transfected under same conditions (arrow). The absence of BSA in comparison with additive of BSA greatly increased the number of the transfected sperm cells (A, BSA absence, B, BSA presence). When DNA concentration of transfected spermatozoa was determined by Laser Scanning Cytometry (LSC) analysis using sample A and B, DNA concentration of transfected sperm cells was increased in case of absence of BSA, indicating that BSA is an inhibitor of cationic liposome. In A' and B', horizontal axis means DNA concentration. Note that transfection efficiency was greatly increased in case of absence of BSA.

was bound onto sperm membranes.

DNA transfer into oocytes by spermatozoa

To investigate the ability of DNA transfer into oocytes, eggs, which have been fertilized by the transfected sperm cells, were investigated by PCR (Figure 5). However, any positive signal was not detected by PCR using cytoplasm alone after removal of zona pellucida (data not shown). Therefore, we have examined whether exogenous DNA transferred under environment of oocyte before pronucleus formation has their integrity without extensive digestion by endonuclease or not. Zona pellucida were removed by using Tyroid solution and zona free oocytes were incubated with transfected sperm cells. Eighteen embryos of 120 oocytes showed a positive signal as shown in Figure 5, suggesting a possibility of ICSI-mediated gene transfer. In case of oocytes with intact zona pellucida, the accumulation of exogenous DNA into the zona pellucida has been shown to
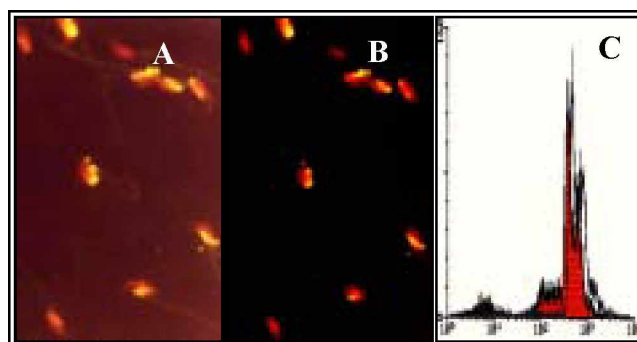

Figure 3. Comparison of labeling patterns and concentration of exogenous DNA when transfected sperm cells was treated or untreated with DNase I. First, foreign DNA from DNase I untreated group was localized onto sperm cells by using *in situ* hybridization after incubation with lipofectin/DNA complexes (A) and measured DNA concentration by using LSC (white line of C). Next, slide of sample A were re-performed in situ hybridization after treatment with DNase I at 37°C for 30 min. Note that DNase I treatment in comparison with DNase I untreated sperm cells slightly reduce the signal of foreign DNA binding, but not for the number of labeled sperm cells. Red color indicates staining of sperm nuclei by Hoechst 33258, while green and yellow means binding or uptake by association with DNA. C) Comparison of DNA concentration in both DNase I treatment and untreatment sperm cell by LSC analysis. Red color indicates DNA concentration of DNase I treated group, but white color means DNA concentration of DNase I untreated group. DNase I untreated sperm cells showed a slightly increased DNA concentration rather than that of DNase I treatment group, indicated some of DNA was bound onto sperm membrane.

begin at 1 h after addition of transfected sperm cells to oocytes. By 8 h postincubation, however, the DNA is rarely localized on cytoplasm of oocytes.

Adoption of foreign DNA into zygote

To examine the integrity of exogenous DNA transferred into oocyte, a spermatozoon was directly microinjected into mouse oocyte, as shown in Figure 6. After activation in a calcium-free medium containing strontium, fertilization and cleavage rates were comparable to those obtained after microinjection of control spermatozoa, but the developmental rate to the blastocyst stage was lower. Embryos harboring foreign DNA were produced by ICSI using fluorescein-tagged DNA encoding the human erythropoietin genomic DNA or lac Z bound on spermatozoa. Both signal of green fluorescence or expression of foreign DNA by ICSI was detected from zygote to blastocyst, indicating the integrity and adoption of exogenous DNA.

DISCUSSION

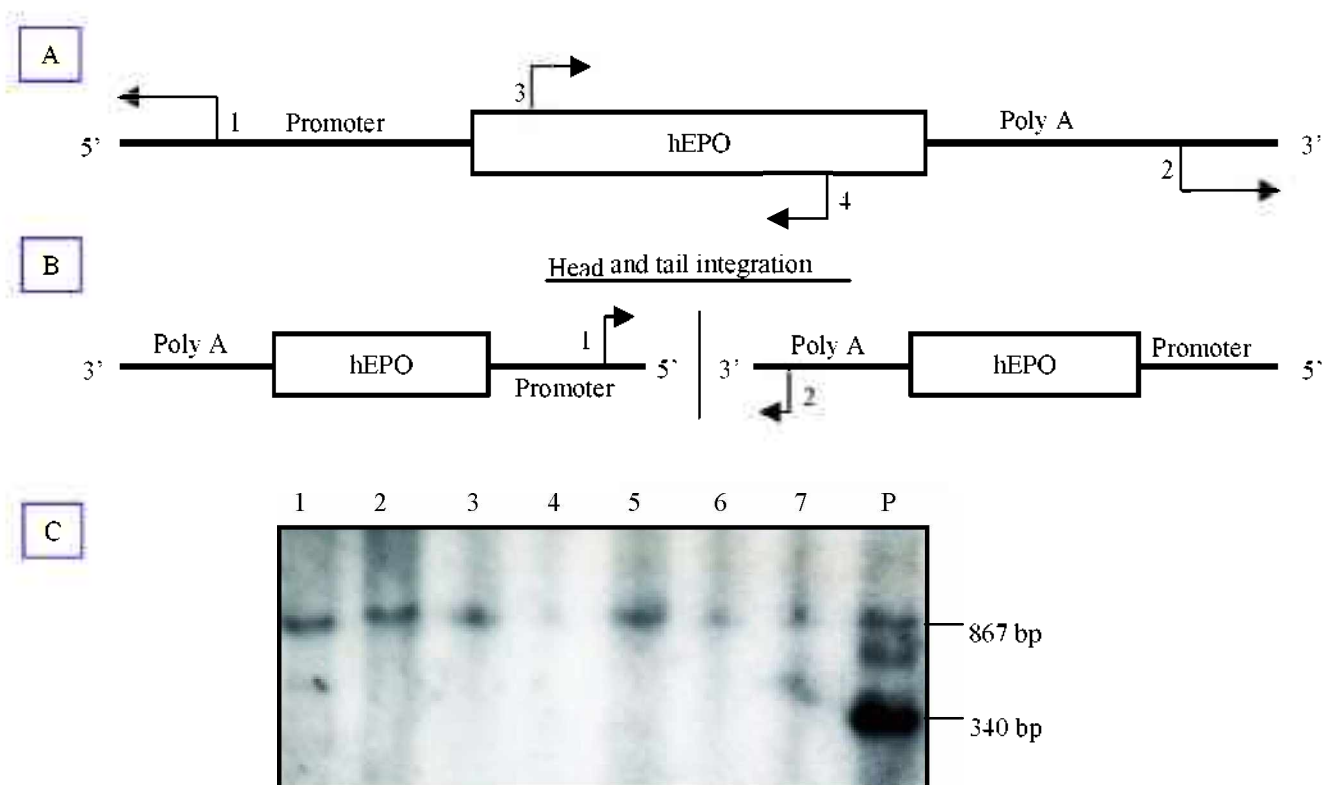The original experiments from Lavitrano et al. (1989)

Figure 4. PCR strategies for the integration analysis of exogenous DNA in the transfected sperm cells. Sperm DNA was extracted by boiling and cooling methods, and subjected into Southern blotting after PCR. DNA purified from the transfected STO cell was used for positive control as described in experiment design III. In case of foreign DNA is integrated into their genome, PCR products will amplify 340 bp. If the PCR products were 867 bp, it means that exogenous DNA was bound onto sperm membrane. Note that sample 1-7 except sample 6 showed a band of 867 bp, while positive control shows 2 bands of 867 bp and 340 bp. A, construction of pZIPhEPO gene; B, strategy for detection of head and tail integration; C, Southern blot analysis.

prompted numerous attempts to reproduce their results and extend the finding to other species. This has been unsuccessful and the conclusion from these experiments is that it is not easier and faster to generate offspring via coincubation of spermatozoa and foreign DNA than through the well established microinjection methods. Thus, most of previous works showed that sperm cells could interact with exogenous DNA and that some of the exogenous DNA could be located into/onto sperm cells (Brackett et al., 1971; Arezzo et al., 1983; Atkinson et al., 1991; Camaioni et al., 1992; Castro et al., 1991; Clausen et al., 1991; Francolini et al., 1993; Gruenbaum et al., 1991; Hochi et al., 1990; Horan et al., 1992; Lavitano et al., 1989, 1992a, 1992b and 1992c; Milne et al., 1989; Perez et al., 1991; Rottmann et al., 1992; Squires and Drake, 1994; Sperandio et al., 1996; Zani et al., 1995). Even though this field is a novel topic, this study is still under examination by several investigator, because of its convenience. Thus, the present study focuses on the association of sperm cells and exogenous DNA, on the possibility of transportation of foreign DNA into oocytes via sperm cells, and on the integration of the foreign DNA into the genome without digestion with endonuclease.

To investigate the possibility of gene transfer via sperm

cells, we have examined the binding and internalization of exogenous DNA into sperm nucleus. The internalization of exogenous DNA into the sperm membrane is especially important, because integration of exogenous DNA into sperm chromatin will participate in the male pronucleus as a component of the zygote genome. However, it is very difficult to prove the incorporation and/or integration of foreign DNA in spermatozoa by using in situ hybridization or LSC analysis. To overcome the technical limitation and to prove the integration of exogenous DNA into sperm cells, we have used inverse PCR. It is well known that injected DNA was integrated into host genome as a tandem array or transiently forms concatemers (Burdon and Wall, 1992). The hypothesis is that if exogenous DNA was taken up or internalized into the sperm cells, the amplified fragments would show the presence of multiple copies such as head and tail arrangement. To avoid concatemers or self-ligation of DNA, the linear DNA was treated with calf intestinal alkaline phosphatase (CIP) and tested for self-ligation by injection of the DNA into 1 cell embryos. We amplified multicopies from embryos injected with CIP-untreated DNA, indicating that some of the DNA was self-ligated or formed concatemers into the nucleus of the embryos. As we
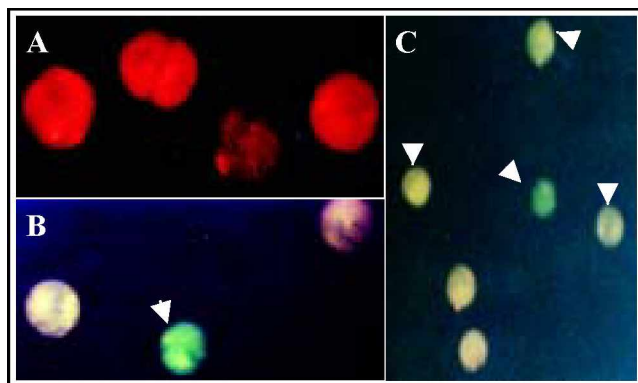
Figure 5. Location of foreign DNA in the embryos by using fluorescent *in situ* hybridization. Zona pellucida of oocyte were removed by Tyroid solution (pH, 3.0) and fertilized by using sperm cells transfected with DNA/lipofectin. A green color indicates that sperm cells transferred exogenous DNA into oocytes. Sperm cells can transfer foreign DNA into cytoplasm of oocytes when removed zona pellucida, but not for case with intact zona pellucida. For labeling of sperm cells, see the Materials and Methods. A, control 2 cells (×200); B, location of foreign DNA in 2 cells embryos (×200); C, location of foreign DNA in 2 cells embryos (×100).
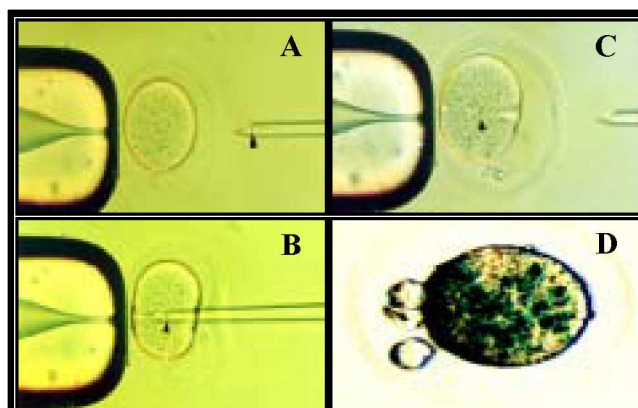

Figure 6. Expression of bacterial β-galactosidase gene in zygote fertilized by ICSI. The transfected spermatozoon was microinjected into cytoplasm of oocyte by using micromanipulator. The zygote and preimplantation embryos was stained with X-gal. Approximately 30% of embryos expressed lacZ gene, but most of expression patterns are mosaic. A. sperm suction (head arrow): B, cytoplasmic injection: C. completion of injection: D. a representative mosaic expression (green color).

expected. we could not find any multi-copies from 1 cell embryos injected with CIP-treated DNA (data not shown). Therefore, we have tested association of sperm cells with foreign DNA by using CIP-treated DNA. As shown in Figure 4. no multiple copies from transfected sperm cells were detected, suggesting that exogenous DNA is simply bound onto sperm cells. To further test this. we studied the change of total DNA concentration in the labeled sperm cells. The transfected sperm cells were treated with DNase I. which digests exogenous DNA attached loosely onto sperm membrane. Using LSC analysis, we were then able to see that the DNA concentration and the number of transfected sperm cells in the DNase I-treated groups were significantly reduced compared to that of the untreated groups (Figure 3). This result was also supported by the observation that the number of labeled sperm was similar to that of the DNase I-untreated groups. but the signal intensity was greatly reduced (Figure 3A and B). Unlike somatic cells. it is well known that the packaging of sperm DNA in mature spermatozoa is highly condensed into a small volume by protamine (Cotten and Chalkley. 1985; Ward and Coffey. 1989 and 1992). Taken together. we surmised that exogenous DNA was not internalized into the sperm nucleus. but simply binds onto the sperm membrane.

Cationic liposome was used to increase the transfection efficiency of sperm cells. Previous data suggested that cationic lipofectin can spontaneously interact with RNA/DNA and fuse with the plasma membrane of a wide variety of cell types in culture to produce transfections (Bachiller et al.. 1991; Felgner et al.. 1987; Francolini et al..

1993; Rottmann et al.. 1992; Stewart. 1991; Wright et al.. 1991) and that association of the cationic liposome/DNA complexes with sperm cells may allow DNA to be carried into oocytes at fertilization (Bachiller et al.. 1991). In agreement with other works. we found that spermatozoa were efficiently bound foreign DNA in the conditions of the cationic liposome/DNA complexes rather than DNA alone. suggested that liposome would be superior to DNA alone. However. sperm motility and fertilizing capability of spermatozoa was lower at the higher concentration of liposome as assessed by microscopic observation. To determine the dose concentration of DNA and cationic liposome required for optimal transfection of sperm cells. nine formulations were prepared at different DNA and liposome concentration. Each formulation was examined by counting the number of labeled spermatozoa using in situ hybridization. In our conditions, we observed high percentage of labeled spermatozoa when used a 1:4 molar ratio of liposome/DNA (4 μg:1 μg). In addition, sperm cells (6×10⁶) were rendered totally infertile after incubation with concentration above 1μg of DNA (data not shown). DNA alone showed a preferential affinity for the apical acrosomal localization of the sperm head. but liposome-mediated gene transfer showed the most frequent of five localization such as whole head containing tail, apical acrosomal. and post-acrosomal localizations (Figure 1).

Recently. Zelphati and Szoka (1996) reported that BSA. a major serum protein. could prevent the cellular uptake of liposome/DNA complexes in cells. In this study. we have tested to see whether the addition of BSA to the transfection

medium inhibited the efficiency of transformation of sperm cells or not. Since BSA is an important inducing factor for capacitation of spermatozoa. BSA was added in the culture medium shortly after transfection. As shown in Table 1 and Figure 2, addition of BSA after transfection greatly enhanced the labeling intensity and the numbers of transfected sperm cells. Furthermore, incubation of sperm cells for up to 3hr did not affect the rate of fertilization and *in vitro* development of embryos. However, the most striking result of these experiments is that the transferred DNA into the oocyte via spermatozoa was not found in the pronucleus. Instead, most exogenous DNA was randomly distributed in the zona pellucida and the cytoplasm. This result suggested that exogenous DNA has been extensively digested during penetration of the zona pellucida or by endonucleases secreted during pronucleus formation.

Even though several methods are available to follow gene distribution within embryos via mature sperm cell, immunofluorescene should be a convenient indicator for the examination of the feasibility of sperm-mediated gene transfer. Because intracellular distributed FITC-labeled DNA is easily detected by commercial available anti-digoxigenin-fluorescein conjugate, it is not limited by availability of substrates. To examine the transportation of foreign DNA into zygotes, the FITC-labeled DNA was prepared with mixture of dUTP-FITC by PCR and oocytes were fertilized by sperm cells incubated with liposome/fluorescein labeled DNA. Exogenous DNA into the zona pellucida and cytoplasm has been detected after addition of transfected sperm cells to oocytes, but exogenous DNA was not detected in the nuclei of embryos. This results suggested that some mature sperm cells could indeed transfer foreign DNA into oocytes at fertilization, but transferred DNA would ultimately be diluted and disappear from the developing embryos.

If it were the case that the DNA bound onto sperm cells was extensively digested during penetration of the zona pellucida, the transfected sperm cells could be enlarged in the production of transgenic animals by using intracytoplasm sperm injection. On the other hand, if the exogenous DNA was extensively digested during formation of the pronuclei, we could not apply this technique in transgenesis, because the transferred DNA will be digested, diluted and ultimately disappearing for the developing embryos. Therefore, it is important to determine whether the foreign DNA has integrity under environment of oocytes before formation of male pronuclei. To test this hypothesis, the zona pellucida of eggs were removed and zona free oocytes were fertilized with sperm cells transfected with exogenous DNA. We have detected some of the exogenous signal in the developing preimplantation embryos (Figure 5). This observation suggests that the exogenous DNA was extensively digested by endogenous

nucleases required for the penetration of zona pellucida. Thus, our results indicated that transgenic animals will be produced by using ICSI-mediated gene transfer.

As discussed in our previous report, the problems of sperm vector may be overcome by the development of an *in vivo* transfection system such as that used for undifferentiated type A spermatogonial stem cells (Kim et al., 1997). In addition, Zelphati and Szoka et al. (1996) have reported that anion liposome/DNA complexes are more effective for transfection of *in vivo* cells than that of cationic liposome/DNA complexes. The merit of anion liposome is that the DNA and liposome separate easily in the cytoplasm, so that the DNA can move into the nucleus. This finding suggests that the development of a new liposome can overcome the limitation of transfection in sperm cells and the transferred DNA/complexes can function efficiently into the cytoplasm of the oocyte (Kukowska-Latallo et al., 1996; Lewis et al., 1996; Wheeler et al., 1996).

In conclusion, we have shown that cationic liposome/DNA complexes were bound onto spermatozoa efficiently, but we have not observed integration of foreign DNA in preimplantation embryos fertilized by transfected and untransfected spermatozoa. Thus, our results lead us to conclude that at least introduction of exogenous DNA into mature spermatozoa may be an impractical technique for producing transgenic animals without further technical improvement.

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