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An Attempt for Sperm-Mediated Gene Transfer by Direct Injection of Foreign DNA into Mouse Testis

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Abstract

The purpose of this study was to demonstrate successful transfection of testicular spermatozoa with foreign DNA by direct DNA injection into testes performed to obtain transgenic animals. The linear plasmid pCAGGS-lacZ DNA [containing the *E. coli* β-galactosidase (β-gal) gene] mixed with nonliposomal lipids, FuGENE™ 6 Transfection Reagent (Boehringer Mannheim GmbH), was injected through a needle into mature ICR mouse testes. At 2 days after injection, the males were mated with superovulated ICR females. When mid-gestational fetuses (E10.5) derived from eggs fertilized by spermatozoa of male mice injected with the foreign DNA were tested for the presence of exogenously introduced plasmid DNA by PCR-Southern analysis, all of the injected males (2 males receiving pCAGGS-lacZ) were found to have transmitted the foreign DNA sequences to their F0 progeny, at frequencies ranging 50.0% to 84.6%. However, no expression of the β-gal gene was observed in mid-gestational fetuses derived from

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eggs fertilized by spermatozoa of males injected with pCAGGS-lacZ, as evaluated by RT-PCR and histochemical staining for β-gal activity. When F1 offspring of two F0 mice carrying pCAGGS-lacZ DNA were tested for gene transmission by PCR Southern analysis, all these F0 mice could transmit their exogenous DNA into F1 offspring at frequencies ranging 16.1% to 23.1%. These transmission rates appeared to be low in view of Mendelian law, suggesting an unusual transmission pattern of DNA introduced by this sperm-mediated gene transfer. This method of direct injection of foreign DNA into the testis appears to be a new approach enabling simple and convenient production of transgenic rodents and other domestic animals, although further improvement of it (especially concerning expression of the introduced gene) will be required.

Introduction

Microinjection of foreign DNA into pronuclei of fertilized 1-cell eggs has been widely used for generation of transgenic animals [1]. However, this technique requires special equipment for DNA microinjection including micromanipulators, in addition to the specific skills needed to perform it.

Gene introduction by fertilization of oocytes by spermatozoa that have been incubated with DNA-containing medium (which is therefore called "sperm-mediated gene transfer") [2] is very simple and rapid, although reproducible results have not yet been obtained with this technique [3]. Bachiller et al. [4] demonstrated that liposome-encapsulated DNA can be incorporated into mouse sperm head after incubation with isolated epididymal spermatozoa, but the sperm which had incorporated DNA failed to generate transgenic animals. Direct injection of Ca-phosphate-precipitated DNA into mouse testes has been performed in an attempt to transfect testicular spermatozoa and spermatogonia; however, it was impossible to produce transgenic mice by this method as well [5]. Ogawa *et al.* [6] recently demonstrated that repeated injection with linearized plasmid DNA [containing β-galactosidase (β-gal) gene] encapsulated with cationic liposome (Lipofectin™ transfection reagent from GIBCO BRL) into testes of adult mouse *via* the scrotum resulted in transmission of the foreign DNA sequences to F0 progeny (blastocysts) through fertilization. They found that 80.0% (16/20) of blastocysts derived

from mating with males receiving the DNA were positively stained with X-Gal, a substrate for β-gal [6].

In this study, we extended the findings of Ogawa *et al.* [6] and showed that (i) a single injection of cationic liposome-circular plasmid DNA complexes into mature mouse testes is sufficient for transfection of testicular spermatozoa, and leads to relatively high efficiency of gene delivery to mid-gestational fetuses obtained by mating of injected males with normal females, and (ii) the introduced DNA in F0 mice can be transmitted to the next generation (F1).

Materials and Methods

Preparation of DNA mixed with FuGENE™ 6 Transfection Reagent and Testis Injection

We used a plasmid DNA, pCAGGS-lacZ [7] (Fig. 1), for direct injection into mouse testes. This plasmid contain cytomegalovirus enhancer/chicken β-actin promoter (hereafter referred to as CAG; 7) for promotion of overexpression of the downstream target gene (lacZ). LacZ (approximately 3.3 kb in size) contains a sequence of the *E. coli* β-gal gene [8]. For injection of DNA into testes, six μl per single testis of nonliposomal solution, FuGENE™ 6 Transfection Reagent (No.1814 443, Boehringer Mannheim GmbH, Mannheim, Germany), was diluted with 23 μl of phosphate-buffered saline without Ca^{2++} and Mg^{2++} [PBS(-)], pH 7.2 and then 11 μl of *Sal*I-linearized pCAGGS-lacZ DNA [4 μg; dissolved in TE (10 mM Tris-Cl, pH 8.0 and 1 mM EDTA)] was then added to the FuGENE™ 6 Transfection Reagent-containing solution to form lipid-DNA complexes. As a control, 29 μl of PBS(-) was mixed with 11 μl of linear plasmid DNA (4 μg). Prior to injection, the solutions (40 μl) were mixed with 3 μl of 0.05% trypan blue dissolved in PBS(-). Trypan blue administration is useful for confirming that a solution has been successfully introduced into a testis under a dissecting microscope.

ICR males [10-15 weeks old; obtained from CLEA Japan, Inc. (Tokyo, Japan)] were anesthetized with pentobarbital, and a lower abdominal incision was made (Fig. 2). The testes were pulled out and exposed. Injection of solutions into testes was performed as previously described [5] with slight modifications. Briefly, a solution containing

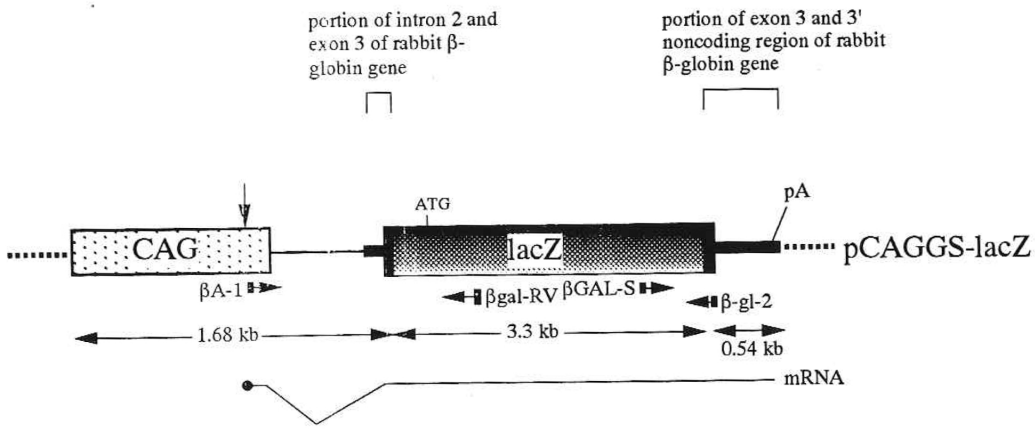

Fig. 1. Schematic representation of the approximately 8.5-kb pCAGGS-lacZ plasmid used for testis injection. In this plasmid, expression of β-gal (lacZ) is driven by the upstream CAG promoter system (cytomegalovirus enhancer/chicken β-actin promoter) (7). The vector backbones (indicated by dotted lines) of pCAGGS-lacZ are pUC13. Arrow indicate transcription initiation site in the chicken β-actin promoter. The one set of primers (βGAL-S/β-gl-2) used for PCR-Southern analysis are indicated below the constructs. The one set of primers (βA-1/βgal-RV) used for RT-PCR analysis is also indicated below the pCAGGS-lacZ construct. Abbreviations: ATG, translation initiation site; lacZ, *E. coli* β-galactosidase gene; pA, poly(A) site of rabbit β-globin gene.

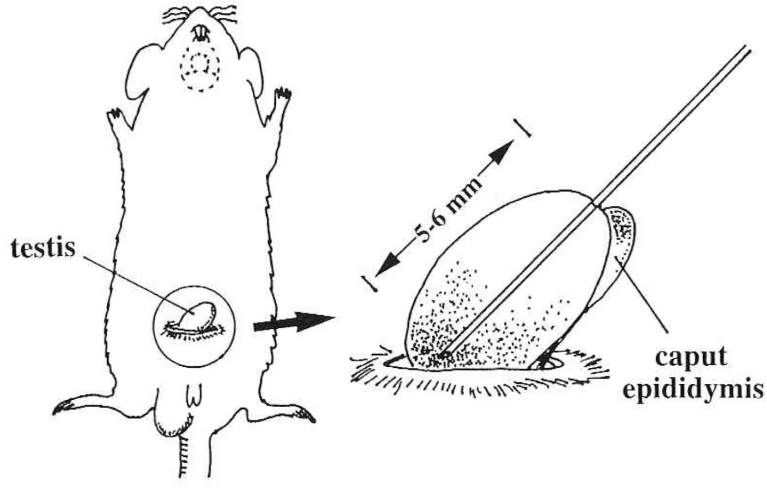

Fig. 2. Schematic representation of DNA injection into mouse testis. Injection of 43 µl of a solution containing plasmid DNA, FuGENE™ 6 Transfection Reagent (in some cases) and trypan blue was performed at the corner of the testis near the caput epididymis to a depth of 5-6 mm.

FuGENE™ 6 Transfection Reagent-plasmid DNA complex was directly and slowly injected with a 30-guage needle (Natsume, Tokyo, Japan) attached to a 1-ml plastic disposable syringe (Termo, Tokyo, Japan) at a depth 5-6 mm through the capsule of the testis near the caput epididymis under a dissecting microscope (Fig. 2). After injection, the needle was slowly removed. At this time, a small number of seminiferous tubules are often observed to have protruded through the injection site outside the capsule, but this does not affect the fertility of males. Both testes were injected. Each of the injected males was continuously mated to two fresh ICR females (4-6 weeks old; obtained from CLEA Japan, Inc.) per cage at 2 days post-injection (dpi). These females had previously been induced to superovulate by two gonadotrophin (PMSG-hCG) treatments spaced about 2 days apart. In the morning after mating, females were inspected for the presence of vaginal plugs, and those with plugs were considered to be at embryonic day 0.4 of gestation (E0.4).

Isolation of Tissues and Preparation of Genomic DNA

Placentas were dissected on E10.5. Tails were dissected from weaned mice. These tissues were subjected to genomic DNA isolation for PCR-Southern analysis, as described below. In some cases, E10.5 fetus with accompanying yolk sac was subjected to RNA isolation or histochemical staining for β-gal activity, as described below.

Genomic DNAs of tails, fetuses with yolk sacs and placentas were extracted, as previously described by Blin and Stafford [9].

PCR-Southern Analysis

One set of PCR primers, βGAL-S/β-gl-2, was designed to yield 431-bp fragments from the 3' region of the β-gal gene (Fig. 1). βGAL-S (5'-GAC CGC TGG GAT CTG CCA TTG-3') corresponds to nucleotides 2,746 to 2,766 in the β-gal gene [8]. β-gl-2 (5'-GTG GTA TTT GTG AGC CAG GG-3') correspond to nucleotides 1,193 to 1,174 in exon 3 of the rabbit β-globin gene [10]. The PCR reactions for detection of the 3' region of the β-gal gene were carried out as previously described [11]. Briefly, nine μl of reaction buffer containing 10 mM Tris-Cl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% (wt/vol) gelatin, 2.5 mM each of dATP, dTTP, dGTP and dCTP, 50 pM of each of two PCR

primers and 0.1 μl of *Taq* polymerase at 5 U/μl (Takara Shuzo Co., Ltd., Shiga, Japan) were mixed with 1 μl of genomic DNA (approximately 0.5 μg), and the reaction mixture was used for PCR. Forty cycles of PCR were performed with cycle times of 1 min at 94°C, 1 min at 58°C, and 4 min at 72°C. The reaction mixture was then analyzed on 2% agarose gels. The gels were stained with ethidium bromide (EtBr), and amplified DNA bands were visualized by ultraviolet transillumination. The gels were then transferred to GeneScreen*Plus* filters (NEN, Boston, MA), which were hybridized with ^{32}P-labelled 3'-β-gal probe (an *Eco*RV and *Bam*HI 1.99-kb fragment of pMC1527 [12]; corresponding to the 3' portion of β-gal gene, as previously described [5]. As a positive control, 5 ng of pCAGGS-lacZ DNA was used.

Direct Sequencing of PCR-Amplified Product

Thirty microliters of the PCR containing the 431-bp product were electrophoresed through a 2% low melting temperature agarose gel (SeaPlaque® GTG® Agarose; FMC BioProducts, Rockland, ME). The gel slice containing the 431-bp PCR product was melted at 65°C for 20 min and then extracted by saturated phenol. After precipitation with ethanol, the gel-purified PCR product was evaluated for automated fluorescent sequencing analysis using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS (PE Applied Biosystems, Foster City, CA) and β-gl-2 primer.

RT (reverse transcription)-PCR Analysis of β-gal mRNAs

Total RNA was isolated by the method of Chomczynski and Sacchi [13] using ISOGEN reagent (Wako Pure Chemical Industry, Tokyo, Japan). cDNA was prepared from total RNA (4 μg) by RT using 10 pmol of each reverse primer, βgal-RV and mβA-RV, in a final volume of 20 μl, as previously described [14]. The cDNA solution (3 μl) was mixed with 17 μl of PCR buffer [final concentrations in 20 μl reaction solution: 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 5 pmol each of the forward and reverse primers, 1 U *Taq* DNA polymerase (Takara Shuzo Co. Ltd.), and 0.2 mM each of dATP, dCTP, dGTP, and dTTP], and was then amplified. The PCR reaction conditions with 24 cycles (except for amplification of mouse β-actin mRNA, for which

15 cycles were employed) were denaturation at 94℃ for 1 min, annealing at 58℃ for 1 min, and extension at 72℃ for 2 min. The PCR primer set for detection of β-gal mRNA derived from transcription from the CAG promoter was as follows: forward primer, βA-1, 5'-TCT GAC TGA CCG CGT TAC TCC CAC A-3', corresponded to the chicken β-actin gene sequence (15) from nucleotides -1,011 to -987 (Fig. 1), while the reverse primer βgal-RV, 5'-AAC AAA CGG CGG ATT GAC CGT AAT G-3', was complementary to nucleotides 342-318 (Fig. 1) of β-gal gene [8]. The PCR primer set (mβA-S/mβA-RV) for detection of mouse β-actin mRNA was used as previously described [14]. These primer sets (βA-1/βgal-RV and mβA-S/mβA-RV) were designed to produce PCR fragments of respectively approximately 670 bp and 310 bp, which included an intron-exon border, thereby eliminating the possibility that DNA contamination was responsible for the resulting target products present in the tissue cDNAs. The resulting products (10 μl) were subjected to electrophoresis in 2% agarose gels and stained with EtBr. After staining with EtBr, gels were blotted onto GeneScreen*Plus* filters for Southern blot hybridization analysis, as previously described [5]. The labeled 5'-β-gal probe (an *Eco*RV and *Bam*HI 1.11-kb fragment of pMC1527; corresponding to the 5' portion of β-gal gene) was used for detection of β-gal mRNA as a probe. As a positive control, total RNA isolated from a transfected ES clone expressing β-gal under CAG promoter was used.

Staining of Fetuses for β-gal Activity

E10.5 fetuses was fixed with 1.25% glutaraldehyde and 2% paraformaldehyde in PBS(-) for 15 min at room temperature. After washing three times with 5 ml solution of 0.05% bovine serum albumin (BSA) in PBS(-), they were transferred to a solution containing 1.2 mM 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), 1 mM $MgCl_2$, 0.1% Triton X-100, 3 mM potassium ferrocyanide (P-3289, Sigma, St. Louis, MO), and 3 mM potassium ferricyanide (P-3667, Sigma) in PBS (-), and incubated at 30℃ for 24 h. Embryos exhibiting β-gal activity are expected to exhibit blue deposits throughout their entire body.

Results

Identification of Plasmid DNA in Mid-Gestational F0 Fetuses Obtained Following Mating with DNA-Injected Males

We examined whether fetuses (E10.5) produced by mating with males which had undergone DNA injection possessed the introduced DNA. On PCR-Southern analysis of genomic DNA isolated from a total of 33 F0 placentas (E10.5) derived from 2 different males (FuZ-2 and -4), 50.0% to 84.6% of the placentas had detectable levels of amplified products of the expected size (Fig. 3A; Table 1). The amounts of the amplified products varied among samples. When only pCAGGS-lacZ DNA (without FuGENE™ 6 Transfection Reagent) was injected into testes of one adult mouse, which were then mated to superovulated females, no transmission of the foreign DNA to their F0 fetuses was observed (Table 1). We also tested the transgenicity in the tails of weaned F0 offspring derived from another two DNA-injected males (FuZ-3 and -6). PCR-Southern analysis revealed that 1 of 3 (33.3%) offspring from FuZ-3 male and 13 of 20 (65.0%) offspring from FuZ-6 male were identified to be transgenic (data not shown). These findings indicate that the exogenous DNA introduced into testes can be transmitted *via* fertilization to mid-gestational fetuses and weaned mice.

Identification of Plasmid DNA in the Tails of F1 Mice Obtained Following Mating between F0 Transgenic Males and Normal Females

We next tested whether F0 mice derived from spermatozoa receiving exogenous DNA can transmit the exogenous DNA to their F1 offspring. Two F0 males (termed FuZ-6-4 and -6-14) carrying pCAGGS-lacZ DNA derived from FuZ-6 male were mated with normal ICR females. Tails were dissected from the resulting weaned F1 mice and subjected to PCR-Southern analysis. Examples of profiles of transmission of DNA into F1 mice obtained by mating with FuZ-6-14 male are shown in Fig. 3B. Several samples (including lanes 3, 13, 25, 26 and 28) possessed the exogenous DNA. The transmission rate was thus 16.1% (5/31). Similarly, 23.1% (3/13) of F1 mice derived from FuZ-6-4 male were identified to be transgenic (data not shown). These findings indicate that (i) injection of FuGENE™ 6 Transfection Reagent-DNA complexes into adult mouse testes

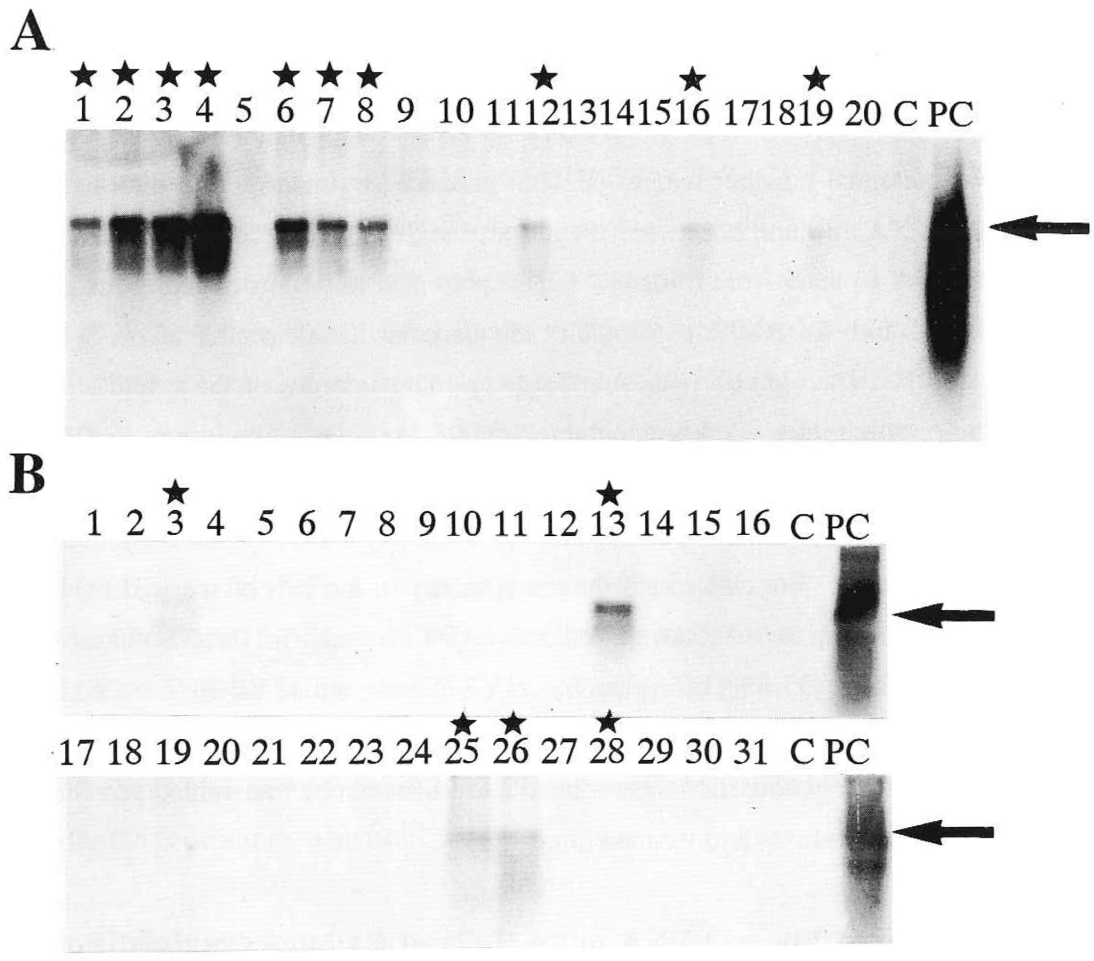

Fig. 3. PCR-Southern analysis of genomic DNA isolated from mid-gestational F0 fetuses (column A) and F1 mouse tails (column B) using primers recognizing the 3' portion of β-gal sequence. Genomic DNAs were extracted, as previously described by Blin and Stafford (9). The PCR reactions using βGAL-S and β-gl-2 primers yielded 431-bp product (indicated by arrows). 3'-β-gal probe (1.99-kb *Eco*RV/*Bam*HI-digested fragment of β-gal gene; corresponding to the 3' portion of β-gal gene) was used for detection of pCAGGS-lacZ. In column A, samples loaded in lanes 1 to 20 were from F0 offspring of one male, FuZ-4. In column B, samples loaded in lanes 1 to 31 were from F1 offspring of F0 male, FuZ-6-14. C, Normal genomic DNA as a negative control; PC, control tail DNA plus 5 ng of pCAGGS-lacZ as a positive control. Several samples were observed to possess the β-gal gene (indicated by asterisks).

Table 1. Transgenicity and gene expression in mid-gestational F0 fetuses derived from FuZ-2 and FuZ-4 males after testis injection with liposome-plasmid DNA complex into mouse testes

F0 Offspring[1]	Males injected with exogenous DNA[2]	Fetuses obtained at E10.5		
		Transgenicity[3]	Expression of b-gal mRNA[4]	Expression of b-gal protein[5]
FuZ-2-1	FuZ-2	Tg	ND	-[6]
FuZ-2-2	FuZ-2	Tg	ND	-
FuZ-2-3	FuZ-2	Tg	ND	-
FuZ-2-4	FuZ-2	Tg	ND	-
FuZ-2-5	FuZ-2	Tg	ND	-
FuZ-2-6	FuZ-2	Tg	ND	-
FuZ-2-7	FuZ-2	Tg	ND	-
FuZ-2-8	FuZ-2	non-Tg	ND	-
FuZ-2-9	FuZ-2	Tg	ND	-
FuZ-2-10	FuZ-2	Tg	ND	-
FuZ-2-11	FuZ-2	non-Tg	ND	-
FuZ-2-12	FuZ-2	Tg	ND	-
FuZ-2-13	FuZ-2	Tg	ND	-
FuZ-4-1	FuZ-4	Tg	-	ND
FuZ-4-2	FuZ-4	Tg	-	ND
FuZ-4-3	FuZ-4	Tg	-	ND
FuZ-4-4	FuZ-4	Tg	-	ND
FuZ-4-5	FuZ-4	non-Tg	-	ND
FuZ-4-6	FuZ-4	Tg	-	ND
FuZ-4-7	FuZ-4	Tg	-	ND
FuZ-4-8	FuZ-4	Tg	-	ND
FuZ-4-9	FuZ-4	non-Tg	-	ND
FuZ-4-10	FuZ-4	non-Tg	ND	-
FuZ-4-11	FuZ-4	non-Tg	ND	-
FuZ-4-12	FuZ-4	Tg	ND	-
FuZ-4-13	FuZ-4	non-Tg	ND	-
FuZ-4-14	FuZ-4	non-Tg	ND	-
FuZ-4-15	FuZ-4	non-Tg	ND	-
FuZ-4-16	FuZ-4	Tg	ND	-
FuZ-4-17	FuZ-4	non-Tg	ND	-
FuZ-4-18	FuZ-4	non-Tg	ND	-
FuZ-4-19	FuZ-4	Tg	ND	-
FuZ-4-20	FuZ-4	non-Tg	ND	-
Control[7]	Z-1	non-Tg (10)[8]	ND	- (10)[8]

[1]F0 offspring were obtained after mating between DNA-njected males (FuZ-2 and -4) and normal females.

[2]Males injected with FuGENE™ 6 Tranafection Reagent-plasmid DNA complex (for FuZ-2 and -4) or plasmid DNA only (for Z-1) were mated at 2 dpi to ICR females that had been induced to superovulate.

[3]Transgenicity was determined by PCR-Southern analysis of genomic DNA isolated from placenta.

[4]Expression of b-gal mRNA from the introduced pCAGGS-lacZ plasmid in fetuses (with yolk sacs) was examined by RT-PCR. "-" indicates samples showing no expression of b-gal mRNA.

[5]Expression of b-gal protein was examined by histochemical staining of fixed fetuses in the presence of X-Gal.

[6]"-" indicates samples showing no positive staining for b-gal activity except for yolk sacs which exhibited non-specific staining for b-gal activity.

[7]F0 offspring was obtained by mating with a male (Z-1) receiving plasmid DNA only.

[8]Parentheses indicate the number of fetuses examined.

ND=not determined.

results in efficient production of fetuses carrying the introduced DNA after injection of males and subsequent mating with superovulated females, and (ii) the introduced DNA can be delivered through generation (at least from F0 to F1), although the effciency of gene delivery appears to be low.

Sequence Fedility of PCR-Amplified Products in F0 Mice Obtained by Sperm-Mediated Gene Transfer

To confirm whether the PCR-amplified products obtained from F0 transgenic mice using βGAL-S/β-gl-2 primer set have the same sequence as does the introduced pCAGGS-lacZ DNA, tail genomic DNA from FuZ-6-14 mouse (F0) was subjected to PCR and the resulting PCR products were directly sequenced. As a positive control, pCAGGS-lacZ plasmid DNA (5 ng) was concomitantly amplified by PCR. The results are shown in Fig. 4. In each sample, the amplified products contained a portion of the exon 3 of rabbit β-globin gene and a 3' portion of β-gal gene, as expected, and shared the same seqeunce.

Expression of β-gal in Mid-Gestational F0 Fetuses Derived from Mating Between DNA-Injected Males and Normal Females

On histochemical staining of a total of 14 E10.5 transgenic fetuses (including 11 F0 offspring of the FuZ-2 male and 3 F0 offspring of the FuZ-4 male; see Table 1), none of the fetuses was positive for β-gal activity (data not shown). Only distinct staining in the yolk sacs was observed in both transgenic and non-transgenic fetal samples (data not shown). When expression of β-gal mRNA was evaluated by RT-PCR for a total of 7 fetal transgenic samples derived from FuZ-4 male, none of the samples expressed β-gal mRNA (Fig. 5; Table 1). Based on these findings, we concluded that there is no expression of the exogenous DNA (if present, its level may be very low) in F0 fetuses obtained by testis injection.

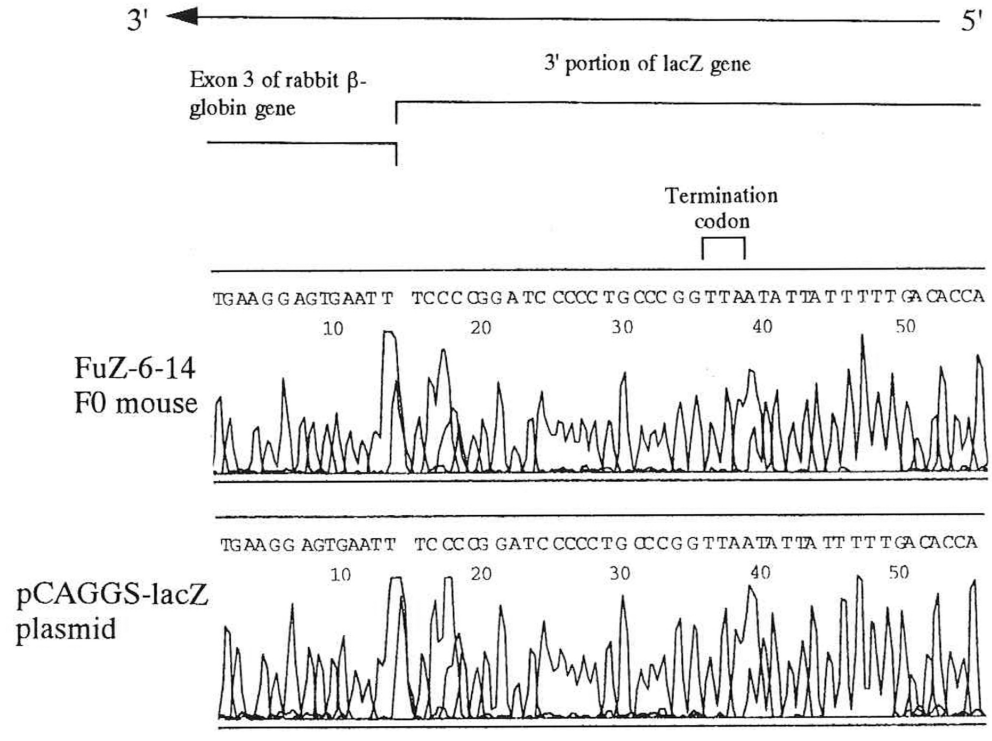

Fig. 4. Direct sequencing of PCR-amplified fragments derived from F0 offspring (FuZ-6-14) of FuZ-6 male and pCAGGS-lacZ DNA (control). The portion spanning a boundary between exon 3 of rabbit β-globin gene and the 3' portion of β-gal gene was amplified by PCR using βGAL-S and β-gl-2 primers, and the PCR products were directly sequenced. FuZ-6-10 mouse exhibited the same sequence profile of pCAGGS-lacZ.

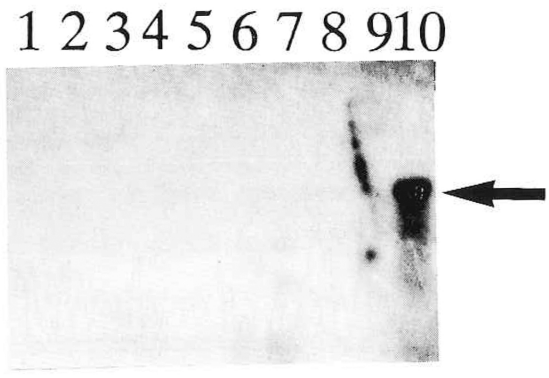

Fig. 5. RT-PCR analysis of total RNAs isolated from mid-gestational F0 fetuses derived from FuZ-4 male. Lanes 1 to 9, F0 fetuses (FuZ-4-1 to -4-9; see Table 1); lane 10, ES clone expressing β-gal mRNA as a positive control. No expression of β-gal mRNA was observed in the experimental group, while a single band with approximately 670 bp (indicated by arrow) was detected in the positive control.

Discussion

We have shown here that direct introduction of liposome-plasmid DNA complexes into mature mouse testis can result in production with relatively high efficiency of fetuses carrying the foreign DNA. This procedure can be successfully performed by mating with superovulated females only 2 days after testis injection. These findings suggest that the foreign DNA injected into mouse testis is uptaken by fully mature spermatozoa (probably epididymal spermatozoa) and then transmitted to oocytes *via* fertilization. It is also possible that the injected DNA was uptaken by testicular sperm such as spermatogonia, spermatocytes and spermatids. However, these transfected testicular sperm appear not to have contributed to the *in vivo* gene delivery observed in this study, since more than 2 days is required for development of fully mature spermatozoa which can transmit DNA to oocytes upon fertilization. It has generally been thought that 30-35 days are required for spermatogonia to develop into fully mature spermatozoa in mice. This suggests that liposome-DNA complex injected into a testis is rapidly transferred to its epididymal portion, where it is uptaken by epididymal spermatozoa.

PCR-Southern analysis demonstrated that certain percentages of the mid-gestational fetuses derived from mating of the DNA-injected males possessed the exogenous plasmid DNA. Although genomic Southern blot analysis of these PCR-Southern-positive DNAs failed to manifest clear and distinct hybridizable bands (data not shown), the exogenous DNA may have been present at a frequency of less than 1 copy per diploid cell, as previously pointed out [16]. Probably, the DNA introduced into adult mouse testis may be present in the fetal tissues mosaically. This possibility may also be supported by the present observation that the transmission rates of the introduced DNA from F0 to F1 generation were between 16.1% and 23.1%. In any event, further experiments will be required to carefully examine the state of the introduced foreign plasmid DNA in the fetal and adult tissues obtained using our method.

In this study, we were unable to detect expression of the exogenous DNA in fetal samples derived from males receiving it even with RT-PCR (a very sensitive method for detection of mRNA). This failure appears to have been due to the small amounts of exogenous DNA present in these samples. Ogawa *et al.* [6] previously demonstrated that 80.0% of blastocysts, derived from mating with males receiving the DNA by repeated

injection with linearized plasmid DNA (containing β-gal gene) encapsulated with cationic liposome into their testes, were positively stained with X-Gal. The main difference in methods used between the study by Ogawa *et al.* [6] and the present study is the number of testis injections [a single injection in our study *vs.* three injections at 4-day intervals by Ogawa *et al.* [6]]. It is conceivable that multiple injections into male testes may result in greater uptake of exogenous DNA by epididymal spermatozoa than does a single injection.

Although the method described here requires further improvement for use as an alternative method for transgenic animal production, it will be of great interest to apply it to the production of other domestic transgenic animals, which has been considered difficult with presently available microinjection techniques [17]. In order to make our technique practically useful, several questions must be answered, including the following: How is the introduced DNA expressed in fetal and adult stages? And why is the DNA introduced present at a frequency of less than 1 copy per diploid cell? Experiments designed to answer these questions are now underway.

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