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Direct Injection of Foreign DNA Into Mouse Testis as a Possible In Vivo Gene Transfer System via Epididymal Spermatozoa

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ABSTRACT We have attempted to transfect testicular spermatozoa with plasmid DNA by direct injection into testes to obtain transgenic animals [this technique was thus termed "testis-mediated gene transfer (TMGT)"]. When injected males were mated with superovulated females 2 and 3 days after injection, (i) high efficiencies (more than 50%) of gene transmission were achieved in the mid-gestational F0 fetuses, (ii) the copy number of plasmid DNA in the fetuses was estimated to be less than 1 copy per diploid cell, and (iii) overt gene expression was not found in these fetuses. These findings suggest the possibility that plasmid DNA introduced into a testis is rapidly transported to the epididymis and then incorporated by epididymal spermatozoa. The purpose of this study was to elucidate the mechanism of TMGT by introducing trypan blue (TB) or Hoechst 33342 directly into testis. We found that TB is transported to the ducts of the caput epididymis via rete testis within 1 min after testis injection, and TB reached the corpus and cauda epididymis within 2–4 days after injection. Staining of spermatozoa isolated from any portion of epididymis was observed 4 days after injection of a solution containing Hoechst 33342. Injection of enhanced green fluorescent protein (EGFP) expression vector/liposome complex into testis resulted in transfection of epithelial cells of epididymal ducts facing the lumen, although the transfection efficiency appeared to be low. In vivo electroporation toward the caput epididymis immediately after injection of EGFP expression vector into a testis greatly improved the uptake of foreign DNA by the epididymal epithelial cells. PCR analysis using spermatozoa isolated from corpus and cauda epididymis 4 days after injection of a DNA/liposome complex into testis revealed exogenous DNA in these spermatozoa even after treatment with DNase I. These findings indicate that exogenous DNA introduced into tesits is rapidly transported to epididymal ducts via the rete testis and efferent ducts, and then incorporated by epithelial cells of epididymis and epididymal spermatozoa. *Mol. Reprod. Dev. 61: 49–56, 2002.*

Key Words: electroporation; epididymis; hoechst 33342; in vivo gene transfer; sperm vector; testis; transfection; trypan blue

INTRODUCTION

Gene transfer via in vitro fertilization of oocytes by spermatozoa that have been incubated with DNA-containing medium ("sperm-mediated gene transfer") is a unique system for efficient production of transgenic animals because of its simplicity (Lavitrano et al., 1989). However, attempts to produce transgenic animals using spermatozoa as vectors of foreign DNA have repeatedly failed, with a group of negative results summarized in a communication by Brinster et al. (1989). Recently, Lavitrano and her collaborators demonstrated that this "sperm-mediated gene transfer" is repeatable (Maione et al., 1998).

We have explored the possibility of in vivo gene transfer through direct introduction of foreign DNA into testes, so-called "testis-mediated gene transfer (TMGT)" (Ogawa et al., 1995; Sato et al., 1999a,b). When a plasmid DNA/liposome complex was singly or repeatedly injected into mature mouse testes, and 2–4 days later injected males were mated with superovulated females, it was found that (i) transfection of spermatozoa (epididymal spermatozoa) leads to relatively high efficiency (50–100%) of gene delivery to F0 mid-gestational fetuses obtained by mating of injected males with normal females (Sato et al., 1999a), (ii) the introduced DNA may have been present mosaically in these fetal tissues, since it was estimated to be present at less than one copy per diploid cell (Sato et al., 1999a), (iii) the introduced DNA was found to be transmitted at least to the second generation (Sato et al., 1999b), and (iv) expression of the introduced DNA was evident only

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in F0 early blastocysts (E 3.4), but almost absent in F0 mid-gestational fetuses (E 10.4) and organs of adult F0 and F1 mice (Ogawa et al., 1995; Sato et al., 1999a,b). Another group (Chang et al., 1999a) demonstrated with use of a TMGT method essentially the same as ours that the plasmid DNA injected into testes was transmitted via fertilization to F0 offspring with efficiencies of 5.6–17.6% for rats and 9.7% for mice, when the injected males were mated with normal females 4 days after testis injection. Notably, Chang et al. (1999a) demonstrated using genomic Southern blot hybridization that these positive samples had more than one copy of the DNA per diploid cell, in contrast with our findings as described above.

The mechanism of TMGT has not been investigated extensively. Chang et al. (1999b) first examined this mechanism using confocal microscopy of frozen sections of an epididymis prepared 4 days after testis injection with fluorescence-labelled DNA, and demonstrated that the exogenous DNA bound to the surface of spermatozoa in the cauda epididymis. This finding suggests that the exogenous DNA introduced into a testis is transferred to epididymis within 4 days and then binds to epididymal spermatozoa. However, it remains unclear how and how long the introduced DNA can reach the epididymal portion or which route is used upon transfer of the DNA from testis to epididymis.

In this study, we examined the fate of a solution introduced into a testis using trypan blue (TB), a dye generally utilized in staining dead cells in cell culture systems, and Hoechst 33342, a fluorescent dye generally utilized in staining cell nuclei, and found that a solution introduced into testis is transported to the ducts of caput epididymis via the rete testis and efferent ducts immediately after testis injection, and reached corpus and cauda epididymis within 3–4 days after injection. We also found using PCR analysis that spermatozoa isolated from the corpus and cauda epididymis 4 days after testis injection of a DNA/liposome complex possessed the introduced DNA. Based on these findings, we describe here a possible mechanism of TMGT: when exogenous DNA is introduced directly into testis, it is rapidly transferred to epididymis via the rete testis and efferent ducts and then incorporated by epididymal spermatozoa, which will subsequently transfer the DNA to oocytes through fertilization.

MATERIALS AND METHODS

Testis Injection With Trypan Blue, Hoechst 33342 or Plasmid DNA

Injection of TB into mouse testes was performed using a previously described method (Sato et al., 1999a,b; Fig. 1a) with slight modification. Briefly, 70 μl of TB (Trypan Blue Stain 0.4%, No. 15250-061, GIBCO BRL, Grand Island, NY) was slowly injected with a 30-guage needle (Natsume, Tokyo, Japan) attached to a 1-ml plastic disposable syringe (Termo, Tokyo, Japan) at a depth of 5–6 mm through the capsule of the testis of ICR males (CLEA Japan, Inc., Tokyo, Japan), aged 10–15 weeks (Fig. 1a). After injection, the needle was slowly removed. The fate of TB injected into a testis was then monitored immediately and 1–4 days after injection by photography using a digital camera (FUJIX HC-300/OL, Fuji Film, Tokyo, Japan) attached to a stereomicroscope (SZX12, Olympus, Tokyo, Japan), and results were printed out using a digital color printer (CP700DSA, Mitsubishi, Tokyo, Japan).

Injection of a PBS(−) solution (70 μl) containing Hoechst 33342 [CALBIOCHEM®, NO. 382065, Calbiochem Co., La Jolla, CA; in a final concentration of 50 μg/ml] and 3 μl of TB into a mouse testis was by the same method as described above. As controls, PBS(−) plus TB were injected. Four days after testis injection, spermatozoa were isolated from the caput epididymis, corpus and cauda epididymis, and washed with PBS (−)/0.05% bovine serum albumin (BSA) (No. A-7906, Sigma Chemical Co., St. Louis, MO) three times. They were then observed for fluorescence using a digital camera attached to a light microscope (BX60, Olympus) with DM400 filters (BP330-385 and BA420, Olympus), and results were printed out using a digital color printer.

Mouse testis was injected with 70 μl of PBS (−) containing a complex comprising 20 μg of circular enhanced green fluorescent protein (EGFP) expression plasmid pCE-29 (Fig. 1b), 10 μl of FuGENE™ 6 (Boehringer Mannheim GmbH, Mannheim, Germany) and 3 μl of TB. Two days later, the caput epididymis and corpus were excised from the DNA-injected males and inspected for EGFP fluorescence using a Olympus SZX12 stereomicroscope under UV illumination with DM505 filters (BP460-490 and BA515IF, Olympus).

In some cases, mouse testis was injected with 70 μl of PBS (−) containing a complex comprising 20 μg of circular plasmid DNA pCETZ-17 (Fig. 1b; Sato et al., 2000), 10 μl of FuGENE6 and 3 μl of TB. Four days later, the corpus and cauda epididymis were excised from the DNA-injected males. Spermatozoa were released in a 300 μl drop (covered with paraffin oil) of TYH medium, a medium used for in vitro fertilization in mice (Toyoda et al., 1971), by mincing the excised corpus or cauda epididymis, washed three times with PBS(−)/0.05% BSA, and then subjected to genomic DNA isolation, as described below.

Injection of Testis With an Enhanced Green Fluorescent Protein Expression Vector and Subsequent In Vivo Electroporation

PBS (−) (70 μl) containing circular pCE-29 (20 μg) and 3 μl of TB was injected into a mouse testis, as described previously. Immediately after the injected solution was confirmed to have been transported to the epididymal ducts, in vivo electroporation (EP) was performed on the caput epididymis using the method described by Yamazaki et al. (1998) with slight modification. Briefly, after DNA injection into testis, EP was performed with an electroporator (Electrosquare

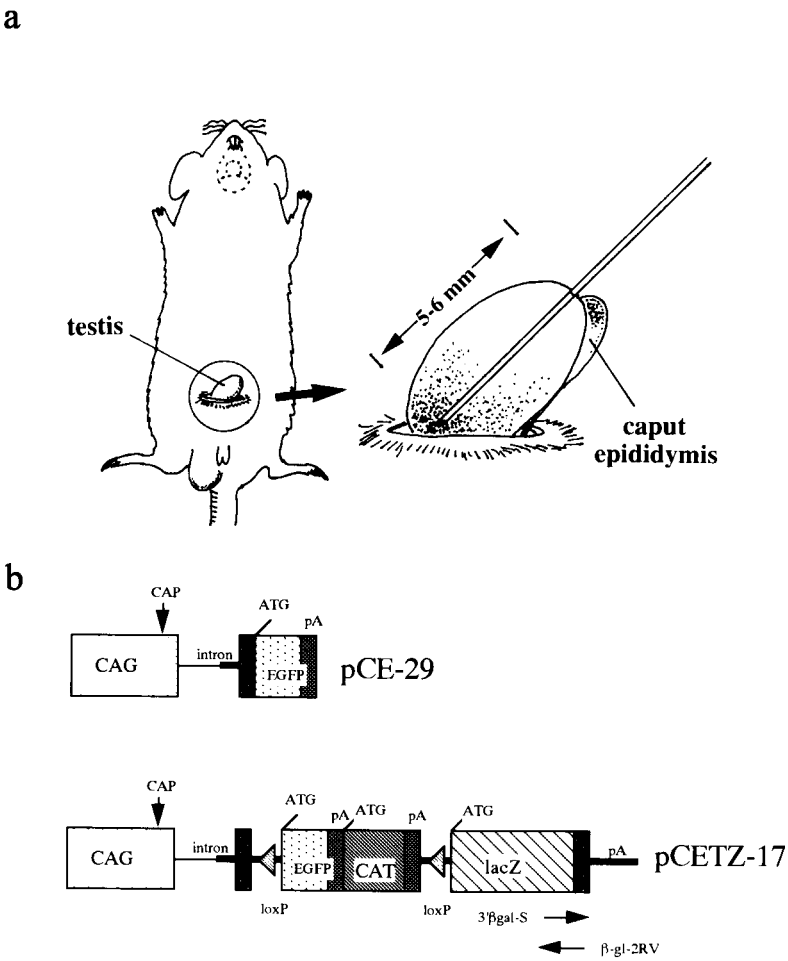

Fig. 1. (**a**) Schematic representation of DNA injection into mouse testis. Injection of 70 μl of a solution was performed at the corner of the testis near the caput epididymis to a depth of 5–6 mm. (**b**) The plasmid constructs [pCE-29 and pCETZ-17 (Sato et al., 2000)] cloned in pBluescript SK(−) vector (Stratagene, La Jolla, CA). pCE-29 consists of CAG (composed of cytomegalovirus enhancer and promoter and a portion of the 1st intron of chicken β-actin gene) (Niwa et al., 1991), a portion of rabbit β-globin gene (including a portion of the 2nd intron and 3rd exon; shown by solid line), and the 1-kb *Sma* I/*Ssp* I EGFP cDNA plus poly(A) signals from SV40 gene [SV40-poly(A)] isolated from pEGFP-N1 (CLONTECH Laboratories, Inc., Palo Alto, CA). pCETZ-17 was constructed by inserting the 1-kb EGFP cDNA and poly(A) signals into the *Xho* I site (the site had been blunted by Klenow treatment) on the 5′ end of the chloramphenicol acetyltransferase (CAT) gene in pCAG-CAT-Z (Araki et al., 1995). Each primer used for PCR is shown below the construct. Abbreviations are: ATG, translation initiation site; CAP, cap site for transcription initiation; pA, poly(A) site; lacZ, gene encoding β-galactosidase.

Porator T820, BTX, SanDiego, CA). The caput epididymis was held between a tweezers-type electrode, and square electric pulses were applied seven times at 50 V with a constant time of 50 msec. These treatments produced no noticeable damage to the epididymis at histopathological level. One day after testis injection and subsequent EP, the epididymis was dissected and inspected for EGFP fluorescence using a Olympus SZX12 stereomicroscope under UV illumination with DM505 filters. After observation, the epididymis was fixed with 4% paraformaldehyde in PBS(−) at 4°C overnight, dehydrated in a sucrose series in PBS(−) and embedded in O.C.T. compound (Tissue-Tek, No. 4583, Miles Scientific, Naperville, IL) for cryostat sectioning. The sections were observed for EGFP fluorescence using a Olympus BX60 microscope under UV illumination.

Treatment With DNase I and Preparation of Genomic DNA

Spermatozoa were isolated from the two corpora or two cauda epididymides 4 days after testis injection of a complex of pCETZ-17 DNA and FuGENE6 into a male. The sperm suspension was divided into two portions (150 μl for each). One portion was mixed with 200 μl of TYH medium containing 20 U of RNase-free DNase I (1,000 U/ml; Promega Co., Madison, WI) and incubated at 37°C for 2 hr in order to remove foreign DNA which might have attached to the surface of spermatozoa, while the other portion was mixed with 200 μl of DNase I-free TYH medium and processed as for the DNase I-treated group. Each portion was then washed with PBS(−)/0.05% BSA three times prior to isolation of genomic DNA. As a control to test the fidelity of

RNase-free DNase I, pCETZ-17 DNA (0.5 μg) was incubated in 200 μl of TYH medium containing 20 U of RNase-free DNase I at 37°C for 2 hr and then phenol-treated to remove protein components. The solution was ethanol-precipitated and dissolved in 10 μl of water to a final concentration of approximately 0.5 μg/μl of DNA. The DNase I-untreated pCETZ-17 DNA (0.5 μg) was also used as a control.

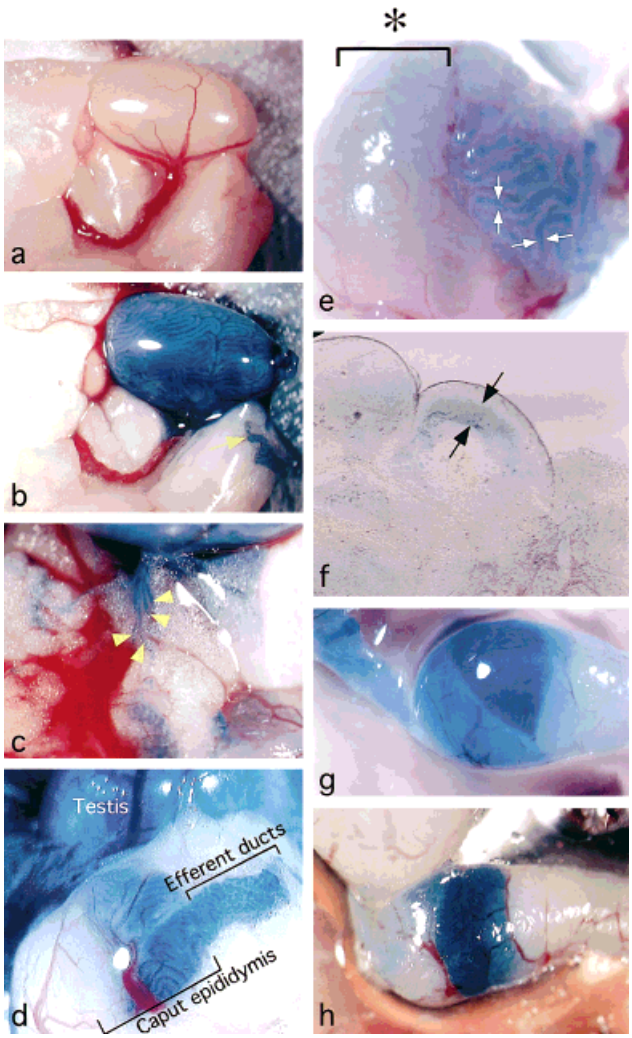

Fig. 2. (**a,b**) Photographs before (a) and after (b) injection of TB into testis. Note that TB injected was widely dispersed within a testis. Arrow indicates the efferent ducts into which TB was rapidly transferred after TB injection. (**c**) Efferent ducts proximal to the rete testis immediately after TB injection. At least four ducts are visible (arrowheads). (**d**) Caput epididymis immediately after TB injection. TB was transferred to the ducts of the 1st segment of caput epididymis via efferent ducts. (**e**) Another caput epididymis immediately after TB injection. TB was observed in the epididymal ducts of the 1st segment of caput epididymis (arrowed). However, only a small amount of TB was observed to have been transferred to the next segment (indicated by *). (**f**) Ducts of the 1st segment of caput epididymis after excision immediately after TB injection and subsequent squashing under a cover glass. TB was observed within a duct (arrowed). (**g,h**) Caput epididymides dissected 1 (g) and 2 days (h) after TB injection. Almost all TB was present in the middle region of the caput epididymis.

Isolation of genomic DNA was performed as previously described by Blin and Stafford (1976) with several modifications (Sato et al., 1994).

PCR Analysis

One set of PCR primers, 3′βGAL-S/β-gl-2RV (Fig. 1b), was used for identification of the introduced pCETZ-17 DNA in spermatozoa isolated from the epididymal portion of the testis-injected males. 3′βGAL-S (5′-CTG CCA TTG TCA GAC ATG TAT-3′) corresponds to nucleotides 2,758–2,778 in the β-galactosidase (β-gal) gene (Kalnins et al., 1983). β-gl-2RV (5′-CTT CTG ATA

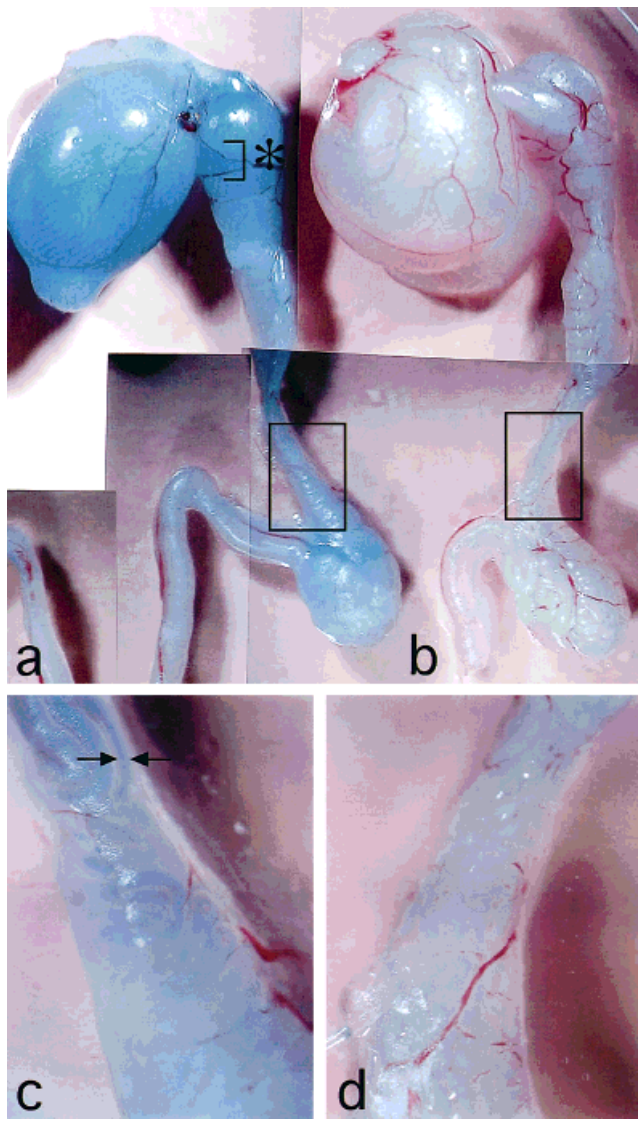

Fig. 3. (**a,b**) Testes, epididymides, and vas deferens dissected 4 days after injection of TB (a) or PBS(−) (b) into a testis. TB was observed in the ducts of caput epididymis, corpus and cauda epididymis, and was maximal in the middle portion (indicated by *) of caput epididymis. (**c,d**) Distal portion of corpus, magnified from the boxed regions (a) and (b). TB was observed in the ducts of corpus derived from the TB-injected male [arrowed in (c)].

GGC AGC CTG CAC-3′) corresponds to nucleotides 1,161–1,141 in exon 3 of the rabbit β-globin gene (van Ooyen et al., 1979). This primer set yields 380-bp fragments from the 3′ region of the β-gal gene.

The general PCR reactions for detection of the 3′ region of the β-gal gene were carried out as previously described (Sato et al., 1995, 1999a). Briefly, 9 μl of reaction buffer containing 10 mM Tris–Cl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% (w/v) gelatin, 200 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., Kyoto, 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 UV transillumination.

RESULTS

Fate of TB and Hoechst 33342 Directly Injected Into Testis

We first examined whether TB injected into mouse testis can be rapidly transferred to the epididymal portion. When 70 μl of TB was introduced into testis, TB was found to disperse into the interstitial space around the seminiferous tubules of the testis, but it seldom entered the seminiferous tubules (Fig. 2a,b). TB was rapidly transferred to the efferent ducts via the rete testis: at least four efferent ducts were visualized soon (within 0.5 min) after TB injection (Fig. 2c). Transfer of TB to the ducts in the 1st segment of caput epididymis was observed within 1 min after testis injection (Fig. 2d–f), but the amount of TB entering the middle portion (probably 2nd/3rd segments) of the caput epididymis appeared to be small even when inspection was continued for 10–15 min after injection of TB (Fig. 2e). This suggests the presence of barriers (probably at the entrance of each epididymal segment) restricting luminal flow in the epididymis. Rapid transfer of TB from rete testis to the 1st segment of the caput epididymis was repeatedly observed even when TB was injected from various sites of testis or a small volume of TB (e.g., 20 μl/testis) was introduced (data not shown). When inspection was performed 1 day after TB injection into testis, a large portion of TB was found to have been transferred to the middle portion of caput epididymis (probably the 2nd/3rd segments) (Fig. 2g,h). At 3–4 days after injection, TB still remained in the middle portion of the caput epididymis, but was often detected in the ducts of the corpus and cauda epididymis (Fig. 3).

We next introduced 70 μl of PBS(−) containing Hoechst 33342 into a testis in order to confirm that a solution injected into testis is transferred to the epididymal portion, where Hoechst 33342 will label epididymal spermatozoa. When spermatozoa were isolated from caput epididymis, corpus and cauda epididymis 4 days after testis injection and then observed for Hoechst 33342-derived fluorescence, more than 90% of spermatozoa in each portion tested exhibited distinct fluorescence in their heads (Fig. 4). The control spermatozoa never expressed fluorescence (Fig. 4). These findings also supported the above result that a solution introduced into testis is rapidly transferred to the epididymal portion via the rete testis and efferent ducts.

Plasmid DNA Transferred to Epididymal Portion After Testis Injection Can be Uptaken By Epithelial Cells of Epididymal Ducts

We first examined whether gene expression occurs in epithelium of epididymal ducts facing the lumen after injection of pCE-29 (Fig. 1b)/FuGENE6 complex into testis, since epididymal spermatozoa are unable to express the exogenous DNA even after successful incorporation of it. As shown in Fig. 5, in almost all cases no fluorescence or only weak fluorescence was detected. This finding suggests that single injection of a pCE-29/FuGENE6 complex into testis is not sufficient to transfect epididymal epithelium and spermatozoa efficiently. To improve the transfection efficiency, we employed in vivo EP, since this method has been proven to be useful for efficient transfection of murine spermatogenic cells (Yamazaki et al., 2000). We injected pCE-29 DNA into a testis and subsequently performed in vivo EP toward the caput epididymis. When the caput epididymis was inspected for EGFP fluorescence 1 day after testis injection and subsequent EP, some portions of efferent ducts and epididymal ducts exhibited distinct fluorescence (Fig. 6a). Histological sections of the fluorescent region revealed that epithelial cells of epididymal ducts facing the lumen exhibited bright fluorescence (Fig. 6c,e). These findings indicate that plasmid DNA is rapidly transferred to epididymal ducts after testis injection and then becomes incorporated by ductal epithelial cells and epididymal spermatozoa after in vivo EP.

In Vivo Uptake of Plasmid DNA by Epididymal Spermatozoa-Evidence From PCR Analysis

We examined whether exogenously introduced plasmid DNA is uptaken by epididymal spermatozoa after testis injection, and whether once uptake has occurred the incorporated DNA is resistant to DNase I digestion. Testes of two males were injected with a complex of circular pCETZ-17 DNA (Fig. 1b) and FuGENE6 and then subjected to sperm isolation 4 days after injection. Spermatozoa isolated from the corpus and cauda epididymides were divided into two portions; one was treated with DNase I and the other was not. When genomic DNA was isolated from the DNase I-treated or -untreated spermatozoa and examined for the presence of pCETZ-17 DNA by PCR, the DNase I-treated spermatozoa isolated from the corpus and cauda epididymides possessed a band with the expected size

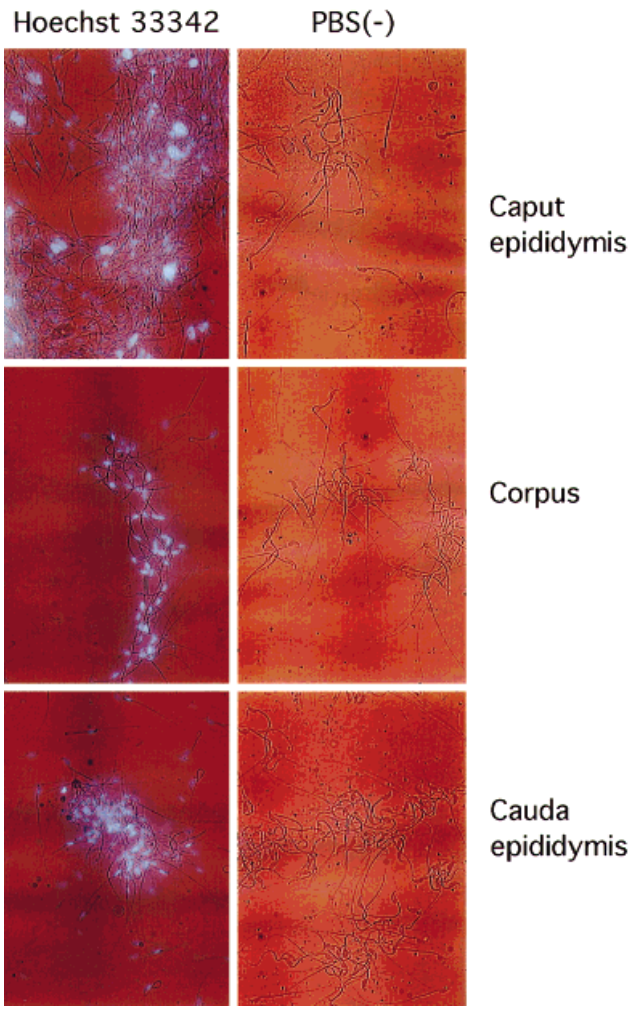

Fig. 4. Spermatozoa isolated from caput epididymis, corpus and cauda epididymis 4 days after injection of Hoechst 33342 or PBS(−) into testes. The isolated spermatozoa were washed three times and inspected for Hoechst 33342-derived fluorescence. Spermatozoa isolated from each portion of epididymis of a male injected with Hoechst 33342 exhibited distinct fluorescence in their heads, while spermatozoa from a male injected with PBS(−) did not.

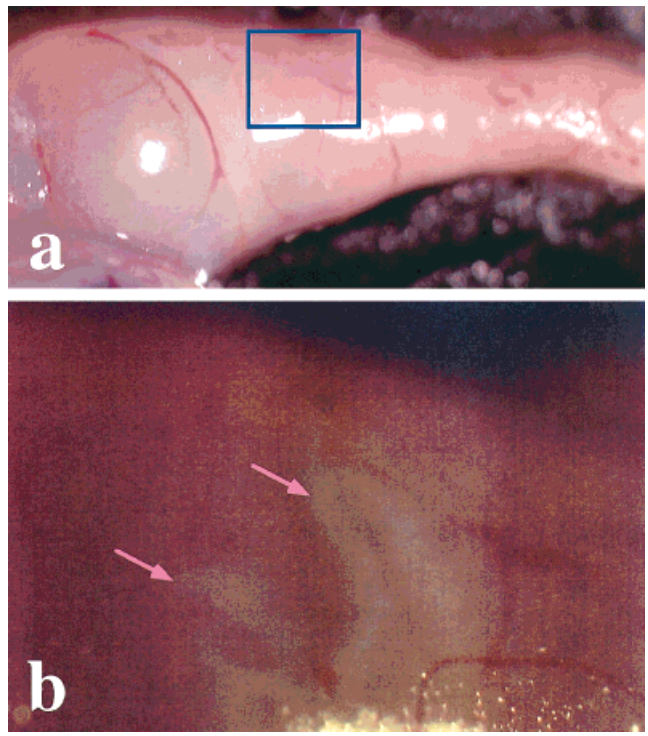

Fig. 5.

Fig. 5. Caput epididymis and corpus 2 days after injection of pCE-29/FuGENE6 complex into a testis. (**a**) A photograph of the junctional portion between caput epididymis and corpus taken by a dissecting microscope. (**b**) A photograph magnified from the boxed region (a) and taken under bright-field illumination to acquire focused images of the objects. Faint EGFP fluorescence was observed along with the epididymal ducts (arrowed).

Fig. 6. Vas efferens and caput epididymis 1 day after injection of pCE-29 DNA into a testis and subsequent in vivo electroporation. (**a,b**) Vas efferens and caput epididymis of a male injected with DNA (a) and of a normal control male (b). Distinct EGFP fluorescence was observed in the vas efferens/caput epididymis of a male receiving the DNA and subsequent in vivo EP, but not in the control sample. (**c–f**) Cryostat sections of vas efferens/caput epididymis of a male receiving DNA (c,e) and an untreated control male (d,f). (e,f) were magnified from the boxed regions (c) and (d), respectively. Some of the epididymal epithelial cells (arrowed) facing the lumen exhibited strong fluorescence (e), while those from a control male did not (f). Photographs were taken under bright-field illumination to acquire focused images of the objects.

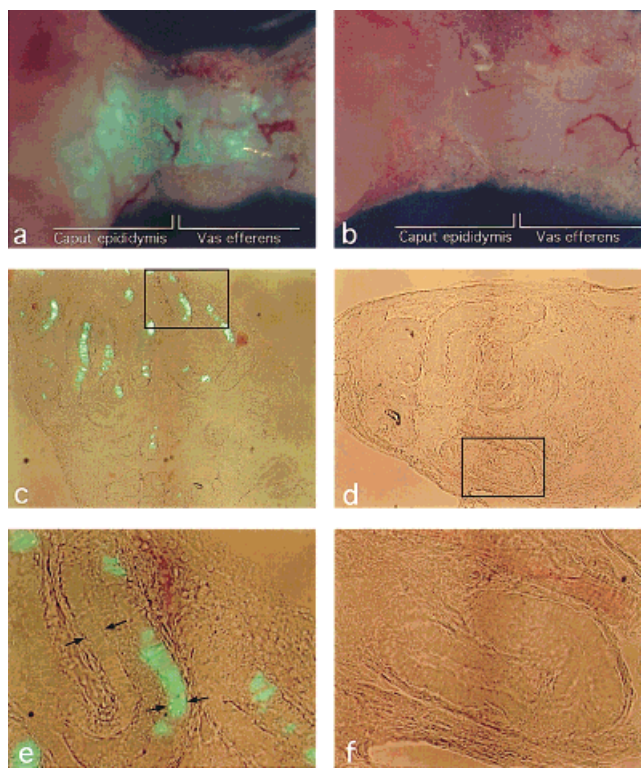

Fig. 6.

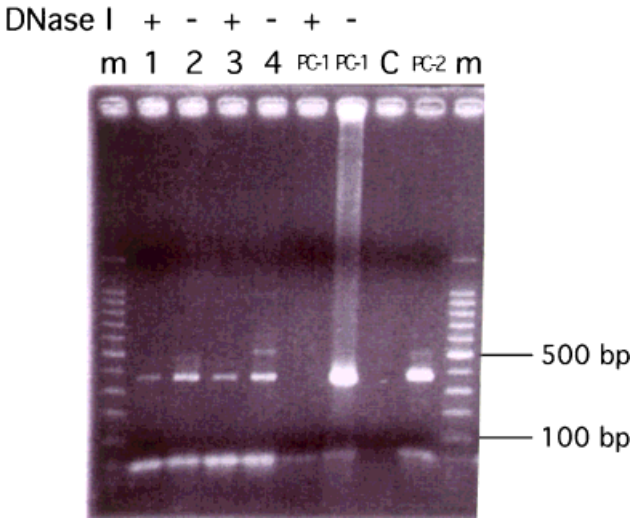

Fig. 7. PCR analysis of genomic DNA of spermatozoa isolated from corpus and cauda epididymides of males 4 days after injection of a pCETZ-17 DNA/FuGENE6 complex into testes, using primers recognizing the 3′ portion of β-gal sequence in pCETZ-17. The PCR reaction using 3′βGAL-S and β-gl-2RV primers yielded a 380-bp product. Staining of a gel with EtBr is shown. Samples loaded in **lanes 1** and **2** were from corpus of the males receiving DNA. **Lanes 3** and **4** were from cauda epididymides of the males receiving DNA. PC-1, 5 ng of pCETZ-17 as a positive control; C, control tail DNA; PC-2, tail DNA from transgenic line 144-5 carrying CETZ-17 transgenes (Sato et al., 2000). "+" and "−" above the lane indicate samples treated or untreated with DNase I prior to PCR, respectively. "m" indicates 100-bp ladder markers (Promega Co.). Note that each experimental sample exhibited a band of expected size of 380-bp, although the samples treated with DNase I exhibited decreased band intensity compared with those untreated with DNase I.

of 380 bp, although the intensity of the band for the DNase I-treated spermatozoa was lower than that for the untreated spermatozoa (Fig. 7). To test the fidelity of the DNase I used here, naked pCETZ-17 DNA was incubated in the absence or presence of DNase I at 37°C for 2 hr and then purified for PCR analysis. The naked plasmid DNA was completely digested by DNase I treatment (Fig. 7), indicating that the DNase I used was enzymatically active. These findings suggest that plasmid DNA encapsulated by FuGENE6 is in part uptaken by epididymal spermatozoa in vivo after testis injection.

DISCUSSION

We and other groups have shown with the TMGT method that direct introduction of plasmid DNA mixed with commercially available transfection reagents into mature mouse testis resulted in production of F0 fetuses (or pups) carrying the foreign DNA (Ogawa et al., 1995; Chang et al., 1999a; Sato et al., 1999a,b). The TMGT method can be performed by mating with superovulated females 2–4 days after single or repeated injection of DNA into testes. These findings suggest that the rate of luminal flow in epididymal ducts becomes very rapid when internal pressure is added through introduction of a solution into testis, and the foreign DNA injected into mouse testis is uptaken by fully mature epididymal spermatozoa and subsequently transmitted to oocytes via fertilization. These conclusions also appear to be supported by the following previous findings: (i) the introduced DNA was detected in caput and cauda epididymal spermatozoa within 1 day after testis injection (Sato et al., 1994), and (ii) the introduced fluorescence-labeled DNA bound to the surface of spermatozoa (including head and in some cases mid-piece and tail) in cauda epididymis 4 days after tesitis injection (Chang et al., 1999b). In the present study, we confirmed these previous findings using TB and Hoechst 33342, and newly found that (i) a solution introduced into testis is transported via rete testis and efferent ducts to the 1st segment of caput epididymis, (ii) flow of solution is restricted, probably at each segment within the epididymis, and (iii) the solution introduced reaches the corpus and cauda epididymis within 3–4 days after testis injection.

Entry of TB into the seminiferous tubules of the testis was seldom observed after testis injection: TB was instead dispersed into a space between the seminiferous tubules and testicular capsule. It is thus unlikely that gene transfer in the TMGT system is mediated by the transfected testicular sperm cells. It remains unclear how TB present between the seminiferous tubules and testicular capsule enters the rete testis. There are probably two "gates" leading to the rete testis, through which testicular sperm cells and fluid are transported: one is that through which testicular spermatozoa are transferred from the seminiferous tubules, while the other is that through which interstitial fluid present between seminiferous tubules and testicular capsule is transported. TB introduced into testis might have been transferred to the rete testis through the latter.

Once exogenous DNA encapsulated by a transfection reagent such as a liposome is introduced into a testis, it will be rapidly transferred to the epididymal portion and then transfect epididymal spermatozoa and epididymal epithelium facing the lumen. Transfection of epididymal spermatozoa was confirmed by PCR analysis in the present study, since exogenous DNA was detectable on the spermatozoa isolated from corpus and cauda epididymis of the DNA-injected males (see Fig. 7). The exogenous DNA appeared to exist not only on the surface of spermatozoa but also in the internal portion of spermatozoa, since digestion with DNase I failed to remove the DNA completely from the spermatozoa isolated from the corpus and cauda epididymis. This finding appears to be consistent with that reported by Huguet and Esponda (1998), who found with an electron microscopic method that in vivo DNA incorporation into mouse spermatozoa occurs after DNA injection into ducts of the vas deferens. However, contradictory findings have been reported by Chang et al. (1999b), who observed that treatment with DNase I resulted in complete loss of exogenous DNA from

ejaculated spermatozoa (intrauterine spermatozoa) derived from males 4 days after injection of liposome-encapsulated plasmid DNA into a testis.

In this study, we observed that the epididymal epithelial cells facing the lumen expressed distinct EGFP fluorescence after introduction of an EGFP expression plasmid and subsequent in vivo EP (see Fig. 6). This finding suggests that with this system the exogenous plasmid DNA may be uptaken efficiently by epididymal spermatozoa. Prior to this trial, we had attempted to detect fluorescence in the epithelium of caput epididymis after introduction of a pCE-29/FuGENE6 complex into testis, but in almost all cases no fluorescence or only weak fluorescence was detected (see Fig. 5). Furthermore, no fluorescence was detected in the epithelium of caput epididymis after introduction of naked pCE-29 plasmid DNA into testis (data not shown). This means that introduction of DNA into testis and subsequent in vivo EP provides better transfection efficiency for epididymal epithelial cells and probably epididymal spermatozoa than simple injection of a liposome/DNA complex or naked plasmid DNA. As previously described (Sato et al., 1999a), with the TMGT system using single introduction of a liposome/plasmid DNA complex, the copy number of plasmid DNA transmitted to F0 descendants is very low (less than one copy per diploid cell). We believe that TMGT combined with in vivo EP can improve the uptake of foreign DNA by epididymal spermatozoa and provide an in vivo gene transfer system useful for introducing a large amount of DNA into epididymal spermatozoa, if EP has no deleterious effects on the survival of these spermatozoa. Furthermore, this procedure will also be useful for targeted gene introduction into epididymal epithelium, through which the microenvironment of the epididymis can be altered. This altered epididymal microenvironment will provide a unique system for study of mechanisms regulating sperm processing.

In conclusion, we clarified in this study a possible mechanism of TMGT using TB and Hoechst 33342. We found that exogenous DNA directly introduced into tesits is rapidly transported to epididymal ducts via the rete testis and efferent ducts, and that 3–4 days after introduction this DNA will be incorporated by epididymal spermatozoa.

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REFERENCES

Araki K, Araki M, Miyazaki J, Vassalli P. 1995. Site-specific recombination of a transgene in fertilized eggs by transient expression of Cre recombinase. Proc Natl Acad Sci USA 92:160–164.

Blin N, Stafford DW. 1976. A general method for isolation of high molecular weight DNA from eukaryotes. Nucleic Acids Res 3:2303–2308.

Brinster RL, Sandgren EP, Behringer RR, Palmiter RD. 1989. No simple solution for making transgenic mice. Letters to the editor. Cell 59:239–241.

Chang K-T, Ikeda A, Hayashi K, Furuhata Y, Nishihara M, Ohta A, Ogawa S, Takahashi M. 1999a. Production of transgenic rats and mice by the testis-mediated gene transfer. J Reprod Dev 45:29–36.

Chang K-T, Ikeda A, Hayashi K, Furuhata Y, Bannai M, Nishihara M, Ohta A, Ogawa S, Takahashi M. 1999b. Possible mechanisms for the testis-mediated gene transfer as a new method for producing transgenic animals. J Reprod Dev 45:37–42.

Huguet E, Esponda P. 1998. Foreign DNA introduced into the vas deferens is gained by mammalian spermatozoa. Mol Reprod Dev 51:42–52.

Kalnins A, Otto K, Rüther U, Müller-Hill B. 1983. Sequence of the *lac Z* gene of *Escherichia coli*. EMBO J 2:593–597.

Lavitrano M, Camaioni A, Fazio VM, Dolci S, Farace MG, Spadafora C. 1989. Sperm cells as vectors for introducing foreign DNA into eggs: genetic transformation of mice. Cell 57:717–723.

Maione B, Lavitrano M, Spadafora C, Kiessling AA. 1998. Sperm-mediated gene transfer in mice. Mol Reprod Dev 50:406–409.

Niwa H, Yamamura K, Miyazaki J. 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene 108:193–200.

Ogawa S, Hayashi K, Tada N, Sato M, Kurihara T, Iwaya M. 1995. Gene expression in blastocysts following direct injection of DNA into testis. J Reprod Dev 41:379–382.

Sato M, Iwase R, Kasai K, Tada N. 1994. Direct injection of foreign DNA into mouse testis as a possible alternative of sperm-mediated gene transfer. Animal Biotech 5:19–31.

Sato M, Kasai K, Tada N. 1995. A sensitive method of testing for transgenic mice using polymerase chain reaction-Southern hybridization. Genetic Anal: Biomol Engin 12:109–111.

Sato M, Gotoh K, Kimura M. 1999a. Sperm-mediated gene transfer by direct injection of foreign DNA into mouse testis. Transgenics 2:357–369.

Sato M, Yabuki K, Watanabe T, Kimura M. 1999b. Testis-mediated gene transfer (TMGT) in mice: Successful transmission of introduced DNA from F0 to F2 generations. Transgenics 3:11–22.

Sato M, Yasuoka Y, Kodama H, Watanabe T, Miyazaki J, Kimura M. 2000. New approach to cell lineage analysis in mammals using the Cre-*loxP* system. Mol Reprod Dev 56:34–44.

Toyoda Y, Yokoyama M, Hoshi T. 1971. Studies on the fertilization of mouse eggs in vitro. I. In vitro fertilization of eggs by fresh epididymal spermatozoa (in Japanese). Jpn J Anim Reprod 16:147–151.

van Ooyen A, van der Berg J, Mantei N, Weissmann C. 1979. Comparison of total sequence of a cloned rabbit β-globin gene and its flanking regions with a homologous mouse sequence. Science 206: 337–344.

Yamazaki Y, Fujimoto H, Ando H, Ohyama T, Hirota Y, Noce T. 1998. In vivo gene transfer to mouse spermatogenic cells by deoxyribonucleic acid injection into seminiferous tubules and subsequent electroporation. Biol Reprod 59:1439–1444.

Yamazaki Y, Yagi T, Ozaki T, Imoto K. 2000. In vivo gene transfer to mouse spermatogenic cells using green fluorescent protein as a marker. J Exp Zool 286:212–218.