

Tn5 Transposase-Mediated Mouse Transgenesis¹

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ABSTRACT

We have developed a novel method for mouse transgenesis. The procedure relies on a hyperactive Tn5 transposase to insert a transgene into mouse chromosomes during intracytoplasmic sperm injection. This procedure integrates foreign DNA into the mouse genome with dramatically increased effectiveness as compared to conventional methods such as pronuclear microinjection and traditional sperm injection-mediated transgenesis. Our data indicate that with this method, transgenic mice, both hybrids and inbreds, can be produced more consistently and with lower numbers of manipulated oocytes required for traditional microinjection methods. The transposase-mediated transgenesis technique is also effective with round spermatids, offering the potential for rescuing the fertility of azoospermic animals using sperm precursor cells.

oocyte development, sperm, spermatid

INTRODUCTION

Gene delivery and production of transgenic animals are becoming increasingly important in every aspect of basic and applied research, with many transgenic mice strains serving as important disease models. There are several approaches for producing transgenic animals by introducing recombinant DNA into their somatic or germ cells [1–4]. To achieve ubiquitous cellular expression of recombinant DNA, most transgenesis efforts have concentrated on the insertion of the transgene (*tg*) at the unicellular stage of developing embryos. The effective insertions of such *tg*'s have been mainly achieved by passive means, as in pronuclear microinjection [1] and traditional intracytoplasmic sperm injection-mediated transgenesis (ICSI-Tr) [2]. However, the most effective means of *tg* insertion to date is exemplified by the active lentiviral transgenesis technique, which makes use of viral sequences and enzymes to increase the efficiency of *tg* insertion [3].

Passive transgenesis entails the injection of linearized *tg* DNA into the pronuclei of single-celled embryos, or its comicroinjection with freeze-thawed spermatozoa into the

cytoplasm of mature unfertilized oocytes by ICSI-Tr. Transgenes in the vicinity or within the nuclei of an individual in its initial stage of development rely for insertion on the DNA repair mechanisms of their newfound environment [5, 6]. When insertion does occur, it is on the order of 1% to at best 5% of oocytes micromanipulated (*o_i*), or 20% to 45% of animals born (*a_b*) being transgenic (*a_t*) [1, 2, 7–10]. In the case of ICSI-Tr, such micromanipulations as freeze-thawing spermatozoa have a detrimental effect on the development of early embryos [11]. It is also common that *tg*'s integrated by passive transgenesis commonly form long concatameric arrays. Such transgene loci are not desirable, because they often have the potential to generate aberrant RNAs that can cause gene silencing [12].

The active lentiviral technique uses disarmed retroviral vectors to insert desirable genes into the host organism, usually a single-celled embryo [3, 13]. However, there are drawbacks to this technique, as exemplified by high embryo lethality rates (70%–80% of *o_i*) and the relatively small size of *tg* DNA (9.5 kb) that can be transported by the vector [3, 14]. Such limitations, further coupled to the required specialized containment facilities for retroviral production, make it prohibitive for most laboratories to exercise the active retroviral transgenesis approach. Despite its potential safety problems, a high percentage (~80%) of the founder (F0) animals born after lentiviral transgenesis carry several (1–20) stably inserted *tg* copies [3]. This high efficiency of transgenic F₀ production (23% of *o_i*) has attracted considerable interest as a new transgenesis method in the livestock industry, in which the cost of producing one transgenic cow by pronuclear microinjection is estimated to be \$300 000 [14, 15].

We sought to develop another active transgenesis method that would be more flexible in terms of *tg* size and less problematic than the lentiviral system in terms of biosafety considerations, while being more effective than conventional ICSI-Tr. To that effect, we employed Tn5 transposase, one of the best-characterized bacterial transposases, for transposon delivery. The structure and mechanism of action for Tn5 transposase have already been established for both wild and mutant types [16–20]. Unlike the wild-type transposases (E54K, M56A, L372P), hyperactive Tn5 mutant transposase (*Tn5p) exhibits a high transposon insertion activity in vitro and has been used to produce DNA:transposase complexes with the transposase protein bound to both ends of transposon DNA [19–21]. Such complexes, called transposomes, are formed by the protein binding to specific 19-bp recognition Mosaic End (ME) sequences of the transposon in the absence of Mg²⁺ ions. Transposomes have subsequently been used for crystallization studies [22] as well as for bacterial gene delivery [23] where, following electroporation, the transposase becomes activated by cellular Mg²⁺ levels and integrates the transposon DNA into a random position

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favoring GATC(A/T)_nGATC sequences in bacterial chromosomes [21, 24].

Here we report the production of hybrid and inbred transgenic mice using a modified transposome-assisted microinjection technique. In vitro-synthesized transposomes were comicroinjected into mouse oocytes together with fresh sperm heads (TN:ICSI) or round spermatids (TN:ROSI). Activation of the transposome complex by the Mg²⁺-rich oocyte cytoplasm results in transgenesis, with transgenic animals born demonstrating EGFP *tg* expression in their tissues. This technique allows the use of unfrozen sperm during transgenesis, resulting in significantly higher percentages of live births and a larger proportion of transgenic animals, with fewer microinjected oocytes used.

MATERIALS AND METHODS

Animals

Females and males of B6D2F1 (B57BL/6 × DBA/2), C57BL/6, and CD1 mice were purchased from the National Cancer Institute. All animals were maintained in temperature- and light-controlled rooms (14L:10D, lights-on from 0500 h). The protocol of animal handling and treatment was reviewed and approved by the Animal Care and Use Committee of the University of Hawaii.

Construction of Transposon DNA

The plasmid pCX-EGFP, expressing EGFP under the control of the CAG promoter, was a kind gift from Dr. Masaru Okabe [25]. The 3179-bp *Sall*/*Bam*HI fragment containing the EGFP gene and its regulatory elements was cloned into *Sall*/*Bam*HI sites present in the MCS of pMOD-3<R6γori/MCS> (Cat. No. MOD1503; Epicentre). The transposon, flanked by its ME sequences, was excised from the resulting plasmid pMOD-3/CX-EGFP by digestion with *Psh*AI, and the 3608-bp fragment containing the active transposon (EZ:TN transposon) was separated in a 1% agarose gel, purified in a QIAquick Gel Extraction Kit (Cat. No. 28704; Qiagen), and used for transposome assembly.

Preparation of Transposome Complex

A 6-μl reaction solution was prepared by mixing together 2 μl of 100 ng/μl EZ:TN transposon DNA containing the pCX-EGFP gene in TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) and 4 μl EZ:TN transposase (1U/μl; Cat. No. TNP92110; Epicentre). Following mixing, the reaction proceeded for 30 min at room temperature. In all experiments, double-stranded DNA (dsDNA) at 16 ng/μl was injected either as transposomes or as DNA-only controls.

Preparation of Microinjection Media

Chatot, Ziomet, and Bavister (CZB) medium supplemented with 5.56 mM D-glucose was used for the culture of mouse oocytes after microinjection. The medium for oocyte collection and subsequent oocyte treatments, including micromanipulation, was a modified CZB medium (HEPES-CZB medium), pH 7.4, containing 20 mM HEPES-HCl, 5 mM NaHCO₃, and 0.1 mg/ml polyvinyl alcohol (PVA; cold-water soluble; Mr 30 000–70 000) instead of bovine serum albumin [26]. CZB medium was used under 5% CO₂ in air, and HEPES-CZB was used under air. The medium used for microinjection was Mg²⁺-free to prevent the premature activation of the Tn5 transposase.

ICSI Microinjection

ICSI was carried out essentially as described by Kimura and Yanagimachi, using a piezoelectric micropipette actuator [26], except that manipulation was carried out at room temperature (about 25°C). Briefly, epididymal spermatozoa and matured oocytes were collected from 8- to 12-wk-old B6D2F1 hybrid or C57BL/6 inbred mice. Oocytes were collected from oviducts of superovulated B6D2F1 or C57BL/6 females after intraperitoneal injection of 5 IU equine chorionic gonadotropin (eCG) followed by injection of 5 IU human chorionic gonadotropin (hCG) 48 h later. Matured oocytes collected 13–15 h after hCG injection were freed from cumulus cells by treatment with 0.1% bovine testicular hyaluronidase (359 U/mg solid) in HEPES-CZB medium. They were rinsed and kept at 37°C in fresh CZB medium before sperm injection. Spermatozoa were collected from the cauda epididymis of B6D2F1 or C57BL/6 males. A dense sperm mass squeezed out of the epididymis was placed at the bottom of 200 μl Mg²⁺-free HEPES-CZB buffered solution in a microcentrifuge tube. After standing for 10 min at 37°C, the upper 20 μl of the sperm

suspension was collected and mixed with an equal volume of 12% PVP solution. A single spermatozoon moving slowly in the solution was drawn, tail first, into the injection pipette in such a way that its neck (the junction between the head and tail) was at the opening of the pipette. The head was separated from the tail by applying a few piezo-pulses to the neck region. To ensure the dilution of any Mg²⁺ ions released during the removal of the tail from the sperm head and prevent the unintended activation of the transposase, the sperm head was transferred to a new drop of 20 μl Mg²⁺-free HEPES-CZB containing 12% PVP and an appropriate concentration of transposome mixture (16.2 ng/μl transposon DNA). It was expected that any Mg²⁺ ions released by the removal of the tail from the sperm head, after dilution in a second drop of PVP, would have minimal effect in activating the *Tn5p. One minute later, sperm heads were individually injected into oocytes. ICSI oocytes were cultured in CZB medium at 37°C under 5% CO₂ in air. Recipients of 2-cell embryos were 8- to 16-wk-old outbred CD-1 females.

ROSI Microinjection

Mouse round spermatids are the smallest cells in the testis and are characterized by a centrally located chromatin mass [27, 28]. They were collected from the testes of B6D2F1 males and microinjected into oocytes according to Kimura and Yanagimachi [28], with some modifications. Briefly, a group of round spermatids were placed in Mg²⁺-free HEPES-CZB medium containing 12% PVP. The round spermatids were then transferred to a 20 μl Mg²⁺-free HEPES-CZB containing 12% PVP and an appropriate amount of transposome mixture, resulting in the final concentration of 16.2 ng/μl transposon DNA. After mixing for 1 min, several round spermatids were drawn into a micropipette. The plasma membrane of each spermatid was broken by sucking it in and out of the pipette, and nuclei were individually injected into mice oocytes that had been previously activated by a 30-min treatment with 10 mM SrCl₂ in a Ca²⁺-free CZB medium [29, 30]. ROSI oocytes were cultured in CZB medium.

Pronuclear and Cytoplasmic Microinjections

Transposomes or a corresponding amount of dsDNA was injected directly into cytoplasm or male pronuclei of B6D2F2 zygotes using an InjectMan microinjection apparatus (Eppendorf).

Embryo Culture and Embryo Transfer

ICSI or ROSI oocytes with two well-developed pronuclei and a distinct second polar body 5 to 6 h after injection of spermatozoa or round spermatid were recorded as being normally fertilized. They were cultured in CZB medium until they reached the 2-cell stage (20–24 h after microinjection). They were then transferred into the oviducts of 8- to 16-wk-old surrogate pseudopregnant CD-1 females that were mated with vasectomized males of the same strain on the day before embryo transfer [26, 28, 31]. Pregnant females were allowed to deliver and raise their pups.

Analysis of Offspring

Genomic DNA obtained from tail-tip biopsies of EGFP-negative 30-day-old F₀ offspring was analyzed by PCR for the presence of transgene sequences. Forward primer (atggtgagcaaggcgaggagctgtcacc, position 0 to 30) for the 5' end of EGFP and reverse 28-bp primer (ctgtgacgcttctctgtctgctgccc, position 490 to 462) for the middle part of EGFP were used to amplify a 490-bp EGFP fragment. Reaction parameters were: 94°C for 3 min (1 cycle); 94°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec (35 cycles), with a final extension at 72°C for 3 min. For Southern blot analyses, 20 μg genomic DNA per sample was cleaved with the restriction enzyme *Nco*I and separated in 1% agarose gels. The DNA was transferred to Immobilon nylon membranes and probed with a 416 bp DIG-labeled Southern probe corresponding to the 3' part of the EGFP gene and its rabbit beta-globin polyA signal. Probe hybridization was detected using the DIG labeling and hybridization kit (Roche). Precise insertion of full-length transposons into the genome of the 23 Southern blot detected TN:ICSI EGFP-positive mice was analyzed by PCR with primers designed to the 5'-end and 3'-end regions of the 3608-bp-long transposon. The forward primer for the 5' end of the transposon (ctgtctcttatacacatctcaaccatcatcg, position 1 to 31), and the 22-bp reverse primer (cctgactactcccagctcatagc, position 339 to 317) were used to amplify the 5'-end region. The 3'-end forward primer (gtgaacatgagagcttagtacg, position 3397 to 3419) and the corresponding reverse primer (ctgtctcttatacacatctcaaccctgaagc, position 3608 to 3578) were used to amplify the 3'-end region. Conditions for the PCR reactions were: 94°C for 3 min (1 cycle); 94°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec (40 cycles), and a final extension at 72°C for 3 min.

RESULTS

In this study we employed ICSI as well as other microinjection-based methods for transgenesis of hybrid (B6D2F1) and inbred (C57BL/6) strains of mice. The delivery and integration of EGFP-coding *tg*'s into the mouse embryo genome was carried out with the help of a hyperactive form of the Tn5 transposase protein [19] (Fig. 1). The *Tn5p:DNA complexes or transposomes resembling natural Tn5 transposition intermediates were formed by allowing the purified transposase to bind to its ME recognition sequences in the absence of Mg²⁺ ions (Fig. 1A). Freshly isolated sperm heads were individually coinjected into mouse MII oocytes with either naked dsDNA alone, as described in previous ICSI-Tr studies [2, 10], or as a *Tn5p:DNA complex (TN:ICSI) (Fig. 1B). The DNA fragment used to construct the transposome contained an EGFP gene driven by a CAG promoter [25] (Fig. 2A). Two-cell embryos were then transferred into oviducts of surrogate females, and all of the resulting F₀ transgenic progeny were recognized for *tg* expression by epifluorescence of EGFP (Fig. 2B). In a control experiment (ICSI using only transposon DNA), only one pup was germline transgenic, showing weak mosaicism (Fig. 2C).

The data in Table 1 is a summary of all micromanipulations employed in this study. Table 1A, row 1 represents the combined data from seven ICSI microinjection repetitions (an average of 29 oocytes per repetition), and two inbred mouse strain attempts (row 3, an average of 47 oocytes per repetition). All such TN:ICSI attempts result in the production of live

transgenic pups, including a microinjection attempt in which only 14 oocytes were subjected to TN:ICSI; this hybrid strain attempt with low oocyte numbers produced a single transgenic live-born pup (Fig. 2B). Panel B, row 1 shows data obtained in the course of ROSI microinjections, with an average of 24 oocytes per microinjection attempt. Each attempt resulted in a live-born transgenic pup, giving a total of five such animals (Fig. 3B). Panel C, row 1 depicts pronuclear microinjection attempts, and Panel D, row 1 contains cytoplasmic microinjection data.

PCR and Southern Blotting

All live-born pups were screened by PCR for EGFP *tg* integration with primers indicated in Figure 2A. The ones found to be positive for *tg* were further analyzed to determine the integrity of the *tg* insertion (Fig. 2D). Finally, the pups'

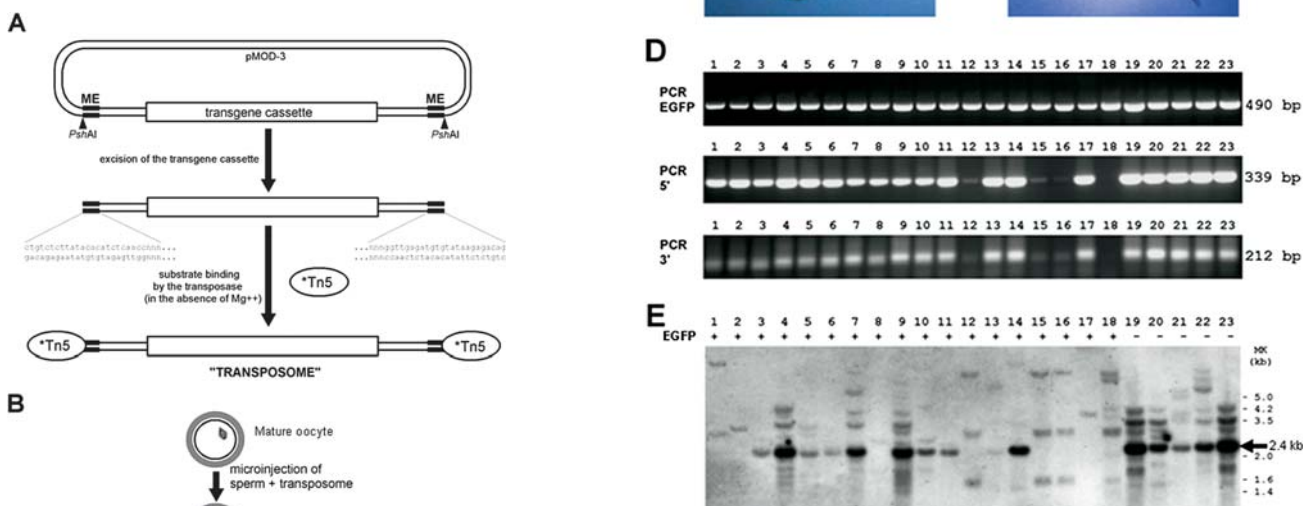


FIG. 2. Analysis of F₀ transgenic animals produced by TN:ICSI. **A**) Scheme of the transposome used in TN:ICSI experiments. Positions of mosaic ends (ME), restriction (*Nco*I) sites and probe used for Southern analysis as well as primer pairs (arrows) used in PCR analysis are indicated. **B**) Typical EGFP-expressing transgenic pup (t) obtained by TN:ICSI together with a nontransgenic control (c) animal. **C**) Weakly EGFP-expressing transgenic mosaic pup (t) obtained by ICSI with naked DNA together with a nontransgenic control (c) animal. **D**) PCR analysis of all transgenic F₀ animals obtained by hybrid TN:ICSI. All animals are positive for the central part of the EGFP transgene (upper panel). PCR fragments corresponding to perfectly preserved 5' (middle panel) and 3' (lower panel) ends of the transposome can be seen in 22 of 23 animals, indicating a high degree of transgene preservation before integration. **E**) Southern analysis of transgenic F₀ animals obtained by hybrid TN:ICSI. Mouse genomic DNA was cleaved with *Nco*I and probed with a 416 bp fragment complementary to the 3' end of the EGFP reporter cassette. Transgenics analyzed in lanes 1–23 were generated by TN:ICSI (Table 1A, row 1), with animals 1–18 showing EGFP fluorescence. Arrow indicates 2.4-kb possible concatemeric insertion.

FIG. 1. TN:ICSI experimental design. **A**) In vitro assembly of transposase:DNA complexes (transposomes). Transgene cassette (EGFP) is cloned between the 19-bp mosaic ends (MEs) of the Tn5 transposon present in the pMOD-3 plasmid. Cleavage with *Psh*AI generates linear transgene fragments flanked by MEs, which can be recognized by the purified, hyperactive Tn5 transposase (*Tn5p). A stable complex similar to extrachromosomal transposition intermediate is formed in the absence of Mg²⁺ ions. **B**) Scheme of TN:ICSI procedure. Transposomes containing the transgene DNA are coinjected into MII oocytes together with a sperm head previously isolated from fresh spermatozoon. Mg²⁺ ions present within the oocyte facilitate the integration of the transposome into the host DNA. Embryo transfer and development typically results in litters containing 10%–40% transgenic offspring.

TABLE 1. Summary of TN:ICSI, TN:ROSI, pronuclear, and cytoplasmic injection experiments.

Transgenesis method	Mouse strain used for sperm and oocyte	No. of oocytes injected (repetitions)*	Total no. of normally fertilized oocytes	No. of embryos transferred (surrogate mothers)	Births (% transferred) [% oocytes]	Transgenic pups*		
						Total	Animals born (% births)	Oocytes micromanipulated (% oocytes)
A								
TN:ICSI	B6D2F1 Hybrid	204 (7) ^a	182	171 (14)	107 (62.6) [52.0]	23 ^a	21.5	11.3
Frozen sperm TN:ICSI	B6D2F1 Hybrid	50 (1)	45	39 (3)	20 (51.0) [40.0]	1	5.0	2.0
TN:ICSI	C57BL/6 Inbred	94 (2)	84	77 (6)	45 (58.4) [47.9]	4	8.8	4.3
DNA-only ICSI control [‡]	B6D2F1 Hybrid	106 (2) ^b	99	87 (6)	40 (46.0) [37.5]	1 ^{b,†}	2.5	0.9
B								
TN:ROSI [§]	B6D2F1 Hybrid	120 (5) ^c	108	86 (7)	31 (36.0) [25.8]	5 ^c	16.1	4.2
DNA-only ROSI control ^{‡,§}	B6D2F1 Hybrid	49 (1) ^d	44	33 (2)	11 (33.3) [22.5]	0 ^d	0	0
C								
Transposome pronuclear microinjection	B6D2F2 Hybrid	160 (4)	117	105 (6)	47 (45.0) [30.0]	1	2.1	0.63
DNA-only pronuclear microinjection control [‡]	B6D2F2 Hybrid	40 (1)	31	26 (2)	10 (38.5) [25.0]	0	0	0
D								
Transposome cytoplasmic injection [¶]	B6D2F2 Hybrid	136 (4)	119	115 (8)	79 (68.7) [58.0]	1	1.3	0.74
DNA-only cytoplasmic injection control [‡]	B6D2F2 Hybrid	59 (1)	54	42 (4)	18 (42.9) [31.0]	0	0	0

*When values within the same column with different superscripts are compared, they differ significantly (^{a,b} $P < 0.001$ Fisher exact probability test and $P < 0.005$ chi-square test; ^{c,d} $P < 0.05$ Student *t*-test).

[†]Very weak chimera: germline transgenic.

[‡]Control experiments performed with naked DNA only.

[§]Activation of oocytes 30 min before ROSI microinjection.

[¶]Embryos at two pronuclei stage.

genomic DNA was subjected to Southern blotting to identify *tg* copy number (Fig. 2E). From these, 23 PCR-positive for EGFP, F₀ B6D2F1 hybrid animals were confirmed for *tg* integration (Fig. 2, D and E). Fragments corresponding to perfectly preserved 5' and 3' ends of the transposome were detected in 22 of 23 animals, indicating a high degree of transgene preservation before integration, probably because of protection of DNA ends by bound transposase molecules (Fig. 2D). In the Southern blot of Figure 2E, the number of *tg*'s ranged from 1 to ~20, with 6 out of 23 animals carrying just 1 or 2 copies of the *tg* (Fig. 2E, lanes 1, 2, 3, 8, 13 and 17). Lanes 4, 7, 9, 14, 19, 20, 22 and 23 of Figure 2E additionally contain a strong band in the region of 2.4 kb that resembles concatamerized fragments produced from head to tail integration. Three animals depict very similar integration pattern (Fig. 2E, lanes 12, 15, and 16). Although the middle 3-kb band seen in these lanes could be the result of a tail-to-tail integration event, the other bands of similar size seen cannot be a product of simple cointegration of transposon molecules. One plausible explanation can be found in the reported sequence preference of Tn5 transposase with respect to the integration sites demonstrated in bacterial transgenesis experiments [21, 23]. The similarity of the pattern can be explained by the transposase's finding such sites on other transposome DNA fragments, which result in unexpected but reproducible integration patterns. Similar integration patterns could also arise with the Tn5 transposase's finding such preferred

integration sites within the repetitive sequences present in the mouse genome.

Meiotic Transmission of Transgene

Analysis of F₁ progeny from crosses between EGFP expressing F₀ hybrid founders and nontransgenic partners established that in most cases, the rate of the *tg* transmission through the germline was approximately 50%, indicating single or closely linked integration sites (Table 2). Southern blots of genomic DNA obtained from biopsies of F₁ progeny mirrored the *tg* insertion patterns of the parents (data not shown).

Alternative Microinjections

Transgenesis success with TN:ICSI encouraged us to try *Tn5p mediated transgenesis by ROSI. Round spermatids, the smallest cells in the testis, were easily recognized by their small size and centrally located chromatin mass. Transposomes were coinjected with a round spermatid into the cytoplasm of mature activated unfertilized oocytes. This new ROSI based transgenesis approach (TN:ROSI) resulted in 5 transgenic EGFP-expressing pups (Table 1B, row 1) corresponding to transgenesis efficiencies of 4.2% of o_i and 16.1% of a_b. Southern analysis done on the first three F₀ TN:ROSI animals born revealed a presence of 1, 7 and 10 copies of the *tg* respectively (Fig. 3A). Just as in the case of TN:ICSI-generated transgenics, the segregation of EGFP expression for 2 F₀ ROSI mice in the

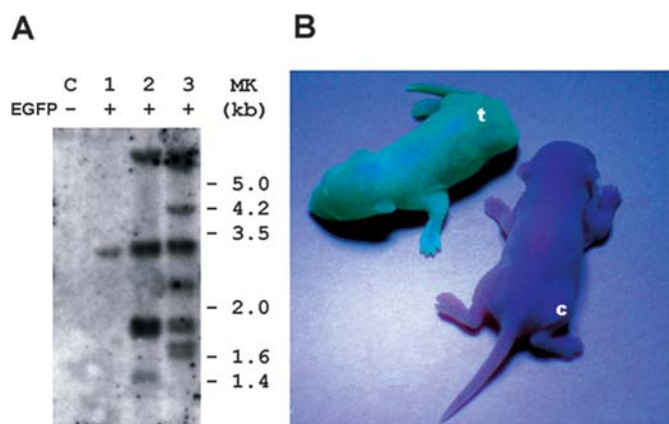


FIG. 3. Analysis of F_0 transgenic animals produced by TN:ROSI. **A**) Southern analysis of a wild-type control DNA (C) and the first three transgenic F_0 animals obtained by TN:ROSI. Mouse genomic DNA was cleaved with *Nco*I and probed with a 416 bp fragment complementary to the 3' end of the EGFP reporter cassette (Fig. 2A). **B**) Typical EGFP-expressing transgenic pup (t) obtained by TN:ROSI together with a nontransgenic control (c) animal.

F_1 generation suggested single-locus integration of the *tg* (Table 2, #R1 and #R2). Southern blots of genomic DNA obtained from biopsies of TN:ROSI F_1 progeny mirrored the transgene insertion patterns of the parents (data not shown).

Acknowledging that for some other laboratories ICSI and ROSI are rather difficult and time-consuming procedures, we injected transposomes into the pronuclei or the cytoplasm of single-cell embryos of B6D2F1 hybrid mice. Somewhat surprisingly, neither pronuclear nor cytoplasmic injection of transposomes into single-celled embryos resulted in efficient transgenesis (Table 1, C and D).

DISCUSSION

We sought to develop a new method of active transgenesis that would be more flexible than the lentiviral system in terms of *tg* size and less problematic in terms of biosafety considerations. Although retroviral vectors have recently become the gold standard in gene delivery—frequently approaching 80% transgenesis efficiencies with respect to a_b [3]—most transgenic animals, especially mice, are still being produced by classical, much less efficient, pronuclear microinjection methods, because of the lentiviral drawbacks listed previously.

Transposons such as Sleeping Beauty have in the past been used to insert transgenes into the mouse genome [32]. However, to our knowledge, in mice, no one has used a transposase protein for microinjection together with a transposon *tg*. The Tn5 transposase of the EZ:TN transposase system is a mutated hyperactive bacterial enzyme that is frequently used for electroporation, complexed with a transposon into bacterial cells [23]. We rationalized that if the complex was stable enough to be electroporated, then it should prove stable enough to be microinjected. Our data in Table 1 indicate that transposomes can indeed be microinjected with transgenic success into oocytes.

Horizontal gene transfer of *tg*'s has been ongoing since the injection of the Simian virus 40 (SV40) viral DNA into the blastocoel cavity of mouse embryos [33]. Safety concerns for *tg* insertions, such as unintentional induction of innate or adaptive immune responses and insertional mutagenesis considerations, for mice are limited. The major safety concerns

TABLE 2. Meiotic transmission of EGFP gene expression in F_1 offspring, from F_0 (+EGFP) X wild-type cross.

Transgenesis method (animal ID no.)	Gender of F_0 mice ^a	No. of EGFP+ pups in F_1 litter	EGFP+ (%)
TN:ICSI			
1	F	2/6	33.3
2	M	9/19	47.4
3	F	4/10	40.0
4	F	2/6	33.3
5	M	5/9	55.6
6	M	4/6	66.7
7	M	6/19	31.6
8	F	3/7	42.9
9	F	3/9	33.3
10	M	6/17	35.3
11	F	5/9	55.6
12	F	2/6	33.3
13	F	1/7	14.3
14	F	2/9	22.2
15	M	4/10	40.0
16	M	10/18	55.6
17	M	7/10	70.0
18	F	3/8	37.5
DNA-only ICSI control^b			
24	F	2/5	40.0
Frozen sperm TN:ICSI			
25	F	3/8	37.5
TN:ROSI			
R1	F	5/9	55.6
R2	M	2/7	28.6

^a F, female; M, male.

^b Control experiments performed with naked DNA only.

expressed in transgenesis research have mostly concentrated in the use of retroviral vectors and risks associated with their *tg* insertion [4]. In common with integrating vectors such as lentiviruses, Moloney leukemia virus (MLV), and adeno-associated virus (AAV), transposases cause chromosomal integration of *tg* DNA. Such assemblages of genes and DNA sequences are combinations that could not be reasonably expected to be found in nature. Therefore, risks represented by transgenesis research and the vectors used in its implementation always have the potential to generate an undesirable event. However, because in this instance we are using a purified bacterial protein as the source of transposase, instead of a DNA-transposase-based-vector such as Sleeping Beauty, long-term transposase expression is not expected, thus preventing ongoing transposition and many different insertion events. Once all the *Tn5p is exhausted, transposition events should cease and no transposase should be available by de novo synthesis.

Fertilization rates obtained using TN:ICSI were comparable to those obtained by ICSI performed with fresh sperm alone [26] (Table 1), indicating that neither naked DNA nor *Tn5p:DNA was deleterious to embryo survival at the concentrations used. All heterozygous mice produced by TN:ICSI were fertile and germline transgenic, the majority of them displaying classical Mendelian segregation of the *tg* (Table 2).

The scoring of transgenesis efficiency is a contentious matter. In techniques for which postembryo transfer development is poor, the preferred method is to score the efficiency as a ratio of a_b/a_a . Thus, if two animals are born and one is transgenic, this form of accounting results in 50% a_b/a_a transgenic efficiency. We chose to employ a scoring efficiency that was reflective of transgenic animals born as a ratio of total oocytes micromanipulated (o_t). We hope that this form of scoring efficiency more accurately reflects the effectiveness of

the methods. Under such conditions, the lentiviral method is the most efficient, with 23.3% o_i and 86% a_b/a_t [3]. Hybrid TN:ICSI follows the lentiviral technique, with values of 11.3% o_i and 21.5% a_b/a_t . Freeze-thawed sperm ICSI-Tr and pronuclear microinjection represent values of 4.6% o_i and 45% a_b/a_t [7] and 3.2% o_i and 24% a_b/a_t [1], respectively. In every instance, when such calculations are considered, transgene sequence, product, and size must be taken into account, because all these factors may affect the final transgenesis efficiency.

TN:ICSI is less technically demanding than TN:ROSI and would be well suited to routine transgenesis. It is efficient in both hybrid and inbred mouse strains and offers the potential of delivering large, gene-sized DNA fragments. The ability to generate transgenic animals with a limited number of oocytes makes it especially well suited for transgenesis attempts on large mammals, such as nonhuman primates, in which oocyte numbers are at a premium and losses because of developmental arrest can be ill afforded.

Low copy number and full-length *tg* integration patterns similar to those obtained by some of the TN:ICSI microinjections are preferable in transgenesis experiments, because expression of multiple *tg* copies or production of aberrant RNA from truncated genes can frequently lead to gene silencing [12]. This notion is also borne out in this study, in which all five B6D2F2 transgenic animals obtained by TN:ICSI carrying a relatively high number of *tg*'s did not express EGFP (Fig. 2E). Future insertion analyses using *tg*'s designed for rescue of insertion sites will address the question of whether the effectiveness of insertion observed is transposase dependent or a random event. This will lead to a better understanding of *Tn5p transposition reactions in mammals. Irrespective of the outcome of such analyses, TN:ICSI results in an elevated effectiveness of *tg* insertion into host chromosomes. Currently there is no information on the *tg* size limitation for *Tn5p mediated insertions. Therefore, it is possible that Tn5 will be successful in the active insertion of larger *tg*'s characterized by bacterial and yeast artificial chromosomes. If successful, this will overcome the *tg* size limitation (9.5 kb) demonstrated by lentiviral vectors.

TN:ROSI offers unique possibilities for transgenesis and genetic rescue of azoospermic animals that do not produce the spermatozoa needed for normal sexual or ICSI-mediated fertilization. To date, the only published study describing presperm transgenesis was performed with freeze-thawed elongating rat spermatids. The transgenic success rate with this attempt was 0.985% for o_i [34].

Using inbred strains of mice to generate transgenic animals for biomedical research can minimize the problem of genetic variation between individuals. However, the efficiency of transgenesis by pronuclear injection in a strain such as C57BL/6 is only one eighth of that obtained using a hybrid strain [35]. TN:ICSI transgenesis attempts with the same strain of inbred mice result in an efficiency that is 38% of the TN:ICSI transgenesis with hybrid strains (Table 1A, row 3). Therefore, the *Tn5p transgenesis method has additionally contributed to an increased effectiveness with inbred mouse strain transgenesis.

None of the procedures mentioned above have been optimized in terms of transposome quantity, incubation conditions, or *tg* size. Such improvement in conditions employed might permit the transposome injection technique to demonstrate more effective insertions of *tg*'s. However, we had no success when we tried to extend transposome injection into the pronuclei or cytoplasm of single-celled embryos. Presently we do not have sufficient information to explain

these low transgenesis rates with pronuclei stage embryos, but it is possible that the chromatin decondensation and remodeling that a spermatozoon or a round spermatid nucleus undergoes after injection into a mature oocyte [26, 36–38], may allow an opportunity for transposase enzymes to integrate the transposon into the embryo genome. The low success rates with transposomes during pronuclear microinjections could be because the injections are usually performed during or after the replication of pronuclear DNA [39]. By that time histones have replaced protamines in the male pronucleus, and the structure of chromatin might be more compacted [37]. Probably these are not conditions that favor insertion of *tg*'s by Tn5 transposases, which usually operate in a bacterial environment lacking a nuclear envelope. We suspect that it is for the same reasons that transposome injections into the cytoplasm do not work, and realize that the control DNA-only injections for pronuclear and cytoplasmic microinjections are low in number (Table 1C, row 2 and 1D, row 2). However, our aim was not to produce transgenic mice by liner DNA microinjection into the pronucleus or cytoplasm. Successful pronuclear microinjections are achieved routinely elsewhere [1]. Instead, our intent was to have transposon controls to compare with transposome injections into the pronucleus and cytoplasm. Because these later microinjection techniques were not efficient with transposome injections, the need to produce a higher number of control animals seems redundant.

Recent advances in embryonic germ cell culture and in vitro differentiation could tap into a plentiful and economical source of germ cells for both TN:ROSI and TN:ICSI transgenesis approaches [40, 41], leading to safe and effective gene delivery in mammals. Extrapolating transposome transgenesis methodology from mice to larger animals would represent a huge improvement in technical ease and effectiveness. Such increases in effectiveness would reduce costs and, when extended to the livestock industry, may significantly facilitate the production of value added commercial animals.

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REFERENCES

1. Nakanishi T, Kuroiwa A, Yamada S, Isotani A, Yamashita A, Tairaka A, Hayashi T, Takagi T, Ikawa M, Matsuda Y, Okabe M. FISH analysis of 142 EGFP transgene integration sites into the mouse genome. *Genomics* 2002; 80:564–574.
2. Perry AC, Wakayama T, Kishikawa H, Kasai T, Okabe M, Toyoda Y, Yanagimachi R. Mammalian transgenesis by intracytoplasmic sperm injection. *Science* 1999; 284:1180–1183.
3. Lois C, Hong EJ, Pease S, Brown EJ, Baltimore D. Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. *Science* 2002; 295:868–872.
4. Wall RJ. New gene transfer methods. *Theriogenology* 2002; 57:189–201.
5. Yanagimachi R. Mammalian fertilization. In: Knobil E, Neill JD (eds.) *The Physiology of Reproduction*, 2nd ed. New York: Raven Press; 1994:189–317.
6. Perry AC. Hijacking oocyte DNA repair machinery in transgenesis? *Mol Reprod Dev* 2000; 56:319–324.
7. Moreira PN, Giraldo P, Cozar P, Pozueta J, Jimenez A, Montoliu L, Gutierrez-Adan A. Efficient generation of transgenic mice with intact yeast artificial chromosomes by intracytoplasmic sperm injection. *Biol Reprod* 2004; 71:1943–1947.
8. Wall RJ. Pronuclear microinjection. *Cloning Stem Cells* 2001; 3:209–220.
9. Hirabayashi M, Takahashi R, Ito K, Kashiwazaki N, Hirao M, Hirasawa K, Hochi S, Ueda M. A comparative study on the integration of exogenous

- DNA into mouse, rat, rabbit, and pig genomes. *Exp Anim* 2001; 50:125–131.
10. Perry AC, Rothman A, de las Heras JI, Feinstein P, Mombaerts P, Cooke HJ, Wakayama T. Efficient metaphase II transgenesis with different transgene archetypes. *Nat Biotechnol* 2001; 19:1071–1073.
 11. Szczygiel MA, Moisyadi S, Ward WS. Expression of foreign DNA is associated with paternal chromosome degradation in intracytoplasmic sperm injection-mediated transgenesis in the mouse. *Biol Reprod* 2003; 68:1903–1910.
 12. Garrick D, Fiering S, Martin DI, Whitelaw E. Repeat-induced gene silencing in mammals. *Nat Genet* 1998; 18:56–59.
 13. Hofmann A, Kessler B, Ewerling S, Weppert M, Vogg B, Ludwig H, Stojkovic M, Boelhaue M, Brem G, Wolf E, Pfeifer A. Efficient transgenesis in farm animals by lentiviral vectors. *EMBO Rep* 2003; 4:1054–1060.
 14. Whitelaw CB. Transgenic livestock made easy. *Trends Biotechnol* 2004; 22:157–159, 159–160 (discussion).
 15. Hofmann A, Zakhartchenko V, Weppert M, Sebald H, Wenigerkind H, Brem G, Wolf E, Pfeifer A. Generation of transgenic cattle by lentiviral gene transfer into oocytes. *Biol Reprod* 2004; 71:405–409.
 16. Reznikoff WS. Tn5 as a model for understanding DNA transposition. *Mol Microbiol* 2003; 47:1199–1206.
 17. Peterson G, Reznikoff W. Tn5 transposase active site mutations suggest position of donor backbone DNA in synaptic complex. *J Biol Chem* 2003; 278:1904–1909.
 18. Goryshin IY, Reznikoff WS. Tn5 in vitro transposition. *J Biol Chem* 1998; 273:7367–7374.
 19. Naumann TA, Reznikoff WS. Tn5 transposase active site mutants. *J Biol Chem* 2002; 277:17623–17629.
 20. Naumann TA, Reznikoff WS. Tn5 transposase with an altered specificity for transposon ends. *J Bacteriol* 2002; 184:233–240.
 21. Goryshin IY, Miller JA, Kil YV, Lanzov VA, Reznikoff WS. Tn5/IS50 target recognition. *Proc Natl Acad Sci U S A* 1998; 95:10716–10721.
 22. Davies DR, Goryshin IY, Reznikoff WS, Rayment I. Three-dimensional structure of the Tn5 synaptic complex transposition intermediate. *Science* 2000; 289:77–85.
 23. Goryshin IY, Jendrisak J, Hoffman LM, Meis R, Reznikoff WS. Insertional transposon mutagenesis by electroporation of released Tn5 transposition complexes. *Nat Biotechnol* 2000; 18:97–100.
 24. Goryshin IY, Naumann TA, Apodaca J, Reznikoff WS. Chromosomal deletion formation system based on Tn5 double transposition: use for making minimal genomes and essential gene analysis. *Genome Res* 2003; 13:644–653.
 25. Ikawa M, Kominami K, Yoshimura Y, Tanaka K, Nishimune Y, Okabe M. A rapid and non-invasive selection of transgenic embryos before implantation using green fluorescent protein (GFP). *FEBS Lett* 1995; 375:125–128.
 26. Kimura Y, Yanagimachi R. Intracytoplasmic sperm injection in the mouse. *Biol Reprod* 1995; 52:709–720.
 27. Ogura A, Matsuda J, Yanagimachi R. Birth of normal young after electrofusion of mouse oocytes with round spermatids. *Proc Natl Acad Sci U S A* 1994; 91:7460–7462.
 28. Kimura Y, Yanagimachi R. Mouse oocytes injected with testicular spermatozoa or round spermatids can develop into normal offspring. *Development* 1995; 121:2397–2405.
 29. Kline D, Kline JT. Repetitive calcium transients and the role of calcium in exocytosis and cell cycle activation in the mouse egg. *Dev Biol* 1992; 149:80–89.
 30. Shamanski FL, Kimura Y, Lavoie MC, Pedersen RA, Yanagimachi R. Status of genomic imprinting in mouse spermatids. *Hum Reprod* 1999; 14:1050–1056.
 31. Kimura Y, Yanagimachi R. Development of normal mice from oocytes injected with secondary spermatocyte nuclei. *Biol Reprod* 1995; 53:855–862.
 32. Dupuy AJ, Clark K, Carlson CM, Fritz S, Davidson AE, Markley KM, Finley K, Fletcher CF, Ekker SC, Hackett PB, Horn S, Largaespada DA. Mammalian germ-line transgenesis by transposition. *Proc Natl Acad Sci U S A* 2002; 99:4495–4499.
 33. Jaenisch R, Mintz B. Simian virus 40 DNA sequences in DNA of healthy adult mice derived from preimplantation blastocysts injected with viral DNA. *Proc Natl Acad Sci U S A* 1974; 71:1250–1254.
 34. Kato M, Ishikawa A, Kaneko R, Yagi T, Hochi S, Hirabayashi M. Production of transgenic rats by ooplasmic injection of spermatogenic cells exposed to exogenous DNA: a preliminary study. *Mol Reprod Dev* 2004; 69:153–158.
 35. Brinster RL, Chen HY, Trumbauer ME, Yagle MK, Palmiter RD. Factors affecting the efficiency of introducing foreign DNA into mice by microinjecting eggs. *Proc Natl Acad Sci U S A* 1985; 82:4438–4442.
 36. Ramalho-Santos J, Sutovsky P, Simerly C, Oko R, Wessel GM, Hewitson L, Schatten G. ICSI choreography: fate of sperm structures after monospermic rhesus ICSI and first cell cycle implications. *Hum Reprod* 2000; 15:2610–2620.
 37. Gao S, Chung YG, Parseghian MH, King GJ, Adashi EY, Latham KE. Rapid H1 linker histone transitions following fertilization or somatic cell nuclear transfer: evidence for a uniform developmental program in mice. *Dev Biol* 2004; 266:62–75.
 38. Chapman JC, Michael SD. Proposed mechanism for sperm chromatin condensation/decondensation in the male rat. *Reprod Biol Endocrinol* 2003; 1:20.
 39. Luthardt FW, Donahue RP. Pronuclear DNA synthesis in mouse eggs. An autoradiographic study. *Exp Cell Res* 1973; 82:143–151.
 40. Marh J, Tres LL, Yamazaki Y, Yanagimachi R, Kierszenbaum AL. Mouse round spermatids developed in vitro from preexisting spermatocytes can produce normal offspring by nuclear injection into in vivo-developed mature oocytes. *Biol Reprod* 2003; 69:169–176.
 41. Geijsen N, Horoschak M, Kim K, Gribnau J, Eggan K, Daley GQ. Derivation of embryonic germ cells and male gametes from embryonic stem cells. *Nature* 2004; 427:148–154.