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Recombinase-mediated mouse transgenesis by intracytoplasmic sperm injection

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Abstract

The low efficiency of current microinjection-based animal transgenesis techniques is largely the result of poor embryo survival. We have developed a new, bacterial recombinase-based transgenesis method. Intracytoplasmic sperm injection (ICSI) of single stranded DNA (ssDNA) complexed with *E. coli* recombinase RecA into mouse metaphaseII (MII) arrested oocytes resulted in RecA-dependent transgenesis. This approach offers significant advantages over pronuclear microinjection and previous ICSI-based transgenesis approaches in terms of improved embryo survival, which translates into greater transgenesis efficiency. It also opens the possibility to attempt experiments, which may affect gene targeting by homologous recombination into DNA of mammalian single celled pre-implantation embryos.

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1. Introduction

Gene delivery and production of transgenic animals are becoming increasingly important aspects of basic and applied research. Successful animal transgenesis implies stable and heritable chromosomal insertion and expression of transgene (*tg*) DNA, and its eventual germline transmission. Despite recent progress achieved with transposon [5] and lentiviral-derived vectors [6,9], the delivery of naked double-stranded DNA (dsDNA) by microinjection is the most frequently used method of generating transgenic animals, especially mice. Pronuclear microinjection of dsDNA, albeit effective, is also inefficient, usually resulting in fewer than 5% of the manipulated oocytes being born as live transgenic pups [14]. Another recent microinjection-based technique is Intracytoplasmic Sperm Injection (ICSI) mediated transgenesis (ICSI-Tr) [19], which relies on incubating linear dsDNA containing the *tg* with mouse spermatozoa, demembrated by either TritonX-100 treatment or freeze-thawing. The DNA-coated sperm is injected into oocytes by ICSI, and the *tg* is incorporated into the genome of embryos. On average, ICSI-Tr has an efficiency of 2–5% of oocytes injected or 20–40% of offspring born being transgenic [13,18,19]. Both techniques are considered as passive means of transgenesis, as the integration of *tg* into the host genome relies on the embryo's DNA repair machinery for integration [16]. It has been shown that many freeze-thawed spermatozoa treated with foreign DNA in the course of ICSI-Tr have increasing incidence of broken paternal chromosomes [21]. Some degree of chromosome breakage is probably necessary for the insertion of the *tg*; however, excessive amounts of chromosomal breakage may lead to developmental arrest. The efficiency of transgenesis could therefore potentially be increased by delivering the *tg* DNA in conjunction with recombinases capable of mediating the integration process, during ICSI-Tr performed with non-frozen spermatozoa.

E. coli RecA is the best-studied bacterial recombinase. It initiates pairing by binding to ssDNA and by forming a helical nucleoprotein presynaptic filament. RecA was shown to protect ssDNA from degradation by creating a protective coating during its binding to it [4]. Such protective mechanism has also been suggested to influence ssDNA stability during pronuclear microinjection mediated transgenesis with RecA:ssDNA complexes during pig and goat transgenesis attempts [11,12]. This RecA coating of ssDNA resulted in higher embryo survival and transgene integration frequencies. Surprisingly however, many of the founder animals generated by this RecA ssDNA coating protocol were mosaic [12].

Pronuclear microinjections of single-celled zygotes is usually performed during or after the replication of pronuclear DNA [10]. Therefore an unequal distribution of *tg*, depending as to when it integrated into the genome of the developing embryo is to be expected, even if it occurred before the first cell division [2]. Such insertions would result in mosaic patterns of expression observed elsewhere [20,22]. To overcome such complications, in this study we employed ICSI to deliver EGFP-coding *tg*'s as RecA:ssDNA complexes (RecA-ICSI) into oocytes before activation, sperm-decondensation and pronuclear formation. Sperm heads freshly isolated from live spermatozoa by a piezo-electric pulse were individually co-injected into mouse metaphase II (MII) oocytes either with RecA:ssDNA complexes or with control ssDNA alone. The resulting zygotes were assayed for development and EGFP fluorescence at the two-cell stage, following which the viable two-cell embryos were transferred into oviducts of pseudopregnant females. The resulting F₀ progeny was also screened for EGFP

expression by analysis of fluorescence in their skin and internal organs, and was tested for the pattern of transgene integration. F₁ progeny of transgenic founders produced by mating F₀ mice with wild type partners were re-tested for transgene expression and integration patterns.

2. Materials and methods

2.1. Production of ssDNA

The EGFP-expressing cassette from pCX-EGFP (a kind gift from Dr. Masaru Okabe) was cloned into the polylinker site of a Y3k-s m13 bacteriophage [15]. This was done by excising the entire EGFP insert spanning the promoter and coding sequence from pCX-EGFP by a double restriction digestion with the enzymes *Bam*HI and *Sal*I and purifying it on a 1% agarose gel. The reporter cassette was cloned into the polylinker site of Y3k-s m13 bacteriophage vector flanked by two different *Pvu*II restriction sites resulting in Y3-CX/EGFP. *E. coli* XL1-Blue F' cells were transformed with dsY3-CX/EGFP phage and ssDNA phagemids were produced. After harvesting of the ssDNA, two 30-mer oligonucleotides Y3-PvuII3' (TCGCTATTACGCCAGCTGGCGAAAGGGGGA) and Y3-PvuII5' (ACCTGTCGTGCCAGCTGCATTAATGAATCG) matching the sequence of the ssY3-CX/EGFP phagemid flanking the reporter cassette and containing the *Pvu*II restriction sites were annealed to the circular ssDNA in *Pvu*II restriction buffer (10 mM TRIS-Cl pH 7.9, 50 mM NaCl, 10 mM MgCl₂, 1 mM DTT). The 3.0 kb ssDNA fragment containing the EGFP expression cassette was excised from the phagemid ssDNA with *Pvu*II and purified on a 0.7% agarose gel.

The gel purified ssDNA transgene cassette was quantified and then used for gel retardation experiments with the RecA protein to demonstrate the binding efficiency of the bacterial recombinase to ssDNA (Fig. 1).

2.2. Preparation of RecA:ssDNA complexes

Preparation of ssDNA + RecA mixture was initiated by removal of the 50% glycerol in which RecA (Epicentre, USA) was shipped, by gel filtration through a Microspin G-25 column (Amersham) as follows: the column was washed by placing 70 μ l of 1X TKM buffer (25 mM Tris-HCl pH8.0, 150 mM KCl, 2 mM MgCl₂) into the center of the column bed and centrifuged at $735 \times g$ for 1 min at 4 °C. This centrifugation step was repeated two more times with each spin lasting for 2 min and the flow through discarded. Thirty-five microliters of RecA was mixed with 35 μ l of 2X TKM buffer, placed to the center of the column bed and centrifuged for 2 min at 4 °C. The 50% diluted RecA flow through, free of glycerol, was then added to the ssDNA mixture in a total volume of 20 μ l at the required concentration to satisfy the 40:1 protein:DNA (wt:wt) ratio. Depending on the experiment 200, 400, 800 or 1600 ng ssDNA was mixed with 8, 16, 32 or 64 μ g RecA respectively, to achieve final ssDNA concentrations of 5, 10, 20 or 40 ng/ μ l while maintaining 1:40 wt:wt DNA:protein ratio deemed necessary to coat all ssDNA. Briefly, the required amount of ssDNA was incubated at 95 °C 5 min in 1X TKM buffer. The denatured ssDNA was quenched on ice and appropriate quantities of 5 μ g/ μ l RecA solution were added to obtain

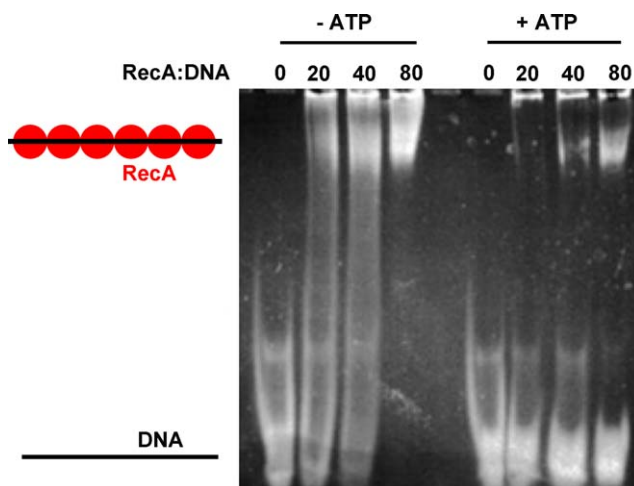


Fig. 1. Gel retardation of ssDNA by RecA. –ATP = reaction mixtures without ATP. +ATP = reaction mixtures containing 5 μ M ATP. Numbers on top of lanes indicate RecA concentrations in nanograms relative to 1 ng of ssDNA (wt.:wt.). Un-retarded ssDNA is indicated by a straight black line. Retarded ssDNA is represented by straight black line surrounded by RecA molecules represented by red circles. For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.

the required protein concentration; the reaction mixture was incubated on ice for 1 h and then mixed with fresh sperm and used for RecA-ICSI-mediated transgenesis.

2.3. Gel retardation experiments

RecA:ssDNA binding reactions containing different amounts of RecA were prepared in TKM buffer containing 10% glycerol. The binding was carried out in the presence (5 μ M) or absence of ATP. The reactions were allowed to proceed on ice for 1 h and run on 4% acrylamide gels (1 mm thick 15 cm \times 15 cm) in 0.25 \times TBE buffer at 4 $^{\circ}$ C and 10 V/cm. The ssDNA was visualized by staining the gel with SYBR Green II RNA dye (Fig. 1).

2.4. Preparation of microinjection media

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). CZB medium was used under 5% CO₂ in air, and HEPES-CZB was used under air.

2.5. RecA-ICSI microinjection

ICSI was carried out essentially as described by Kimura and Yanagimachi [8]. MII Oocytes were collected from superovulated 8–12-week-old B6D2F₁ females induced by

intraperitoneal injection of 5 IU pregnant mare serum gonadotropin (PMSG), followed by injection of 5 IU human chorionic gonadotrophin (hCG) 48 h later. Matured oocytes were collected from the ampullary region of oviducts 13–15 h after hCG injection. Oocytes were freed from cumulus cells by treatment with 0.1% bovine testicular hyaluronidase (359 U/mg solid) in HEPES-CZB medium, rinsed and kept at room temperature (26 °C) in fresh HEPES-CZB medium before sperm injection. Epididymal spermatozoa were collected from 8- to 12-week-old B6D2F₁ males by “swim-up” procedure in HEPES-CZB medium. A 10 µl aliquot of the ssDNA + RecA solution was mixed with 10 µl of sperm suspension. Motile spermatozoa were collected and placed in another 20 µl droplet containing a mixture of the appropriate concentration of RecA + ssDNA solution and 12% (w/v) polyvinylpyrrolidone (PVP; Mr 360,000) prepared in HEPES-CZB. A single motile spermatozoon was drawn, tail first, into the injection pipette in such a way that its neck was at the opening of the pipette. The head was separated from the tail by applying a few piezo-pulses to the neck region. The tail-less spermatozoon was injected into an oocyte. RecA-ICSI oocytes were cultured in CZB medium at 37 °C under 5% CO₂ in air.

2.6. Embryo culture and embryo transfer

Oocytes with two well-developed pronuclei and a distinct second polar body at 5 h after RecA-ICSI were recorded as being activated and cultured in CZB medium until they reached the two-cell stage (20–24 h after ICSI). Following culture, the embryos were transferred into the oviducts of 8–16-week old surrogate CD-1 females, mated with vasectomized males of the same strain 1 day before embryo transfer.

3. Results

3.1. Gel retardation experiments

The RecA protein was shown to cooperatively bind ssDNA, with a stoichiometry of one RecA protein monomer for every three nucleotides of DNA [1] and to protect the DNA from digestion by nucleases and phosphodiesterases [24]. Since RecA is a DNA-dependent ATPase we tested whether its ssDNA binding activity is affected by the presence of ATP.

Gel retardation experiments performed with ssDNA in the presence and absence of ATP showed, somewhat surprisingly, that the stable RecA:ssDNA complexes are formed more readily in the absence of ATP (Fig. 1). This finding could be explained by the more dynamic nature of the complex in the presence of ATP or simply by the negatively charged ATP molecules interfering with the electrophoretic mobility of the RecA + ssDNA complex. Our gel retardation data showed that all of the DNA is incorporated into the complex at a 80:1 RecA:DNA ratio (wt:wt). Preliminary ICSI microinjections with the complex showed optimal *tg* integration rates when 40:1 or 80:1 RecA:ssDNA ratios are used (data not shown); consequently complexes formed with the 40:1 RecA:DNA ratio were employed for microinjection, reducing the amounts of foreign substances injected into oocytes and resulting in better embryo survival.

Table 1
Summary of RecA-ICSI transgenesis experiments

Complex	DNA concentration (ng/ μ l)	Oocytes injected (=n)	Oocytes activated	Number of two-cell embryos EGFP positive (% activated)	Embryos transferred (surrogates)	Pups born (% transferred)	Transgenic pups Total (% births) (% oocytes)			Transgene copy number ^a
ssDNA	5	84 (2)	56	10 (18)	54 (4)	29 (54)	0	0	0	NA
ssDNA	10	55 (2)	30	11 (37)	30 (3)	15 (50)	0	0	0	NA
ssDNA + RecA	5	90 (3)	74	19 (26)	69 (6)	45 (65)	6	13.3	6.7	2.15
ssDNA + RecA	10	69 (2)	58	21 (36)	56 (4)	27 (48)	2	7.4	2.9	8.17
ssDNA + RecA	20	52 (2)	41	12 (29)	36 (3)	10 (28)	2	40.0	7.7	3.10
ssDNA + RecA	40	55 (2)	51	13 (26)	51 (4)	34 (67)	2	5.9	3.6	4.8

^a Transgene copy numbers as determined by Southern analysis. Two independent F₀ transformants were analyzed for each DNA concentration used.

Our ability to produce large quantities of ssDNA and to visualize it without radioactive tagging is an improvement over methods used previously [11,25]. Additionally, the production of large quantities of linear ssDNA represents an improved experimental advance over pronuclear microinjection experiments, in which linear dsDNA is heat-denatured and added to a protein coating mixture containing buffer, nucleotide cofactors and RecA protein [11]. The mere presence of the opposing strand of dsDNA, in the absence of readily available ssDNA, will necessitate the addition of extra RecA molecules to satisfy the stoichiometric needs of the DNA:RecA binding requirements. This will lead to the microinjection of considerable more excessive foreign material into the pronucleus, with the resulting outcome remaining unknown.

3.2. Transgene delivery by ICSI

Coinjection of fresh sperm heads and either ssDNA or RecA:ssDNA complexes into MII oocytes resulted in fertilization and developmental rates comparable to those obtained by ICSI performed with fresh sperm alone [8] (Table 1). This result suggests that neither naked ssDNA nor RecA:ssDNA were deleterious to embryo survival at the concentrations used. EGFP fluorescence was detected in 18–37% of two-cell embryos, regardless of the amount of ssDNA injected, or the presence or absence of the RecA protein. The resulting birth rates varied from 28 to 67% of embryos transferred. Such embryo survival rates exceeded those obtained with “frozen-sperm” ICSI-TR, which typically lie in the 20–36% range [18,19]. The most probable explanation for these improved values is the omission of the sperm freezing or Triton X-100 treatment step, which maintains the integrity of the sperm genome. Somewhat surprisingly, only experiments performed with RecA:ssDNA complexes resulted in birth of EGFP-expressing pups. This finding suggests that, despite the presence of *tg* ssDNA inside the nucleus of the developing embryo, as indicated by two-cell EGFP fluorescence, only embryos into which the ssDNA was injected as a complex with RecA could give rise to transgenic animals (Table 1). Transgenesis efficiency, as determined by the percentage of transgenic animals born, varied from 5.9 to 40%, with the complex containing 20 ng/ μ l ssDNA giving the best results. However, in these RecA-ICSI mediated transgenesis experiments all of the offspring where true transgenics and did not demonstrate a mosaic pattern of expression (Fig. 2B).

3.3. Transient transgene expression in embryos versus stable transgene integration in F_0 progeny

The presence of EGFP fluorescence at the blastocyst stage has been suggested as evidence of stable *tg* integration in some cases [7,17]. Even though we could only obtain transgenic animals by ICSI with RecA:ssDNA complexes (Table 1), both embryos injected with ssDNA and RecA:ssDNA complexes showed EGFP fluorescence prior to transfer into surrogate mothers. We followed the EGFP expression during embryo development subsequent to ICSI with either ssDNA alone or RecA:ssDNA complex. We found in both cases that embryos demonstrating EGFP fluorescence in the early stages of development maintained it to the blastocyst stage (Table 2, rows 1 and 2). We then investigated whether EGFP fluorescence at embryo stage is tantamount to producing a transgenic animal. We

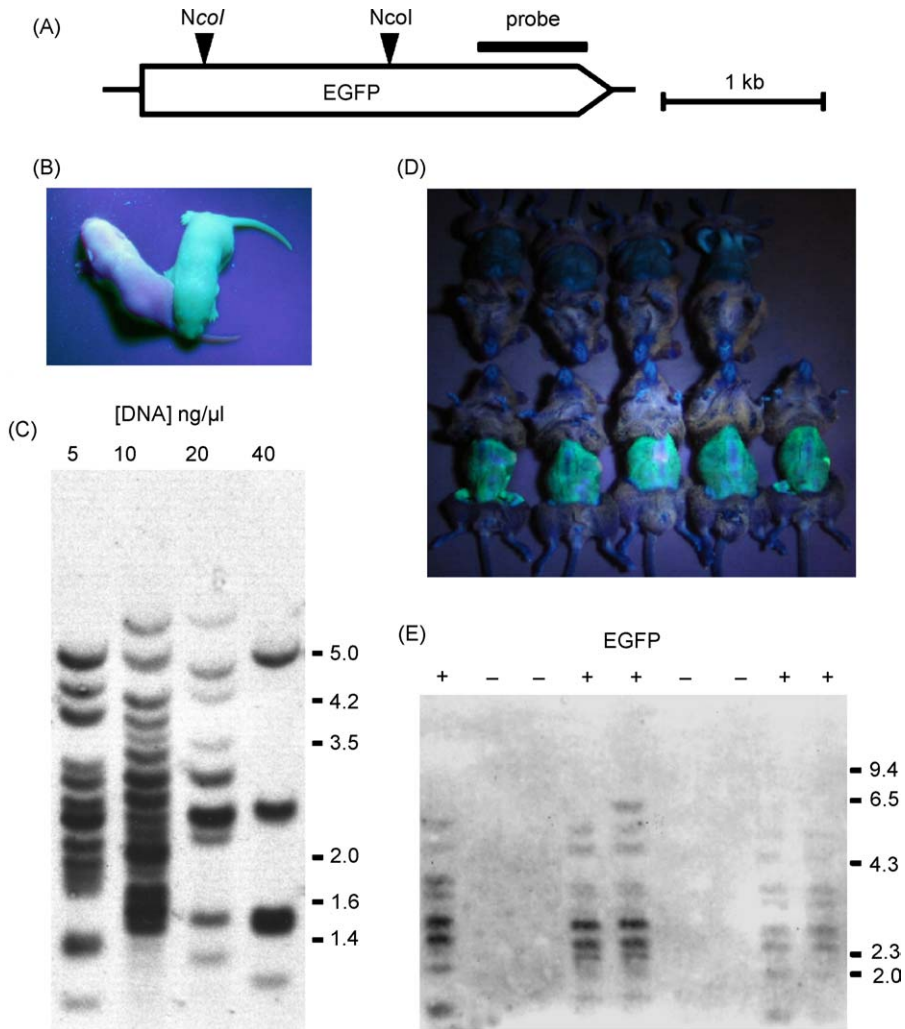


Fig. 2. Analysis of transgenic F_0 founder animals and their F_1 offspring. Mouse genomic DNA was cut with *NcoI* and probed with a 416 bp fragment complementary to the 3' end of the EGFP reporter cassette. (A) Scheme of the probe and restriction enzymes used in Southern analysis. (B) Transgenic pup (right) obtained by RecA-ICSI and its non-transgenic littermate viewed under UV light. (C) Southern analysis of four typical F_0 founder animals obtained following RecA-ICSI procedure. Genomic (20 μ g) DNA from four transgenic animals obtained by ICSI with RecA:ssDNA complexes containing 5, 10, 20 and 40 ng/ μ l DNA, respectively, was cleaved with *NcoI* and probed for the presence of the EGFP gene. (D) Segregation of EGFP expression in F_1 progeny of one of the transgenic F_0 founder animals following a back-cross with a non-transgenic mate. The litter of nine pups contained four wild-type (top) and five EGFP-expressing (bottom) littermates. Due to skin and hair pigmentation only the fluorescence of internal organs can be observed. (E) Transgene segregation analysis by Southern blotting performed on the DNA of the F_1 progeny from Fig. 1D. Genomic DNA (10 μ g) from each animal was cleaved with *NcoI* and probed for the presence of the EGFP gene.

Table 2
Developmental visualization of embryos under differing treatment conditions

Experiment	Complex	DNA concentration (ng/μL)	Oocytes injected	Oocytes activated	Green fluorescence				Embryos transferred (surrogates)	Pups born	EGFP + pups
					Two-cell	Four-cell	Morula	Blastocyst			
1	ssDNA	2.0	49	34	0 of 34	0 of 34	2 of 34	3 of 27	NA	NA	NA
2	ssDNA + RecA	5.0	22	16	5 of 23	5 of 23	5 of 23	5 of 23	NA	NA	NA
3	ssDNA + RecA	5.0	39	30	6 of 30	6 of 30	–	–	Six green (1) 24 non-green (2)	3 9	0 0

performed ICSI on 32 oocytes with 5 ng/ μ l ssDNA:RecA complex (Table 2, row 3) and followed the persistence of EGFP fluorescence both at the time of transfer at the four-cell stage and at birth. Six of 30 embryos demonstrated EGFP fluorescence at the four-cell stage. Based on previous observation (Table 2, rows 1 and 2), we assumed that the fluorescence would persist at least till the blastocyst stage in these embryos. The six EGFP-expressing and the 24 non-fluorescing embryos were transferred into separate surrogate mothers. Three and nine non-transgenic pups were obtained from the fluorescing and non-fluorescing embryos, respectively. Therefore, it can be inferred that in the RecA:ssDNA experiments undertaken here most of the embryos demonstrating fluorescence at the blastocyst stage following microinjection do so in a transient manner with only some of them destined to integrating the *tg* and thus giving rise to truly transgenic animals.

It is not clear how microinjection of ssDNA alone results in transient transgene expression. Perhaps some cellular machinery converts ssDNA to dsDNA ectopically which is then transcribed in the nuclear milieu. While evidence for such machinery is lacking at present, the fact that ssDNA can be expressed just as efficiently as the RecA:ssDNA complex further support the notion that RecA mediates the stable insertion of the *tg* DNA into the genome of the developing embryo, rather than just protecting ssDNA as reported elsewhere [11,12].

Tg integration in EGFP-expressing mice could be confirmed by Southern blotting experiments (Table 1, Fig. 2C and E). The number of *tg* copies in different animals, ranged from 2 to almost 20. Some of the F₀ founders were mated with non-transgenic partners and their progeny (F₁) was analyzed, allowing the number of *tg* loci to be determined. Both the crossing results and subsequent Southern analysis pointed to close linkage of *tg* copies, indicative of multiple copies of the *tg* residing in single loci for all cases analyzed (Fig. 2E).

4. Discussion

The RecA-ICSI method outlined above appears to be more effective than the pronuclear microinjection-based transgenesis method done with RecA:ssDNA [12]. Furthermore, unlike in the case of pronuclear injections of RecA:ssDNA, all transgenic animals produced by RecA-ICSI were fully transgenic with all of them passing the trait to the next generation. This lack of mosaicism in case of RecA:ICSI may be due to the *tg* integrating into the host genome before the first cell division, during sperm chromatin decondensation. We have observed similar facilitated *tg* insertion during a transposase enzyme mediated insertion of a transposon during ICSI microinjections as a complex named transposome (TN:ICSI, submitted). Pronuclear microinjection transposome attempts were unsuccessful in that instance, perhaps due to the re-compaction of the chromatin during pronuclear formation. RecA-coated ssDNA integration during pronuclear microinjection might be a victim of such chromatin rearrangements that contribute to the mosaic expression patterns observed [12]. The RecA-ICSI method described herein, with its resulting true transgenic animals, may be especially suited to species in which either the number of oocytes is limited or in which the traditional methods of transgene delivery proved to be difficult either due to poor visualization of the pronucleus or other unknown factors. Embryo survival rates of RecA-ICSI also exceeded those reported for Lentiviral transgenesis [9]

and although not demonstrated here, should not suffer from *tg* size limitations particular to this approach.

Our results show that the RecA-ICSI transgenesis process is RecA dependent. The role of RecA in transgenesis could be the protection of ssDNA from degradation, as previously suggested for pronuclear injection of RecA:ssDNA complexes [12]. However, the EGFP fluorescence of embryos injected with either naked ssDNA or RecA:ssDNA clearly demonstrates that *tg* stability, as monitored by transient expression, is not RecA dependent during ICSI, suggesting that RecA plays a different, possibly enzymatic, role in *tg* integration. The exact mechanism by which RecA may facilitate random *tg* integration is not clear.

One possibility is that the *tg* DNA is directed into the genome through the use of microhomology search. Such a mechanism has been described in the case of integration of *Agrobacterium tumefaciens* T-DNA into the host plant genome [23]. The use of a RecA mutant that retains its ssDNA binding properties but is deficient in strand exchange activity [3] may shed light on the role of RecA in the transgenesis process. However, the most elegant way of testing and exploiting the homologous exchange properties of RecA may be by targeting of homologous sequences in the mouse genome. This approach may not only confirm the active role of RecA in the RecA-ICSI process but also lead to a simple alternative to ES cell-derived “knock-out” mice.

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