No Effect of Recombinase-Mediated DNA Transfer on Production Efficiency of Transgenic Rats

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Abstract: It was reported that recombinase-A protein (RecA)-coated exogenous DNA was more likely to be integrated into mouse, goat and pig genomes. The objective of this study was to investigate whether integration of exogenous DNA into the rat genome is improved by the recombinase-mediated DNA transfer. Pronuclear microinjection of RecA-coated EGFP or OAMB DNA resulted in a production efficiency of transgenic rats of 1.4–2.9%, comparable with 0.9–2.6% when non-coated control DNA was used. Intracytoplasmic injection of the sperm heads exposed to RecA-coated EGFP DNA did not produce any transgenic rats (0 vs. 0–2.8% in control groups). Thus, the recombinase-mediated DNA transfer contributed very little to the production of transgenic rats by means of pronuclear microinjection and intracytoplasmic sperm injection.

Key words: rat, RecA, transgenesis

Although pronuclear DNA microinjection is the most convenient approach to produce transgenic animals, the low integration of exogenous DNA into genomes is still an obstacle to the widening of the transgenic technology. In general, the proportion of microinjected zygotes developing into transgenic offspring is <5% in rodents [1, 5], and <1% in large domestic species [17, 22]. Another approach to production of transgenic animals is intracytoplasmic sperm injection (ICSI)-mediated transgenesis (ICSI-Tg) [16], in which <7% of inseminated oocytes develop into transgenic mice [16, 20] and rats [7, 9]. Maga et al. [11] recently reported that recombinase-A protein (RecA)-coated ssDNA was more likely to be integrated into goat and pig genomes after pronuclear microinjection (6- and 10-fold higher, respectively). Very recently, Kaneko et al. [8] have also reported that the ICSI-Tg using RecA-coated ssDNA produced transgenic mice at efficiencies of 2.9–7.7% of inseminated oocytes, while ICSI-Tg using the same ssDNA without RecA-coating resulted in no transgenic offspring. However, the mechanism by which the RecA-coating of exogenous DNA facilitates its integration into genomes remains unclear. The objective of this study was to investigate whether integration of exogenous DNA into the rat genome is promoted by recombinase-mediated DNA transfer us-
ing RecA protein in pronuclear microinjection and ICSI-Tg.

The dsEGFP DNA (3.0 kb) was prepared according to a previously described method [6]. The EcoRI-EcoRI cDNA fragment encoding EGFP was introduced into the EcoRI site of a pCXN2 expression vector [13] containing chicken β-actin enhancer-promoter and cytomegalovirus enhancer, β-actin intron and rabbit β-globin poly-A signal. The OAMB DNA (4.6 kb) was prepared by introducing a fragment encoding Drosophila octopamine receptor [4] into the EcoRI site of the pGG vector [1] containing human glial fibrillary acidic protein promoter and poly-A signal, followed by digestion with the Sa/I and NotI sites of the vector. Both linearized plasmid DNAs were separated by 1.2% agarose gel electrophoresis, extracted from the gel using the QIAEX II (Qiagen Inc., Valencia, CA, USA), dissolved in 10 mM Tris-HCl (pH 7.6) / 0.1 mM EDTA, and stored at 4°C until use. The recombinase protein, RecA, was bought from Roche Diagnostics (Basel, Switzerland). According to the method reported by Maga et al. [11], linear dsEGFP DNA (100 ng) was heat-denatured at 98°C for 5 min, and cooled on ice for 1 min. Then, the ssEGFP DNA was mixed at 4°C with 4.2 µg RecA protein in 10 mM Tris-acetate buffer containing 2 mM magnesium acetate and 2.4 mM ATPγS. The mixture was incubated at 37°C for 15 min and then the concentration of magnesium acetate was increased to 11 mM to terminate the reaction.

In Experiment 1, pronuclear DNA microinjection was performed as reported previously [21]. Briefly, Crlj:WI female rats (8 to 10 weeks old; Charles River Japan, Inc., Kanagawa, Japan) were superovulated by ip injections of 20 IU eCG (Nippon Zenyaku Kogyo, Co., Fukushima, Japan) and 10 IU hCG (Sankyo Yell Yakuhin, Co., Tokyo, Japan) at an interval of 48 h. The pronuclear-stage zygotes were collected from the oviductal ampullae 30 h after the hCG injection using modified rat 1-cell embryo culture medium (mR1ECM) [15] containing 110 mM NaCl and 4 mg/ml fatty acid-free BSA (Sigma-Aldrich Corp., St. Louis, MO, USA) instead of polyvinylalcohol: the mixture is referred to hereafter as mR1ECM/BSA. The zygotes were microinjected with dsEGFP and dsOAMB DNA (final concentration 5 ng/µl) or ssEGFP/RecA and ssOAMB/RecA (5 ng/µl), and cultured in 100 µl microdrops of mR1ECM/BSA under mineral oil at 37°C in 5% CO₂ in air. Morphologically normal zygotes at the 2-cell stage and non-degenerating 1-cell stage, harvested at 14 h post-DNA-microinjection, were transferred into the oviducts of recipient rats which had been previously mated with a vasectomized male rat. The embryo transfer to the pseudopregnant recipients was performed on the day that the vaginal plug was detected. The pregnant recipients were then allowed to deliver the offspring. The expression of EGFP DNA in the offspring was examined by fluorescence under 480 nm UV light. The integration of the DNA into genome was determined in the ear tissue of the offspring by PCR analysis [6, 7, 9], with a primer set for EGFP (EGFPF: 5’-GTT CAG CGT GTC CGG CGA-3’, EGFRP: 5’-GCG GTC ACG CCT GCC GCG C-3’) and with a primer set for OAMB (OAMBf: 5’-ATG GTT TCC CTT TGG CTG CTG ATC-3’, OAMBr: 5’-ATG GTT TCC CTT TGG ATA GGT CGG C-3’).

The RecA-coating of the exogenous DNAs resulted in higher cleavage rates of microinjected oocytes (91 to 98 vs. 52 to 56% in the non-coated groups), while post-injection survival of rat zygotes (86 to 99%) was not influenced by coating of the DNAs with RecA (Table 1). The protective action of RecA-coating agreed well with the results of Maga et al. [11]. However, the offspring rate from oocytes injected with the ssEGFP/RecA (20%) was comparable with that from those injected with dsEGFP (26%), when non-cleaved as well as cleaved oocytes were transferred into the recipients. The proportion of EGFP-carrying transgenic offspring in the ssEGFP/RecA group (18%, 5/28) tended to be higher (P=0.063) than that in the dsEGFP group (4%, 2/47). Thus, the overall production efficiency of EGFP-carrying transgenic rats per microinjected oocyte was 3-fold, but not significantly (P=0.150) higher than when exogenous DNA was coated with RecA recombinase (2.9 vs. 0.9%). In our previous larger-scale study using the same EGFP construct [9], similar proportions of oocyte survival (89% per injected oocyte), cleavage (63% per survivor), full-term development (30% per transferred oocyte) and offspring carrying the EGFP DNA (3% per newborn offspring) were obtained from a conventional microinjection procedure, resulting in the same overall production efficiency of EGFP-carrying transgenic rats (0.9%) as in the present result.

In contrast, there was a harmful effect of OAMB DNA on the full-term development of microinjected
THE EFFECT OF RECOMBINASE-MEDIATED RAT TRANSGENESIS

oocytes that was masked by coating with the RecA (46 vs. 11% in the dsOAMB group, *P* < 0.05; Table 1). The toxicity of exogenous DNA solution has been associated with the DNA concentration [2, 5, 14] and components of solvent buffer [2]. The design of exogenous DNA is responsible for the developmental loss of pronuclear zygotes after microinjection, but the design of the promoter region controlling the timing, specificity and level of structural gene expression must be important for the survival of injected zygotes. Coating of exogenous DNA with the RecA protein would be recommended especially in case the DNA itself was found developmentally harmful for the pronuclear zygotes. However, regardless of this positive action of the RecA, only two OAMB DNA-carrying transgenic offspring were detected among the resultant 56 offspring (4%, 2/56). The overall production efficiency of OAMB DNA-carrying transgenic rats per microinjected oocyte (1.4%) did not exceed the value in the non-coated control group (2.6%, 9/345).

In Experiment 2, ICSI-Tg was performed using a protocol reported previously [7, 9]. Briefly, a suspension of cauda epididymal spermatozoa was treated for 10 s using a 10% power output from an ultrasonic cell disruptor (Sonifier250, Branson, Danbury, CT, USA), and was then cryopreserved in liquid nitrogen until use. Nine microliters of sperm suspension, thawed in a 25°C water bath, were mixed with 1 µl of exogenous DNA solution, and kept for 1 min at ambient temperature (23 ± 2°C). The final concentration of exogenous DNA was 0.1 or 5 ng/µl. Oocytes were collected 14–17 h after hCG injection from Slc:SD female rats (4 to 5 weeks old; Japan SLC, Inc., Shizuoka, Japan) that were superovulated with 30 IU eCG, and 30 IU hCG at an interval of 48 h. The oocytes were freed from cumulus cells by a 5-min treatment with 0.1% hyaluronidase (Sigma-Aldrich) in mR1ECM/BSA. DNA-bound sperm heads were placed in 12% PVP solution and injected into the denuded oocytes in Hepes-mR1ECM medium (supplementation of Hepes at 22 mM and reduction of sodium bicarbonate to 5 mM) at an ambient temperature using a piezo impact-driving unit (PMM-140FU; Prime Tech, Co., Ibaraki, Japan) with a pulse controller (PMAS-CT150; Prime Tech). The ICSI oocytes were cultured *in vitro* and transferred into recipient rats 24 h post-ICSI. The presence and expression of EGFP DNA in the offspring rats were analyzed by PCR and fluorescence, respectively, as mentioned above.

When EGFP DNA at 0.1 ng/µl was used, the cleavage rate of oocytes injected with RecA-coated DNA (57%) was higher than that with non-coated DNA (29%, Table 2). However, the post-ICSI survival (85 to 89%) and the *in vivo* survival of rat oocytes (31 to 39%) were not influenced by the coating of DNA with RecA. There was no transgenic rat among 39 offspring that were born after transfer of 126 oocytes injected with ssEGFP/RecA-bound sperm heads, while four transgenic rats were produced from transfer of 103 oocytes by the standard ICSI-Tg procedure. Kaneko *et al.* [8] used 5 to 40 ng/µl EGFP DNA in the recombinase-mediated mouse transgenesis, with low cleavage rates (26 to 36%) but high offspring rates (28–67%).

### Table 1. Recombinase-mediated rat transgenesis by pronuclear DNA microinjection

<table>
<thead>
<tr>
<th>DNA</th>
<th>No. (%) of zygotes</th>
<th>No. (%) of offspring</th>
<th>Overall efficiency*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Injected / Survived / Cleaved / Transferred</td>
<td>Born / Transgenic / Expressed</td>
<td></td>
</tr>
<tr>
<td>ssEGFP/RecA</td>
<td>173/148 (86)/135 (91)</td>
<td>141/28 (20)/5 (18)</td>
<td>2.9</td>
</tr>
<tr>
<td>dsEGFP</td>
<td>211/183 (87)/102 (56)</td>
<td>182/47 (26)/2 (4)</td>
<td>0.9</td>
</tr>
<tr>
<td>ssOAMB/RecA</td>
<td>140/122 (87)/119 (98)</td>
<td>122/56 (46)/2 (4)</td>
<td>1.4</td>
</tr>
<tr>
<td>dsOAMB</td>
<td>345/343 (99)/180 (52)</td>
<td>308/33 (11)/9 (27)</td>
<td>2.6</td>
</tr>
</tbody>
</table>

*Calculated as; (No. of transgenic offspring / No. of injected oocytes) × 100. *Significantly different from the control dsEGFP group (Fisher’s exact probability test; *P* < 0.05). *Not analyzed.
ent in first cleavage (0%) and in vivo survival (0%) if the DNA was not coated with RecA protein (Table 2). This result agreed well with our previous report that ICSI after exposure to 5 ng/µl DNA resulted in no development beyond the 4-cell stage in vitro [7]. However, even with the protection of RecA-coating, there was no EGFP-carrying transgenic rat among the 26 newborn offspring produced by this procedure. The concentration of exogenous DNA solution used for exposure to mouse sperm heads before ICSI was 5 ng/µl [20] or 5–10 ng/µl [16]. Due to the larger surface area of the sperm heads in rats than in mice, exposure to a higher concentration of exogenous DNA may lead to excess DNA association with the sperm heads; the introduction of sperm heads with excess DNA into oocytes by ICSI may have a toxic effect on the development of the oocytes. RecA binds to single-strand DNA to form a nucleoprotein filament with a stoichiometry of one RecA monomer for every 3–4 nucleotides of DNA [3], and completely protects DNA from digestion by phosphodiesterases and nucleases [23]. These characteristics of RecA may explain the higher cleavage rate (in case of pronuclear DNA microinjection) and in vivo survival of microinjected rat oocytes (in case of ssOAMB/RecA and ICSI-Tg of 5 ng/µl ssEGFP/RecA) observed in the present study.

As to the expression of EGFP DNA, 4 out of 7 PCR-positive offspring in Experiment 1 (Table 1) and 3 out of 4 PCR-positive offspring in Experiment 2 (Table 2) were green-fluorescent transgenic rats (67% in total). Three out of five (60%) PCR-positive offspring derived from the recombinase-mediated DNA transfer method were the green rats. A high incidence of mosaicism has been reported in transgenic goats and pigs produced by RecA-coated DNA injection [11]. The true mosaicism of founder rats as assessed by transmission of the transgene to progeny was not investigated in the present study, and because of the small sample number, it remains to be clarified whether integration of transgenes into rat genomes is likely to be mosaic. In general, 70% of transgenic founders produced by pronuclear DNA microinjection are not mosaic and transmit their transgene to progeny.

There was no effect of recombinase-mediated transfer of exogenous DNA on rat genomes, by means of ICSI-Tg as well as pronuclear microinjection. The difference from earlier successful reports of this method in the mouse [8], pig and goat [11] may be due to a different cytoplasmic environment in rat oocytes. At the local site where the sperm head was deposited, a lower concentration of magnesium ion and/or a higher concentration of ATP would dissociate the RecA from ssDNA/RecA, resulting in degradation of the ssDNA by cytoplasmic phosphodiesterases and nucleases. In addition, the magnesium concentration in Hepes-mR1ECM medium used during rat ICSI-Tg (0.49 mM MgCl₂) [7, 9] was lower than that in CZB medium used during mouse ICSI-Tg (1.18 mM MgSO₄) [8]. While ATPγS leads to stable formation of ssDNA/RecA, ATP stimulates the release of RecA from ssDNA [12]. It remains to be clarified whether these factors are involved in the low integration efficiency of ssDNA/RecA into rat genomes.

In conclusion, the recombinase-mediated DNA transfer contributed very little to the production efficiency of transgenic rats by means of pronuclear microinjection and intracytoplasmic sperm injection, but coating of exogenous DNA with RecA protein reduced the toxic effect of exogenous DNA on rat embryos.

<table>
<thead>
<tr>
<th>DNA</th>
<th>Conc.</th>
<th>DNA Injected</th>
<th>No. (%) of oocytes</th>
<th>No. (%) of offspring</th>
<th>Overall efficiency*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Survived</td>
<td>Cleaved</td>
<td>Transferred</td>
<td>Born</td>
</tr>
<tr>
<td>ssEGFP/RecA</td>
<td>0.1</td>
<td>145</td>
<td>129 (89)</td>
<td>74 (57)%</td>
<td>126</td>
</tr>
<tr>
<td>dsEGFP</td>
<td>0.1</td>
<td>144</td>
<td>122 (85)</td>
<td>35 (29)</td>
<td>103</td>
</tr>
<tr>
<td>ssEGFP/RecA</td>
<td>5</td>
<td>129</td>
<td>113 (88)</td>
<td>38 (34)%</td>
<td>109</td>
</tr>
<tr>
<td>dsEGFP</td>
<td>5</td>
<td>131</td>
<td>106 (81)</td>
<td>0 (0)</td>
<td>102</td>
</tr>
</tbody>
</table>

*Calculated as: (No. of transgenic offspring / No. of injected oocytes) × 100. *Significantly different from the control dsEGFP group (Fisher’s exact probability test; P<0.05).

Table 2. Recombinase-mediated rat transgenesis by intracytoplasmic sperm injection
Acknowledgments

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