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A hyperactive *piggyBac* transposon system is an easy-to-implement method for introducing foreign genes into mouse preimplantation embryos

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Abstract. Transgenic mice are important tools for genetic analysis. A current prominent method for producing transgenic mice involves pronuclear microinjection into 1-cell embryos. However, the total transgenic efficiency obtained using this method is less than 10%. Here, we demonstrate that highly efficient transgenesis in mice can be achieved by cytoplasmic microinjection using a hyperactive *piggyBac* system. In embryos in which *hyPBase* mRNA and pPB-CAG-TagRFP DNA were co-injected into the cytoplasm, TagRFP fluorescence was observed after the 2-cell stage; when 30 ng/μl pPB-CAG-TagRFP DNA and 30 ng/μl *hyPBase* mRNA were co-injected, 94.4% of blastocysts were TagRFP positive. Furthermore, a high concentration of *hyPBase* mRNA resulted in creation of mosaic embryos in which the TagRFP signals partially disappeared. However, suitable concentrations of injected DNA and *hyPBase* mRNA produced embryos in which almost all blastomeres were TagRFP positive. Thus, the hyperactive *piggyBac* transposon system is an easy-to-implement and highly effective method that can contribute to production of transgenic mice.

Key words: Cytoplasmic injection, Hyperactive *piggyBac* transposase, Transgenic mice

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Genetic analysis in mice is essential for biology and medicine. Transgenic mice are important tools for genetic analysis. A current general method for producing transgenic mice is pronuclear microinjection of linear DNA into 1-cell embryos. However, pronuclear microinjection is technically challenging, and the survival percentage after pronuclear microinjection is very low [1]. Consequently, the final transgenesis efficiency (i.e., the rate at which individual animals are produced from manipulated embryos) obtained using this method is less than 10% (% of pups) [2]. To improve transgenesis efficiency, DNA transposons such as *Sleeping beauty*, *Tol2* and *piggyBac* have been used to produce transgenic mice [3–6]. The transposon system has several advantages: the transposase recognizes DNA regions flanked by terminal repeat sequences, so inserted DNAs tend to contain full sequences; in addition, the system is nonviral and thus avoids the risk of insertional mutagenesis. In the *Tol2* transposon system, a transgene donor plasmid and a *Tol2* transposase mRNA are microinjected into the cytoplasm of 1-cell embryos [3]; this manipulation is much easier than pronuclear microinjection. Moreover, the total transgenesis efficiency of the *Tol2* transposon system is more than 60% (% of pups). Recent studies showed

that a hyperactive (mutant) *piggyBac* transposase (*hyPBase*) had higher activity in both excision and integration assays than other types of transposases [7, 8]; consequently, this system has often been used to generate induced pluripotent stem (iPS) cells [9, 10] and to introduce transgenes in mouse iPS cells [11]. However, this method has not yet been used to generate transgenic mice. In this study, we designed a method using the hyperactive *piggyBac* transposon system to produce transgenic mice. As shown in Fig. 1, after microinjection of a donor transgene DNA (pPB-CAG-TagRFP) containing the inverted terminal repeats (ITRs; *hyPBase* recognition sequences) and the *hyPBase* mRNA, ITRs of the donor transgene DNA are cut, transported from the cytoplasm into the nucleus [12] and integrated into genomic DNA by the *hyPBase* protein. Therefore, the presence of TagRFP fluorescence in embryos indicates that the target transgene has been successfully integrated into genomic DNA by the hyperactive *piggyBac* transposon system.

To develop our transgenic method, we examined several conditions by testing different concentrations of the *hyPBase* mRNA. In addition, by comparing the signal observed in embryos not subjected to *hyPBase* mRNA injection, we confirmed that the TagRFP fluorescence observed in embryos microinjected with *hyPBase* mRNA originated from the integrated transgene. In embryos co-injected with pPB-CAG-TagRFP DNA (30 ng/μl) and *hyPBase* mRNA (30 ng/μl), the percentage of TagRFP-positive blastocysts was 94.4%, while the percentage was only 2.7% in embryos injected without *hyPBase* mRNA (Fig. 2A and Table 1), suggesting that the *hyPBase* system is a very effective method. However, since a small amount of embryos injected without *hyPBase* mRNA exhibited the TagRFP signals (2.7%), we cannot

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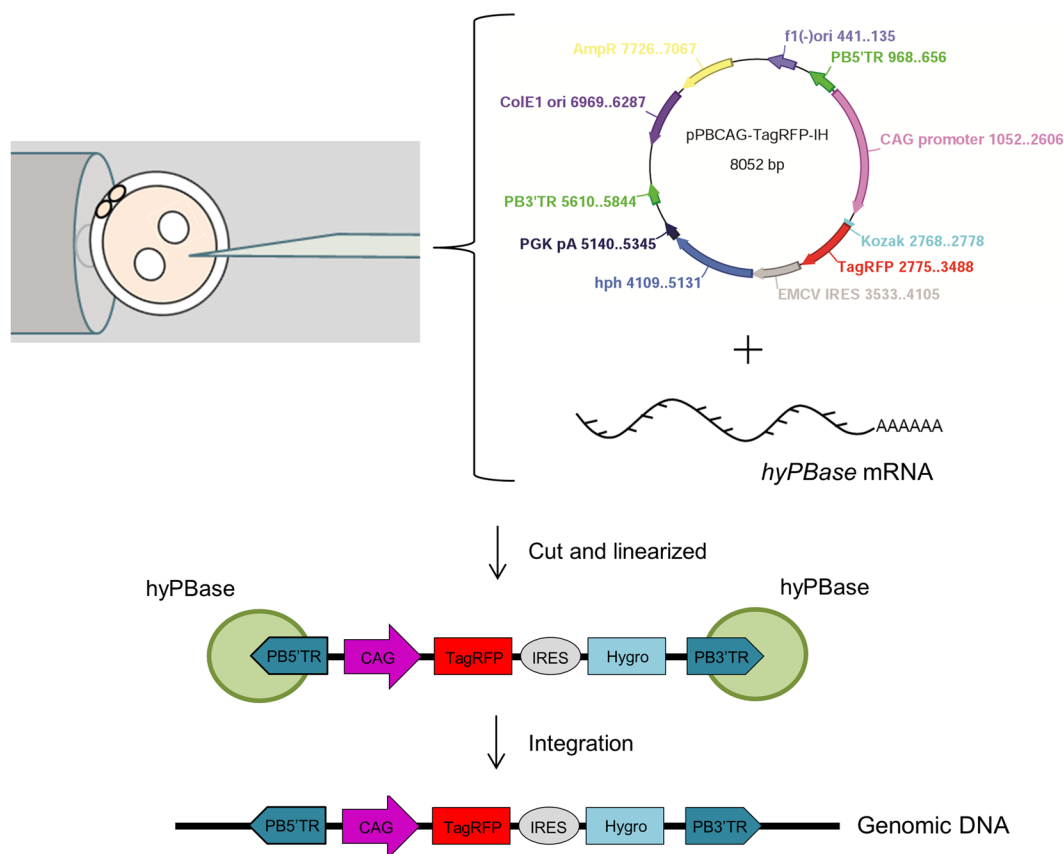


Fig. 1. A schematic illustration of microinjection into the cytoplasm of 1-cell embryos and target DNA integration into genomic DNA mediated by hyPBase proteins.

exclude the possibility that part of the TagRFP signals are derived from donor plasmid DNA. Furthermore, we determined when TagRFP fluorescence starts to emerge. Weak TagRFP fluorescence was already observed in 2-cell embryos co-injected with pPB-CAG-TagRFP DNA (30 ng/ μ l) and *hyPBase* mRNA (10, 30, 50 and 100 ng/ μ l), whereas it was scarcely observed in embryos injected without *hyPBase* mRNA (Fig. 2B). However, in embryos co-injected with pPB-CAG-TagRFP DNA (30 ng/ μ l) and low concentrations of *hyPBase* mRNA (10 and 30 ng/ μ l), TagRFP signals were observed in almost all blastomeres at the blastocyst stage; by contrast, in embryos co-injected with pPB-CAG-TagRFP DNA (30 ng/ μ l) and high concentrations of *hyPBase* mRNA (50 and 100 ng/ μ l), the TagRFP signals partially disappeared (Fig. 3). Thus, it is probable that excess hyPBase proteins act to remove integrated DNAs from the genome, resulting in the production of mosaic embryos. Because a second generation of transgenic mice could be obtained from mosaic embryos only when cells containing the target gene contributed to the germline, even mosaic embryos could be utilized to produce transgenic progeny. Additionally, TagRFP fluorescence in embryos injected with pPB-CAG-TagRFP DNA (30 ng/ μ l) and *hyPBase* mRNA (30 ng/ μ l) was stronger than that in embryos injected with pPB-CAG-TagRFP DNA (30 ng/ μ l) and *hyPBase* mRNA (10 ng/ μ l) (Fig. 2A and 3), suggesting that the copy number of donor DNA integrated into the genomic DNA depends

on the concentration of *hyPBase* mRNA.

On the basis of these findings, we conclude that the hyperactive *piggyBac* transposon system is an easy and highly effective method for contributing to production of transgenic mice.

Methods

Superovulation and embryo collection

Eight- to ten-week-old ICR female mice (Japan SLC, Hamamatsu, Japan) were superovulated by injection of 5 IU of equine chorionic gonadotropin (eCG; ASUKA Pharmaceutical, Tokyo, Japan), followed by 5 IU of human chorionic gonadotropin (hCG; ASUKA Pharmaceutical) 48 h later. Unfertilized eggs were harvested 14 h after hCG injection and placed in a 90- μ l droplet of HTF supplemented with 4 mg/ml BSA (A3311; Sigma-Aldrich, St. Louis, MO, USA) [13]. Spermatozoa were collected from the cauda epididymis of 11- to 15-week-old ICR male mice (Japan SLC) and cultured for 2 h in 100- μ l of HTF supplemented with 4 mg/ml BSA. After preincubation, sperm were introduced into fertilization droplets at a final concentration of 1×10^6 cells/ml. After a 3-h incubation, fertilized 1-cell embryos were collected and washed 3 times in KSOM supplemented with amino acids [14] and 4 mg/ml BSA and then used for microinjection [15].

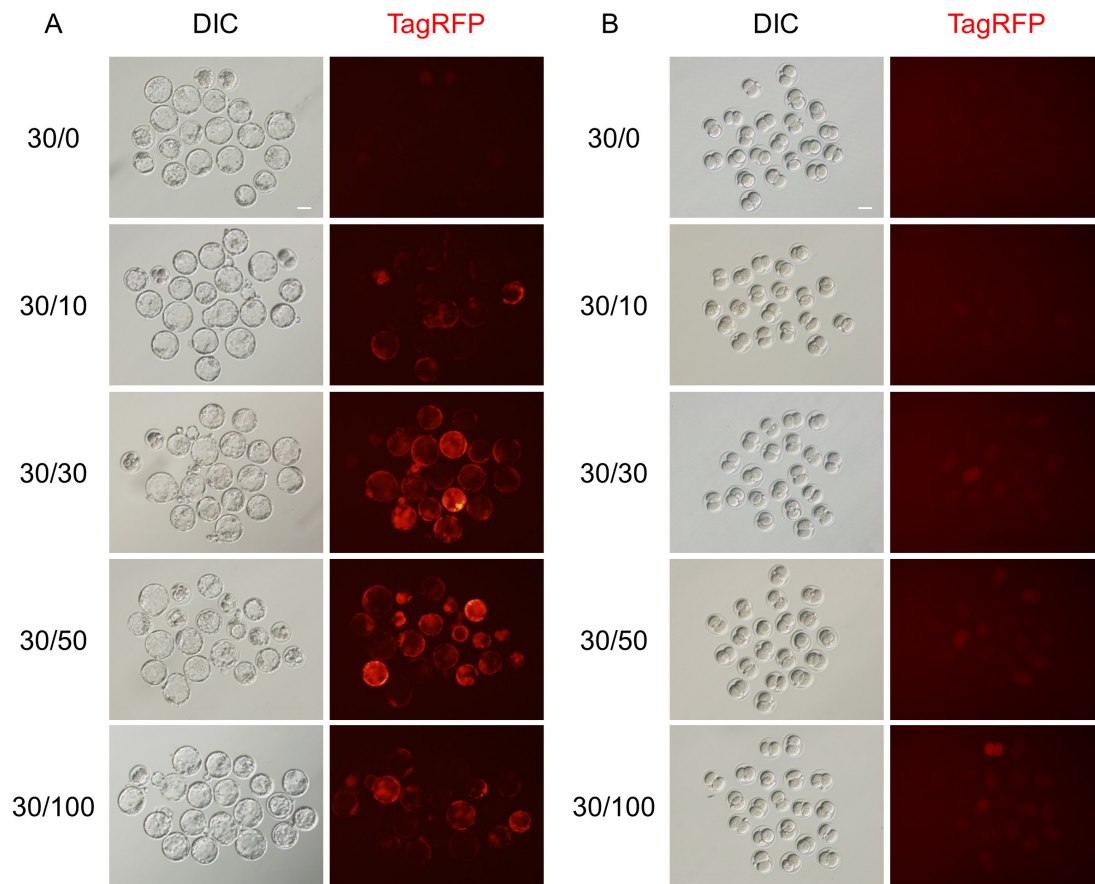


Fig. 2. Fluorescence of TagRFP in embryos co-injected with pPB-CAG-TagRFP DNA and *hyPBase* mRNA. (A) Representative photos showing TagRFP fluorescence in embryos co-injected with pPB-CAG-TagRFP DNA (30 ng/μl) and *hyPBase* mRNA (0, 10, 30, 50 and 100 ng/μl) at the blastocyst stage. Embryos were photographed 108 h after *in vitro* fertilization. Scale bar, 100 μm. (B) Representative photos showing TagRFP fluorescence in embryos co-injected with pPB-CAG-TagRFP DNA (30 ng/μl) and *hyPBase* mRNA (0, 10, 30, 50 and 100 ng/μl) at the 2-cell stage. Embryos were photographed 38 h after *in vitro* fertilization. Scale bar, 100 μm.

Table 1. Summary of the hyperactive *piggyBac* transposon system

pPB-CAG-TagRFP DNA/ <i>hyPBase</i> mRNA (ng/μl)	No. of 2-cell stage embryos (no. of trials)	No. of blastocyst stage embryos (% 2-cell)	No. of TagRFP-positive blastocyst stage embryos (% 2-cell) [% Blastocyst]
30/0	94 (3)	75 (79.8)	2 (2.1) ^a [2.7] ^a
30/10	138 (4)	110 (79.7)	95 (68.8) ^b [86.4] ^b
30/30	147 (4)	124 (84.4)	117 (79.6) ^b [94.4] ^b
30/50	104 (3)	79 (76.0)	70 (67.3) ^b [88.6] ^b
30/100	123 (3)	103 (83.7)	91 (74.0) ^b [88.3] ^b

^{a,b} Values with different superscripts within the same column are significantly different ($P < 0.05$).

In vitro transcription, microinjection, embryo culture and observation

For construction of a *hyPBase* expression vector, the *hyPBase* ORF was amplified from pCMV-*hyPBase* [8] by PCR using specific primers (5'-GGGACCGGTTAATACGACTCACTA TAGGGAATTCGCCGCCACCATGGGC-3', 5'-GGGGGTACC GAAACAGCTCTGGCACATGT-3'), and the SV40 polyadenylation signal was added to the amplicon. The resultant DNA fragment was

used as a template for *in vitro* transcription. RNA synthesis and poly(A) tailing were performed with a MEGAscript T7 kit (Invitrogen, Carlsbad, CA, USA). Approximately 5–10 pl of 0, 10, 30, 50 and 100 ng/μl *hyPBase* mRNA and 30 ng/μl pPB-CAG-TagRFP [10] in DEPC water (Invitrogen) were microinjected into the cytoplasm of 1-cell embryos between 3 and 4 h after insemination. After injection, the embryos were cultured in KSOM medium supplemented with amino acids [14] and 4 mg/ml BSA under mineral oil (Sigma-Aldrich)

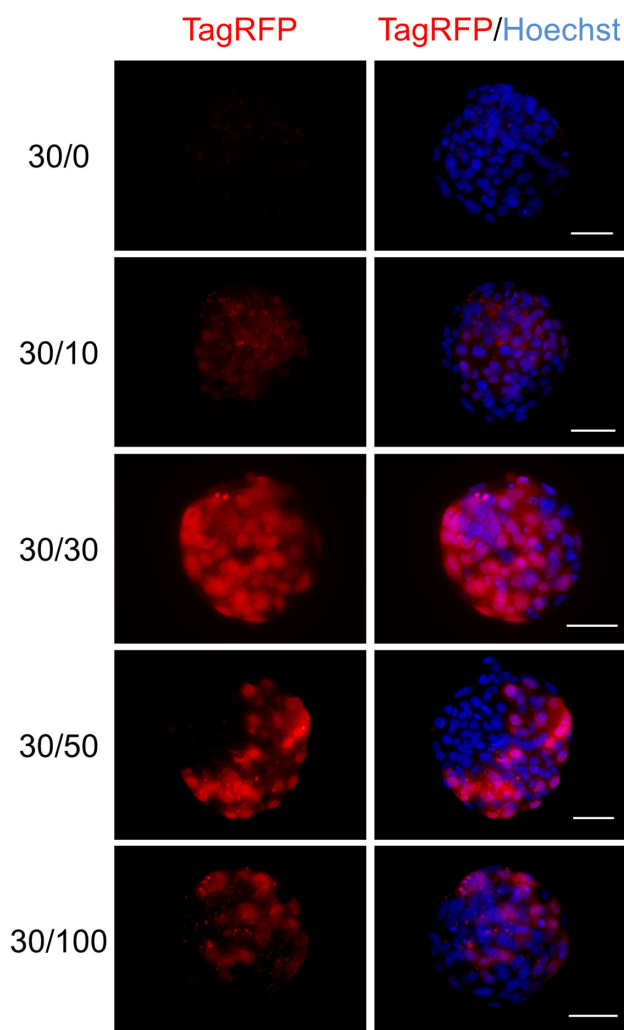


Fig. 3. Fluorescence of TagRFP in embryos co-injected with pPB-CAG-TagRFP DNA and *hyPBbase* mRNA at the blastocyst stage. Detection of TagRFP fluorescence in embryos co-injected with pPB-CAG-TagRFP DNA (30 ng/μl) and *hyPBbase* mRNA (0, 10, 30, 50 and 100 ng/μl) (red, Tag-RFP; blue, chromatin). Scale bars, 100 μm.

at 37 °C in an atmosphere containing 5% CO₂. To examine TagRFP fluorescence, embryos were observed at 38 and 108 h after insemination. At 108 h after insemination, embryos were collected and fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde (Sigma-Aldrich) for 20 min at room temperature. After washing three times in PBS, nuclei were stained in PBS containing 10 μg/ml Hoechst 33342 (Sigma-Aldrich) for 10 min. Stained embryos were mounted on slides in 50% glycerol/PBS, and fluorescent signals were detected using a fluorescence microscope (BX50, Olympus, Tokyo, Japan).

Statistical analysis

Each experiment was repeated at least three times. Statistical analysis of the data was performed by analysis of variance (ANOVA)

with the Student's *t*-test. *P* values < 0.05 were considered to be statistically significant.

Ethical approval for the use of animals

All animal experiments were approved by the Animal Research Committee of Kyoto University (permit number: 24–17) and performed in accordance with the guidelines of the committee.

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