Transposition of the *Tol2* Element, an *Ac*-Like Element From the Japanese Medaka Fish *Oryzias latipes*, in Mouse Embryonic Stem Cells

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ABSTRACT

The *Tol2* transposable element of the Japanese medaka fish belongs to the hAT family of transposons including *hobo* of Drosophila, *Ac* of maize, and *Tam3* of snapdragon. To date, *Tol2* is the only natural transposon in vertebrates that has ever been shown to encode a fully functional transposase. It has not been known, however, whether *Tol2* can transpose in vertebrates other than fish. We report here transposition of *Tol2* in mouse embryonic stem (ES) cells. We constructed a transposon donor plasmid containing a nonautonomous *Tol2* element with the neomycin resistance gene and a helper plasmid capable of expressing the transposase and introduced the donor plasmid with various amounts of the helper plasmid by electroporation into mouse ES cells. The number of G418-resistant ES colonies increased as the amount of helper plasmid was increased, in a dose-dependent manner, indicating that the transposase activity elevated the integration efficiency. These G418-resistant ES colonies were cloned and the structure of the junction of the integrated *Tol2* element and the genomic DNA was analyzed by inverse PCR. In those clones, *Tol2* was surrounded by mouse genomic sequences and an 8-bp direct repeat was created adjacent to both ends of *Tol2*, indicating that *Tol2* was integrated in the genome through transposition. The *Tol2* transposon system is thus active in mouse as well as in fish. We propose that it should be used as a genetic tool to develop novel gene transfer, transgenesis, and mutagenesis methods in mammals.

THE *Tol2* element was isolated from the genome of the Japanese medaka fish, *Oryzias latipes*, a small freshwater teleost inhabiting East Asia. Laboratory strains of the medaka fish contain ~10 copies of the *Tol2* element per haploid genome. The DNA sequence of the *Tol2* element is similar to those of transposons belonging to the hAT family, *i.e.*, *hobo* of Drosophila, *Ac* of maize, and *Tam3* of snapdragon (Koga *et al.* 1996). The *Tol2-tyr* element, a particular element cloned from the tyrosinase gene locus of a medaka albino mutant strain (Koga *et al.* 1996), has been shown to be an autonomous member that encodes an active transposase capable of catalyzing transposition in the zebrafish germ lineage (Kawakami *et al.* 1998, 2000; Kawakami and Shima 1999). Thus, to date, the *Tol2* element is the only natural transposon derived from a vertebrate from which an autonomous member has been established and a fully functional transposase has been identified. It has not been known, however, whether the *Tol2* transposon system is active in vertebrates other than fish.

Transposons have been used as powerful tools in molecular biological and genetic studies. Although their usefulness and importance have been described in bacteria, plants, and invertebrates, transposon systems have not been developed in mammals because of the lack of an active transposable element. Recently, a synthetic transposon system, *Sleeping Beauty* (*SB*), was constructed by analyzing a consensus sequence of nonautonomous transposons of the *Tc1/mariner* family identified in salmonid genomes and has been shown to be active in human, mouse, and other vertebrates (Ivics *et al.* 1997; Dupuy *et al.* 2001, 2002; Fischer *et al.* 2001; Horie *et al.* 2001; Yant *et al.* 2001). While the gene transfer, transgenesis, and mutagenesis methods using the *SB* transposon system are promising, such a transposon tool is still limited in mammals.

In this study, we aim to test whether the *Tol2* transposon system is active in mouse. For this purpose, we developed a two-component assay system consisting of a transposon donor plasmid containing a nonautonomous *Tol2* transposon with the neomycin resistance gene and a helper plasmid capable of expressing the *Tol2* transposase cDNA and measured transposition in mouse embryonic stem (ES) cells. It is expected that if transposition occurs in the mouse cells, the transposon on the donor plasmid will be excised and integrated in the genome upon cotransfection with the helper plasmid. Here we describe how the *Tol2* element, a natural autonomous transposon from a fish genome, can transpose in mouse ES cells.

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MATERIALS AND METHODS

Plasmids: An *Xho*I site was created in the *Tol2* element by inserting double-stranded synthetic oligonucleotides (5′-GAC AGA TCT CAT ATG CTC GAG GGC CC-3′ and 5′-GGG CCC TCG AGC ATA TGA GAT CT-3′) between the *Eco*O109I and *Eco*RV sites of the (*Tol2-tyr*)ΔRV plasmid (Kawakami *et al.* 1998), resulting in pTol2000; and the PGK-neo expression cassette, composed of the phosphoglycerate kinase (PGK) promoter, the neomycin phosphotransferase (*neo*) gene, and the poly(A) signal of the PGK gene (Nakai *et al.* 1995), was inserted at the *Xho*I site of pTol2000, resulting in the T2KPKneo plasmid. In T2KPKneo, the direction of *neo* transcription is opposite to that of the transposase gene. The *Xho*I-*Sal*I fragment containing the full-length *Tol2* transposase cDNA was obtained from the plasmid described previously (Kawakami and Shima 1999) and inserted at the *Xho*I site of pCAGGS (Niwa *et al.* 1991), giving rise to the pCAGGS-T2TP plasmid. The structures of these plasmids are shown in Figure 1. These plasmids for the transposon system are available upon request to K.K.

Transfection to ES cells: The mouse ES cell line, cultivation of ES cells, and transfection by electroporation were described previously (Nakai *et al.* 1995). In each transfection experiment, 2×10^7 ES cells were transfected with pT2KPKneo and pCAGGS or pCAGGS-T2TP and divided and 3×10^6 cells per dish were plated on three 10-cm dishes. G418-resistant colonies were formed in the presence of 175 μg/ml G418. After 10–14 days of cultivation, G418-resistant colonies were picked, divided into two, and cultured as duplicates in 96-well plates. Cells in one well were used for a frozen stock and the duplicated cells in the other well were expanded in a well of 24-well plates for DNA preparation. The colonies formed on two 10-cm dishes in each cotransfection condition were fixed with methanol, stained with 10% Giemsa, and counted. The numbers of colonies on two dishes were added and then divided by 2 and multiplied by 20/3 to calculate the total number of G418-resistant colonies per 2×10^7 cells (Figure 2).

Southern blot hybridization and PCR analysis of the genomic DNA: Genomic DNA from the ES cells was prepared as described previously (Nakai *et al.* 1995). The genomic DNA was digested with *Bgl*II, separated on a 1% agarose gel, transferred to a nylon membrane, and hybridized with the ^{32}P-labeled *neo* gene probe (Figure 1). PCR was performed by 30 cycles of 20 sec at 94°, 20 sec at 56°, and 20 sec at 72°. The primers used in the PCR analysis are: r7 (Kawakami *et al.* 2000); 3′f2, 5′-ACT TGT ACT TTC ACT TGA GTA-3′; tyr1, 5′-AAG GCT CTT GGA TAC GAG TAC GCC-3′; and PGKr1, 5′-GGT GGA TGT GGA ATG TGT GCG AGG-3′.

Inverse PCR: Inverse PCR was carried out as described (Kawakami *et al.* 2000) with some modifications. The genomic DNA was digested with *Mbo*I and self-ligated. DNA containing either 5′ or 3′ end of *Tol2* was amplified by two rounds of PCR using nested primers. For the 5′ end, f21 (5′-AGT ACT TTT TAC TCC TTA CA-3′) and r11 (5′-GAT TTT TAA TTG TAC TCA AG-3′) were used for the first-round PCR (30 cycles of 30 sec at 94°, 30 sec at 56°, and 2 min at 72°), and CUA-5′f3 (5′-CUA CUA CUA CUA TTA CAG TCA AAA AGT ACT TA-3′) and CUA-5′r1 (5′-CUA CUA CUA CUA CAA GTA AAG TAA AAA TCC CCA-3′) were used for the second-round PCR (25 cycles of 30 sec at 94°, 30 sec at 56°, and 2 min at 72°). For the 3′ end, f20 (5′-TTT ACT CAA GTA AGA TTC TAG-3′) and r10 (5′-CTC CAT TAA AAT TGT ACT TGA-3′) were used for the first-round PCR, and CUA-3′f2 (5′-CUA CUA CUA CUA ACT TGT ACT TTC ACT TGA GTA-3′) and CUA-3′r2 (5′-CUA CUA CUA CUA GCA AAG AAA GAA AAC TAG AGA-3′) were used for the second-round PCR. The amplified fragments were cloned using the pAmp10 system (Life Technologies, Grand Island, NY) and sequenced with BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA).

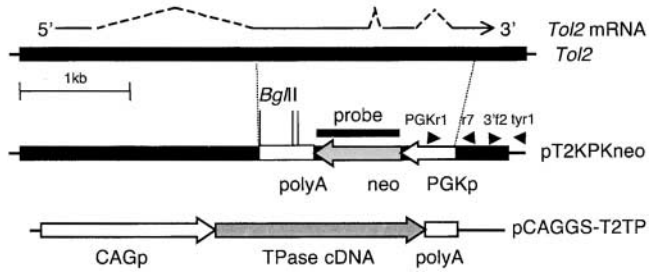

Figure 1.—Structures of the *Tol2* element and plasmids. The thick black line and thin line on the top indicate the full-length *Tol2* element and its transcript, respectively. An arrow and broken lines in the transcript show the direction of the transcript and introns, respectively. pT2KPKneo: the region between the *Eco*O109I and *Eco*RV sites of the (*Tol2-tyr*)ΔRV plasmid (Kawakami *et al.* 1998) as indicated by dotted lines was replaced by the PGK-neo expression cassette (Nakai *et al.* 1995). The direction of *neo* transcription is opposite that of the transposase transcript. The *Bgl*II sites used for the Southern blot analysis are shown. pCAGGS-T2TP: the full-length transposase (TPase) cDNA (Kawakami and Shima 1999) was cloned into pCAGGS (Niwa *et al.* 1991). A solid bar above pT2KPKneo indicates a probe used for Southern blot hybridization. Arrowheads indicate primers used for the PCR analysis.

RESULTS

Construction of the *Tol2* transposon system in mouse: To test whether the *Tol2* transposon system is active in mouse ES cells, we constructed a transposon donor plasmid containing a modified *Tol2* transposon, T2KPKneo, with the neomycin resistance (*neo*) gene expression cassette, and a helper plasmid, pCAGGS-TP, containing the full-length *Tol2* transposase cDNA under the control of the CAG promoter, a hybrid cytomegalovirus enhancer/chicken β-actin promoter, which could direct strong gene expression in mouse ES cells (Niwa *et al.* 1991; Araki *et al.* 1997; Figure 1). The T2KPKneo element contains 2754 bp of sequence derived from the original *Tol2-tyr* element; *i.e.*, 2228 bp on the left-hand side and 526 bp on the right-hand side of the inserted PGKneo cassette (Figure 1). In previous studies using zebrafish, we showed that the (*Tol2-tyr*)ΔRV element, which contained nearly the same portions of the *Tol2* sequence carried by T2KPKneo, could not transpose by itself but retained *cis*-sequences necessary for transposition (Kawakami and Shima 1999; Kawakami *et al.* 2000). To test whether the T2KPKneo element also retains *cis*-elements necessary for transposition, we coinjected circular DNA of the pT2KPKneo plasmid with the transposase mRNA synthesized *in vitro* into zebrafish fertilized eggs and performed an excision assay, which detects excision of the transposon from the plasmid DNA by a simple PCR experiment (Kawakami and

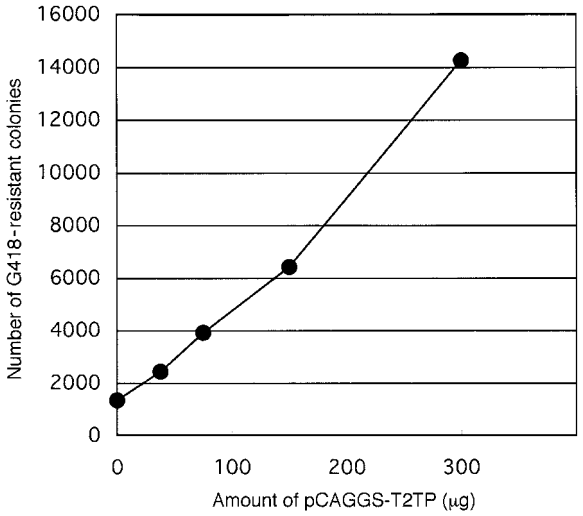

FIGURE 2.—Dependence of integration of T2KPKneo on dose of pCAGGS-T2TP. ES cells were transfected with 50 μg of the pT2KPKneo DNA and 0, 37.5, 75, 150, or 300 μg of the pCAGGS-T2TP DNA. The total numbers of G418-resistant colonies are 1300, 2400, 3900, 6400, and 14,000, respectively.

SHIMA 1999). The T2KPKneo element was excisable from the plasmid DNA in zebrafish (data not shown), indicating that the insertion of the PGK-neo cassette sequence did not interfere with the *cis*-activity, at least for the excision reaction.

Transposase activity increases the integration efficiency: We first introduced 50 μg of circular DNA of the donor plasmid, pT2KPKneo, by electroporation into mouse ES cells with 300 μg of circular DNA of the helper plasmid, pCAGGS-T2TP. As a control, ES cells were cotransfected with 50 μg of circular DNA of pT2KPKneo and 300 μg of circular DNA of the vector plasmid, pCAGGS. In this initial experiment, we observed a significant increase in the number of G418-resistant colonies when cells were cotransfected with pCAGGS-T2TP.

We then performed cotransfection experiments using various amounts of pCAGGS-T2TP. ES cells were cotransfected with 50 μg of circular pT2KPKneo DNA and 0, 37.5, 75, 150, or 300 μg of circular pCAGGS-T2TP DNA. A total of 300, 262.5, 225, 150, or 0 μg of circular pCAGGS DNA, respectively, was added to the DNA samples prepared for cotransfection to keep the amount of DNA used for electroporation equal. The transfected cells were plated and cultivated in the presence of G418. As shown in Figure 2, the number of G418-resistant colonies increased as the amount of pCAGGS-T2TP increased. These results strongly suggest that the transposase activity synthesized from pCAGGS-T2TP can enhance the efficiency of integration of T2KPKneo through transposition.

PCR and Southern blot analysis: To determine whether the integration that had occurred in the presence of the helper plasmid was indeed generated through transposition, we isolated and analyzed genomic DNA from

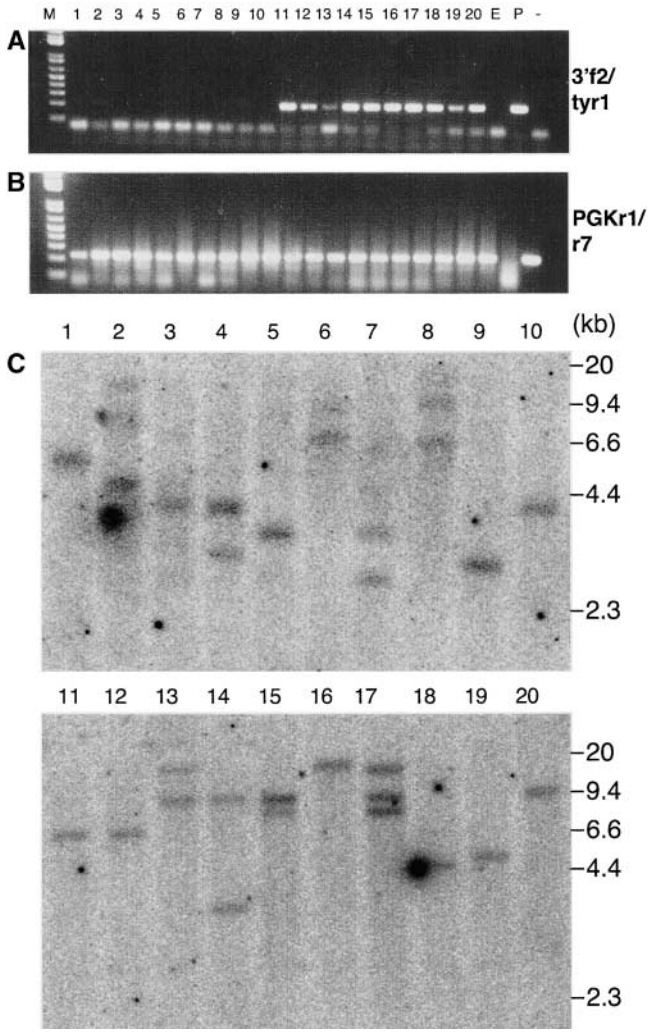

FIGURE 3.—Southern blot and PCR analysis of genomic DNA from G418-resistant ES clones. (A) ES cell clones 1–10 (lanes 1–10) were obtained from cotransfection of pT2KPKneo and pCAGGS-T2TP and clones 11–20 (lanes 11–20) were obtained from cotransfection of pT2KPKneo and pCAGGS. Genomic DNA of these clones was digested with *Bgl*II and hybridized with a ^{32}P-labeled probe shown in Figure 1. (B) Genomic DNA of clones 1–20 was analyzed by PCR using 3′f2 and tyr1 primers. M, size marker; E, genomic DNA from nontransfected ES cells was used; P, 1 pg of pT2KPKneo plasmid DNA was used; dash indicates no DNA. (C) PCR was carried out using primers PGKr1 and r7 as positive controls.

the G418-resistant colonies. Ten G418-resistant colonies generated by cotransfection using 50 μg of pT2KPKneo and 300 μg of pCAGGS-T2TP were picked at random and expanded (clones 1–10 in Figure 3). Also, 10 G418-resistant colonies generated following cotransfection using 50 μg of pT2KPKneo and 300 μg of pCAGGS were picked at random and analyzed (clones 11–20 in Figure 3). Genomic DNA was extracted from these clones and analyzed by PCR using the 3′f2 primer, which was located in the 3′-end region of the *Tol2* element, and tyr1, which was located adjacent to the 3′-end region of *Tol2* in the vector portion (Figure 1). No DNA band was

TABLE 1

Cloning and sequencing analysis of the junction fragments

Clone ID	Target sequence[a]	Chromosome	Putative features	Accession no.
1	CCTGATGCTCCTTAACTTT	6	Intergenic region, ~20 kb apart from the *ret* oncogene	AB119128
2	CCCAGCCTGTGAACCCTC	11	Intergenic region, between slc2295 and LOC327929	AB119129
4	ACCGCCAAAGCTGCATAT	No BLAST hit against the mouse genome		AB119130
5	ACTTGGCGCCAGCCCACT	7	Exon of LOC244082	AB119131
7	CCGGTGTTAACTGTGCGG	4	Intron of LOC329929	AB119132
8	AAGCTCAAAGCACACATT	No BLAST hit against the mouse genome		AB119133
9	AATCTCTGCCATTCCAAC	6	Intron of Mgl1 (monoglyceride lipase)	AB119135
10	AGGGAGGGCTGCAGAGTG	5	Exon of 0610039P13Rik	AB119134

[a] The 8-bp sequence duplicated upon insertion of the *Tol2* element is underlined.

amplified from the ES clones 1–10 (Figure 3A, lanes 1–10) while a DNA band of 203 bp was amplified by PCR using the same primers from the ES clones 11–20 (Figure 3A, lanes 11–20). These results suggested that, in the presence of pCAGGS-T2TP (ES clones 1–10), the T2KPKneo sequence was integrated in the genome through transposition, and, in the absence of pCAGGS-T2TP (ES clones 11–20), the T2KPKneo DNA was integrated with the vector DNA through nonhomologous recombination. The PCR analysis was carried out using primers PGKr1 and r7 as positive controls (Figure 3B).

Then these genomic DNA were digested with *Bgl*II, which should generate one fragment corresponding to each insertion detected by hybridization with the *neo* gene probe (Figure 1). The Southern blot analysis revealed that T2KPKneo was integrated at different loci in the genome, and, in some cases, a single copy of the T2KPKneo insertion was detected (Figure 3C, top).

Cloning of the junction fragments by inverse PCR: To characterize the integration at the sequence level, we cloned the junctions of the integrated T2KPKneo element and the surrounding genomic DNA by inverse PCR. The junction fragments containing 5′ and 3′ ends of the *Tol2* element were isolated from the ES clones 1, 2, 4, 5, 7, 8, 9, and 10. In the cases of clones 1, 2, and 7, inverse PCR for the 3′ end did not work for unknown reasons. To clone the 3′ junction fragments from these ES clones, the sequence of the 5′ junction was used for the BLAST analysis against the mouse genomic database (http://www.ncbi.nlm.nih.gov/genome/seq/MmBlast.html). From this analysis, the sequence adjacent to the 3′ end of *Tol2* could be predicted, and 3′ genomic primers that matched with the predicted 3′ sequence were designed, respectively. The 3′ junction fragments were successfully isolated from the genomic DNA of those ES clones by performing PCR using the 3′ genomic primer and a primer in the 3′ end of *Tol2* (f20) and sequenced. In all of the ES clones, the T2KPKneo sequence was surrounded by the mouse genomic sequence and an 8-bp direct repeat, which was always created at the target site of the *Tol2* element (Table 1; Koga *et al.* 1996; Kawakami *et al.* 2000), indicating that T2KPKneo was integrated through transposition.

The flanking sequences, except for clones 4 and 8, could be mapped on the mouse genome by the BLAST analysis. The results are summarized in Table 1. The sequences obtained from clones 4 and 8 could not be mapped, probably because the greater part of these sequences was occupied by repetitive sequences. Before integration of the *Tol2* element, each locus contained only one copy of the 8-bp sequence. From these results, we concluded that the transposase could catalyze transposition of the *Tol2* element in mouse ES cells.

DISCUSSION

This study revealed that the *Tol2* transposon system is active in mouse as well as in fish. The *Tol2* transposon system we developed here consists of two components: the transposon donor plasmid, pT2KPKneo, containing a modified *Tol2* element with the PGK-neo expression cassette, and the helper plasmid, pCAGGS-T2TP, expressing the transposase. These *cis*- and *trans*-elements have been generated from a natural autonomous member (Kawakami and Shima 1999), and our results indicate that T2KPKneo contains *cis*-sequences necessary for transposition and that pCAGGS-T2TP can produce a fully functional transposase in mouse. While the *Sleeping Beauty* transposon system, which was constructed artificially, has been shown to be active in vertebrate species (Ivics *et al.* 1997), our study for the first time establishes a transposon system constructed from a natural transposon of vertebrates, which is active in mammals. Host

factors required for transposition of the *Tol2* element, such as DNA repair enzymes, etc., have not been elucidated. Such factors appear to be conserved from fish to mammals, and thus the *Tol2* transposon system may be used as a genetic tool in a wide variety of vertebrate animals.

We also found that transposition could increase the efficiency of chromosomal integration in mouse ES cells. The efficiency could become >10-fold higher in comparison to integration through nonhomologous recombination. Further, the transposon DNA can be integrated in the genome as a single copy without causing deletions or rearrangements of the genome surrounding the insertion site, which are often associated with chromosomal integration of a foreign DNA through nonhomologous recombination. These indicate that the *Tol2* transposon system should be a useful system for gene transfer in mammalian cells.

The *Tol2* transposon system may also be used to mutate the genome. We found in several cases that the transposon was inserted within a gene or a hypothetical gene, in either an intron or an exon (Table 1). Although we do not know whether these insertions disrupted the function of the gene, they could be more mutagenic if the transposon construct was designed so that it can interrupt the endogenous transcript (*i.e.*, a gene trap construct, etc.).

The *Sleeping Beauty* transposon was constructed on the basis of the sequences of transposons of the *Tc1/mariner* family, and it always integrates at a specific sequence, TA dinucleotides, which is duplicated upon insertion (Ivics *et al.* 1997). In contrast, the *Tol2* element belongs to the hAT family of transposons, a different family from the *Tc1/mariner* family, and such a specific sequence in the target site has not been detected (Table 1). It will be important to know whether the *Tol2* element preferably integrates in either transcribed loci or nontranscribed loci, either an exon or an intron, or either 5′ or 3′ ends of a transcribed region and to determine whether these two different transposon systems have different characteristics in efficiency and preference for the target site. It would be beneficial if we could use these transposon systems complementarily; for instance, we could use different transposon systems to introduce more than one foreign gene into a cell or we could target a larger variety of loci in the genome by using these two systems to perform a genome-wide screen for insertional mutations. Therefore we propose that the *Tol2* transposon system should be used as a genetic tool to develop novel gene transfer, transgenesis, and insertional mutagenesis strategies in mouse and other vertebrates and, possibly, to develop a novel nonviral vector for gene transfer in humans.

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LITERATURE CITED

Araki, K., T. Imaizumi, K. Okuyama, Y. Oike and K. Yamamura, 1997 Efficiency of recombination by Cre transient expression in embryonic stem cells: comparison of various promoters. J. Biochem. **122:** 977–982.

Dupuy, A. J., S. Fritz and D. A. Largaespada, 2001 Transposition and gene disruption in the male germline of the mouse. Genesis **30:** 82–88.

Dupuy, A. J., K. Clark, C. M. Carlson, S. Fritz, A. E. Davidson *et al.*, 2002 Mammalian germ-line transgenesis by transposition. Proc. Natl. Acad. Sci. USA **99:** 4495–4499.

Fischer, S. E. J., E. Wienholds and R. H. A. Plasterk, 2001 Regulated transposition of a fish transposon in the mouse germ line. Proc. Natl. Acad. Sci. USA **98:** 6759–6764.

Horie, K., A. Kuroiwa, M. Ikawa, M. Okabe, G. Kondoh *et al.*, 2001 Efficient chromosomal transposition of a Tc1/mariner-like transposon Sleeping Beauty in mice. Proc. Natl. Acad. Sci. USA **98:** 9191–9196.

Ivics, Z., P. B. Hackett, R. H. Plasterk and Z. Izsvák, 1997 Molecular reconstruction of *Sleeping Beauty*, a *Tc1*-like transposon from fish, and its transposition in human cells. Cell **91:** 501–510.

Kawakami, K., and A. Shima, 1999 Identification of the *Tol2* transposase of the medaka fish *Oryzias latipes* that catalyzes excision of a nonautonomous *Tol2* element in zebrafish *Danio rerio*. Gene **240:** 239–244.

Kawakami, K., A. Koga, H. Hori and A. Shima, 1998 Excision of the *Tol2* transposable element of the medaka fish, *Oryzias latipes*, in zebrafish, *Danio rerio*. Gene **225:** 17–22.

Kawakami, K., A. Shima and N. Kawakami, 2000 Identification of a functional transposase of the *Tol2* element, an *Ac*-like element from the Japanese medaka fish, and its transposition in the zebrafish germ lineage. Proc. Natl. Acad. Sci. USA **97:** 11403–11408.

Koga, A., M. Suzuki, H. Inagaki, Y. Bessho and H. Hori, 1996 Transposable element in fish. Nature **383:** 30.

Nakai, S., H. Kawano, T. Yudate, M. Nishi, J. Kuno *et al.*, 1995 The POU domain transcription factor Brn-2 is required for the determination of specific neuronal lineages in the hypothalamus of the mouse. Genes Dev. **9:** 3109–3121.

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

Yant, S. R., L. Meuse, W. Chiu, Z. Ivics, Z. Izsvak *et al.*, 2001 Somatic integration and long-term transgene expression in normal and haemophilic mice using a DNA transposon system. Nat. Genet. **25:** 35–41.

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