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

Koichi Kawakami*†, Akihiro Shima‡, and Noriko Kawakami*

*Department of Tumor Biology, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan; and ‡Department of Integrated Biosciences, Graduate School of Frontier Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

The *Tol2* element of the medaka fish *Oryzias latipes* belongs to the hAT family of transposons (*hobo*/*Ac*/*Tam3*). We report here identification of a functional transposase of *Tol2* that is capable of catalyzing its transposition in the germ line of zebrafish *Danio rerio*. A transcript produced from *Tol2* encodes a putative transposase. Zebrafish fertilized eggs were coinjected with mRNA transcribed *in vitro*, using cDNA of the *Tol2* transcript as a template and a plasmid DNA harboring a mutant *Tol2*, which had a deletion in the putative transposase gene but retained necessary cis sequences. The injected fish were raised to adulthood and mated to noninjected fish, and genomic DNA of the progeny fish were analyzed by PCR and Southern hybridization. Half of F$_1$ fish obtained from one of eight injected fish contained the *Tol2* DNA in their genomes but not the vector portion. Among these F$_1$ fish, *Tol2* insertions at four different loci were identified, and some F$_1$ fish carried two or three different *Tol2* insertions, indicating that the germ line of the founder fish is highly mosaic. Sequencing analyses revealed that, in all cases, *Tol2* was surrounded by zebrafish genomic sequences, and an 8-bp duplication was created at the target site, indicating that *Tol2* was integrated in the zebrafish genome through transposition. This study identifies an autonomous member of a DNA-based transposable element from a vertebrate genome. The *Tol2* transposon system should thus be used to develop novel transgenesis and insertional mutagenesis methods in zebrafish and possibly in other fishes.

T he DNA-based transposable elements (or transposons) are repetitive sequences that move from one locus in the genome to another and have been used as powerful tools to study model animals and plants. The members of transposable elements fall into two classes: 1) autonomous elements, such as the *Ac* element in maize, encode a functional transposase and can transpose by itself; 2) nonautonomous elements, such as the *Ds* element in maize, carry mutations or deletions in the transposase gene and can be mobilized only in the presence of autonomous members (1–7). In vertebrates, several repetitive sequences similar to transposons of the *Tc1*/*mariner* elements of *Caenorhabditis elegans* and *Drosophila* have been identified (8–12). Although a synthetic *Sleeping Beauty* transposase, which was reconstructed based on a consensus sequence derived from sequences of nonautonomous salmonid *Tc*-like elements, has been shown to be capable of catalyzing transposition in vertebrate cells (13), an endogenous autonomous element has not yet been identified from a vertebrate genome.

The Japanese medaka fish, *Oryzias latipes*, is a freshwater teleost inhabiting East Asia. More than 80 mutations that affect pigmentation and body shapes have been isolated mainly from wild populations in Japan (ref. 14; see also http://biol1.bio.nagoya-u.ac.jp:8000/). Among them, mutations in the *i* locus, which encodes a gene for tyrosinase, cause amelanotic skin and red-colored eyes (15). In one of the *i* alleles, *i⁴*, an insertion of about 4.7 kilobases (kb) of DNA was found in the fifth exon of the tyrosinase gene. The sequence of the insertion is similar to those of transposons of the hAT family; i.e., *hobo* of *Drosophila* (16, 17), *Ac* of maize (4, 5), and *Tam3* of snapdragon (18, 19). The element was named *Tol2* (20). Laboratory strains of the medaka fish contain about ten copies of the *Tol2* element per haploid genome. The *Tol2-tyr* element, a particular *Tol2* element which resides in the tyrosinase gene, has been shown by PCR to be excisable from the target locus during embryogenesis, suggesting the presence of at least one autonomous member somewhere in the genome (20). Until now, neither a functional transposase nor an autonomous member of the *Tol2* element, however, had been identified.

Zebrafish, *Danio rerio*, is another small teleost that has been used as a model animal to study vertebrate genetics and development. The zebrafish genome does not contain the *Tol2* element (21). To determine whether the *Tol2* element encodes a functional transposase, we have been investigating the activities of the *Tol2-tyr* element using zebrafish (21, 22). In our previous studies, we cloned cDNAs of the *Tol2-tyr* element from zebrafish embryos injected with a plasmid DNA harboring the *Tol2-tyr* element and developed a transient embryonic excision assay. In this assay, zebrafish fertilized eggs were coinjected with mRNA transcribed *in vitro* using the full-length cDNA as a template and a plasmid DNA harboring a mutant *Tol2* element, which has a deletion in the putative transposase coding region; DNA extracted from the injected embryos at 50–100% epiboly stages was analyzed by PCR. The mutant nonautonomous *Tol2* element is excisable from the vector plasmid only when coinjected with the mRNA, indicating that the mRNA can produce a putative transposase activity that catalyzes the excision reaction.

In the present study, we aimed to determine whether the *Tol2-tyr* element encodes a fully functional transposase that catalyzes a complete transposition process, excision and reintegration, in zebrafish. We show here that mRNA transcribed from the *Tol2-tyr* element produces a fully functional transposase, which is capable of catalyzing transposition of a nonautonomous *Tol2* element in the zebrafish germ lineage. The *Tol2-tyr* element is thus shown to encode a functional transposase, and, hence, to be autonomous. We also suggest that the *Tol2* transposon system

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should be useful to develop transgenesis and insertional mutagenesis methods in zebrafish and possibly in other fish species.

Materials and Methods

Fish. Zebrafish strains, Tübingen, TL (leo^{t1}, lof^{dt2}) and *brass*, were used for microinjection and mating.

Plasmids and Primers. The structures of the (*Tol2-tyr*)ΔRV plasmid (21) and the *Tol2* cDNA (22) are shown in Fig. 1. The cDNA of 2149 bp was cloned into the *Xho*I site of pBlueScript II SK+ (Stratagene) and used for *in vitro* transcription. The positions and directions of primers used in this study are shown in Fig. 1 and Fig. 3 *A* and *C*. Nucleotide sequences of tyr-ex5r, tyr-ex4f, and the zebrafish wnt5A primers were described previously (21–24). The sequences of other primers are as follows. Tol2f1, 5′-CUACUACUACUAAGACAUUCCGCUGCACUUGCC-3′;. Tol2f2, 5′-CUACUACUACUAACUUGUACUUUCACUU-GAGUA-3′; Tol2f5, 5′-CTGCTCTGATCATGAAACAG-3′; Tol2f20, 5′-TTTACTCAAGTAAGATTCTAG-3′; Tol2r1, 5′-CUACUACUACUACAAGUAAAGUAAAAAUCCCCA-3′; Tol2r2, 5′-CUACUACUACUAGCAAAGAAAGAAAAC-UAGAGA-3′; Tol2r7, 5′-CCGATGCGGGAAGAGGTGTAT-TAG-3′; Tol2r9, 5′-GGAAAATAGAATGAAGTGATCTCC-3′; Tol2r10, 5′-CTCCATTAAAATTGTACTTGA-3′; 7a5′, 5′-GACGTAGTGTCCTGCTGGTGTA-3′; 7a3′, 5′-CGAGA-AGCAGCGCCTGGAGCGA-3′; 7b5′, 5′-GTTTTATG-CAAAAGTTTGGCC-3′; 7b3′, 5′-TCTCGGCCTGTTGCAG-CAGAG-3′; 7c5′, 5′-CAATATTGTGCTGAAGGCTACA-3′; 7c3′, 5′-CTGGTTAGTCAATGCCATAGT-3′; 7d5′, 5′-CAAATCATTGTGCATGCATGTA-3′; 7d5′-2, 5′-AGAG-CAAATTTGTTCTTCTGG-3′; and 7d3′, 5′-CCTTCCCCT-TCACACACTTGGTT-3′.

***In Vitro* Transcription and Microinjection.** *In vitro* transcription of *Tol2* mRNA using T7 RNA polymerase and microinjection of the mRNA and the (*Tol2-tyr*)ΔRV plasmid DNA were carried out as described previously (22).

Southern Hybridization and PCR Analyses of F_1 Fish. For PCR analysis of pooled F_1 embryos, 20–30 embryos of 1 day old were pooled in one microtube and lysed in 300 μl of DNA extraction buffer (10 mM EDTA/10 mM Tris·HCl, pH 8.0/200 μg/ml proteinase K) at 50°C for 3 h or overnight. Genomic DNA was extracted with phenol/chloroform, precipitated with ethanol, and resuspended in 50 μl of 10 mM Tris·HCl (pH 8.0), 1 mM EDTA. An aliquot of 1 μl was used for PCR: 35 cycles of 20 s, 94°C; 20 s, 56°C; 20 s, 72°C with Tol2f5 and Tol2r7. For PCR analysis of adult F_1 fish, caudal fins were clipped and lysed in 200 μl of DNA extraction buffer at 50°C for 3 h or overnight. Genomic DNA was extracted with phenol/chloroform, precipitated with ethanol, and resuspended in 50 μl of 10 mM Tris·HCl (pH 8.0), 1 mM EDTA. An aliquot of 1 μl was used for PCR: 30 cycles of 20 s, 94°C; 20 s, 56°C; 20 s, 72°C with primers described in the text. For Southern hybridization, 5 μg of caudal fin DNA of F_1 fish was digested either with *Eco*RV or *Eco*RI, separated on a 1% agarose gel, transferred on a nylon membrane, and hybridized with the ^{32}P-labeled probe shown in Fig. 1.

Inverse PCR and DNA Sequencing. One microgram of caudal fin DNA isolated from F_1 fish was digested with *Eco*RV, *Pst*I, *Hin*dIII, or *Sau*3A I, diluted to 2 ng/ml, and then circularized by ligation. The ligated DNA was concentrated by ethanol precipitation and used for two rounds of PCR; i.e., first PCR: 30 cycles of 30 s, 94°C; 30 s, 58°C; 5 min, 72°C with Tol2f5 and Tol2r9 (for 5′-end) or with Tol2f20 and Tol2r10 (for 3′-end); an aliquot was used for the second PCR: 30 cycles of 30 s, 94°C; 30 s, 58°C; 5 min, 72°C with Tol2f1 and Tol2r1 (for 5′-end) or with Tol2f2 and Tol2r2 (for 3′-end). The amplified DNA were gel-extracted and
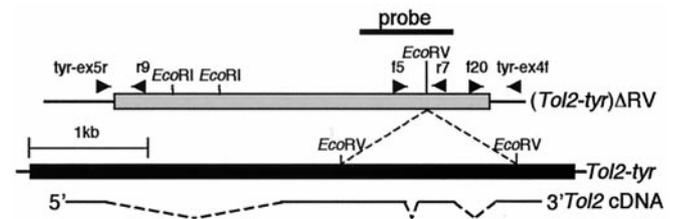

Fig. 1. Structures of the *Tol2* elements and cDNA. The black and gray boxes indicate the *Tol2-tyr* and (*Tol2-tyr*)ΔRV elements, respectively, and thin lines flanking these boxes represent plasmid DNA (the medaka fish tyrosinase sequence). Self-ligation at two *Eco*RV sites in *Tol2-tyr* generated (*Tol2-tyr*)ΔRV. 5′ and 3′ in *Tol2* cDNA indicate the direction of transcription, and dotted lines indicate introns. Arrowheads show positions and directions of primers used in the experiment described in Fig. 2*A*. A thick bar shows the probe used in Southern hybridization analyses. Note that the total length of the *Tol2-tyr* element, previously reported as 4681 bp (20), has been corrected to 4682 bp (D84375), by addition of 1 bp in the first intron, by the authors of the first report. r9, Tol2r9; f5, Tol2f5; r7, Tol2r7; f20, Tol2f20.

cloned using pAmp10 system (GIBCO/BRL). For cloning of wild-type DNA, 100 ng of genomic DNA was used for PCR: 30 cycles of 30 s, 94°C; 30 s, 56°C; 5 min, 72°C with primers shown in Fig. 3*C*. The amplified DNA were gel-extracted and cloned using TOPO TA Cloning (Invitrogen). The plasmid DNA was prepared from bacteria and sequenced using BigDye terminator cycle sequencing kit (Applied Biosystems) and ABI PRISM 310 genetic analyzer.

Results

Identification of Founder Fish. A transcript of 2156 nt is synthesized from the *Tol2-tyr* element in zebrafish embryos injected with the *Tol2-tyr* plasmid DNA (Fig. 1) (22). The cDNA of 2149 bp was inserted into a pBlueScript II plasmid, which lacked the first 7 bp of the 5′-end (5′-*ACGTCAT*GTC . . .). This eliminates the possibility for the first ATG, located at nucleotides 6–8, to be used as a start codon, which would produce a short peptide of 11 aa. The resulting cDNA has the capacity to encode a protein of 649 aa when the first ATG was used as a start codon. The (*Tol2-tyr*)ΔRV element has a deletion of ≈1.5 kb, which inactivates the putative transposase by disrupting the exon sequences and hence becomes a nonautonomous element (Fig. 1) (21, 22).

To test whether the *Tol2* transcript encodes a functional transposase that catalyzes transposition in the zebrafish germ lineage, zebrafish fertilized eggs were coinjected with mRNA transcribed *in vitro* using the plasmid DNA carrying the *Tol2* cDNA as a template and the (*Tol2-tyr*)ΔRV plasmid DNA. It is expected that a transposase is synthesized from the mRNA, excises the (*Tol2-tyr*)ΔRV element from the plasmid DNA, and reintegrates it into the genome of the zebrafish germ cells during embryogenesis. Such transposition events could be detected by analyzing F_1 progeny fish from the injected fish. In every injection experiment, aliquots of the injected embryos were subjected to a transient excision assay (22), and the excision activity was verified (data not shown). The rest of the injected fish were raised to adulthood, and pair mating to noninjected fish of opposite sexes yielded F_1 offspring. Eight injected fish mated successfully, and at least 50 embryos from each mating were pooled and analyzed by PCR using the Tol2f5 and Tol2r7 primers for the presence of the (*Tol2-tyr*)ΔRV DNA. Pools of F_1 embryos from two of eight fish were PCR positive (data not shown). Therefore, the F_1 progeny fish from these two founder fish, referred to as fkk-1 and fkk-7, were raised to adulthood and studied further.

Analysis of F_1 Fish with Insertions of *Tol2*. Genomic DNA isolated from a caudal fin of individual F_1 fish was analyzed by PCR using

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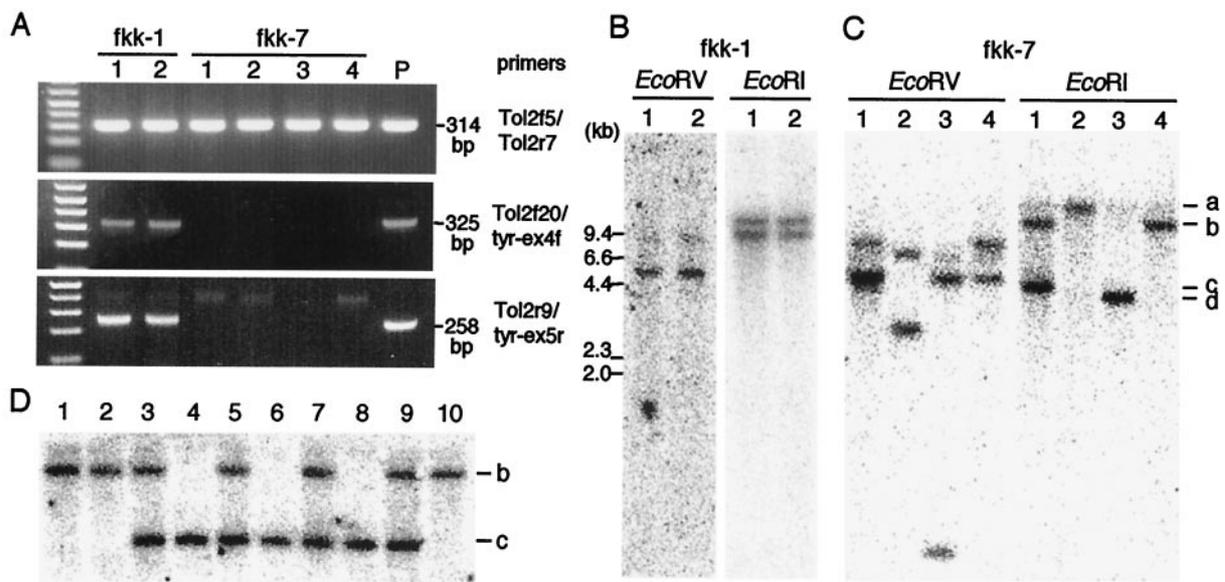
Fig. 2. PCR and Southern hybridization analyses of F_1 fish. (*A*) PCR analyses of genomic DNA extracted from caudal fin clippings of F_1 fish from fkk-1 and fkk-7. Primer pairs used for PCR and the sizes of the PCR products are shown on the right. Two (fkk-1, lanes 1 and 2) and 25 F_1 fish were found positive for PCR using Tol2f5/Tol2r7 out of 68 and 50 F_1 fish from fkk-1 and fkk-7, respectively. PCR analyses of the 25 F_1 fish from fkk-7 yielded the same results, and four representatives are shown (fkk-7, lanes 1–4). The same four samples were used also for Southern hybridization (described in *C*). In the control (P), 100 ng of zebrafish genomic DNA plus 1 pg of the (*Tol2-tyr*)ΔRV plasmid DNA were used as a PCR template. (*B*) Southern hybridization analysis of F_1 fish from fkk-1. DNA samples of two fish (*A*) were digested with either *Eco*RV or *Eco*RI and hybridized with the probe shown in Fig. 1. (*C*) Southern hybridization analysis of F_1 fish from fkk-7. DNA samples of four fish (*A*) were digested with either *Eco*RV or *Eco*RI and hybridized with the probe (Fig. 1). *Eco*RI bands of four different sizes, shown as a–d on the right, correspond to the fkk-7a, fkk-7b, fkk-7c, and fkk-7d insertions described in the text. The lower bands in *Eco*RV lane 1 are probably a triplet. (*D*) Southern hybridization analysis of F_2 fish from the lane 1 F_1 fish (fkk-7). Ten fish out of 14 F_2 fish, which were positive for PCR using Tol2f5 and Tol2r7, were analyzed. DNA samples were digested with *Eco*RI and hybridized with the probe (Fig. 1). Fish with fkk-7b alone (lanes 1, 2, 10), fkk-7b alone (lanes 4, 6, 8), and both fkk-7b and fkk-7c (lanes 3, 5, 7, 9) were identified.

primer pairs Tol2f5 and Tol2r7, Tol2f20 and tyr-ex4f, and Tol2r9 and tyr-ex5r (Figs. 1 and 2*A*). Two fish out of 68 F_1 fish from the fkk-1 founder fish were positive for the *Tol2* sequence (fkk-1, lanes 1 and 2 of Tol2f5/Tol2r7, Fig. 2*A*), and these fish were shown to carry the tyrosinase gene sequences of the medaka fish flanking the *Tol2* sequence (fkk-1, lanes 1 and 2 of Tol2f20/tyr-ex4f and Tol2r9/tyr-ex5r, Fig. 2*A*). In contrast, 25 fish out of 50 F_1 fish from the fkk-7 founder fish, which were positive for the *Tol2* sequence, did not carry the tyrosinase gene sequences flanking *Tol2* (four representatives of the PCR analyses are shown in fkk-7, lanes 1–4 of Tol2f5/Tol2r7, Tol2f20/tyr-ex4f, and Tol2r9/tyr-ex5r, Fig. 2*A*). The (*Tol2-tyr*)ΔRV DNA injected into fertilized eggs could integrate into the genome either through nonhomologous recombination, independently of a transposase activity (25), or, as expected, in a transposase-dependent manner. In the former case, the *Tol2* sequence is likely to be flanked by sequences of the vector plasmid, the tyrosinase gene sequences of the medaka fish; in the latter case, it should be surrounded by zebrafish genomic sequences. Thus, in F_1 fish from fkk-1, the integration was likely to occur independently of transposition.

Digestion of the genomic DNA containing the (*Tol2-tyr*)ΔRV DNA with *Eco*RV and *Eco*RI should give rise to two bands and a single band in Southern hybridization using the probe shown in Fig. 1, respectively. Southern hybridization analysis of the genomic DNA of the two transgenic F_1 fish from fkk-1 showed the same hybridization pattern either by digestion with *Eco*RV or *Eco*RI (Fig. 2*B*). Although these fish might have the *Tol2* DNA at two distinct loci and one of them might integrate through transposition, these possibilities were not examined further. On the other hand, Southern hybridization analyses of the F_1 fish from fkk-7 showed DNA bands of four different sizes by digestion with *Eco*RI (Fig. 2*C*), indicating that at least four

different integration events of the *Tol2* DNA took place in the germ line of fkk-7. These *Tol2* insertions were designated as fkk-7a, fkk-7b, fkk-7c, and fkk-7d with respect to the sizes of the *Eco*RI bands (Fig. 2*C*). Although the F_1 fish on lanes 2, 3, and 4 carried a single *Tol2* insertion, fkk-7a, fkk-7d, and fkk-7b, respectively, the F_1 fish on lane 1 was likely to carry two different *Tol2* insertions (Fig. 2*C*, lane 1). To verify this observation, the lane 1 F_1 fish was crossed with wild-type fish, and F_2 offspring were raised. Ten fish out of 14 F_2 fish were positive for PCR using Tol2f5 and Tol2r7 (data not shown), and these 10 fish were then analyzed by Southern hybridization (Fig. 2*D*, lanes 1–10). The result indicates that the lane 1 F_1 fish carried both the fkk-7b and fkk-7c insertions, which were generated at distinct loci in the zebrafish genome.

Evidences for Transposition of *Tol2*. The structures of the insertions identified among the F_1 fish from fkk-7 were analyzed by inverse PCR (Fig. 3*A*). For fkk-7a, DNA fragments containing the 5′- and 3′-ends (with respect to the direction of transcription) of the (*Tol2-tyr*)ΔRV element were amplified from genomic DNA of the lane 2 fish (Fig. 2*C*) digested with *Eco*RV and *Sau*3A I, respectively, and sequenced (Fig. 3*B*, fkk-7a). For fkk-7b, DNA fragments containing the 5′- and 3′-ends of the *Tol2* element were amplified from genomic DNA of the lane 4 fish (Fig. 2*C*) digested with *Hind*III and *Pst*I, respectively, and sequenced (Fig. 3*B*, fkk-7b). For fkk-7c, DNA fragments containing the 5′- and 3′-ends of the *Tol2* element were amplified from genomic DNA of the lane 1 fish (Fig. 2*C*) digested with *Eco*RV and *Pst*I, respectively, and sequenced (Fig. 3*B*, fkk-7c). Although this fish carries both fkk-7b and fkk-7c, the sequences of the DNA fragments amplified by inverse PCR using those restriction enzymes are distinct from those obtained for the fkk-7b insertion and hence are thought to correspond to fkk-7c. For fkk-7d, DNA

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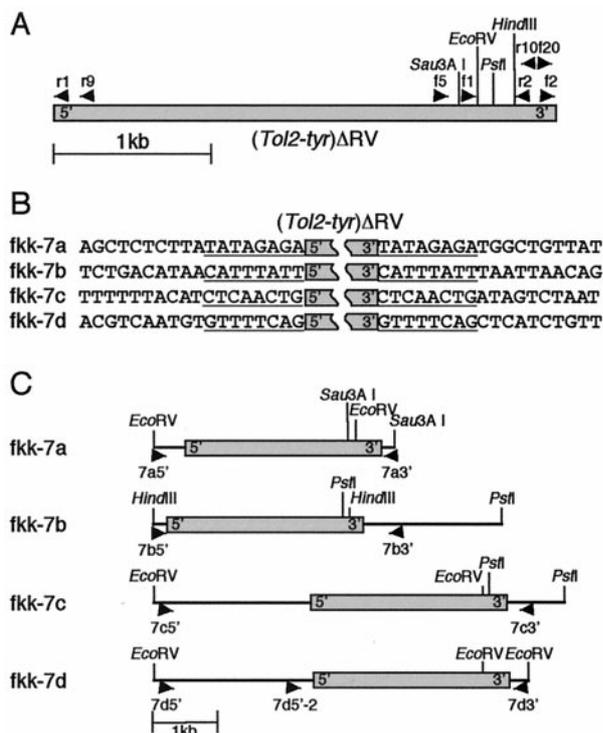
Fig. 3. Inverse PCR and DNA sequencing analysis of the *Tol2* insertions. (*A*) Primers and restriction enzyme sites used for inverse PCR. The gray box indicates (*Tol2-tyr*)ΔRV. Arrowheads indicate positions and directions of primers. *Eco*RV and *Hin*dIII are unique sites. *Sau*3A I and *Pst*I cut more than twice, and only most 3′ sites used to clone the 3′ junctions are drawn. r1, Tol2r1; r9, Tol2r9; f5, Tol2f5; f1, Tol2f1; r2, Tol2r2; r10, Tol2r10; f20, Tolf20; f2, Tol2f. (*B*) DNA sequences adjacent to (*Tol2-tyr*)ΔRV. DNA fragments were amplified by inverse PCR from fish with fkk-7a, fkk-7b, fkk-7c, and fkk-7d insertions (Fig. 2*C*, lanes 1–4), and sequenced. Eight-base pair duplications, created upon transposition, are underlined. (*C*) Structures of *Tol2* insertions. These structures were drawn based on DNA sequences from inverse PCR products and the wild-type DNA fragments. The gray box indicates (*Tol2-tyr*)ΔRV. Restriction enzyme sites used for inverse PCR are shown. Arrowheads (7a5′ and 7a3′, 7b5′ and 7b3′, 7c5′ and 7c3′, and 7d5′ and 7d3′) indicate positions and directions of primers used to amplify wild-type DNA fragments, which extend across the integration sites for *Tol2*. 7d5′-2 primer was used in the experiment described in Fig. 4.

fragments containing the 5′- and 3′-ends of the *Tol2* element were amplified from genomic DNA of the lane 3 fish (Fig. 2*C*) digested with *Eco*RV and sequenced (Fig. 3*B*, fkk-7d). All of these 5′ and 3′ sequences contained the same 8-bp sequences adjacent to the *Tol2* sequence. It has been shown that an 8-bp duplication is created at the target site upon transposition of transposons of the hAT family (4, 5, 16, 18, 20). Thus, these results strongly suggest that, in all four cases, the integration of the *Tol2* element was carried out through transposition.

To confirm this notion, we characterized wild-type alleles of these target loci. We constructed primer pairs 7a5′ and 7a3′, 7b5′ and 7b3′, 7c5′ and 7c3′, and 7d5′ and 7d3′ (Fig. 3*C*), based on the DNA sequences obtained from the inverse PCR analyses, so as to amplify DNA fragments extending across the integration sites of the *Tol2* element. PCR using these primer pairs and genomic DNA isolated from a wild-type zebrafish strain successfully amplified DNA fragments. Sequencing analyses of these DNA fragments revealed that each wild-type locus contained a single 8-bp sequence (wild-type sequences for fkk-7a, fkk-7b, fkk-7c, and fkk-7d are AB045573, AB045574, AB045575, and AB045576, respectively), which was duplicated in the F₁ fish, and the integration of the *Tol2* element did not cause any
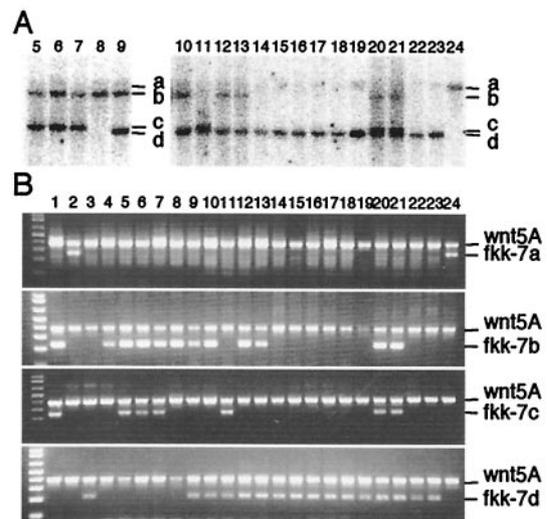

Fig. 4. Identification of the *Tol2* insertions in F₁ fish. (*A*) Southern hybridization analysis of F₁ fish from fkk-7. DNA samples from 20 F₁ fish were digested with *Eco*RI and hybridized with the probe (Fig. 1). The numbering of the lanes (fish) is continued from Fig. 2*C*. Bands of four different sizes, a (lane 24), b (lanes 5–10, 12, 13, 20, 21), c (lanes 5–7, 11, 20, 21), and d (lanes 9–23), were detected. The lower bands on lanes 11, 20, and 21 are doublets. (*B*) PCR analysis of 24 F₁ fish from fkk-7. DNA samples on lanes 1–4 correspond to those on lanes 1–4 of Fig. 2*C*. PCR reactions were carried out using the wnt5A primers (positive control) and 7a3′ and Tol2f20 (fkk-7a), 7b5′ and Tol2r1 (fkk-7b), 7c3′ and Tol2f20 (fkk-7c), or 7d5′-2 and Tol2r1 (fkk-7d). On these photos, the presence of the lower band indicates that the fish carries the specific *Tol2* insertion. The results are consistent with those obtained by Southern hybridization analysis and are summarized in Table 1.

chromosomal rearrangement at the target locus. Therefore, we conclude that these *Tol2* insertions were generated through transposition and that the mRNA injected with the (*Tol2-tyr*)ΔRV DNA can produce a fully functional transposase, which catalyzes a complete transposition process, excision and reintegration reactions, in the zebrafish germ lineage. The structures of the *Tol2* insertions are summarized in Fig. 3*C*. The ≈3-kb, ≈5-kb, and ≈5-kb and ≈0.8-kb *Eco*RV fragments in fkk-7a, fkk-7c, and fkk-7d shown in Fig. 3*C* are consistent with the sizes of the DNA bands observed in the Southern hybridization analysis (Fig. 2*C*, *Eco*RV lanes 1–3).

Multiple *Tol2* Insertions in F₁ Fish. Genomic DNA of 24 F₁ fish (25 fish were originally identified as PCR positives as described above, but one fish was lost before this analysis) from fkk-7 were analyzed by Southern hybridization (Fig. 2*C*, lanes 1–4, and Fig. 4*A*, lanes 5–24). The individual F₁ fish had one (Fig. 2*C*, lanes 2–4, and Fig. 4*A*, lanes 8, 14–19, 22–24), two (Fig. 2*C*, lane 1, and Fig. 4*A*, lanes 5–7, 9–13), or three (Fig. 4*A*, lanes 20 and 21) insertions, which should correspond to any of the insertions identified above. To confirm the identities of the insertions, PCR analyses were performed using primers designed to detect specific *Tol2* insertions; i.e., for fkk-7a, 7a3′ and Tol2f20; for fkk-7b, 7b5′ and Tol2r1; for fkk-7c, 7c3′ and Tol2f20; for fkk-7d, 7d5′-2 and Tol2r1 (Fig. 4*B*). The results are also summarized in Table 1. Thus, the four *Tol2* insertions were segregated independently in the germ line of the fkk-7 founder fish, giving rise to the highly mosaic germ line, and we could obtain F₁ fish with multiple insertions, up to three insertions.

Discussion

In the present study, we have demonstrated that a nonautonomous *Tol2* element could transpose in the genome of the

Table 1. Classification of transgenic F$_1$ fish

Founder fish	Transgenic fish*/F$_1$ progeny fish	Type of integration	Grouping by Southern hybridization and PCR	
fkk-1	2/68	Illegitimate†	Same	2/2
fkk-7	25/50	Transposition	a alone	2/24‡
			b alone	2/24
			d alone	9/24
			b + c	4/24
			b + d	4/24
			c + d	1/24
			b + c + d	2/24

*Transgenic fish were identified as positives for PCR using Tol2f5 and Tol2r7.
†The structure of the insertions in F$_1$ fish from fkk-1 has not been analyzed by DNA cloning.
‡One fish, which was counted as transgenic fish, was lost accidentally before this analysis. The actual data are shown in Figs. 2 and 4. The numbers of fish classified per the number of fish analyzed are shown as the ratios.

zebrafish germ lineage when coinjected with mRNA transcribed from the *Tol2-tyr* element. Therefore, we concluded that the *Tol2-tyr* element is an autonomous member that encodes a functional transposase capable of catalyzing transposition. In vertebrates, several repetitive sequences similar to transposons of the *Tc1/mariner* family have been reported (8–12). Although a synthetic *Sleeping Beauty* transposase, which was reconstructed based on a consensus sequence obtained from nonautonomous salmonid *Tc*-like elements, has been shown to be capable of catalyzing transposition in vertebrate-cultured cells (13), neither an autonomous member nor a functional transposase has yet been identified from endogenous transposable elements in vertebrates. This study identified an autonomous member of a DNA transposable element from a vertebrate genome. Also, we have demonstrated that controlled germ-line transformation in zebrafish can be mediated by using the *Tol2* transposable element.

We have previously shown by a transient embryonic excision assay that the (*Tol2-tyr*)ΔRV element is excisable but the *Tol2* element lacking the first intron sequence of the transposase gene cannot be excised, and we have suggested the presence of essential cis elements in this region (22). It has, however, not been clear whether the (*Tol2-tyr*)ΔRV sequence is sufficient for the reintegration reaction. Our present result clearly indicates that the (*Tol2-tyr*)ΔRV element contains cis elements necessary for transposition. Further studies on dissecting and defining cis requirements will be needed to develop useful transposon vectors. Host factors necessary for the transposition reaction of the *Tol2* element other than the transposase itself (e.g., polymerase, exonuclease, ligase, etc.) have not been known. Our study suggests that such host factors are conservatively present in both zebrafish and the medaka fish, which diverged from a common ancestor some 150 million years ago (26). Therefore, the *Tol2* transposon system may possibly work also in other fish species.

The members of the hAT family are known to comprise complete and defective copies in the genome, which correspond to autonomous and nonautonomous elements, respectively. The defective copies are often shorter, as they have deletions of different sizes in the transposase coding region (3–7). This is not the case with *Tol2* because it has been shown that members of the *Tol2* element in the medaka fish genome are highly homogeneous in their structures, and some of them are identical to the *Tol2-tyr* element at the sequence level (27). These observations, together with our present results, raise a question whether all, or most, of the *Tol2* element in the

medaka fish genome encodes a functional transposase. It has been shown that the excision frequency of an endogenous *Tol2* element is relatively low (20). In this regard, it should be noted that Koga *et al.* isolated mRNA from the medaka fish cells and identified a longer (2319 nt) and a shorter (1946 nt) transcript transcribed from anonymous *Tol2* elements (28). Given the first ATG was used for translation initiation, the longer transcript has the capacity to encode a protein of 685 aa, and the shorter one encodes a protein of 576 aa. These are different from the protein of 649 aa, which could be synthesized from mRNA used in this study and was shown to be fully functional. The activities of the mRNAs transcribed endogenously in the medaka fish cells have not yet been reported. Studies on those activities using the zebrafish system described here should give an insight into the regulation of the *Tol2* element in the medaka fish.

Construction of transgenic animals is an important technology to study functions of genes and also to develop insertional mutagenesis methods. Germ-line transmission of foreign DNA has been achieved in zebrafish by microinjection of naked DNA into fertilized eggs (25), by infection of a pseudotyped retrovirus to blastula-stage embryos (23), and by using transposon systems derived from *Tc3* of *C. elegans* (29) and *mariner* of *Drosophila* (30), both members of the *Tc1/mariner* family. Microinjection of DNA is the most popular way to construct transgenic zebrafish and has been applied to study the function of promoters *in vivo* (31, 32). The pseudotyped retrovirus system can generate a large number of insertions at different loci very efficiently (33) and has made it possible for a large-scale insertional mutagenesis to be performed (34–38). In contrast to these, development of transposon technologies in zebrafish is still in its early stages, and neither highly efficient transgenesis nor insertional mutagenesis using a transposon has been established yet. In the case of *Tc3*, the *Tc3* vector DNA and mRNA for the *Tc3* transposase were coinjected into fertilized eggs, and only one transposase-mediated integration event was identified among F$_1$ fish from 40 injected fish (29). In the case of *mariner*, the *mariner* (*peach*) vector DNA and mRNA for the *mariner* transposase were coinjected into fertilized eggs, and four transposase-mediated integration events were identified among F$_1$ fish from 12 injected fish by Southern hybridization analysis, but not at the DNA sequence level (30). Our present study clearly identified four different transposition events among F$_1$ fish from eight injected fish. Although the relatively small number of fish characterized here and in the previous studies by others precludes a precise evaluation of the frequencies of the transposition events in these different transposon systems, we think that the *Tol2* system is potentially useful as we could find fish with multiple insertions, which had not been reported in the studies using either the *Tc3* or *mariner* element.

This study has provided the basis for developing novel transgenesis and insertional mutagenesis methods using the *Tol2* transposon system in zebrafish. The next important steps toward the establishment of transgenesis and insertional mutagenesis methods using the *Tol2* element are (*i*) to determine the frequency of transposition in the germ line more precisely and develop a protocol to increase the frequency, (*ii*) to determine whether integration of *Tol2* can cause mutations efficiently, and (*iii*) to develop transposon vectors and put useful reporter genes into the vector. Efforts are in progress along these lines.

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