Review

Transposon mediated transgenesis in a marine invertebrate chordate: *Ciona intestinalis*

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Abstract

Achievement of transposon mediated germline transgenesis in a basal chordate, *Ciona intestinalis*, is discussed. A *Tc1/mariner* superfamily transposon, *Minos*, has excision and transposition activities in *Ciona*. *Minos* enables the creation of stable transgenic lines, enhancer detection, and insertional mutagenesis.

Introduction

DNA transposons are powerful tools for genetic analyses. Transposons are employed for creation of stable transgenic lines, enhancer detection, gene trapping, and insertional mutagenesis. These transposon-mediated techniques have been facilitated by the discovery and reconstruction of active transposons in several organisms [1-11]. Despite their utility, transposon technologies are restricted to a few model organisms. Marine invertebrates include most of the phyla whose study is crucial to elucidating the evolutionary molecular mechanisms of diversification in metazoans. To date, transposon technologies have been introduced for only a few marine invertebrate species. Because of the scarcity of refined genetic techniques, research into gene functions in marine invertebrates has remained limited.

Recent achievement of germline transgenesis in a marine invertebrate chordate, *Ciona intestinalis*, has altered this situation [12,13]. *C. intestinalis* has several characteristics that make it amenable for genetics research. In this basal chordate, a *Tc1/mariner* superfamily transposon, *Minos*, has the complete activity required for its transposition [14]. *Minos* introduced into *Ciona* is excised from a plasmid vector by transposase and is integrated into TA dinucleotides of another DNA molecule. The TA dinucleotides are known as target sequences of *Tc1/mariner* transposons [15]. Transposition occurs in *Ciona* germ cells, and *Minos* is inserted into the chromosomes of germ cells [12]. The insertions are inherited stably by subsequent generations, thereby creating stable transgenic lines. Using this transformation technique, genetic techniques such as enhancer detection and insertional mutagenesis have been introduced into *Ciona* using *Minos* [13,16,17]. In this article, recent achievements with transposon techniques in *Ciona*, as well as characteristics of *Ciona* as a new genetic model, are discussed.

Characteristics of *Ciona intestinalis* as an experimental system for genetics

Ascidians, or sea squirts, are members of the subphylum Tunicata [18]. Tunicata belong to the phylum Chordata along with Cephalochordata (amphioxus) and Vertebrata (Figure 1a) [19,20] (Kawashima T, Putnam N, personal communication). As this phylogenetic position suggests, ascidians possess a simplified chordate body plan. This characteristic is most apparent in their larval stage. The larvae of ascidians are typical tadpole larvae and swim like fish (Figure 1b). Each larva has a dorsal hollow neural tube and notochord, both of which represent common characteristics of chordates. In contrast to those apparent similarities, the

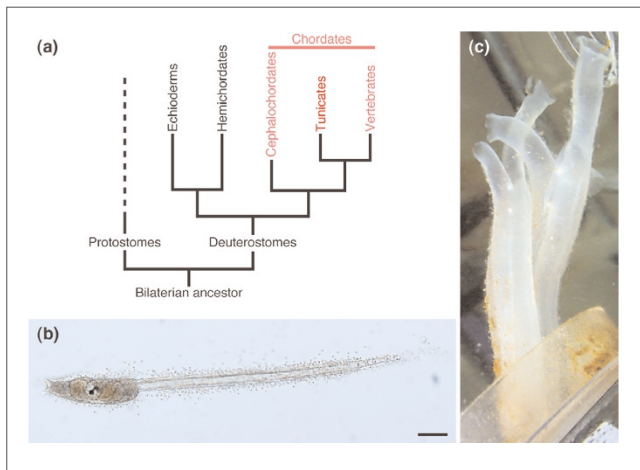

Figure 1
An ascidian - *Ciona intestinalis*. **(a)** Phylogenetic relationships of chordates. Ascidians are included in the subphylum Tunicata. **(b)** A *Ciona intestinalis* larva. This photograph was constructed by merging three photographs of the same individual. Scale bar: 100 µm. **(c)** *Ciona intestinalis* adults. After metamorphosis, *Ciona* loses its tail and starts to settle. Most ascidians are filter feeders.

ascidian larval body is strikingly simple compared with that of vertebrate tadpoles. The typical ascidian larva consists of numerous, but countable, cells [18]. For example, the larvae of *C. intestinalis* consist of approximately 2,600 cells, of which 40 constitute the notochord, 26 make up the muscle, and about 330 the nervous system [18,21]. Ascidians develop rapidly; many ascidians complete embryogenesis within 1 day. This simplicity and rapid embryogenesis aid detailed cell-by-cell analyses of the mechanisms of tadpole body formation. In fact, ascidians are the only chordates for which cell lineages have been described [22-24].

Ascidian larvae change their structure through metamorphosis and become sessile adults (Figure 1c) [25]. After metamorphosis, ascidians start to take in food by filter feeding. During metamorphosis, the larval tail is lost and adult tissues grow rapidly, which include characteristic chordate structures such as pharyngeal gills and an endostyle (the endostyle is homologous to vertebrate thyroid gland [26,27]). Metamorphosis of typical ascidians is completed within several days. Metamorphosis is a dramatic event in ascidian development and provides a good experimental system in which to uncover the mechanisms of metamorphosis and their conservation among vertebrates.

Ciona intestinalis (hereafter referred to as *Ciona*) is a cosmopolitan ascidian [28-31]. It is hermaphroditic and self-fertile. This characteristic represents a great advantage when performing mutant screens because creating animals that are homozygous with respect to mutation sites is possible without genotyping [32,33]. An adult *Ciona* bears thousands of eggs; eggs and sperm can be collected surgically. Surgically collected eggs can be fertilized with sperm from a different individual. They subsequently exhibit normal, synchronized development. Natural spawning can be induced by simple dark-light adjustment to facilitate self-fertilization. Moreover, sperm can be stored on ice for 1 week without loss of fertility. Cryopreservation of sperm is also established to store mutants or transgenic lines semi-permanently [34]. The easy handling of germ cells enables reduction in labor associated with mutant screening and preservation of lines.

The most striking characteristic that distinguishes *Ciona* from other ascidians is the availability of a draft genome sequence [35]. The *Ciona* genome size is approximately 166 megabases per haploid, which contains 15,852 protein coding genes [36]. The genome size and gene number are comparable to those of *Drosophila melanogaster*, and much smaller than those of most vertebrates. In addition, the *Ciona* genome is less redundant than those of vertebrates [37,38], which is probably related to the twofold to threefold duplication of genomes during vertebrate evolution [39]. Therefore, *Ciona* possesses the basic set of genes for a chordate body plan. Because of its compact genome, *Ciona* provides a simple experimental system in which to uncover genetic mechanisms that specify the chordate body plan as well as mechanisms of chordate evolution.

An unusual aspect of the *Ciona* genome with respect to transposons is that an extensive search of the *Ciona* draft genome identified no *Tc1/mariner* transposon (Table 1). Taking into consideration the global conservation of this transposon family [40], the absence of *Tc1/mariner* transposon in *Ciona* genome is curious. Two possibilities are readily apparent. One is that *Tc1/mariner* transposon has been lost in the *Ciona*, ascidians, or tunicates branch by accumulation of mutations. Another possibility is that a hypothetical suppressor of this transposon family interfered with the lateral transfer of transposons. This might be related to the weak transposon activity of several *Tc1/mariner* transposons in *Ciona*, as discussed below.

In *Ciona*, techniques to support the practice of genetics research have been developed. The *Ciona* life cycle is about 2 to 3 months. An inland culture system has been established [17,34]. Settlement after metamorphosis enables retention of several lines in the same aquarium. Introduction of DNA and RNA into eggs by microinjection or electroporation is performed routinely [41,42]. The latter technique can introduce DNA and RNA into hundreds of eggs within 1 hour, thereby facilitating creation of transgenic lines.

There are three major obstacles to use of *Ciona* to conduct genetics studies. First, no inbred strain has been created; most experiments are dependent on natural populations. Creating strains had been difficult because of complications in culturing. Recent improvements of inland culture systems

Table 1

BLAST search of *Tc1/mariner* superfamily transposons in five eukaryotic genomes

	Ciona intestinalis ver 1.0		*Brachiostoma belcheri* ver 1.0		*Fugu rubripes* ver 4.0		*Xenopus tropicalis* ver 4.1		*Nematostella vectensis* ver 1.0	
Query	Scaffold no.	Highest hsp_e-value	Scaffold no.	Highest hsp_e-value	Scaffold no.	Highest hsp_e-value	Scaffold no.	Highest hsp_e-value	Scaffold no.	Highest hsp_e-value
Mos1	no hit	-	44	$1.1 \times e^{-29}$	no hit	-	78	$3.2 \times e^{-6}$	no hit	-
SB	no hit	-	532	0	299	0	314	0	2,141	0
Minos	no hit	-	532	$8.6 \times e^{-26}$	144	$1.69 \times e^{-39}$	566	0	2,141	$2.9 \times e^{-28}$
Tc1	no hit	-	55	0	9	0	1,139	0	2,141	$1.6 \times e^{-41}$

Databases released from JGI [82] were used for the analyses. Tblastn search at the threshold of $1 \times e^{-5}$ was done with the amino acid sequences of the transposases shown in the 'Query' columns. Scaffold numbers that exhibited the top hit are described with the highest e values. If there was no hit, it is shown as 'no hit'. SB, *Sleeping Beauty*.

are expected to resolve this problem [43]. Second, natural *Ciona* harbor many single nucleotide polymorphisms. The genome project reported that 1.2% of nucleotide differences were observed between alleles of the single individual [35]. This score is 15 times higher than that in humans, and three times higher than that in pufferfish. Such highly frequent polymorphism would render it difficult to perform systematic fine mapping of point mutations. On the contrary, high polymorphism might allow retention of highly frequent natural mutants, which are a valuable resource for mutant screening. In *C. intestinalis*, and its related species *C. savignyi*, several mutants have been isolated through screening of wild populations [34,44,45]. The third obstacle to genetics studies is the requirement for seawater for culture. Large-scale culturing requires a considerable amount of seawater, which limits the culturing of *Ciona* to laboratories that are near to the sea. Recently, *Ciona* culture with artificial seawater has been achieved [34,43], which will promote the spread of *Ciona* studies to inland laboratories.

Activity of *Minos* transposon in *Ciona*

Minos is a member of the *Tc1/mariner* superfamily of transposons isolated from *Drosophila hydei* [5]. *Minos* exhibited both excision and transposition activity from protostomes to deuterostomes [46-54], suggesting a wide host range. *Minos* is the only transposon whose activity has been described in *Ciona* [12-14,55,56]. Its excision is observed in almost all embryos when *Minos* is injected into *Ciona* embryos together with transposase mRNA (Figure 2). Footprint sequences indicate that *Minos* is excised correctly by transposase. The typical footprint sequences of *Minos* are 5'-TACTCGTA-3' or 5'-TACGAGTA-3'; both typical and atypical footprint sequences are observed in *Ciona* [14,57]. The atypical footprint sequences might be related to the endogenous repair system of *Ciona*. Neither excision nor transposition occurs without transposases, suggesting that no *Ciona* protein mimics *Minos* activity. Interplasmid transposition assay using donor and recipient plasmids (Figure 2) has revealed that *Minos* has slightly lower transposition activity in *Ciona* than in insects [14,49]. The manner of insertion of *Minos* into the recipient plasmid is identical to that previously reported; the target sequences are TA dinucleotides and the duplication of the TA sequences occurs, which flanks two inverted repeats. The frequency of excision and transposition activity suggests that *Minos* has sufficient activity to cause germline transgenesis in *Ciona*, as shown by microinjection of transposase mRNA with recombinant *Minos* containing a promoter-green fluorescent protein (GFP) cassette. The scheme of screening transgenic lines is shown in Figure 3. About 30% to 36% of *Minos*-injected *Ciona* become founders and transmit *Minos* insertions to progeny. The average insertion number inherited from a founder was estimated at around two (Sasakura Y, unpublished data). This transgenesis frequency is comparable to that of *Sleeping Beauty* (SB) in zebrafish [58]. Thermal asymmetric interlaced (TAIL)-polymerase chain reaction (PCR) is used to identify *Minos* insertion sites [12,59]. *Minos* was preferably inserted into TA-rich sequences such as introns [12].

Another convenient transgenesis technique of *Ciona* with *Minos* was achieved using electroporation [56]. As described above, electroporation enables rapid and reproducible transgenesis of early *Ciona* embryos. This technique simultaneously electroporates *Minos* DNA and *in vitro* synthesized *Minos* transposase mRNA in *Ciona* embryos. The transformation frequency by electroporation mediated transgenesis is about 20% to 30%, which is lower than that by microinjection mediated transgenesis, perhaps because of a lower amount of mRNA introduced into embryos by electroporation. By microinjection, 5 to 10 ng/μl of *Minos* DNA and 50 to 200 ng/μl of transposase mRNA are included in the injected solution. The current electroporation method requires 60 μg of *Minos* DNA and 60 μg of transposase mRNA, which would correspond to 5 to 10 ng/μl of DNA and

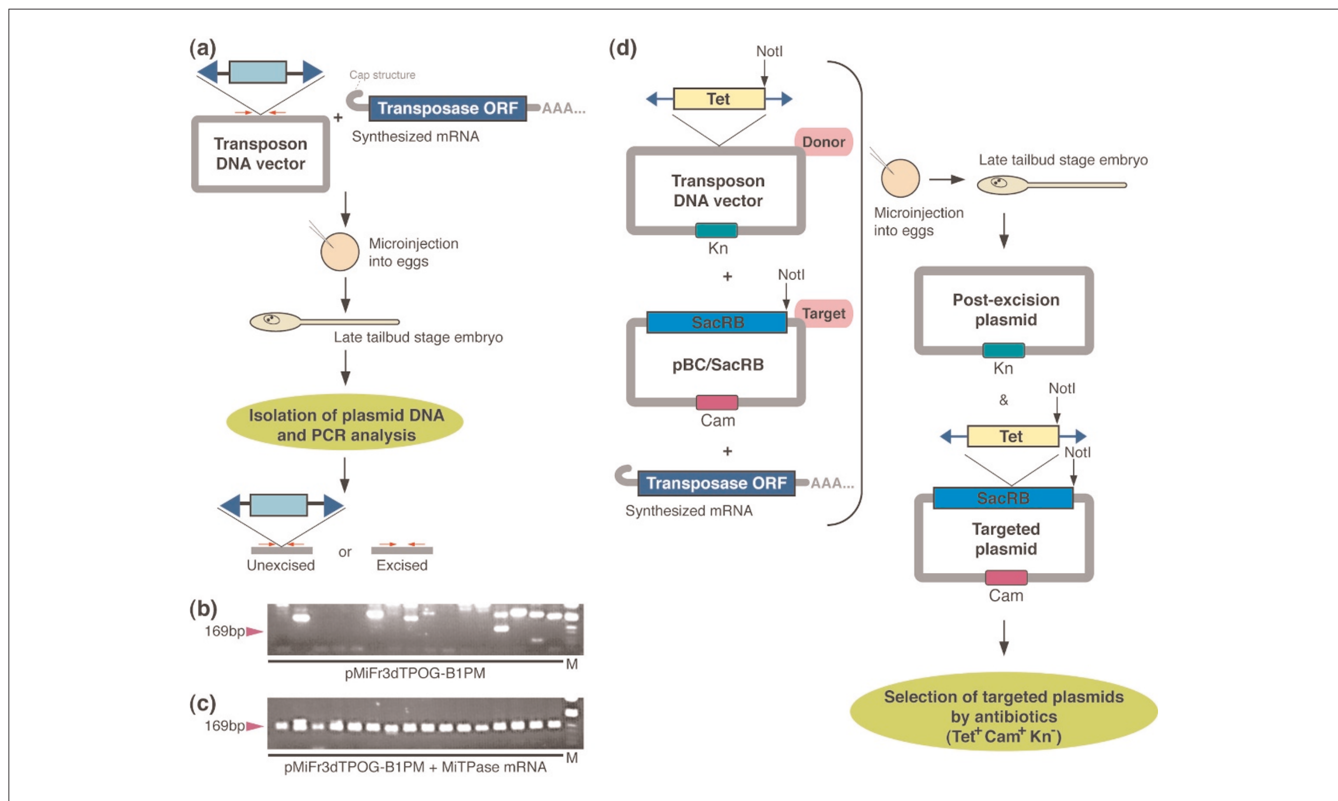

Figure 2
Procedures of experiments testing transposon activity in *Ciona*. **(a)** Excision assay. A mixture of transposon vector and *in vitro* transcribed transposase mRNA was injected into *Ciona* one-cell embryos. After these embryos reach the late tailbud stage, DNA was extracted from embryos and was subjected to polymerase chain reaction analyses to identify the excision. **(b,c)** An example of excision assay: a *Minos* vector (pMiFr3dTPOG-B1PM) with an insert longer than 12 kilobases was examined. Excised bands are detected only when the transposase mRNA is co-injected. **(d)** Interplasmid transposition assay. A donor vector containing a *Minos* construct and a target vector are introduced into *Ciona* embryos with transposase mRNA. Plasmids are recovered from *Ciona* embryos and are used to transform *Escherichia coli*. The occurrence of transposition was monitored by selection of *E. coli* using antibiotics. bp, base pairs; ORF, open reading frame.

RNA in the injection solution. Nevertheless, electroporation mediated transgenesis is now the main strategy of *Ciona* transformation because of its convenience.

Minos exhibited constant excision and transformation activity, even when the length of insertion is sufficiently long to suppress transposition of another *Tc1/mariner* transposon, namely SB [60]. So far, an insert size of up to 10 kilobases has been found to have no adverse effect on insertion efficiency (Sasakura Y, unpublished data). Such flexibility of *Minos* with respect to insert length allows the creation of various transposon constructs that are appropriate for experimental purposes.

Activity of other *Tc1/mariner* transposons in *Ciona*

The identification of other active transposons would make transposon technology more versatile in *Ciona*, because it would be useful to create 'jump starter' lines of *Minos*. Modifier screens of mutants generated by *Minos* must be done using a different transposon. Different transposons can be expected to have different insertion site preferences. Therefore, execution of large-scale mutagenesis with two transposons would be effective for saturation mutagenesis. In addition to these technical innovations, description of activity of transposons in various organisms is necessary to elucidate cross-species activity of transposons and the mechanisms that determine transposon activity in nonhost organisms. Such knowledge would be valuable for further improvement of transposon technologies. Transposon activity in marine invertebrates has not been described, except for *Minos* in *Ciona* and in a crustacean [12,53]. *Ciona* is the pioneer organism of transposon technology among marine invertebrates; testing of various transposons in this organism is important.

The *Tc1/mariner* superfamily includes many transposons whose consistent activity in several protostomes and vertebrates has been described [61-64]. We have tested some of these transposons, including SB, *Frog Prince* (FP), and *Mos1*, in *Ciona*. The former two transposons are resurrected

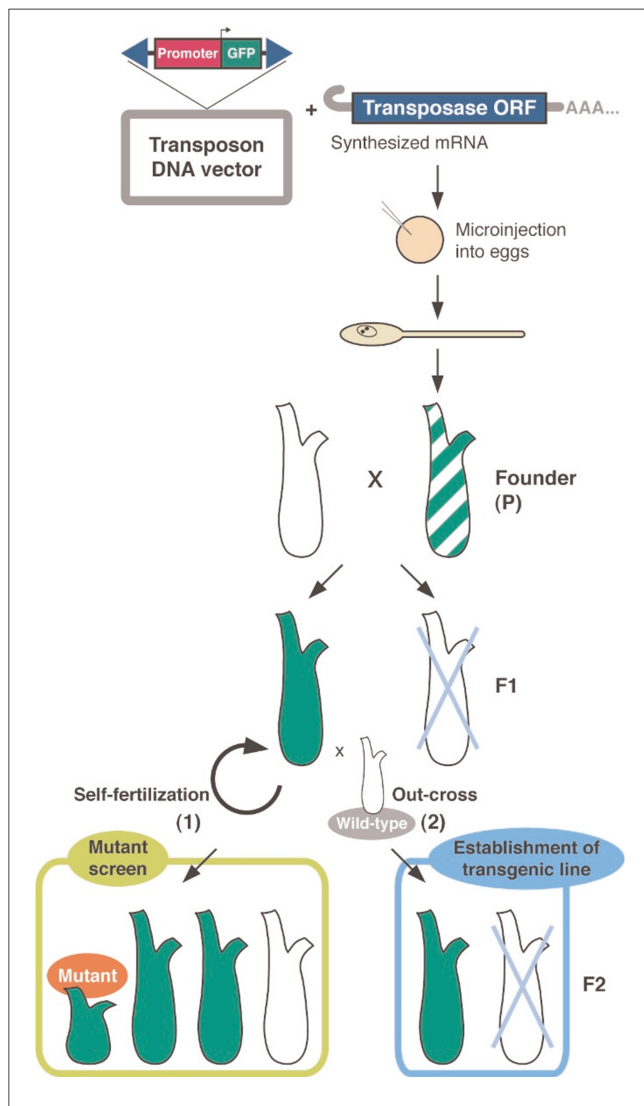

Figure 3
Creation of stable transgenic lines with *Minos* and mutant screening. Mixture of a transposon vector containing a promoter-*gfp* cassette and transposase mRNA is introduced into *Ciona* one-cell embryos by microinjection or electroporation. These animals are cultured until gamete maturation. Generally, sperm from *Minos*-introduced individuals are used to fertilize wild-type eggs to obtain F_1 family individuals, which are screened to find green fluorescent protein (GFP)-positive animals. The GFP-positive animals are selected for further culture, thereby establishing transgenic lines. The F_1 animals with matured eggs and sperm are subjected to self-fertilization to obtain F_2 family individuals, which are screened to identify mutant phenotypes. ORF, open reading frame.

transposons that are derived from vertebrate genomes [8,11]; *Mos1* was isolated from the insect *Drosophila mauritiana* [3,4]. All three transposons are active in vertebrates [11,61,63,64]. Excision activity of the three transposons was examined using a PCR-based assay (Figure 2a), and SB, FP, and *Mos1* exhibited excision in *Ciona* (Figure 4). Their excisions have been supported by the presence of footprint sequences (Figure 4). However, the excision efficiency of these transposons was lower than that of *Minos*. When the excision efficiencies of transposons were compared with the same condition (5 to 10 ng/μl of transposon DNA and 50 to 200 ng/μl of transposase mRNA in the injection solution), *Minos* showed excision in almost all embryos, whereas *Mos1*, SB, and FP showed excision in only a few embryos. For example, nine out of 16 embryos exhibited excision in the case of *Mos1*, two out of 16 in the case of SB, and eight out of 32 in the case of *FP*. Interplasmid transposition assay and germline transformation of *Ciona* with *Mos1*, SB, and FP were also tested, but no transposition was detected (Awazu S, Sasakura Y, unpublished data).

What might restrict *Mos1*, SB, and FP activity in *Ciona*? One possibility is that co-factors that are required for transposase activity are incompatible or absent in *Ciona*. In fact, SB and FP are transposons derived from vertebrates [8,11], and therefore they retain high activity in vertebrates, indicating that all sets of co-factors required for SB and FP activity are present in vertebrates. Recent studies have revealed necessary co-factors for SB transposases [65,66]. Although *Ciona* contains the basic set of genes for the chordate body plan, many genes are specific to vertebrates. The supply of such co-factors may be necessary to make transposons active in *Ciona* if SB and FP transposases require such vertebrate-specific co-factors. An alternative possibility is that a factor is present that inhibits transposases. Inhibition of the transposase activity has been reported in the *Tn5* transposon of *Escherichia coli*, in which an inhibitor of the transposition protein (a truncated form of Tn5 transposase that does not possess DNA-binding activity) forms a complex with Tn5 transposase and interferes with transposition [67]. The presence of such an inhibitor has not been demonstrated in *Tc1/mariner* transposons, but the possibility remains that there is a *Ciona* protein that binds transposases and inhibits their activity.

The inefficiency of *Mos1*, SB and FP in *Ciona* implies that activity of transposons must be tested in each animal model to seek an active transposon. Identification of factors that restricts the activity of transposons is necessary to make them more valuable tools for genetics research in various organisms.

Enhancer detection

The compact genome of *Ciona* is a convenient feature for studying regulatory elements of gene expression [68,69]. High density of enhancer elements is expected in the *Ciona* genome, facilitating efficient enhancer detection, which is necessary to identify enhancers that cannot be identified using conventional *cis* element analyses. This technique is also useful in creating marker lines that express reporter genes in a tissue-specific manner. In *Ciona*, techniques of germline transgenesis were established recently, but to date only a few marker transgenic lines are available. Enhancer

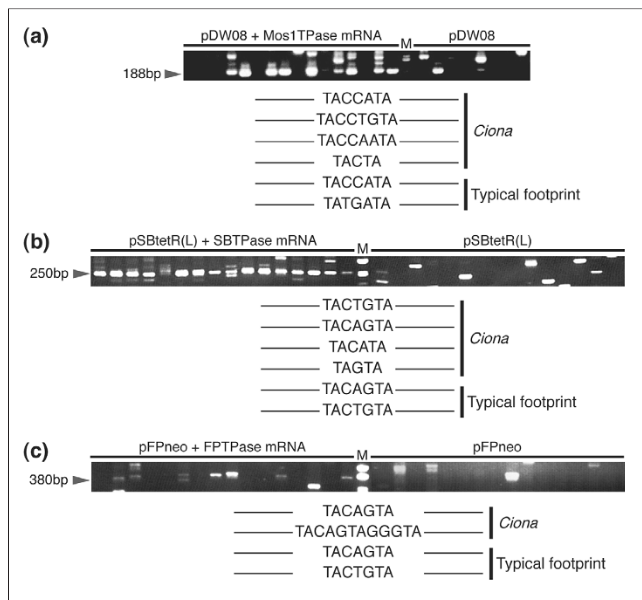

Figure 4
Excision activity of *Mos1*, SB, and FP transposons in *Ciona*. **(a)** (top part) Excision of *Mos1*. The left panel shows the polymerase chain reaction result of *Mos1* transposon and transposase-injected embryos; the right panel shows the results of *Mos1* transposon-injected control embryos. The expected sizes of correct excision events are shown by arrowheads. (bottom part) Footprint sequences of *Mos1* observed in *Ciona* and typical footprint sequences reported previously (typical footprint). **(b)** Excision of *Sleeping Beauty* (SB). Note that three to six times more transposon DNA was injected in this experiment, which resulted in the detection of excised bands from every embryo. **(c)** Excision of *Frog Prince* (FP). bp, base pairs; M, marker lane.

detection will provide useful marker lines for future genetics studies. In addition, novel tissues or subpopulations of tissues are identifiable by enhancer detection that were previously unidentifiable by simple observation or *in situ* hybridization (Awazu S, Sasakura Y, unpublished data).

In *Ciona*, enhancer detection by microinjection mediated transgenesis has been reported [16]. An enhancer detection line near the *musashi* orthologous gene (*Ci-musashi* [16]) has been identified among 21 *Minos* injected animals [12]. A recent enhancer detection screen using a promoter of the *Ciona* thyroid peroxidase orthologous gene (*Ci-TPO*) yielded six enhancer detection lines from 110 injected animals [70]. The frequency of enhancer detection in *Ciona* is therefore estimated at 4.7% to 5.4% per injected animal. This frequency is higher than that of SB mediated enhancer detection in zebrafish (2.5% per injected animals [71]), but lower than that of *Tol2* mediated enhancer detection (12% [72]).

Thus far, the promoter of *Ci-TPO* is the only promoter that has been used for enhancer detection in *Ciona*. It includes 860 base pairs of upstream sequence from the initiation codon of the gene and exhibits weak expression in endodermal tissues [72]. This might not be an ideal promoter for enhancer detection in all tissues. There might be enhancers to which *Ci-TPO* promoter could not respond, because minimal promoters exhibit different responsiveness to enhancers (Lemaire P, personal communication). In fact, most enhancer detection lines with *Ci-TPO* promoter showed reporter gene expression in endodermal tissues [70]. Comparing the efficiency of enhancer detection between the *Ci-TPO* promoter and a basal promoter or a minimal promoter derived from a housekeeping gene may be necessary to identify an ideal promoter for enhancer detection in *Ciona*.

In the *Ci-musashi* enhancer detection line, *Minos* was inserted into an intron [16]. Detailed analysis of the line revealed that expression of *Ci-musashi* is regulated by many enhancers located at the 5' upstream region and in introns [16]. These enhancers have both redundant and distinct functions for gene expression. Such an enhancer complex is probably necessary to ensure the appropriate spatial and temporal expression of *Ci-musashi*. Enhancer identification in the context of chromosomes is necessary to understand the *in vivo* function of these enhancers. Enhancer detection is a viable method for this purpose.

Remobilization of *Minos* in *Ciona* genome

Non-autonomous transposons in the genome can be re-mobilized by providing transposase mRNA (Figure 5). This technique is useful for creating new insertions and 'local hopping', and for creating new mutant alleles by deletions. Remobilization of *Minos* within the *Ciona* genome was achieved by microinjection of transposase mRNA into embryos whose respective genomes contain tandem arrays of *Minos* (Figure 5a [70]). This method has been used for enhancer detection [70].

We created a transgenic 'mutator' line harboring a tandem array of *Minos* vector for enhancer detection, which contains a promoter of *Ci-TPO* [70]. The tandem array in the mutator line was estimated to include as many as 255 transposons. In this study, remobilization of a few copies of *Minos* copies probably occurred from the concatemer. Screening enhancer detection using the remobilization technique was conducted as follows. Transposase mRNA was injected into unfertilized wild-type eggs. These eggs were fertilized with sperm from the mutator line. Because our enhancer detection vector shows GFP expression in a part of somatic cells, these transposase-introduced *Ciona* were selected to remain as GFP positive, transposon containing animals. These GFP positive animals were crossed with wild-type individuals; then, the GFP expression pattern in the next generation was monitored to screen families exhibiting altered GFP expression.

The results indicated that 79% of transposase-injected animals transmitted enhancer detection insertions (Figure 5b). This frequency is considerably higher than that seen in the microinjection mediated approach. Although many of the

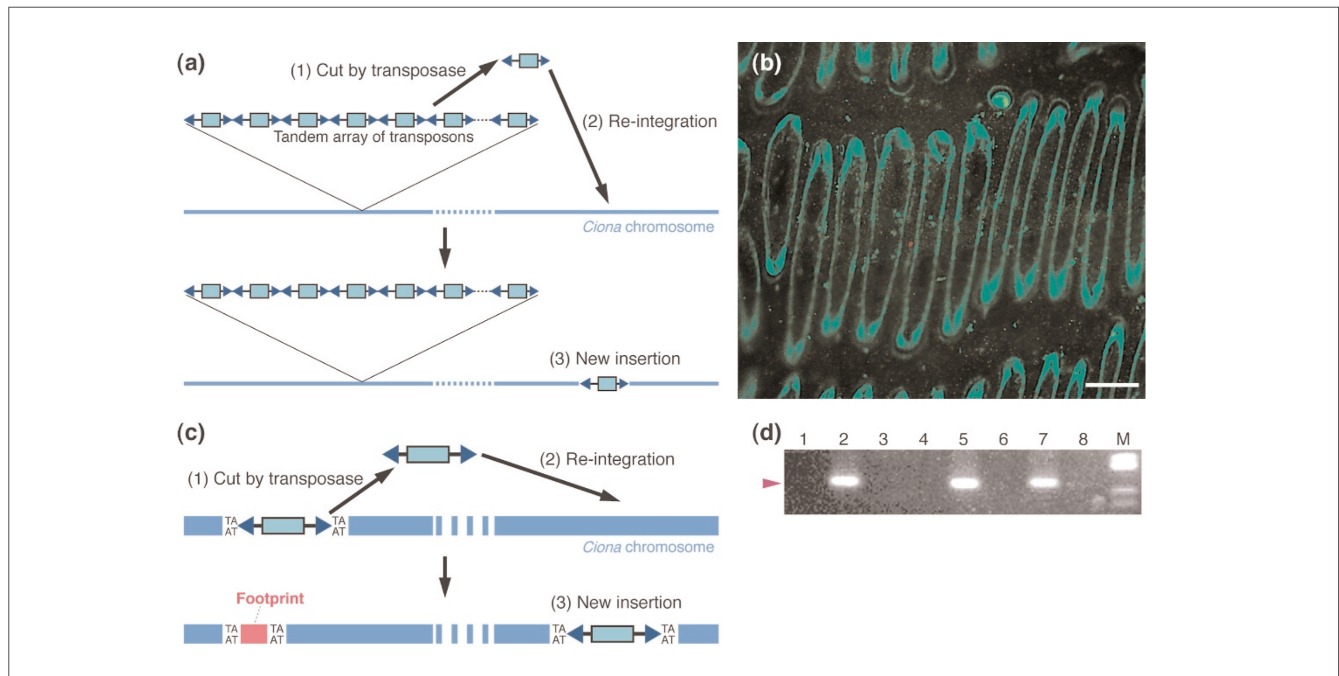

Figure 5
Remobilization of *Minos* in the *Ciona* genome. **(a)** Remobilization from a tandem array. **(b)** An example of enhancer trap lines created using remobilization technique. Green fluorescent protein is expressed in the pharyngeal gill. Bar = 100 μm. **(c)** Remobilization of *Minos* from a single inserted site. **(d)** Excision of single *Minos* insertion in the somatic cells. The polymerase chain reaction bands show *Minos* excision from the genome (arrowhead). M, marker lane.

enhancer detection lines showed GFP expression in endodermal tissues, a few lines showed expression in ectodermal or mesodermal tissues. Therefore, this method could be more efficient for large-scale enhancer detection with creation of many valuable lines. The tandem array interferes with detailed analyses of insertions by Southern blot and TAIL-PCR. In fact, Southern blot was done to show the presence of novel insertions created by remobilization. However, the signal was not conspicuous in many individuals, and as a result the number of new insertions is likely to have been underestimated. Identification of new insertion sites by TAIL-PCR was performed after digestion of genomic DNA with restriction enzymes to suppress PCR amplification within the concatemer [73]. Numerous lines have insertion sites that were unidentifiable, even after restriction enzyme treatment. The enhancer detection insertions can be segregated from the original tandem array by passing through several generations. This may result in the establishment of transgenic lines that have a single insertions of enhancer detection in their genome. Characterization of their insertion sites may increase the efficiency of identification of the causal insertions that were obtained using the remobilization technique.

Remobilization of a single *Minos* insertion might reduce these problems (Figure 5c). Several tests of remobilization of a single insertion have been carried out using microinjection of transposase mRNA into embryos of transgenic lines (Sasakura Y, unpublished data). In somatic cells excision events were observed (Figure 5d). However, the frequency of excision appeared to be low, and evidence of excision or transposition in the germ cells was not obtained. The primordial germ cells of ascidians are suggested to be two small cells, called B7.6, in early embryogenesis [74]. Thus, germ cells are derived from a small number of primordial cells. Less injected transposase mRNA would be delivered to germ cells than to somatic cells. Therefore, the frequency of excision and transposition in the germ cells would be much lower than in the somatic cells. A technical innovation, such as generation of 'jump starter' lines, is necessary to achieve highly frequent jumping of a single *Minos* copy in germ cells [75].

Insertional mutagenesis

Insertions of *Minos* can disrupt gene function to create mutants. Insertional mutants are distinguishable from background mutations by the fact that they segregate with the insertions. In *Ciona*, a small-scale mutagenesis screen was carried out using self-fertilization (Figure 3), and two insertional mutants were isolated from 120 transgenic lines, which are estimated to correspond to 240 insertions; one mutant can be isolated for every 120 insertions. The mutant frequency is lower than with insertional mutagenesis with pseudotyped retrovirus in zebrafish (one mutant per 85

insertions [76,77]). Taking into consideration the compact genome of *Ciona*, which has less redundancy, it is curious that insertional mutagenesis in *Ciona* would be less efficient than in zebrafish. There are two possible explanations. One is that the preference of the insertion sites in the gene, such as 5' end, introns, exons, or 3' end, might be different between *Minos* and pseudotyped retrovirus. In the zebrafish approach, approximately 60% of the mutagenic insertions reside in the promoter, first exon, or first intron [77]. As mentioned above, *Minos* is preferably inserted into TA-rich sequences such as introns and intergenic regions. The second possible explanation is that pseudotyped retrovirus would be more mutagenic than *Minos*. Introduction of a gene trap cassette into the pseudotyped retrovirus vector did not affect the mutation frequency [77,78]. Pseudotyped retrovirus might interfere with splicing to produce truncated proteins, even without such a cassette. In contrast, the single *Minos* insertions into introns appeared to be insufficient to cause mutations (Sasakura Y, Awazu S, unpublished data). The mutant frequency would therefore reflect the difference between two vectors.

From mutant screening, one insertional mutant has been characterized in detail [17]. In this mutant, an insertion at the promoter of a gene encoding cellulose synthase (*Ci-CesA* [79]) disrupts expression of this gene. Animals homozygous for this insertion exhibit abnormalities in the process of metamorphosis. At the larval stage, their trunks show post-metamorphosed states, although they retain tails, which would normally be lost during metamorphosis. The trunk-metamorphosed larvae continue to swim vigorously. This mutant was named *swimming juvenile* (*sj*). This mutant showed a novel function of animal cellulose synthase for the process of normal metamorphosis as well as for the biosynthesis of cellulose. As described above, a concatemer of *Minos* is inserted into the promoter of *Ci-CesA*. In another insertional mutant (Matsuoka T, Sasakura Y, unpublished data), a concatemer of *Minos* is inserted into an intron. Such concatemers are very long and may therefore disrupt promoters or introns. However, mutations by a single insertion are superior to concatemers; some refinement of transposon vectors, such as gene trap, is necessary to produce highly mutagenic *Minos*. Recently, we attempted to introduce a gene trap method into *Ciona* (Oogai Y, Sasakura Y, unpublished data).

Because insertional mutagenesis with *Minos* has been achieved, the next step will be saturation mutagenesis using this transposon. *Ciona* contains a smaller set of genes with less redundancy than in vertebrates. This characteristic renders this ascidian a suitable organism for saturation mutagenesis. It is necessary to estimate the frequency of essential genes for development in order to calculate the number of transgenic lines that are necessary for saturation mutagenesis. For such estimation, isolation of more mutants is necessary. In addition, several obstacles to *Ciona* genetics must be overcome in order to conduct saturation mutagenesis. One is the need to create a mutagenic *Minos* construct. Other obstacles are associated with the primitive state of *Ciona* genetics, resulting from its short history. Although we take only *Ciona* into consideration here, most of these points also pertain to other marine invertebrates.

In the mutant screen, we used a *Minos* construct with a GFP reporter. The expression of GFP was used to judge whether mutations are associated with insertions. However, a correlation between a mutation and GFP expression does not always indicate that the mutation is caused by a *Minos* insertion. A wild population of *Ciona* was used to create insertional mutants. Wild populations maintain frequent background mutations. Sometimes these natural mutation sites are located very close to *Minos* insertion sites, and therefore natural mutants, so-called associated mutants, appear to be related to the *Minos* insertions. These associated mutants must be discriminated from insertional mutants because transposon insertion sites in the associated mutants are close, but not identical, to the actual sites of mutations. In the recent small-scale mutagenesis studies [13], four mutants exhibited strong correlation with GFP expression (>90% of homozygous mutants showed GFP expression). Two of them were associated mutants and two were insertional mutants. Associated mutants are distinguishable from insertional mutants by imperfect correlation between mutations and GFP expression. Reporter gene expression is a good marker for this purpose, because hundreds of mutants can be examined through simple observation. The mutants showing perfect correlation with GFP expression are candidates for insertional mutants. Several experiments must be performed to conclude that they actually are insertional mutants. Identification of the insertion sites is primarily required. It is also necessary to demonstrate perfect homozygosity of mutants with respect to the insertions, which is evidence that recessive insertional mutants have been created. Finally, to establish a causal link, it is necessary to identify those genes that are responsible for mutants; this may be achieved through rescue experiments or knockdown of genes by microinjection of antisense morpholino oligonucleotides or dominant negative forms [17,42].

The second disadvantage of *Ciona*, after its suboptimal mutation frequency, is that its embryos sometimes develop poorly compared with those of other model organisms. Typical unhealthy development includes kinked tails at the larval stage. Families showing such unhealthy development are omitted from screens. Such omission might cause the loss of mutants that would show the kinky-tail phenotype. Several insertional mutants might have been lost through this technical limitation. Therefore, the mutation frequency with *Minos* described above might have been underestimated. Recent improvements in culturing systems will enable continual production of healthy embryos. If a family

shows unhealthy development in this setting, then the phenotype is probably derived from mutations.

Forward genetics is a powerful technique in which to identify gene function; it is possible to identify gene functions that are neglected by reverse genetics. This approach has recently been employed in *Ciona* with the chemical mutagen *N*-ethyl-*N*-nitrosourea and *Minos* transposon [17,32,33,80,81]. Causal genes have been identified in only a few mutants. Most of the mutants generated in the near future would therefore be novel ones. Insertional mutagenesis provides an ideal system in *Ciona* because the causal genes are identifiable in a short period of time, without time consuming fine mapping.

Conclusion

In this article we review recent achievements in germline transgenesis with the *Minos* transposon in *Ciona intestinalis*. These studies have revealed that *Minos* is a highly active transposon in this organism, as shown by establishment of techniques such as stable transgenic lines, enhancer detection, and insertional mutagenesis. These technical innovations will be of great value to future genetic analyses in *C. intestinalis*. Frequent enhancer detection by remobilization will provide useful transgenic lines. Insertional mutagenesis allows the identification of novel functions of genes during development, as shown by the example of cellulose synthase. Taking into consideration the advantages of *Ciona* as a subject of genetics research, future genetic analyses in this organism will provide unique insights into chordate gene function. In addition to these technical innovations with *Minos*, we describe several technical hurdles that *Ciona* researchers must overcome if they are to conduct large-scale mutagenesis studies.

Minos is a valuable transposon, and its activity may be the first to be tested in organisms for which no genetic approach has yet been introduced. We also provide evidence that some other *Tc1/mariner* superfamily transposons have excision activity in *Ciona*. However, these transposons have not been found to be efficient in causing germline transgenesis in *Ciona*. This information may be useful in elucidating the mechanisms that determine transposon activity in different organisms. Resolving these issues would make these transposons further valuable tools in *Ciona* genetics research.

Competing interests

The authors declare that they have no competing interests.

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