Germline transgenesis and insertional mutagenesis in the ascidian Ciona intestinalis

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[Review]

Germline transgenesis and insertional mutagenesis in the ascidian *Ciona intestinalis*

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Abstract

Stable transgenesis is a splendid technique that is applicable to the creation of useful marker lines, enhancer/gene trappings, and insertional mutagenesis. Recently, transposon-mediated transformation using a Tc1/*mariner* transposable element *Minos* has been reported in two ascidians: *Ciona intestinalis* and *C. savignyi*. The transposon derived from an insect, *Drosophila hydei*, has high activity for excision in *Ciona* embryos and transposition in their genome. As much as 37% of *Minos*-injected *C. intestinalis* transmitted transposon insertions to the subsequent generation. *Minos*-mediated germline transgenesis has also been achieved *via* electroporation method. *Minos* techniques have been applied to enhancer trappings and insertional mutagenesis in *Ciona*. For those reasons, *Minos* offers the high potential for use as a powerful tool for future genetic studies. This review specifically addresses recent achievements of transformation techniques in *Ciona,* as exemplified using the *Minos* system.

Keywords: transgenic techniques; *Minos*; Tc1/*mariner*; enhancer trap; insertional mutagenesis

INTRODUCTION

Transgenesis is routinely carried out in many model organisms from bacteria to higher eukaryotes. The basis of this technique is the introduction of exogenous DNA into organisms. The introduced DNA molecules are frequently lost during cell division. Their integration into the genomes is required to maintain these DNA stably. These individuals show a mosaic state of transgene-positive and transgene-negative cells when transgenes are introduced into multicellular organisms. If the transgenes are integrated into chromosomes of primordial germ cells, the progeny derived from these germ cells show integration of transgenes in whole cells. These non-mosaic individuals transmit the transgenes to the next generations stably, thereby establishing transgenic lines. The stable transgenic organisms generated by germline transgenesis provide useful tools to analyze gene functions. For example, introduction of various reporter constructs produces easy markers for specific tissues, organs, and subcellular components. Trapping techniques, as exemplified by enhancer and gene trappings, are useful to discover endogenous enhancers and genes. Integration of transgenes into genes sometimes destroys their functions, thereby creating insertional mutants.

Genomic integration of transgenes occurs occasionally by the endogenous system of double-strand break repair. Transgenes that have double-strand breaks are introduced into eggs to create stable transgenic organisms (Constantini and Lacy, 1981; Gordon and Ruddle, 1981; Gordon and Ruddle, 1986; Etkin and Pearman, 1987; Stuart et al., 1988; Higashijima et al., 1997). However, integration by such an endogenous system occurs probabilistically: the frequency is not high. Specific elements that facilitate DNA integration are applied to overcome this problem. In vertebrates, some viral vectors are

used (Roe et al., 1993; Lin et al., 1994). However, viral vectors can be difficult to package and handle; moreover, they require special facilities because they are also harmful to humans. In contrast to them, transposable elements (transposons) have simpler structures and are easy to handle in the normal facilities of laboratories. Therefore, germline transformation with transposons is used frequently in animals.

Ascidians represent the most basic chordate body plan (Satoh, 1994; Corbo et al., 2000; Satoh, 2003; Satoh et al., 2003; Nishida, 2005). In these marine organisms, extensive analyses of gene functions during development, gene regulatory networks and functional genomics have been performed. These studies are supported by the availability of techniques of embryology and molecular biology (Ettensohn et al., 2003). Microinjection and electroporation enable us to introduce DNA, RNA and oligonucleotides into embryos to observe effects of these treatments (Hikosaka et al., 1994; Corbo et al., 1997; Satou et al., 2001). However, in ascidians, germline transgenesis has not been developed until recently. Several laboratories have made attempts to generate stable transgenic ascidians through simple introduction of linearized DNA into eggs, but those trials failed. Recently, stable transgenic lines of two ascidians, *Ciona intestinalis* and *C. savignyi*, have been established through two independent techniques (Deschet et al., 2003; Sasakura et al., 2003a, b). One uses a transposable element, *Minos*; the other is meganuclease I-SceI-mediated transgenesis. This review describes these transgenic techniques in ascidians.

TC1/*MARINER* SUPERFAMILY TRANSPOSONS

Transposons are components of DNA that display autonomous mobilization between DNA (Alberts et al., 2002; Craig et al., 2002). There are two major types of transposons, DNA transposons and retrotransposons. The DNA transposons are mobilized between DNA molecules as DNA, whereas retrotransposons are transcribed and are mobilized *via* reverse transcription. Although both types of transposons have been used in transposon technologies, DNA transposons are described in this review.

Most DNA transposons are mobilized or transposed using a 'cut and paste' mechanism (e.g. van Luenen et al., 1994). DNA transposons have two inverted repeats at each end, and several genes are present between each set of inverted repeats (Fig. 1A). Transposase enzymes, which are encoded in the transposons themselves, excise transposons from DNA at the inverted repeats, and the enzymes integrate transposons again at the specific sites of DNA called '*target sequences*' (Fig. 1B). The double-strand breaks generated by the excision of transposons are repaired by the endogenous repair system (Gloor et al., 2000; Raizada et al., 2001; Izsvak et al., 2004). As a result of the repair, characteristic sequences called '*footprints*' remain. Various DNA transposons are used for transformation technologies. The most famous example is the *P* element of *Drosophila melanogaster* (Grigliatti, 1998). With the *P* element, many splendid genetic techniques have been established in this organism. *Drosophila* is an excellent organism with which to study gene functions through genetic approaches. Although the *P* element is hyperactive in *Drosophila melanogaster*, this transposon is not active even in other insects (Handler et al., 1993). The major reason is considered that the *P* element requires cofactors for its transposition. Because of its absence of cofactors, the *P*

element might not be active in other animals. For that reason, the choice of a transposon should be made carefully when we introduce transposon-mediated transformation in animals in which such attempts have not been done.

Tc1/*mariner* superfamily transposons are strong candidates for such purposes because they are active in various animals (reviewed in Plasterk et al., 1999; Miskey et al., 2005). This family of transposons, including Tc1 of *C. elegans*, *mariner* of *Drosophila mauritiana*, and *Sleeping Beauty* of fish, has been identified in many species of different phyla (Ivics et al., 1997; Plasterk and van Luenen, 1997; Plasterk et al., 1999). They are very small (ca. 2000 bp) and simple. Tc1/*mariner* transposons have two inverted repeats at each end, which flank one open reading frame encoding transposase (Fig. 1A; Rosenzweig et al., 1983; Jacobson et al., 1986; Franz et al., 1994; Ivics et al., 1996). Their target sequences are TA dinucleotides (Fig. 1B; Plasterk and van Luenen, 1997); consequently, they can be integrated with less preferable insertion sites (Fig. 1B; van Luenen and Plasterk, 1994; Liu et al., 2004). The region encoding transposase can be replaced by any DNA sequence without the loss of transposon activities. These recombinant transposons can be mobilized only when transposases are provided from other sources. This flexibility enables changing of transposons to accommodate researchers' purposes. In addition to these advantages, the most important aspect of the Tc1/*mariner* transposons is that *in vitro* experiments have shown that transposases are the only component necessary for transposition of two members of Tc1/*mariner* transposons (Lampe et al., 1996, 1998; Vos et al., 1996). The mechanisms of the transposition of Tc1/*mariner* transposons seem to be similar (van Luenen et al., 1994). This similarity implies that most of the Tc1/*mariner* transposons are potentially active only if transposases are provided together with transposon DNA. For this reason,

activities of Tc1/*mariner* transposons in a wide range of non-host organisms have been reported. Although a cofactor of *SB* transposase has been reported recently (Zayed et al., 2003; Walisko et al., 2006), Tc1/*mariner* transposons are good candidates to achieve transposon-mediated transformation in an organism.

MINOS-MEDIATED TRANSFORMATION IN *CIONA*

Minos is a member of Tc1/*mariner* transposons isolated from *Drosophila hydei* (Franz and Savakis, 1991). *Minos* has shown consistent activity in various non-host organisms including insects, crustaceans, and mammals (Loukeris et al., 1995a; Arca et al. 1997; Klinakis et al., 2000; Shimizu et al., 2000; Drabek et al., 2002; Zhang et al., 2002; Pavlopoulos et al., 2005), suggesting its low host-specificity and potential utility as transformation vectors in various organisms.

In two ascidians, *Ciona intestinalis* and *C. savignyi*, *Minos* is sufficiently active to cause germline transformation (Sasakura et al., 2003a, b; Matsuoka et al., 2004). When *Minos* is provided in *Ciona* eggs together with transposase mRNA by microinjection or electroporation, *Minos* is excised from the vector DNA; then the excised transposon is integrated in the *Ciona* genome. This event occurs in both somatic and germ cells, and the transposon-introduced F0 animals show the mosaic state of the genome; some of the cells have *Minos* insertion, but other cells do not. Genomic integration of transgenes maintains their stability. When both *Minos* DNA containing a GFP cassette and transposase mRNA are introduced into embryos, the GFP expression is maintained for a longer period than when DNA alone is provided. In one experiment, when *Minos* DNA and transposase mRNA were electroporated, as many as 94.1% (n=34) of animals

showed GFP expression after culturing until sexual maturation, whereas only 21.7% (*n*=23) of *Minos*-DNA-electroporated animals showed GFP expression at the same period. Most of these latter animals showed GFP signals in very small populations of cells. Because of longer maintenance of transgenes in somatic cells than simple DNA introduction, the *Minos* system will be useful to analyze gene expressions in later development.

When *Minos* is inserted into chromosomes of primordial germ cells of F0 animals, they transmit *Minos* insertions to the next generation. These F0 animals are called 'founders'. The frequency of F1 progeny with *Minos* insertion varies among founders. In one case, over 50% of progeny show reporter gene expression, although in an extreme case, only a few GFP-positive animals were found among more than 1,000 progeny. This transmission rate variation reflects the mosaicism of the germ cells. If *Minos* is inserted into the chromosomes of the primordial germ cells in earlier stages, more progeny inherit *Minos*, whereas if *Minos* insertion occurs at later stages of the germ cell proliferation, only a few F1 progeny inherit the insertion. The number of insertions inherited to the F1 family also differs among founders. On average, the number of insertions is 1–2, according to the method to create transgenic lines. The mosaicism of the germ cells would be improved by optimizing the parameters of DNA introduction so as to achieve high efficiency of germline transformation (Zeller et al., 2006).

The F1 animals having *Minos* insertion in their genome show non-mosaicism with respect to the insertion. They show consistent expression of reporter genes in a non-mosaic fashion. The non-mosaicism is the major technical advantage of stable transgenesis. Without performing artificial treatments, which sometimes engender

defective development, researchers can obtain hundreds or even thousands of animals showing reporter gene expression that exhibit no differences in the state of transgenes between cells. This non-mosaicism facilitates detailed analyses of reporter gene expression throughout the life cycle. In addition, establishment of transgenic lines will provide reproducible tissue-specific marker lines. For analyzing gene functions, detections of marker gene expression are frequently undertaken. Most such experiments are currently carried out using *in situ* hybridization. Unfortunately, *in situ* hybridization is time-consuming and difficult in some developmental stages. The introduction of reporter constructs by microinjection or electroporation sometimes causes side effects; moreover, mosaic expression of reporter genes would render data analyses confusing. The utilization of marker lines, which express reporter genes in specific tissues and organs, will provide a means for their easy and reproducible detection.

Minos insertions in the ascidian genome are transmitted stably between generations. Several transgenic lines have been maintained over 10 generations. Those lines do not show change in the intensity of reporter gene expression. Apparently, transgenic lines that have an insertion of a tandem array of transgenes are also maintained stably. We can select appropriate progeny according to the intensity of reporter gene expression. For that reason, the stable maintenance of the transgenes might be accomplished easily.

EFFICIENCY OF GERMLINE TRANSGENESIS BY *MINOS*: COMPARISON OF MICROINJECTION AND ELECTROPORATION

Two methods, microinjection-mediated and electroporation-mediated transformation, reportedly cause germline transgenesis by *Minos* (Sasakura et al., 2003b; Matsuoka et

al., 2005). Both methods entail advantages and disadvantages. Microinjection method shows higher transformation frequency than electroporation method. In one experiment using a *Minos* vector pMiTFr3dTPOG (Sasakura et al., 2005), 37.4% (*n*=456) of the *Minos*-injected *C. intestinalis* became founder animals by microinjection; each founder was estimated to have transmitted two insertions in average. Transformation by electroporation with the same vector yielded 20–26% of founders; each founder was estimated to provide only a single insertion to the next generation. Microinjection method has a very low cost, whereas electroporation method is much more expensive because of the requirement of much amount of DNA and *in vitro*-transcribed transposase mRNA (80 μg per electroporation). The technical advantages of electroporation over microinjection are its easy handling, shorter work time, and the number of embryos that can be processed simultaneously. We can introduce transposons into thousands of embryos within one hour. Researchers can use the best method available to accommodate their purposes.

FACTORS AFFECTING THE TRANSFORMATION FREQUENCY

Numerous factors determine the limitations of the efficiency of transgenesis. The amount of the introduced transposon and transposase, temperature, the length of constructs, and the activity of *Minos* might each affect the overall efficiency. Among them, the concentration of transposon DNA introduced in *Ciona* seems to be difficult to increase because of the DNA toxicity. In the first report of germline transgenesis in *Ciona* (Sasakura et al., 2003b), the concentration of transposons in the injection medium was 50 ng/μl, and 30% of injected *Ciona* became founders. In recent experiments, the

DNA concentration was reduced to 10 ng/μl; as many as 37% of *Ciona* became founders. Although different vectors were used in these experiments and the copy number inherited from one founder was not compared, the DNA concentration of 10 ng/μl in the injection medium seems to be sufficient to create transgenic lines without strong toxicity against development. Our recent experiment suggested that different concentrations of transposase mRNA (50–500 ng/μl) in the injection medium showed only a little difference in transgenesis efficiency. However, an overly low amount of transposase mRNA will lessen the transformation frequency, thereby lowering the frequency of transformation by electroporation. Higher temperature (21°C) engenders higher activity of *Minos* than lower temperature (18°C) in the transposition assay between plasmids (Sasakura et al., 2003a). If *Ciona spp.* embryos can be cultured at higher temperatures, it would increase the transformation frequency, though that effect has not been examined. The insert length of transposons might also affect the transformation frequency, as reported for other Tc1/*mariner* transposons (Lampe et al., 1998; Karsi et al., 2001). *Minos* seems to be tolerant of long insertions. An approximately 10-kbp-long DNA fragment was inserted between *Minos* inverted repeats without affecting the transformation frequency (Sasakura, unpublished data).

Among several parameters, the potential activity of *Minos* itself is expected to affect the transformation frequency most. Improving the transposon activity requires changing the sequences of inverted repeats and amino-acid substitutions of transposases. In several Tc1/*mariner* transposons, these attempts have yielded hyperactive forms of transposons (Ivics et al., 1997; Lampe et al., 1999; Pledger and Coates, 2005). The creation of hyperactive *Minos* mutants will raise the efficiency of transgenesis in *Ciona*.

LOCAL EFFECT OF REPORTER GENE EXPRESSION

The transgenic lines created by the same *Minos* vectors almost always show reporter gene expression in the same patterns. However, the expression intensity of the reporter gene expression often differs among transgenic lines of the same vector (Fig. 2A). That difference might arise from the local effect of the insertion sites. Condensation of chromatin or difference in the state of methylation might be the causes. In extreme and rare cases, mosaic expression of the reporter gene was observed (Figs. 2C and 2D). Two possibilities exist to explain this mosaicism. One possibility is that *Minos* is lost in some cells; the other is that gene expression is strongly repressed at the site of *Minos* insertion. Because *Minos* insertions seem to be stable in the *Ciona* genome, the latter might be the case, though it has not been investigated in detail. In these transgenic lines, *Minos* might be inserted in the position of heterochromatin. Similar phenomena have been reported for *Drosophila melanogaster* (Weiler and Wakimoto, 1995). Because of local effects, it is better to isolate several transgenic lines and select them to meet one's purpose. Enhancer trapping, an extremely prominent local effect, is discussed later in this review.

COMPARISON OF REPORTER GENE EXPRESSIONS OF TRANSIENT AND STABLE TRANSGENIC ANIMALS

Transient transgenesis is a prominent technique of *Ciona* study because this strategy eases performance of *cis*-element analyses (Corbo et al., 1996; Zeller et al., 2006). Within a couple of days, a researcher can uncover the necessary elements for gene expression. Reporter gene expression almost always reflects the endogenous expression

patterns. Although stable transgenesis has been achieved, transient transgenesis remains the dominant method in *Ciona*.

Almost always, reporter genes show consistent expression between transient and stable transgenic animals. However, inconsistency of reporter gene expression is observed, although rarely. In these cases, reporter gene expression, which is observed constantly in transient transgenesis, is lost in the stable transgenic animals. Although the mechanism has not been proved, overly high copy numbers of the transgenes, concatemerization and rearrangement of vectors, or damage during manipulation would affect gene expression in the transient system. When analyzing gene expression, care must be taken to avoid artificial gene expression based on transgenic techniques.

ENHANCER TRAPPING

Enhancer trapping is an excellent technique to generate tissue/organ-specific marker lines, to identify endogenous enhancers that are sometimes located on distal positions of genes, and to create insertional mutants with information of expression patterns of candidate causal genes (O'Kane, 1998). *Minos* techniques are applicable to enhancer-trap screening. For example, one enhancer trap line has been isolated among 10 transgenic lines (Sasakura et al., 2003b; Awazu et al., 2004). This enhancer trap line revealed the presence of enhancers in the introns of *Ci-Musashi*. In our recent screening, about 5.4% (n=111) of a *Minos* construct-injected *Ciona*, which is estimated to correspond to 14.6% of founder animals, transmitted enhancer-trapped insertions (Fig. 2B). Because the *Ciona* genome is simple, i.e. the genome size is small and genes are located densely, *Minos* might have a greater probability of being inserted close to an

enhancer. Therefore, enhancer-trapping is an effective technique to establish marker lines and to identify novel enhancers.

Our screenings of enhancer trap lines were performed using a promoter of *thyroid peroxidase* (*Ci-TPO*; Ogasawara et al., 1999); *Ci-TPO* is expressed in endostyle, and our promoter used for enhancer trapping also shows expression at both ends of the endostyle (Sasakura et al., 2003b). Because of such endoderm-specific expression, this promoter responds preferably to enhancers for endodermal tissues, such as intestine, pharyngeal gill, and endostyle. The promoter of *Ci-TPO* is responsible for enhancers for neural tissues, blood cells, muscle cells and epidermis, but the frequency of the entrapment of these enhancers is lower than that for the endodermal enhancers (Awazu and Sasakura, manuscript in preparation). The different characteristics and preferences of basal promoters can be revealed through computational comparison (Kusakabe et al., 2004). Utilization of promoters of housekeeping genes that show ubiquitous expression will be better to isolate enhancer trap lines for every tissue.

MINOS-MEDIATED INSERTIONAL MUTAGENESIS

Minos insertion sometimes destroys genes to create insertional mutants. Insertional mutagenesis by transposon tagging provides several advantages over chemical mutagenesis, which has been carried out in *C. intestinalis* and *C. savignyi* (Moody et al., 1999; Nakatani et al., 1999; Sordino et al., 2000; Sordino et al., 2001). First, the mutation site is easily identifiable using PCR. It is possible to identify the mutated sites within one week. Second, heterozygous carriers of the mutants can be selected easily from wild-type animals by reporter gene expression, even when these carriers do not

show any phenotype. This ease of selection reduces the labor associated with the maintenance of mutants without balancer chromosomes. The easy maintenance system of the mutant loci is important for large-scale mutagenesis. Third, jumping of transposons by transposases often causes the rescue of the mutations. Because *Minos* transposons cause a 6-bp addition to the target sequences when they are excised precisely (Fig. 1B; Arca et al., 1997), excision of *Minos* will rescue mutant phenotypes even though they are inserted in the coding region. Furthermore, transposition of the transposons occasionally engenders the deletion of the flanking DNA, thereby generating another mutant allele with stronger phenotypes.

In *Ciona intestinalis*, we carried out small-scale mutant screening to show that *Minos* can generate mutants and to investigate their frequency. Ascidians, including *Ciona*, are hermaphrodite, and *C. intestinalis* is self-fertile. Therefore, most screening was performed using a self-fertilization system according to mutant screening in *C. savignyi* (Moody et al., 1999). Transgenic F1 progeny derived from one founder sometimes have different insertions because founders can transmit several insertions. Consequently, in animals in which males and females are separate, Southern blot analyses of F1 animals are necessary to reveal which animals have identical insertions (e.g., Amsterdam et al., 1999; Amsterdam, 2003). The F1 animals that have identical insertions must be mated to create homozygous animals with respect to insertions for mutant screening. The self-fertilization system in *Ciona* assures us that sperm and eggs contain the same insertions without such experiments. Therefore, the F2 family generated by self-fertilization always includes homozygous animals according to Mendel's law. This advantage greatly reduces the labor that is necessary for mutant screening.

We screened 120 transgenic lines of a *Minos* construct, pMiTFr3dTPOG, which contains transcription termination sequences at both strands (Sasakura et al., 2005). The transgenic lines were estimated to correspond to ca. 240 insertions. From these lines, we identified four mutants that show strong correlation between phenotypes and *Minos* insertions. Further investigation allowed classification of these mutants into two groups: one has mutations caused by *Minos* insertions (insertional mutants); the other shows a strong but imperfect correlation between *Minos* insertions and mutations. We call this second group '*associated mutants*'. Among associated mutants, *Minos* vectors may be inserted very close to naturally maintained mutations. Although identification of the causal genes for such associated mutants requires fine positional cloning, the sites close to the mutations are readily identifiable using *Minos* as a tag. Using the assembled whole genome sequences and annotated genes (Dehal et al., 2002; Shoguchi et al., 2006), the candidate genes for the mutations will be listed by referring the genes close to the *Minos* insertions, which might facilitate identification of the causal genes.

Among the four mutants, two have been shown to be insertional mutants. The remaining two are putatively associated mutants. Therefore, the mutation frequency of *Minos* by the vector pMiTFr3dTPO-G is estimated as 0.83% per insertion. Large-scale insertional mutagenesis by a pseudotype retrovirus was conducted in zebrafish (Amsterdam et al., 1999). In this mutagenesis, the mutation frequency is estimated as ca. 1–1.25%. Therefore, *Minos* might have slightly weaker efficiency for mutagenesis. Our screening particularly concerns mutants with easily recognizable phenotypes for the limited schedule. A certain number of mutants with subtle phenotypes might be lost during screening. The actual frequency of *Minos* mutagenesis would be higher. By performing screenings with higher throughput, the efficiency of mutant recovery using

the *Minos* system would be increased.

Both insertional mutants have an insertion of a tandem array of transposons and plasmids. Although tandem arrays were formed, both ends of the transgenes were inverted repeats. Perhaps such long transposon insertions destroy gene function. One mutant, *swimming juvenile*, has an insertion at the 5' upstream region of *Ci-CesA*; the other has the insertion in the intron of a gene (Sasakura et al., 2005; Matsuoka and Sasakura, unpublished data). *Minos* insertion in such non-coding sequences as well as exons can disrupt gene functions. Because the target sequences of *Minos* are TA dinucleotides (Loukeris et al., 1995b), *Minos* is often inserted in TA-rich regions such as introns. Transposons inserted in introns do not disrupt splicing in most cases. Therefore, some optimization of *Minos* sequences for mutagenesis, such as introduction of splicing donor/acceptor sequences is necessary to disrupt genes efficiently.

SWIMMING JUVENILE' MUTANTS

Metamorphosis is an extremely dramatic event that occurs during ascidian development (Cloney, 1982). This event converts swimming tadpole larvae to sessile adults. Extensive studies of the mechanisms of metamorphosis have been performed (Arnold et al., 1997; Degnan et al., 1997; Eri et al., 1999; Bishop et al., 2002; Chambon et al., 2002; Davidson et al., 2003; Kimura et al., 2003; Nakayama et al., 2005). Especially, gene expression during metamorphosis has been investigated in various ascidians (Davidson and Swalla, 2001, 2002; Nakayama et al., 2001, 2002; Woods et al., 2004). However, the overall mechanisms remain largely unknown, such as the regulation of timing of these metamorphic events, the relationships between each event, and the

molecules that function in the regulation of the metamorphosis.

One mutant generated by a *Minos* insertion has phenotypes in metamorphosis. The mutant named '*swimming juvenile* (*sj*)' undergoes trunk metamorphic events, although they continue swimming with intact tails. The phenotypes of this mutant suggest that metamorphic events in the trunk can occur independently of the tail regression. These events might therefore be regulated through different mechanisms. In the normal procedure of metamorphosis, tail regression always precedes trunk metamorphic events (Cloney, 1982). Therefore, a mechanism might be present which links these events; that mechanism might be disrupted in *sj* mutant. Similar perturbation of the process of metamorphic events was also caused by dechorionation (Sato and Morisawa, 1997), suggesting that the mechanism disrupted in *sj* mutant is related to the functions of test cells, follicle cells and chorion.

The causal gene for *sj* mutant is *Ci-CesA*, which encodes cellulose synthase, thus suggesting the involvement of cellulose in metamorphic events. Some trunk metamorphic events, namely the initial phase of body axis rotation, retraction of adhesive papillae and conversion of the preoral lobe, take place in *sj* larvae without tail regression. Consequently, they are regulated by a certain mechanism in which cellulose synthase is involved. Maybe cellulose is involved in the mechanism that inhibits the initiation of these trunk metamorphic events until settlement. Among the metamorphic events in the trunk, the later phase of the body axis rotation and growth of adult organs seem to be regulated normally in *sj* mutants, suggesting that these events are regulated by a cellulose synthase-independent mechanism. Currently, two more mutants that show phenotypes similar to *sj* have been isolated by *Minos*-based mutant screening. Further analyses of these *sj* mutants will reveal the mechanisms regulating *Ciona* metamorphic

events.

Cellulose seems to be dispensable for *Ciona* viability because *sj* mutants grow to be adults with sexual maturation. Adults of *sj* mutants have very soft tunics. Those of the *sj* mutants adhere less efficiently to the substrates: they are easily stripped off (Sasakura, unpublished data). Cellulose in the tunics might be required for firm settlement. The defect in the settlement implies that the acquisition of the cellulose synthesis in the tunic would have affected the acquisition of the life style of the settlement as well as the metamorphosis during ascidian evolution. Adults of *sj* mutants seem to be less resistant to algae growth on their tunics. Cellulose might have an important function of protection from algae growth. Through observations of *sj* mutants, multiple functions of cellulose are inferred. Considering that all tunicates maintain a system of cellulose synthesis, acquisition of cellulose synthesis in the lineage of tunicates might have imparted a great impact upon their evolution. Their lifestyles might have thereby been adjusted to one in which they use cellulose effectively. Such evolutional aspects of tunicates might be revealed by analyzing and comparing the phenotypes of mutants of cellulose synthases in other tunicates.

OTHER APPROACHES TO CREATE STABLE TRANSGENESIS

I-SceI is a kind of meganuclease that is derived from *Saccharomyces cerevisiae* (Monteilhet et al., 1990). The restriction site of this nuclease is 18 bp sequences. With such a large restriction site, I-SceI can be introduced into cells of some organisms without cutting their genomes (Rouet et al., 1994; Thermes et al., 2002). Recently, germline transgenesis with this endonuclease has been reported in *Ciona savignyi*

(Deschet et al., 2003). The basis of this technique is the introduction of mixture of I-SceI enzyme and a DNA construct, which has several I-SceI restriction sites. Although the mechanism of I-SceI-mediated germline transgenesis is not yet clearly understood, the creation and maintenance of double-strand breaks of transgenes *in vivo* might increase the probability of DNA integration in genomes. Using this technique, the integration of concatemer of exogenous DNA is expected (Deschet et al., 2003).

Electroporation, which introduces exogenous DNA into hundreds of eggs at a time, sometimes causes germline transgenesis without that assistance of transposases or endonucleases (Matsuoka et al., 2005). The frequency of transformation by this 'electroporation-mediated transgenesis' is not high and this technique often inserts a large tandem array of transposons at a site. Despite these demerits, this technique will be useful because special factors for genomic integration are not required.

FUTURE PROSPECTS

Great improvement in the transgenic techniques of ascidians has been achieved during the last three years. Transposon *Minos*-mediated germline transgenesis provides us high transformation frequency, enhancer trap lines, and insertional mutants, all of which will be powerful tools to study gene functions. Both I-SceI-mediated and electroporation-mediated transgenesis enable the creation of many transgenic lines, which can provide reproducible and useful markers for analyses of tissue/organ formation. Transgenic lines generated by these approaches might also be useful for live imaging of developmental events (Rhee et al., 2005). These techniques support sophisticated genetic analyses of gene functions based on transgenic techniques.

To enhance these techniques' efficiency, further refinement of the techniques themselves is required. Regarding transposon-mediated transgenesis, four techniques that have been established in several model organisms should be introduced: (1) re-mobilization of *Minos* within the *Ciona* genome; (2) trapping techniques for highly frequent mutagenesis; (3) the UAS-Gal4 system; and (4) utilization of transposons that show higher activity than *Minos*. The re-mobilization or jumping technique will be applicable to rescue experiments, creation of new mutant alleles by deletion, and local hopping technique. Local hopping utilizes the nature of transposons that they move locally at a frequency that is higher than transposition to another chromosome (Grigliatti, 1998). This characteristic will increase the chance to disrupt a gene-of-interest. Jumping might be carried out by introduction of transposase mRNA in eggs of transgenic lines. However, establishment of jump-starter lines that express transposase in the germ cells will be ideal for jumping techniques. *Minos* cannot be used to create jump-starter transgenic lines of *Minos* transposase. Therefore, I-SceI-mediated transgenesis, electroporation-mediated transgenesis and other transposon systems are useful for this purpose.

More efficient mutagenesis by *Minos* will be achieved by gene trapping techniques such as promoter trapping and poly (A) trapping (Ishida and Leder, 1999; Zambrowicz et al., 1999; Wurst and Gossler, 2000; Lukacosovich et al., 2001; Morin et al., 2001; Clark et al., 2004; Kawakami et al., 2004; Skarnes, 2005). In these techniques, expression of reporter genes requires help from elements of endogenous genes. Therefore, transgenic animals that have insertions in the genes can be screened easily using signals of reporter genes. In addition, vectors for gene trapping techniques contain splicing acceptor/donor sequences. These elements often disrupt splicing of endogenous

genes, even though transposons are inserted into introns. These technical advantages will greatly improve the efficiency of mutant screening.

The UAS-Gal4 system, which enables tissue-specific overexpression of genes-of-interest, will be a powerful tool in studying functions of genes. The UAS-Gal4 is active in *Ciona* (Sasakura, unpublished data). In conventional overexpression techniques from DNA constructs, a gene-of-interest must be fused with each tissue-specific promoter. Many vectors should be created to analyze effects of overexpression in various tissues. By establishing Gal4 driver lines through enhancer trap screening, overexpression and misexpression in any tissue will be possible through creation of one UAS construct. We recently established a method to isolate many enhancer trap lines efficiently (Awazu and Sasakura, manuscript in preparation). By applying this technique to Gal4 constructs, many enhancer trap lines will be generated in a short time.

Attempts to find a transposon that shows comparable or higher activity than *Minos* in *Ciona* have been carried out. Unfortunately, we have not found a transposon that is sufficiently active for application to *Ciona* germline transgenesis. However, we are continuing this search because utilization of transposons with high activity will increase the efficiency of germline transgenesis and insertional mutagenesis. In addition, another transposon is necessary for modifier screening in the mutant background created by *Minos*.

For the utilization and improvement of transgenic techniques in *Ciona*, researchers should design and create many *Minos* vectors. Construction of DNA vectors is very time-consuming. Therefore, an easy system to create transposon vectors is necessary. The application of the Gateway system, which uses the Lambda phage recombination

mechanism, is an easy technology for construction (Earley et al., 2006); this system is being introduced in *Minos*. A set of Gateway-compatible vectors for *Ciona* studies will be released soon (Roure, Rothbacher, Lemaire, unpublished data), and these vectors will be combined to *Minos*-Gateway vectors to facilitate vectors construction. Together with the simple *Ciona* transformation by electroporation, the systematic creation of transformation vectors enables the routine creation of many transgenic lines, which will support the establishment of useful marker lines and insertional mutants.

Sophisticated genetic techniques are available in *Ciona* now. Transgenic lines and mutants are valuable resources for future studies. Making effective use of these techniques, novel aspects of *Ciona* development will be uncovered that cannot be discovered through conventional techniques. *Ciona* has a small and compact genome that is comparable to that of *Drosophila melanogaster* (Dehal et al., 2002). This is an excellent advantage to perform forward genetics because the compact genome assures disruption of genes with high probability, and consequently, high frequency. Generation of many mutants with rapid identification of mutated sites using the *Minos* system will give us convenient resources to analyze gene function.

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REFERENCES

Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. 2002. Molecular Biology of the Cell, Fourth Edition. New York, NY: Garland Publishing, Inc. 1616p.

Amsterdam A. 2003. Insertional mutagenesis in zebrafish. Dev Dyn 228:523-534.

Amsterdam A, Burgess S, Golling G, Che, W, Sun Z, Townsend K, Farrington S, Haldi M, Hopkins N. 1999. A large-scale insertional mutagenesis screen in zebrafish. Genes Dev 13:2713-2724.

Arca B, Zabalou S, Loukeris T, Savakis C. 1997. Mobilization of a *Minos* transposon in *Drosophila melanogaster* chromosomes and chromatid repair by heteroduplex formation. Genetics 145:267-279.

Arnold JM, Eri R, Degnan BM, Lavin MF. 1997. Novel gene containing multiple epidermal growth factor-like motifs transiently expressed in the papillae of the ascidian tadpole larvae. Dev Dyn 210:264-273.

Awazu S, Sasaki A, Matsuoka T, Satoh N, Sasakura Y. 2004. An enhancer trap in the ascidian *Ciona intestinalis* identifies enhancers of its *Musashi* orthologous gene. Dev Biol 275:459-472.

Bishop CD, Bates WR, Brandhorst BP. 2002. HSP90 function is required for morphogenesis in ascidian and echinoid embryos. Dev Genes Evol 212:70-80.

Chambon JP, Soule J, Pomies P, Fort P, Sahuquet A, Alexandre D, Mangeat PH,

Baghdiguian P. 2002. Tail regression in *Ciona intestinalis* (Protochordate) involves a caspase-dependent apoptosis event associated with ERK activation. Development 129:3105-3114.

Clark KJ, Geurts AM, Bell JB, Hackett PB. 2004. Transposon vectors for gene-trap insertional mutagenesis in vertebrates. Genesis 39:225-233.

Cloney RA. 1982. Ascidian larvae and events of metamorphosis. Amer Zool 22:817-826.

Constantini F, Lacy E. 1981. Introduction of a rabbit β-globin gene into the mouse germ-line. Nature 294:92-94.

Corbo JC, Levine M, Zeller RW. 1997. Characterization of a notochord-specific enhancer from the *Brachyury* promoter region of the ascidian, *Ciona intestinalis*. Development 124:589-602.

Corbo JC, Di Gregorio A, Levine M. 2001. The ascidian as a model organism in developmental and evolutionary biology. Cell 106:535-538.

Craig NL, Craigie R, Gellert M, Lambowitz AM. 2002. Mobile DNA II. NW, Washington, DC: American Society for Microbiology, Inc. 1204 p.

Davidson B, Swalla BJ. 2001. Isolation of genes involved in ascidian metamorphosis, epidermal growth factor and metamorphic competence. Dev Genes Evol 211:190-194.

Davidson B, Swalla BJ. 2002. A molecular analysis of ascidian metamorphosis reveals activation of an innate immune response. Development 129:4739-4751.

Davidson B, Shammon E, Wallace S, Howsmon RA, Swalla BJ. 2003. A morphological and genetic characterization of metamorphosis in the ascidian *Boltenia villosa*. Dev Genes Evol 213:601-611.

Degnan BM, Souter D, Degnan SM, Long SC. 1997. Induction of metamorphosis with potassium ions requires development of competence and an anterior centre in the ascidian *Herdmania momus*. Dev Genes Evol 206:370-376.

Dehal P, Satou Y, Campbell RK, Chapman J, Degnan B, De Tomaso A, Davidson B, Di Gregorio A, Gelpke M, Goodstein DM, Harafuji N, Hastings KE, Ho I, Hotta K, Huang W, Kawashima T, Lemaire P, Martinez D, Meinertzhagen IA, Necula S, Nonaka M, Putnam N, Rash S, Saiga H, Satake M, Terry A, Yamada L, Wang HG., Awazu S, Azumi K, Boore J, Branno M, Chin-Bow S, DeSantis R, Doyle S, Francino P, Keys DN, Haga S, Hayashi H, Hino K, Imai KS, Inaba K, Kano S, Kobayashi K, Kobayashi M, Lee BI, Makabe KW, Manohar C, Matassi G, Medina M, Mochizuki Y, Mount S, Morishita T, Miura S, Nakayama A, Nishizaka S, Nomoto H, Ohta F, Oishi K, Rigoutsos I, Sano M, Sasaki A, Sasakura Y, Shoguchi E, Shin-I T, Spagnuolo A, Stainier D, Suzuki MM, Tassy O, Takatori N, Tokuoka M, Yagi K, Yoshizaki F, Wada S, Zhang C, Hyatt PD, Larimer F, Detter C, Doggett N, Glavina T, Hawkins T, Richardson P, Lucas S, Kohara Y, Levine M, Satoh N, Rokhsar DS. 2002. The draft genome of *Ciona intestinalis*: Insight into chordate and vertebrate origins. Science 298:2157-2167.

Deschet K, Nakatani Y, Smith WC. 2003. Generation of *Ci-Brachyury-GFP* stable transgenic lines in the ascidian *Ciona savignyi*. Genesis 35:248-259.

Drabek D, Zagoraiou L, deWit T, Langeveld A, Roumpaki C, Mamalaki C, Savakis C, Grosveld F. 2003. Transposition of the *Drosophila hydei Minos* transposon in the mouse germ line. Genomics 81:108-111.

Earley KW, Haag JR, Pontes O, Opper K, Juehne T, Song K, Pikaard CS. 2006. Gateway-compatible vectors for plant functional genomics and proteomics. The

Plant J 45:616-629.

Eri R, Arnold JM, Hinman VF, Green K, Jones M, Degnan BM, Lavin MF. 1999. Hemps, a novel EGF-like protein, plays a central role in ascidian metamorphosis. Development 126:5809-5818.

Etkin LD, Pearman B, 1987. Distribution, expression and germ-line transmission of exogenous DNA sequences following microinjection into *Xenopus laevis* eggs. Development 99:15-23.

Ettensohn CA, Wessel GM, Wray GA, editors. Methods in cell biology: Developments of sea urchins, ascidians, and other invertebrate deuterostomes: Experimental approaches. San Diego, California: Elsevier Academic Press Inc. 912p.

Franz G, Savakis cc. 1991. *Minos*, a new transposable element from *Drosophila hydei*, is a member of the *Tc1*-like family of transposons. Nucleic Acid Res 19:6646.

Franz G, Loukeris TG, Dialektaki G, Thompson CRL, Savakis C. 1994. Mobile *Minos* elements from *Drosophila hydei* encode a two-exon transposase with similarity to the paired DNA-binding domain. Proc Natl Acad Sci USA. 91:4746-4750.

Geurts AM, Yang Y, Clark KJ, Liu G, Cui Z, Dupuy AJ, Bell JB, Largaespada DA, Hackett PB. 2003. Gene transfer into genomic of human cells by the *Sleeping Beauty* transposon system. Mol Ther 8:108-117.

Gloor GB, Moretti J, Mouyal J, Keeler KJ. 2000. Distinct P-element excision products in somatic and germline cells of Drosophila melanogaster. Genetics 155:1821-1830.

Gordon JW, Ruddle FH. 1981. Integration and stable germ-line transmission of genes injected into mouse pronuclei. Science 214:1244-1246.

Gordon JW, Ruddle FH. 1986. Gene transfer into mouse embryos. Dev Biol 4:1-36.

Grigliatti TA. 1998. Transposons-gene tagging and mutagenesis. In: Roberts DB, editor.

Drosophila Second Edition. New York: Oxford University Press. p. 85-107.

Handler AM, Gomez SP, O'Brochta DA. 1993. A functional analysis of the *P*-element gene transfer vector in insects. Arch Insect Biochem Physiol 22:373-384.

Higashijima S, Okamoto H, Ueno N, Hotta Y, Eguchi G. 1997. High-frequency generation of transgenic zebrafish which reliably express GFP in whole muscles or the whole body by using promoters of zebrafish origin. Dev Biol 192:289-299.

Hikosaka A, Kusakabe T, Satoh N. 1994. Short upstream sequences associated with the muscle-specific expression of an actin gene in ascidian embryos. Dev Biol 166:763-769.

Ivics Z, Izsvak Z, Minter A, Hackett PB. 1994. Identification of functional domains and evolution of Tc1-like transposable elements. Proc Natl Acad Sci USA. 93:5008-5013.

Ishida Y, Leder P. 1999. RET: a poly A-trap retrovirus vector for reversible disruption and expression monitoring of genes in living cells. Nucleic Acids Res 27:e35.

Ivics Z, Hackett PB, Plasterk RH, Izsvak Z. 1997. Molecular reconstruction of *Sleeping Beauty*, a *Tc1*-like transposon from fish, and its transposition in human cells. Cell 91:501-510.

Izsvak Z, Stuwe EE, Fiedler D, Katzer A, Jeggo PA, Ivics Z. 2004. Healing the wounds inflicted by Sleeping Beauty transposition by double-strand break repair in mammalian somatic cells. Mol Cell 13:279-290.

Jacobson JW, Medhora MM, Hartl DL. 1986. Molecular structure of a somatically unstable transposable element in Drosophila. Proc Natl Acad Sci USA 83:8684-8688.

Karsi A, Moav B, Hackett P, Liu Z. 2001. Effects of insert size on transposition

efficiency of the *Sleeping Beauty* transposon in mouse cells. Mar Biotechnol 3:241-245.

Kawakami K, Takeda H, Kawakami N, Kobayashi M, Matsuda N, Mishina M. 2004. A transposon-mediated gene trap approach identifies developmentally regulated genes in zebrafish. Dev Cell 7:133-144.

Kimura Y, Yoshida M, Morisawa M. 2003. Interaction between noradrenaline or adrenaline and the beta 1-adrenergic receptor in the nervous system triggers early metamorphosis of larvae in the ascidian, *Ciona savignyi*. Dev Biol 258:129-140.

Klinakis AG, Zagoraiou L, Vassilatis DK, Savakis C. 2000. Genome-wide insertional mutagenesis in human cells by the *Drosophila* mobile element *Minos*. EMBO reports 1:416-421.

Kusakabe T, Yoshida R, Ikeda Y, Tsuda M. 2004. Computational discovery of DNA motifs associated with cell type-specific gene expression in Ciona. Dev Biol 15:563-580.

Lampe DJ, Churchill MEA, Robertson HM. 1996. A purified mariner transposase is sufficient to mediate transposition *in vitro*. EMBO J 15:5470-5479.

Lampe DJ, Grant TE, Robertson HM. 1998. Factors affecting transposition of the *Himar1 mariner* transposon in vitro. Genetics 149:179-187.

Lampe DJ, Akerley BJ, Rubin EJ, Mekalanos JJ, Robertson HM. 1999. Hyperactive transposase mutants of the Himar1 mariner transposon. Proc Natl Acad Sci USA 96:11428-11433.

Lin S, Gaiano N, Culp P, Burns JC, Friedmann T, Yee J-K, Hopkins N. 1994. Integration and germ-line transmission of a pseudotyped retroviral vector in zebrafish. Science 265:666-669.

Liu G, Geurts AM, Yae K, Srinivasan AR, Fahrenkrug SC, Largaespada DA, Takeda J, Horie K, Olson WK, Hackett PB. 2005. Target-site preferences of *Sleeping Beauty* transposons. J Mol Biol 346:161-173.

Loukeris TG, Livadaras I, Arca B, Zabulou S, Savakis C. 1995a. Gene transfer into the Medfly, *Ceratitis capitata*, with a *Drosophila hydei* transposable element. Science 270:2002-2005.

Loukeris TG, Arca B, Livadaras I, Dialektaki G, Savakis C. 1995b. Introduction of the transposable element Minos into the germ line of Drosophila melanogaster. Proc Natl Acad Sci USA 92:9485-9489.

Lukacosovich T, Asztalos Z, Awano W, Baba K, Kondo S, Niwa S, Yamamoto D. 2001. Dual-tagging gene trap of novel genes in *Drosophila melanogaster*. Genetics 157:727-742.

Matsuoka T, Awazu S, Satoh N, Sasakura Y. 2004. *Minos* transposon causes germline transgenesis of the ascidian *Ciona savignyi.* Dev Growth Differ 46:249-255.

Matsuoka T, Awazu S, Shoguchi E, Satoh N, Sasakura Y. 2005. Germline transgenesis of the ascidian *Ciona intestinalis* by electroporation. Genesis 41:61-72.

Miskey C, Izsvak Z, Kawakami K, Ivics Z. 2005. DNA transposons in vertebrate functional genomics. CMLS Cell Mol Life Sci 62:629-641.

Monteilhet C, Perrin A, Thierry A, Colleaux L, Dujon B. 1990, Purification and characterization of the *in vitro* activity of I-*Sce* I, a novel and highly specific endonuclease encoded by a group I intron. Nucleic Acids Res 18:1407-1413.

Moody R, Davis SW, Cubes F, Smith WC. 1999. Isolation of developmental mutants of the ascidian *Ciona savignyi*. Mol Gen Genet 262:199-206.

Morin X, Daneman R, Zavortink M, Chia W. 2001. A protein trap strategy to detect

GFP-tagged proteins expressed from their endogenous loci in *Drosophila*. Proc Natl Acad Sci USA 98:15050-15055.

Nakatani Y, Moody R, Smith WC. 1999. Mutations affecting tail and notochord development in the ascidian *Ciona savignyi*. Development 126:3293-3301.

Nakayama A, Satou Y, Satoh N. 2001. Isolation and characterization of genes that are expressed during *Ciona intestinalis* metamorphosis. Dev Genes Evol 211:184-189.

Nakayama A, Satou Y, Satoh N. 2002. Further characterization of genes expressed during *Ciona intestinalis* metamorphosis. Differentiation 70:429-437.

Nakayama A, Satoh N, Sasakura Y. 2005. Tissue-specific profile of DNA replication in the swimming larvae of *Ciona intestinalis.* Zool Sci 22:301-309.

Nishida H. 2005. Specification of embryonic axis and mosaic development in ascidians. Dev Dyn 233:1177-1193.

Ogasawara M, Di Lauro R, Satoh N. 1999. Ascidian homologs of mammalian thyroid peroxidase genes are expressed in the thyroid-equivalent region of the endostyle. J Exp Zool 285:158-169.

O'Kane CJ. 1998. Enhancer traps, In: Roberts DB, editor, Drosophila Second Edition. New York: Oxford University Press. p.131-178.

Pavlopoulos A, Averof M. 2005. Establishing genetic transformation for comparative developmental studies in the crustacean *Parhyale hawaiensis*. Proc Natl Acad Sci USA 102:7888-7893.

Plasterk RHA, van Luenen HGAM. 1997. Transposons, In: Riddle DL, Blumenthal T, Meyer BJ, Priess JR, editors, *C. elegans* II. New York: Cold Spring Harbor Laboratory Press. p.97-116.

Plasterk RH, Izsvak Z, Ivics Z. 1999. Resident aliens: The Tc1/*mariner* superfamily of

transposable elements. Trends Genet 15:326-332.

Pledger DW, Coates CJ. 2005. Mutant *Mos1 mariner* transposons are hyperactive in *Aedes aegypti*. Insect Biochem. Mol Biol 35:1199-1207.

Raizada MN, Nan GL, Walbot V. 2001. Somatic and germinal mobility of the RescueMu transposon in transgenic maize. Plant Cell 13:1587-1608.

Rhee JM, Oda-Ishii I, Passamaneck YJ, Hadjantonakis A-K, Di Gregorio A. 2005. Live imaging and morphometric analysis of embryonic development in the ascidian *Ciona intestinalis*. Genesis 43:136-147.

Roe T, Reynold TC, Yu G, Brown PO. 1993. Integration of murine leukemia virus depends on mitosis. EMBO J 12:2099-2108.

Rouet P, Smih F, Jasin M. 1994. Expression of a site-specific endonuclease stimulates homologous recombination in mammalian cells. Proc Natl Acad Sci USA 91:6064-6068.

Rosenzweig B, Liao LW, Hirsh D. 1983. Sequence of the *C. elegans* transposable element Tc1. Nucleic Acids Res 11:4201-4210.

Sasakura Y, Awazu S, Chiba S, Kano S, Satoh N. 2003a. Application of *Minos*, one of the Tc1/*mariner* superfamily transposable elements, to ascidian embryos as a tool for insertional mutagenesis. Gene 308:11-20.

Sasakura Y, Awazu S, Chiba S, Satoh N. 2003b. Germ-line transgenesis of the Tc1/*mariner* superfamily transposon *Minos* in *Ciona intestinalis*. Proc Natl Acad Sci USA 100:7726-7730.

Sasakura Y, Nakashima K, Awazu S, Matsuoka T, Nakayama A, Azuma J, Satoh N. 2005. Transposon-mediated insertional mutagenesis revealed the functions of animal cellulose synthase in the ascidian *Ciona intestinalis*. Proc Natl Acad Sci USA

102:15134-15139.

Sato Y, Morisawa M. 1999. Loss of test cells leads to the formation of new tunic surface cells and abnormal metamorphosis in larvae of *Ciona intestinalis* (Chordata, Ascidiacea). Dev Genes Evol 209:592-600.

Satoh N. 1994. Developmental Biology of Ascidians. New York, NY: Cambridge University Press. 234 p.

Satoh N. 2003. The ascidian tadpole larva: comparative molecular development and genomics. Nat Rev Genet 4:285-295.

Satoh N, Satou Y, Davidson B, Levine M. 2003. Ciona intestinalis: an emerging model for whole-genome analyses. Trends Genet 19:376-381.

Satou Y, Imai K, Satoh N. 2001. Action of morpholinos in *Ciona* embryos. Genesis 30:103-106.

Skarnes WC. 2005. Two ways to trap a gene in mice. Proc Natl Acad Sci USA 102:13001-13002.

Shimizu K, Kamba M, Sonobe H, Kanda T, Klinakis AG, Savakis C, Tamura T. 2000. Extrachromosomal transposition of the transposable element *Minos* in embryos of the silkworm *Bombyx mori*. Insect Mol Biol 9:277-281.

Shoguchi E, Kawashima T, Satou Y, Hamaguchi M, Sin-I T, Kohara Y, Rokhsar DS, Satoh N. 2006. Chromosomal mapping of 170 BAC clones in the ascidian *Ciona intestinalis*. Genome Res 16:297-303.

Sordino P, Heisenberg GP, Cirino P, Toscano A, Giuliano P, Marino R, Pinto MR, De Santis R. 2000. A mutational approach to the study of development of the protochordate *Ciona intestinalis* (Tunicata, Chordata). Sarsia 85:173-176.

Sordino P, Belluzzi L, De Santis R, Smith WC. 2001. Developmental genetics in

primitive chordates. Phil Trans R Soc Lond B 356:1573-1582.

Stuart GW, McMurray JV, Westerfield M. 1988. Replication, integration and stable germ-line transmission of foreign sequences injected into early zebrafish embryos. Development 103:403-412.

Thermes V, Grabher C, Ristoratore F, Bourrat F, Choulika A, Wittbrodt J, Joly J-S. 2002. *I-SceI* meganuclease mediates highly efficient transgenesis in fish. Mech Dev 118:91-98.

Van Luenen HGAM, Plasterk RHA. 1994. Target site choice of the related transposable elements Tc1 and Tc3 of *Caenorhabditis elegans*. Nucleic Acids Res 22:262-269.

van Luenen HG, Colloms SD, Plasterk RH. 1996. The mechanism of transposition of Tc3 in *C. elegans*. Cell 79:293-301.

Vos JC, De Baere I, Plasterk RH. 1996. Tc1 transposase is the only nematode protein required for *in vitro* transposition of Tc1. Genes Dev 10:755-761.

Walisko O, Izsvak Z, Szabo K, Kaufman CD, Herold S, Ivics Z. 2006. Sleeping Beauty transposase modulates cell-cycle progression through interaction with Miz-1. Proc Natl Acad Sci USA 103:4062-4067.

Weiler KS, Wakimoto BT. 1995. Heterochromatin and gene expression in *Drosophila*. Annu Rev Genet 29:577-605.

Wurst W, Gossler A. 2000. Gene trap strategies in ES cells In: Joyner AL editor, Gene Targeting, Second Edition. New York: Oxford University Press. p. 207-254.

Zambrowics BP, Friedrich GA, Buxton EC, Lilleberg SL, Person C, Sands AT. 1998. Disruption and sequence identification of 2,000 genes in mouse embryonic stem cells. Nature 392:608-611.

Zayed H, Izsvak Z, Khare D, Heinemann U, Ivics Z. 2003. The DNA-bending protein

HMGB1 is a cellular cofactor of Sleeping Beauty transposition. Nucleic Acids Res 31:2313-2322.

Zeller RW, Virata MJ, Cone AC. 2006. Predictable mosaic transgene expression in ascidian embryos produced with a simple electroporation device. Dev Dyn 235:1921-1932.

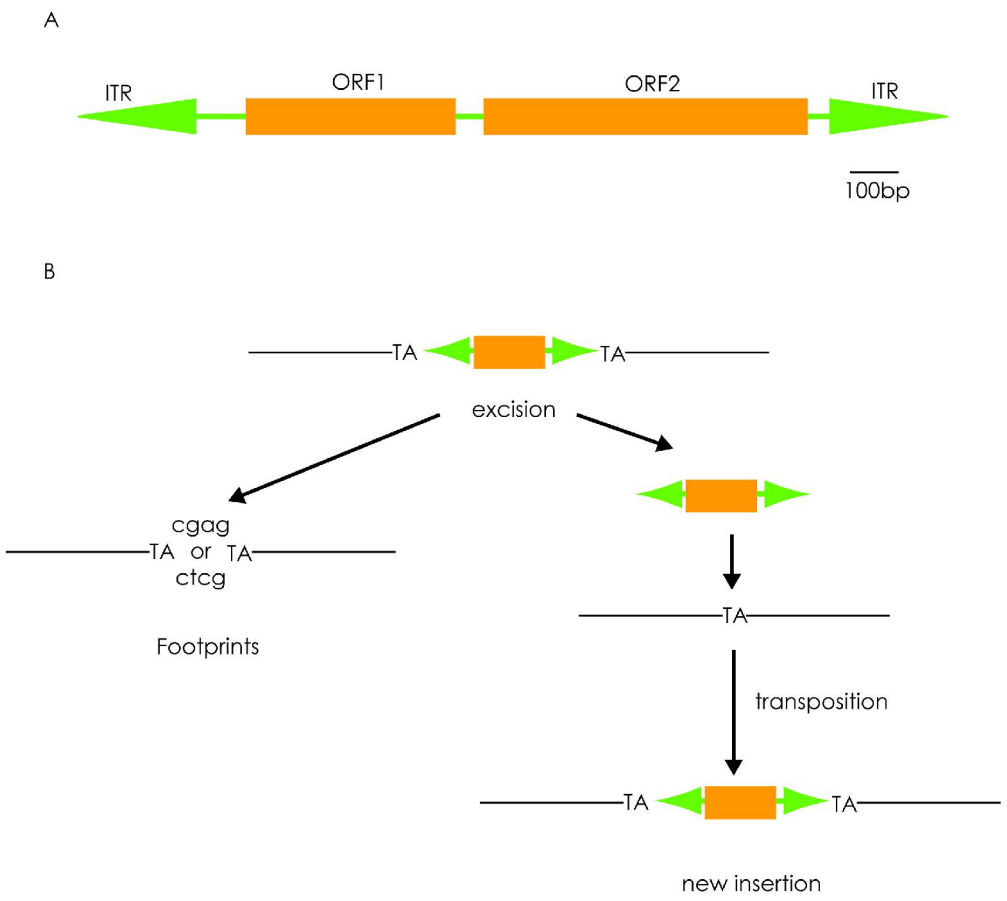
Zhang H, Shinmyo Y, Hirose A, Inoue Y, Ohuchi H, Loukeris TG, Eggleston P, Noji S. 2002. Extrachromosomal transposition of the transposable element *Minos* in embryos of the cricket *Gryllus bimaculatus*. Develop Growth Differ 44:409-417.

FIGURE LEGENDS

Fig. 1. (A) Structure of the *Minos* transposon. A gene encoding a transposase consists of two exons (ORF1 and ORF2). The transposase gene is flanked by two inverted terminal repeats (ITRs). (B) Mechanisms of excision and transposition of *Minos*. A *Minos* is excised from DNA by transposases. At the excised position, characteristic footprint sequences (5'-TACTCGTA-3' or 5'-TACGAGTA-3') are formed during double-strand break repair. Excised *Minos* is re-integrated into TA dinucleotides. During the integration, targeted TA is duplicated.

Fig. 2. Local effects of transgene expression in transgenic lines. (A) Two F1 progeny (arrows) derived from the same founder showed different GFP expression intensities. The difference might be attributable to the difference in insertion sites. (B) An enhancer trap line shows GFP expression in pharyngeal gills. (C and D) Mosaic reporter gene

expression observed in a transgenic line. Although both (C) and (D) are stable transgenic animals of the same vector, a juvenile in (D) showed a mosaic GFP expression pattern.



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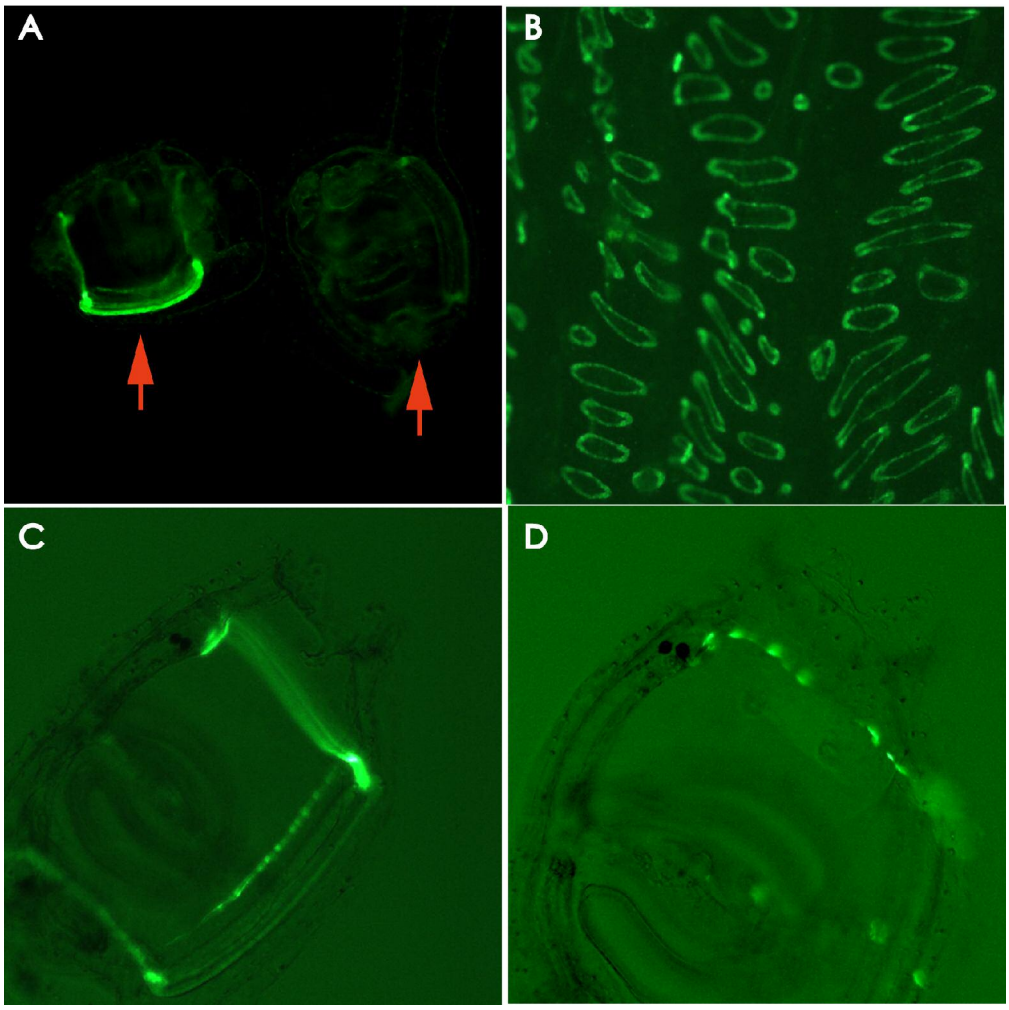

Figure 2 Local effects of transgene expression in transgenic lines. (A) Two F1 progeny (arrows) derived from the same founder showed different GFP expression intensities. The difference might be attributable to the difference in insertion sites. (B) An enhancer trap line shows GFP expression in pharyngeal gills. (C and D) Mosaic reporter gene expression observed in a transgenic line. Although both (C) and (D) are stable transgenic animals of the same vector, a juvenile in (D) showed a mosaic GFP expression pattern.