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Long and short mRNAs transcribed from the medaka fish transposon *Tol2* respectively exert positive and negative effects on excision

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Summary

The medaka fish transposable element, *Tol2*, is a member of the *hAT* family of transposons. It has been directly demonstrated to be active and two mRNAs, differing in length, have been isolated. They cover exons 1–4 and exons 2–4 and the longer form has already been proven to catalyse transposition reactions. However, the function of the shorter mRNA in medaka cells has hitherto remained unclear. In the present study, first we constructed a quantitative system to detect *Tol2* excision using an indicator plasmid carrying a non-autonomous *Tol2* within its *lacZ* gene; second we injected mRNAs with the plasmid into medaka eggs. Excision of *Tol2* was detected as *E. coli* blue colonies caused by the recovery of *lacZ* activity. Addition of the longer mRNA increased excision, but the shorter did not. Moreover, co-injection of both mRNAs greatly lowered the frequency compared with the case of treatment with the longer mRNA alone. These results indicate that the shorter mRNA has an inhibitory effect on the excision reaction, and that the N-terminal region of the transposase encoded by exon 1, including a BED zinc finger, presumably plays an important role in excision. Here, we suggest a regulatory mechanism of *Tol2* transposition involving the expression of these mRNAs.

1. Introduction

Autonomous inverted-terminal-repeat transposable elements each carry a gene encoding a transposase, the key enzymes for cut-and-paste transposition of themselves or their non-autonomous counterparts. The medaka fish *Tol2* is one such element that belongs to the *hAT* (*hobo*/*Ac*/*Tam3*) family of transposons (Calvi *et al.*, 1991; Atkinson *et al.*, 1993). It has been shown to be active (Koga *et al.*, 1996) and autonomous (Kawakami *et al.*, 1998), and if *in vivo* transposition of *Tol2* could be manipulated, it would be a powerful genetic tool for gene tagging as a vector in vertebrates.

In our previous study (Koga *et al.*, 1999), two mRNAs, covering exons 1–4 (mRNA-L; means long) and exons 2–4 (mRNA-S; means short), were isolated (Fig. 1). The protein deduced from mRNA-L has already proven to be a *Tol2* transposase (Koga & Hori, 2000; Kawakami *et al.*, 2000). However, it has remained unclear whether mRNA-S has any transposition functions, although both proteins include the three distinctive amino acid blocks conserved among *hAT* family transposases (Feldmar & Kunze, 1991).

To investigate the effects of mRNA-S on excision, we constructed a simple quantitative method to assess the *Tol2* excision rate, employing an *E. coli lacZ* gene recovery system. This can detect not only precise but also imprecise excision. Although catalysis of excision has been demonstrated by qualitative analysis using PCR (Koga *et al.*, 1996; Kawakami & Shima, 1999), the degree of activity can only be compared with a quantitative approach. Excision rates were determined by injecting these mRNAs along with the *lacZ* marker into eggs. As a result the injection of mRNA-S was found to inhibit the excision reaction, providing clues as to how *Tol2* transposition might be regulated.

2. Materials and methods

(i) *Fish*

Medaka fish, *Oryzias latipes*, purchased from a pet shop in Nagoya, were used. Each harbours about 20 copies of *Tol2* in its genome (Koga & Hori, 1999).

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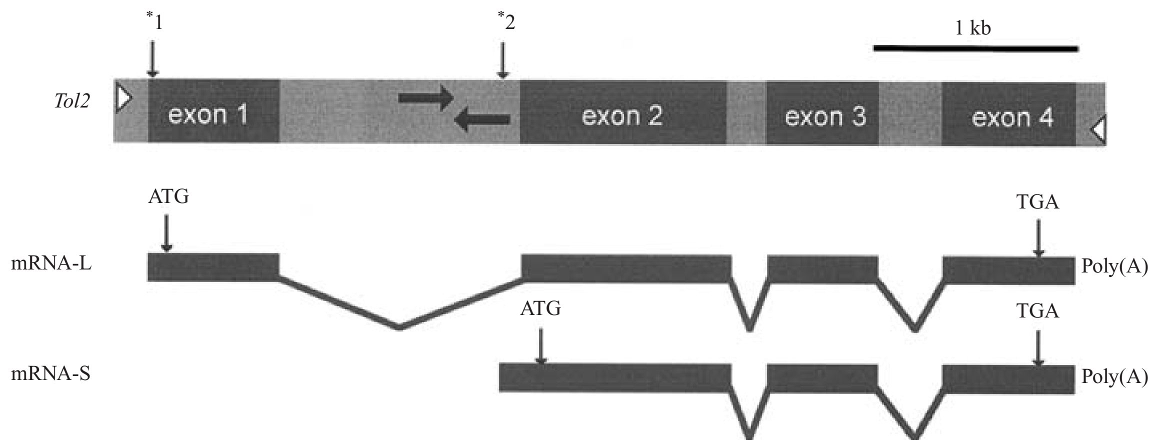

Fig. 1. *Tol2* structure and mRNA expression. *Tol2* is 4·7 kb in length including four exons. mRNA-L and mRNA-S are composed of exons 1–4 and exons 2–4, respectively. *1 (183rd nucleotide from the 5′ end of *Tol2*) and *2 (2077th nucleotide from the 5′ end of *Tol2*) are transcription initiation sites for mRNA-L and mRNA-S, respectively (Koga *et al.*, 1999). The open arrowheads are the inverted terminal repeats. Arrows between exons 1 and 2 are the *Angel* internal inverted repeats (Iszvak *et al.*, 1999; Koga *et al.*, 2000).

(ii) *Indicator plasmid*

The plasmid pDon01 which had been constructed as a donor plasmid to provide a non-autonomous *Tol2* element carrying an antibiotic-resistant gene in its sequence (Koga & Hori, 2000) was used as an indicator for the excision of *Tol2* (Fig. 2). pDon01 has the *Tol2* insertion in the multicloning site of the *lacZ* gene.

(iii) *Frameshift assay*

Since the reading frame could be shifted after *Tol2* excision (Koga *et al.*, 1996), we verified whether the *lacZ* gene with a frameshift mutation can produce an active β-galactosidase, using the plasmids pHSG399plus1 and pHSG399plus2, whose reading frames of *lacZ* were shifted because of one base pair and two base pair insertions, respectively.

The plasmids were constructed as follows. The *Nco*I and *Bam*HI fragments of pHSG399 (Takeshita *et al.*, 1987) amplified by PCR were cloned into the same restriction sites of pHSG399, which include an extra 1 or 2 bp between *Xba*I and *Bam*HI sites. The PCR primers used were 5′ AACGTGGCCAATATGGACAACTTCTTCGCC 3′ (nucleotides 667–696 on pHSG399) for the reverse and 5′ AAGGATCCCTCTAGAGTCGACCTGCAGGC 3′ (nucleotides 1223–1198, pHSG399plus1) or 5′ AAGGATCCACTCTAGAGTCGACCTGCAGGC 3′ (nucleotides 1223–1198, pHSG399plus2) for the forward direction. The extra base pairs between *Xba*I and *Bam*HI are underlined in the primer sequences. Multicloning sites of the final constructs were checked by sequencing, and the reading frames of *lacZ* were found to be shifted because of the insertions. The plasmids were individually introduced into the *E. coli* SURE strain (*lacZ*Δ*M15*, STRATAGENE), plated on LB agar containing 80 μg/ml chloramphenicol, 30 μg/ml of X-gal and 10 μg/ml of IPTG (isopropylthiogalactoside). After overnight incubation at 37 °C, colours of the colonies were checked.

(iv) *Excision assay*

The pDon01 plasmid was diluted to a final concentration of 400 ng/μl in 10 mM Tris–HCl (pH 8·0) with or without mRNAs, and 1–2 nl was injected into fertilized medaka eggs at the 1- to 2-cell stage. The mRNAs were prepared by *in vitro* transcription from *Tol2* cDNAs as templates using a MEGAscript SP6 Kit and a Cap Analog (Ambion), which were checked on agarose gel electrophoresis and diluted with nuclease-free water. Injected embryos were incubated at 25 °C for 20 h. The plasmids were recovered from 30 surviving embryos by homogenizing them with 300 μl of extraction buffer (0·6% SDS, 10 mM EDTA) followed by phenol–chloroform extraction. The aliquots were introduced into the *E. coli* SURE strain by electroporation. After spreading on X-gal plates as detailed above and overnight incubation at 37 °C, white and blue colonies were counted.

(v) *Sequencing*

The sizes of the plasmids from the colonies were checked by *Nco*I digestion, which cuts the vector at a single site, followed by agarose gel electrophoresis. Then, the original insertion site of *Tol2* in *lacZ* was sequenced.

3. Results

(i) *Construction of a system to detect excision*

The design of the detection system for *Tol2* excision is illustrated in Fig. 2. Bacteria harbouring pDon01

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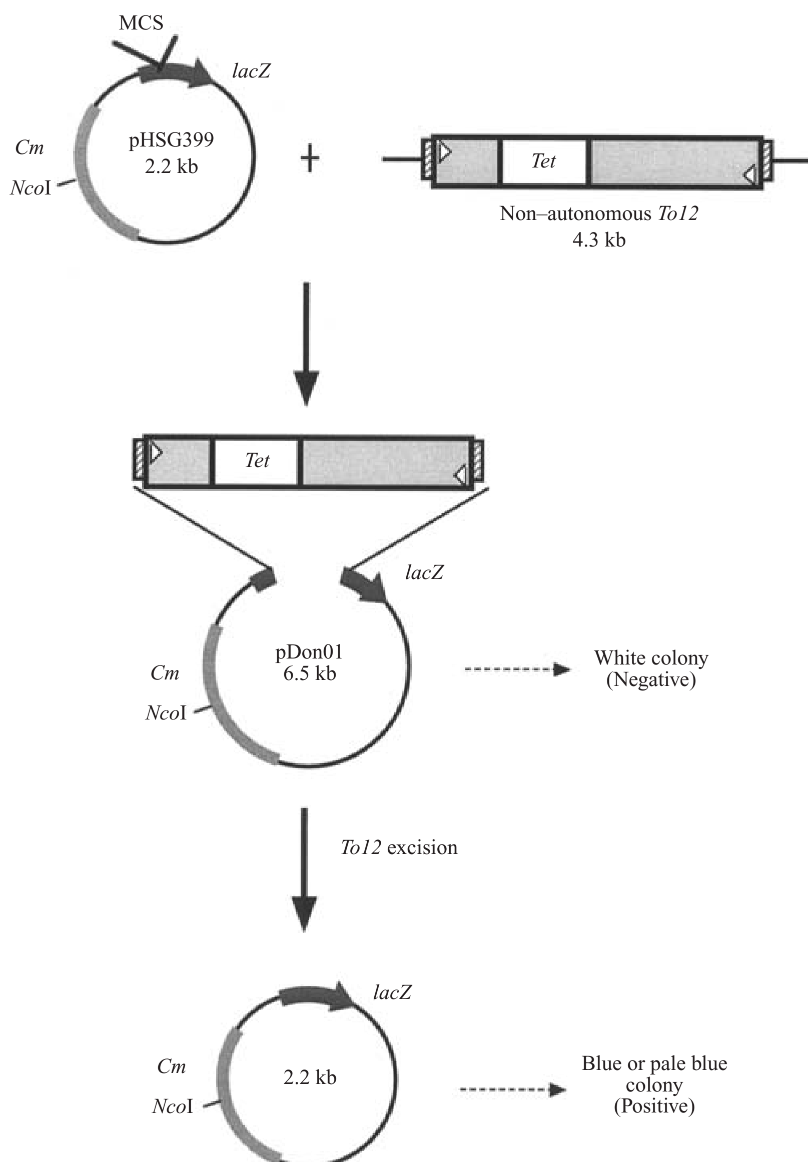

Fig. 2. Structure of pDon01 and a schematic illustration of the detection system for *Tol2* excision. The large rectangle is a non-autonomous *Tol2* whose exons are replaced by the tetracycline-resistant gene (open rectangle). Shaded rectangles are target site duplications. The open arrowheads are the inverted terminal repeats. The bold arrow and the grey rectangle in the plasmid are the *lacZ* gene and the chloramphenicol resistant gene (*Cm*), respectively. *Tol2* was inserted into the *lacZ* multicloning site (MCS) of pHSG399 (see Fig. 4). pDon01 leads to negative (white) colonies when it is introduced into bacteria. If *Tol2* has been excised from pDon01, positive (blue or pale blue) colonies are formed.

form white (=negative) colonies in the presence of X-gal, because the *lacZ* gene is interrupted by the insertion of a *Tol2* into its multicloning site. When the *Tol2* in *lacZ* on pDon01 is excised, blue colonies are formed.

The number of nucleotides left as a footprint after excision is thought to vary (Koga *et al*., 1996), so that footprints with numbers of nucleotides which are not simple multiples of 3 are expected to arise. These 'out-of-frame' excisions shift the reading frame of the *lacZ* gene, and then an active β-galactosidase is not expressed. However, inaccuracies of *lacZ* have been reported in that a gene with an in-frame stop codon on a plasmid, constructed as an expression vector to produce a hybrid β-galactosidase protein, generates weak β-galactosidase activity in *E. coli*. One of the causes might be frameshifting errors in bacteria (Shapira *et al*., 1983). Therefore, we tested whether a plasmid with an out-of-frame mutation in its *lacZ* gene could produce a blue colony. The plasmids pHSG399plus1 and pHSG399plus2, with 1 and 2 bp of extra nucleotides, respectively, between the *Xba*I and *Bam*HI sites in pDon01 instead of *Tol2* were constructed, the reading frame of *lacZ* becoming shifted because of the insertions. The plasmids were individually introduced into bacteria and the colonies were pale blue in both

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Table 1. *Rates of* in vivo *excision of* Tol2 *from pDon01 with different treatments*[a]

No. of colonies			
Negative	Positive	Excision rate ($\times 10^{-5}$)[c]	Ave. ($\times 10^{-5}$)[d]
Direct introduction[b]			
1 500 000	0	0	–
No mRNA			
434 000	16	3·7	3·4 ± 1·6
465 000	6	1·3	
217 000	7	3·2	
310 000	16	5·2	
mRNA-L (100 ng/μl)			
186 000	128	69	65 ± 19
310 000	177	57	
155 000	68	44	
341 000	304	89	
mRNA-S (100 ng/μl)			
217 000	11	5·1	4·7 ± 0·97
348 000	14	4·0	
155 000	9	5·8	
403 000	15	3·7	
mRNA-L (100 ng/μl) + mRNA-S (100 ng/μl)			
155 000	8	5·2	6·4 ± 1·4
217 000	16	7·4	
217 000	17	7·8	
465 000	24	5·2	

[a] Results of four independent experiments are shown, except for the Direct introduction case.
[b] pDon01 was directly introduced into bacteria, not through embryos.
[c] Defined as rate = positive/(negative + positive).
[d] Averages of the excision rates.

cases under conditions when pDon01 produced white colonies (described below). The development of pale blue colonies with these out-of-frame plasmids is presumably due to frameshifting errors in bacteria, which might correct the reading frame so that a little β-galactosidase is produced. The experiments indicate that imprecise and out-of-frame excisions can also be detected by colony colour selection. Hereafter, for the present study both blue and pale blue colonies were regarded as positive.

We introduced pDon01 into bacteria directly to confirm that all colonies transformed by the plasmids before injecting into embryos would be negative. A total of 1 500 000 colonies were screened and all confirmed to be negative (Direct introduction in Table 1). This indicates that recovery reactions for the *lacZ* gene, such as excision, do not occur in bacteria.

(ii) In vivo *excision assay*

To investigate whether excision of *Tol2* causes recovery of *lacZ* to produce positive colonies, we injected pDon01 into medaka eggs with *in vitro* synthesized mRNA-L which catalyses transposition. The plasmids were collected from the embryos for introduction into bacteria. After spreading on X-gal^{+} plates and incubation (Fig. 3), some positive colonies arose among the many negative colonies. Plasmids were digested with a restriction enzyme, and analysed by electrophoresis. All those from 16 positive colonies were sized 2·2 kb, while all 10 clones of plasmids from negative colonies examined were 6·5 kb (data not shown). This means that 4·3 kb of *Tol2* had been excised from the 6·5 kb of pDon01 in medaka embryos, but not in bacteria, to form positive colonies.

The excision footprints of sixteen 2·2 kb plasmids were sequenced (Fig. 4). Some nucleotides of the target site duplication were retained and the numbers of footprint nucleotides from the pale blue colonies were not simple multiples of 3 as expected, thus creating frameshifts (excision products A, B, C and F), while the blue colonies had footprints that maintained the *lacZ* open reading frame (excision products D, E and G). The ratio of blue to pale blue was about 1 to 10 (data not shown), indicating the excision footprint patterns to be biased.

(iii) *Excision frequency*

It was investigated whether the frequency of appearance of the positive colonies reflects the excision activity (Fig. 5). mRNA-L at different concentrations was injected with pDon01 into medaka eggs, and then the resultant colonies were screened. As the amount of mRNA-L increased, the percentage of positive colonies also increased, roughly in proportion. Thus, excision activity can be determined as a rate of *Tol2* excision from pDon01 by counting colonies.

Table 1 shows the excision frequencies for the *Tol2* element from pDon01 in medaka embryos with or without mRNA treatment. Injection of pDon01 alone caused a few positive colonies (No mRNA in Table 1). These colonies without mRNA might be due to background excisions catalysed by an endogenous transposase, because there are 20 copies of *Tol2* in the medaka genome, and almost all of them are thought to be autonomous (Koga & Hori, 1999). Injection of mRNA-L raised the frequency about 20 times (Table 1). On the other hand, when mRNA-S was injected, the frequency differed little from that after no mRNA injection (Table 1). In the case of co-injection of equal amounts of both mRNAs, the excision rate was essentially the same as occurred with no mRNA injection (Table 1). It is unlikely that the decreased rates with both mRNAs compared with mRNA-L alone were due to the overproduction inhibition of transposase that has been observed in *Ac* (Scofield *et al.*, 1993) where the transposases aggregate to form inactive complexes (Heinlein *et al.*, 1994), because doubling of the amount of mRNA-L increased the

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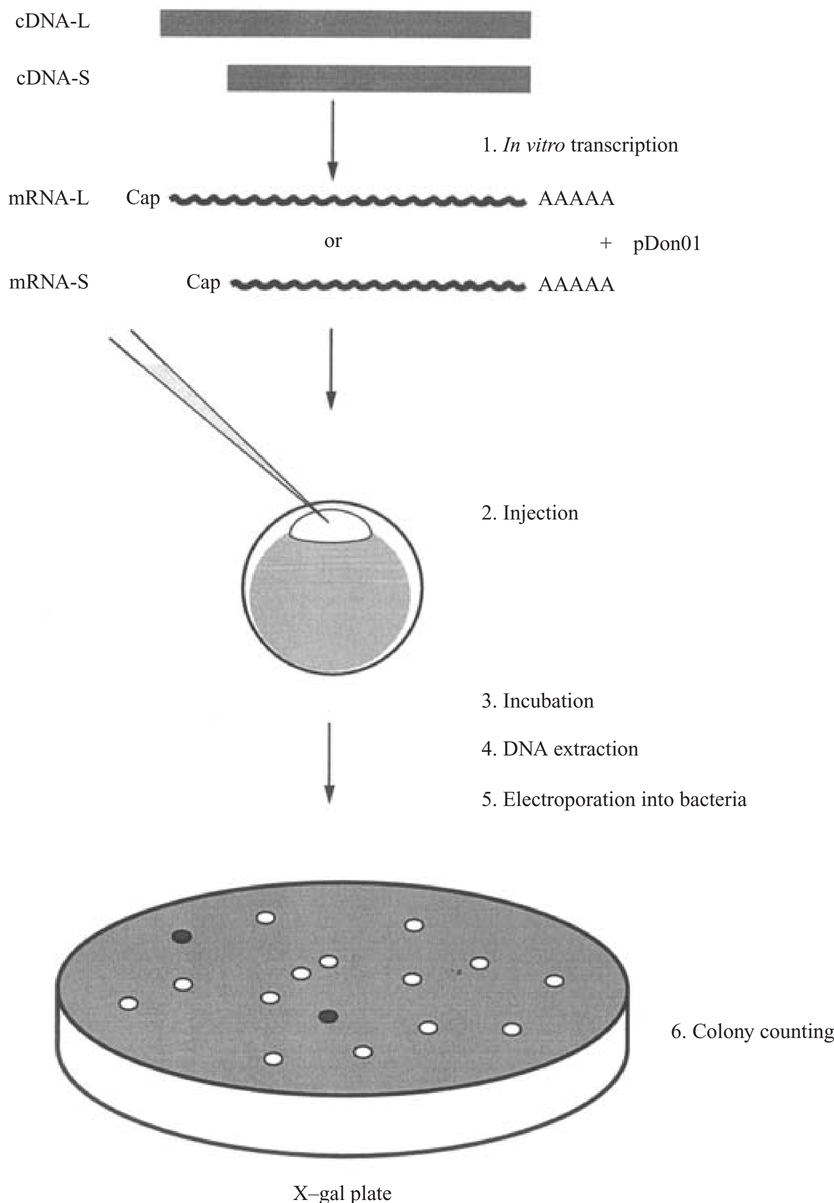

Fig. 3. Experimental processes to detect *Tol2* excision *in vivo*. (1) mRNAs are synthesized *in vitro*. (2) pDon01 and mRNA are co-injected into eggs. (3) Injected eggs are incubated for 20 h. (4) Plasmid DNAs are extracted from the embryos. (5) They are introduced into bacteria by electroporation. (6) Colonies of each colour are counted.

rate at these concentrations (Fig. 5). These results thus suggest that injection of mRNA-S can block the excision reaction. The fact that the excision activity with mRNA-S alone was not lower than the case without mRNA might have been caused by a low level of background excision activity.

4. Discussion

The *Tol2* transposon has been a focus for the development of a new genetic tool since its discovery in a naturally occurring albino mutant medaka fish (Koga *et al.*, 1996). In vertebrates, the artificially reconstructed transposon, *SB*, has attracted a great deal of interest and attempts at application as a gene vector have already been reported (Yant *et al.*, 2000; Izsvak *et al.*, 2000). *Tol2* can also carry DNA fragments to code a protein inside its sequence (Koga & Hori, 2000). Because *SB* belongs to the *Tc1/mariner* family with different features, *Tol2* may provide a useful alternative. Transposition of *Tol2* in zebrafish has already been reported (Kawakami *et al.*, 2000). Moreover, *Tol2* excision has been demonstrated in cultured cells of human and mouse (Koga *et al.*, 2003) and also insertion (unpublished data).

Our excision detection system confirmed the anticipated effect of the mRNA-L in raising the excision rate. Both the mRNAs and pDon01 were likely to

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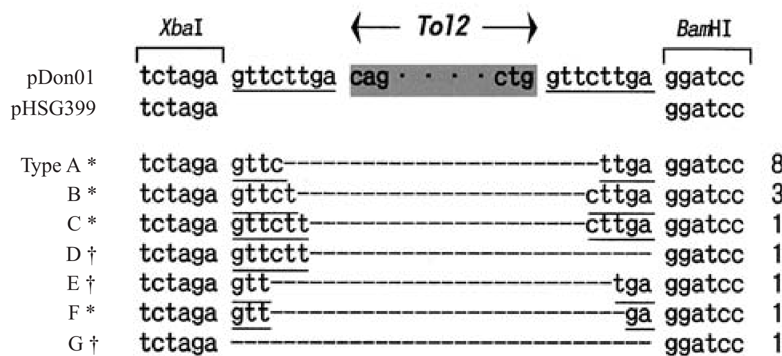

Fig. 4. A part of the sequence of each plasmid and excision footprints (A to G) in the *lacZ* gene. Bold letters are part of the *Tol2* sequence. Nucleotides of target site duplications in both pDon01 and the excision products are underlined. Regions lost by excision are shown as broken lines. Numbers of clones identified are given on the right. Daggers (†), excision products from blue colonies; asterisks (*), excision products from pale blue colonies.

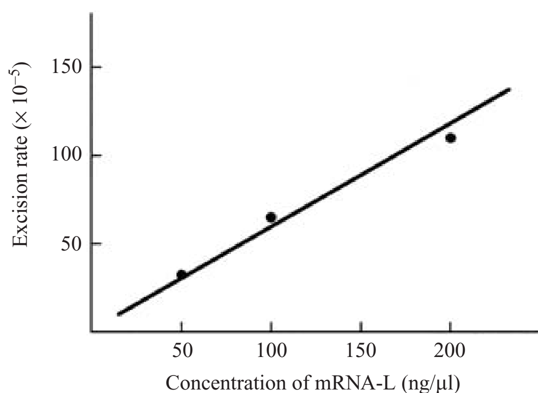

Fig. 5. Excision rates of *Tol2* from pDon01 with mRNA-L treatments at different concentrations. The concentrations of mRNA-L injected were 50 ng/μl, 100 ng/μl and 200 ng/μl. Rates were defined as: positive/(negative + positive).

have entered into eggs to be translated or reside as substrates of excision reactions at a constant rate, since the injection of double or half the amount of mRNA-L resulted in a roughly doubled or halved frequency of excision. The approach adopted allows quantification of excision activity by counting positive and negative colonies selected by colour, although not all excisions may be detectable because *lacZ* activity might not be recovered under all circumstances. Using this system, *cis* and *trans* elements for more efficient excision reactions are now being investigated to study excision mechanisms and facilitate development of genetic tools.

Since it has been demonstrated that endogenous transposase catalyses the excision of *Tol2-tyr* (Koga *et al*., 1996), the excision reaction in this study without *Tol2* mRNA treatment would be catalysed in this way.

In our present experiments, mRNA-S was revealed to have an inhibitory effect on excision. Its deduced protein lacks 109 amino acids of the N-terminal region compared with the functional transposase encoded by mRNA-L. Alignment of the N-terminal regions for *Tol2* and other *hAT* family transposases is shown in Fig. 6. This region of *Tol2* contains aromatic residues including tryptophan, and cysteines and histidines, showing a typical zinc finger motif called the BED finger, which is a DNA binding domain shared by proteins responsible for critical cellular functions (Aravind, 2000). This suggests that the N-terminal region encoded by exon 1 is essential for transposase activity, and possibly has some role in binding to DNA, although the possibility of other functions in excision reactions cannot be eliminated. The probable DNA-binding-deficient short transposase might dominantly inhibit excision not by competing with the long transposase for DNA-binding but rather by some other mechanism. It has been reported that the *Ac* transposase is active as an oligomer, and that an N-terminal truncated inactive transposase, without the DNA-binding domain, interacts with normal transposase to inhibit its functions (Kunze *et al*., 1993). *Ac* transposase has a dimerization domain in the C-terminal conserved region of the *hAT* family (Essers *et al*., 2000) which resides in both *Tol2* transposases, too. *Tol2* short transposases may form inactive hetero-oligomers with long transposases deficient for excision, but the transposition mechanism of *hAT* family transposons is not well understood at present.

As an example of transposition regulation by transposase and its truncated protein, the P element of *Drosophila* is well-known in eukaryotes. The 87 kDa protein has transposase activity, while the 66 kDa counterpart, produced by differential splicing of the mRNA with tissue specificity, acts as a repressor (Rio *et al*., 1986; Laski *et al*., 1986). In somatic cells, the transposition is repressed by the 66 kDa protein due to splicing loss of the third intron that forms the C-terminal region of the 87 kDa protein (Siebel & Rio, 1990; Misra & Rio, 1990). In exon

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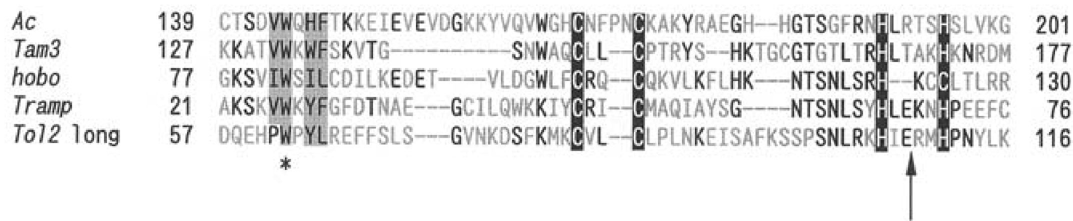

Fig. 6. Alignment of the BED finger domains in the N-termini of transposases from the *hAT* family, including the *Tol2* long transposase. The numbers at each side are the positions of the first and last amino acid residues. Gaps were introduced (broken lines) to optimize the alignments. The metal-chelating residues, i.e. cysteines and histidines, are white on a black background. The residues on a grey background are aromatic and the asterisk indicates the conserved tryptophan. In the other regions, identical residues are black, and the others are grey. The arrow indicates the boundary between exons 1 and 2 of *Tol2*. Alignment was performed as previously described (Hori *et al.*, 1998).

3 of the P element, however, there is neither a BED finger nor any other DNA binding domain (Rio *et al.*, 1986).

As shown in our previous study (Koga *et al.*, 1999), the transcription initiation site for the mRNA-L is located at the 183rd nucleotide position from the 5′ end of *Tol2*, and the site for the mRNA-S is located at the 2077th, that is within the internal inverted repeats (Fig. 1). Since, the expression levels of these two mRNAs are too minor to allow comparison of amounts, we performed a promoter assay for each mRNA using a luciferase gene as a reporter. When 183 bp and 156 bp upstream sequences from the transcription initiation sites of mRNA-L and mRNA-S, respectively, were tested, the results showed that the promoter activity for mRNA-S was several times higher than that for mRNA-L (unpublished data). This suggests that the transcript of mRNA-S may act to inhibit the transposition of *Tol2* in medaka cells.

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