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Targeted reduction of the DNA methylation level with 5-azacytidine promotes excision of the medaka fish *Tol2* transposable element

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Summary

The *Tol2* element of the medaka fish *Oryzias latipes* is a member of the *hAT* (*hobo*/*Activator*/*Tam3*) transposable element family. There is evidence for rapid expansion in the genome and throughout the species in the past but a high spontaneous transposition rate is not observed with current fish materials, suggesting that the *Tol2* element and its host species have already acquired an interactive mechanism to control the transposition frequency. DNA methylation is a possible contributing factor, given its involvement with many other transposable elements. We therefore soaked embryos in 5-azacytidine, a reagent that causes reduction in the DNA methylation level, and examined amounts of PCR products reflecting the somatic excision frequency, obtaining direct evidence that exposure promotes *Tol2* excision. Our results thus suggest that methylation of the genome DNA is a factor included in the putative mechanisms of control of transposition of the *Tol2* element.

1. Introduction

Transposable elements are repetitive sequences that are, or were at some time in the past, capable of changing their chromosomal locations. Too high a transposition activity is obviously deleterious to host organisms, and it is a widely accepted idea concerning the evolution of transposable elements that only examples that acquire some mechanism to suppress activity are likely to survive (Hartl *et al.*, 1997; Brookfield, 2005). A good example is the *P* element of *Drosophila*, which invaded the fly genome, spread throughout the species and became tame. The sequence of these events proceeded in a period as short as 30–40 years (Engels, 1992). The main mechanism for control of its transposition frequency is alternative splicing, leading to the production of a transposase and a repressor of mRNAs transcribed from its internal gene (Misra & Rio, 1990). Another clear example is the *Activator* element of maize, whose transposition is suppressed by DNA methylation of its terminal regions, causing inhibition of transposase binding (Ros & Kunze, 2001).

The *Tol2* element of the medaka fish is a member of the *hAT* transposable element family (Calvi *et al.*, 1991) that includes *hobo* of *Drosophila*, *Activator* of maize and *Tam3* of snapdragon. It is 4·7 kb in length, has terminal inverted repeats of 17 bp and 19 bp, carries a gene for its own transposase and is flanked by an 8 bp target site duplication (Koga *et al.*, 1996; Koga & Hori, 2000). About 20 copies are present in the diploid genome of the medaka fish (Koga *et al.*, 2000). The chromosomal locations of copies differ from fish to fish even in a single local population, suggesting that they are continuously moving (Koga & Hori, 1999). However, transposition is not so frequent as to be detectable over one generation with laboratory strains (Koga & Hori, 1999). From this result and other findings described below, we infer that the *Tol2* element and its host, the medaka fish, have already acquired an interactive mechanism to control the transposition frequency.

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Table 1. *PCR primers used in the present study*

Primer pair	Sequence (5′ to 3′)	Expected product size
P(tyr)	CCTCCCTTGAGGACGAGTCGCATGGTTAAC TTGACATTCATATCCAGGGACAATCTAGAG	3124 bp
P(i^b)	TGAACACTTGGTCATGCGTAAATTCCTGGC GACAATGAAAAATCCCCAAATCTCTTGGTG	425 bp on excision
P(i^4)	ATTGGCCACAATGACGGCTACTACATGGTG TATTAGAACCGCATGGCACAGGGAGCTGTG	499 bp on excision
P(*Tol*)	CCGCTGCACTTGCCTGCAAAAGATTAATGG CCTGGTGTCTGAAACACAGGCCAGATATCC	1100 bp from mRNA, 1572 bp from DNA

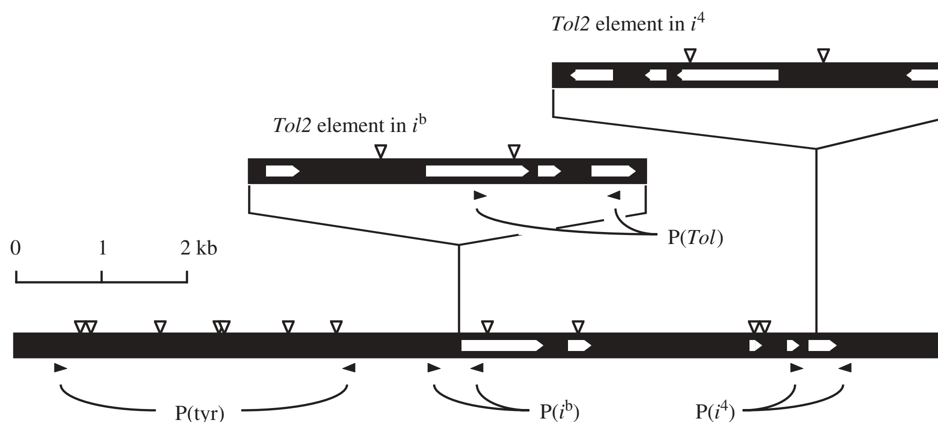

Fig. 1. Structure of the medaka fish tyrosinase genes. Exons of the tyrosinase gene and the *Tol2* transposase gene are shown by white arrows. The mutant tyrosinase genes for the i^b and i^4 alleles carry insertions of the *Tol2* element at the positions indicated. The nucleotide sequence of the wild-type tyrosinase gene and its neighbouring regions can be cited from GenBank with accession number AB010101. The *Tol2* element creates a target site duplication of 8 bp upon insertion, and the locations of the duplications for the i^b and i^4 alleles are nt 3089–3096 and 7589–7596, respectively. The locations and directions of the PCR primers are indicated by arrowheads. Their sequences are listed in Table 1. The white triangles are locations of possible *Msp*I/*Hpa*II recognition sites.

DNA methylation is a possible contributary factor with the *Tol2* element because it plays a role in control mechanisms for many transposable elements, including the maize *Activator* element (Ros & Kunze, 2001). In this study, we therefore examined whether changes in the DNA demethylation level affect the excision frequency in somatic cells, by exposing embryos to 5-azacytidine, an agent that induces a decrease in the methylation level of genome DNA. A clear positive correlation was observed between the reduction in the methylation level and the excision frequency, suggesting that DNA methylation indeed contributes to the mechanisms controlling the frequency of transposition of the *Tol2* element.

2. Materials and methods

(i) *Fish strains*

The *i* locus of the medaka fish includes the gene for tyrosinase, which is the key enzyme for melanin biosynthesis. Alleles i^b (Iida *et al.*, 2004*a*) and i^4 (Koga *et al.*, 1996) at this locus correspond to mutant tyrosinase genes carrying insertions of the *Tol2* element at different locations in the gene (Fig. 1), and are both recessive to the wild-type allele i^+ that does not have an insertion of this element. Mutant lines of genotypes i^b/i^b and i^4/i^4 (denoted i^b and i^4, respectively) obtained from Y. Wakamatsu of the National Bioresource Project of Japan were used in this study. The *Tol2* elements in these mutant genes have been proven to undergo spontaneous excision in somatic cells that is detectable by PCR using primer pairs encompassing their insertion sites (Iida *et al.*, 2004*a*).

(ii) *5-Azacytidine treatment*

Fish were maintained at 26 °C under a 14 h light and 10 h dark photoperiod cycle. Fertilized eggs were collected within 1 h of fertilization and soaked in 2·0 ml of 5-azacytidine which had been prepared by

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dissolving powder in water. After 72 h (3 days) of incubation at 26 °C, embryos were washed with water and cultured in water at the same temperature for 2 more days. Hatching of embryos usually occurs at 9 days post-fertilization (DPF) at 26 °C. No embryos reached the hatching stage by 5 DPF in this experiment, as expected.

(iii) *Preparation of genome DNA*

Twelve 5 DPF embryos for each 5-azacytidine concentration class were put into microfuge tubes and washed with distilled water. Genome DNA was extracted from these embryos using a DNeasy Tissue Kit (QIAGEN, Hilden, Germany) and finally eluted into 10 mM Tris (pH 8·0). The DNA concentration was determined by measurement of the optical density and adjusted to 100 ng/μl by diluting with the solvent.

(iv) *Endonuclease treatment*

Genome DNA, 200 ng for each assay, was treated with 10 units of restriction endonuclease *Hpa*II or *Msp*I (Takara Bio, Otsu, Japan) at 37 °C for 60 min. This ratio of DNA to enzyme provides an excess enzyme state. The entire reaction mixture was then used for agarose gel electrophoresis.

(v) *PCR*

PCR was performed using the Ex Taq enzyme (Takira Bio) and a GeneAmp PCR System 9700 machine (Applied Biosystems, Foster City, CA, USA), with 200 ng of template DNA in a reaction mixture of 50 μl. When nuclease-treated DNA was to be used as the template, inactivation of the nuclease by incubation at 70 °C for 15 min was carried out before incorporation into the PCR reaction. The primers used and the PCR conditions are described for each case.

(vi) *RT-PCR analysis*

For each 5-azacytidine concentration class, 20 embryos of the i^b strain, which had been treated with 5-azacytidine in a different batch from that for excision assays, were homogenized in 500 μl of the homogenizing buffer of an RNeasy Kit (QIAGEN). The homogenate was then divided into two portions of equal volume (250 μl). One was treated with RNase A, and genome DNA was extracted using a DNeasy Tissue Kit and the concentration determined with reference to optical density. The other half was diluted with the solvent, to adjust the DNA concentration to the lowest among the five 5-azacytidine concentration classes. Aliquots of an equal volume (250 μl) of these solutions were then transferred into other tubes and, after DNase I treatment, total RNA was extracted using an RNeasy Kit. These procedures were performed to standardize the number of cells contributing to the final RNA samples. This is because the amount of RNA per cell may be affected by 5-azacytidine treatment but that of DNA is expected to be far less affected.

With the RNA samples, reverse transcription using an oligo dT primer and subsequent PCR amplification using primer pair P(*Tol*) were carried out using an RNA PCR Kit (Takara Bio).

3. Results

(i) *5-Azacytidine causes disorder of development*

As a preliminary experiment, we treated embryos with various concentrations of 5-azacytidine and checked embryos for disorders of development. Morphological changes or lack of organs were observed at concentrations of more than 0·5 mM (data not shown). The rate for heartbeat cessation with 7 DPF embryos was about 80% at 4 mM, but less than 5% at 3 mM. Based on this information, we chose 0, 0·5, 1, 2 and 3 mM as concentrations to be tested in endonuclease and PCR analyses.

(ii) *5-Azacytidine causes decrease in the methylation level*

The recognition sequence of *Hpa*II is 'CCGG' but DNA molecules are not cleaved at this site if the internal cytosine is methylated. *Msp*I is an isoschizomer of *Hpa*II, which cleaves DNA irrespective of the methylation status of the internal cytosine. Therefore, differences in the extent of fragmentation of genome DNA between these two endonucleases indicate variation of the methylation level at 'CG' sites (Singer *et al*., 1979).

Embryos of the i^b and i^4 strains were treated with 5-azacytidine at five different concentrations (0, 0·5, 1, 2 and 3 mM), and genome DNAs were extracted from the embryos. The upper panels of Fig. 2 illustrate results of gel electrophoresis with no endonuclease treatment, or after treatment with *Msp*I or *Hpa*II. No detectable differences in the amounts of DNA are apparent among the five 5-azacytidine concentrations (see 'No endonuclease' lanes). With *Msp*I, the extent of fragmentation did not vary with the 5-azacytidine concentration, in contrast to the *Hpa*II case. These results indicate that, as with mammalian cells (Jones & Taylor, 1980), exposure of medaka fish embryos to 5-azacytidine causes concentration-dependent reduction in CG methylation, at least in the range examined.

To further confirm the effects of 5-azacytidine, we performed PCR analysis of 3·1 kb segment located

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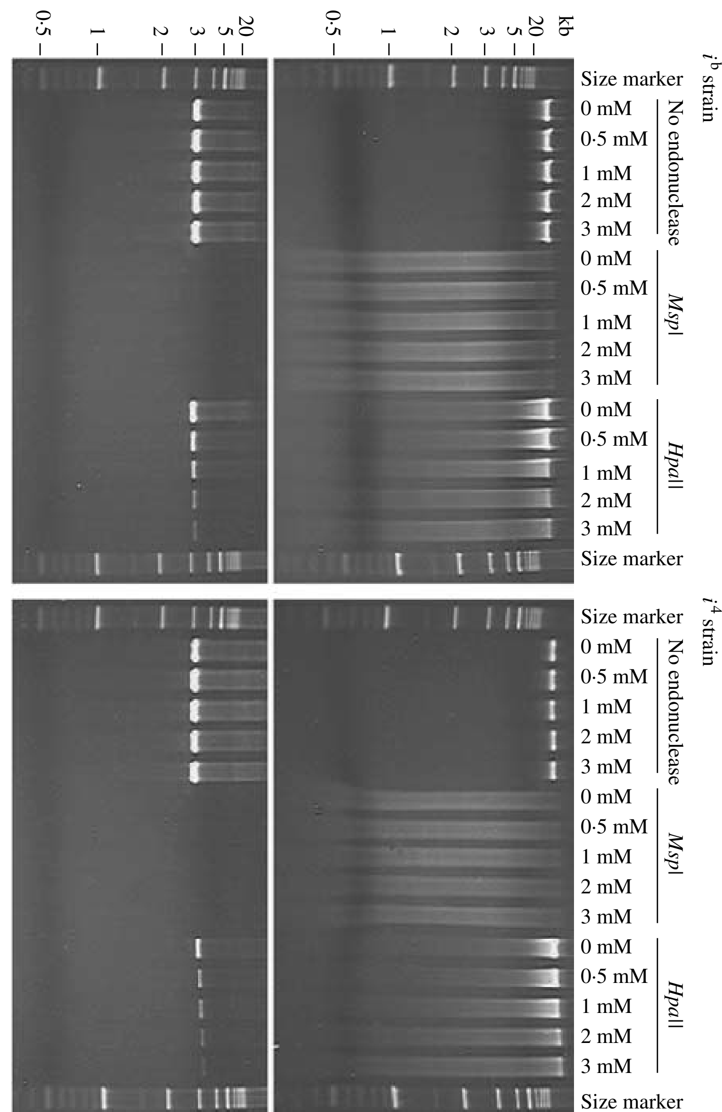

Fig. 2. Analysis of methylation levels. The upper panels show results of endonuclease analysis. DNAs without endonuclease treatment or after treatment with *Msp*I or *Hpa*II were electrophoresed on 1·0% agorose gels. The lower panels show results of further analysis by PCR. The PCR reaction mixtures were electrophoresed on 1·0% agarose gels.

in the tyrosinase gene region and carrying seven possible *Msp*I/*Hpa*II recognition sites (see Fig. 1). The primer pair P(tyr) was applied with genomic DNAs as template untreated or treated with *Msp*I or *Hpa*II, under conditions of [120 s at 94 °C], 25 cycles of [20 s at 94 °C, 20 s at 64 °C, 180 s at 72 °C] and [180 s at 72 °C]. The lower panels of Fig. 2 illustrate the results of gel electrophoresis of PCR reaction mixtures. Amplification of fragments of the expected size was efficient with DNAs not treated with an endonuclease, and a smaller amount of products was observed with *Msp*I-treated DNAs. There were no detectable differences in the product amounts among the 5-azacytidine concentrations in these two cases. However, clear decline of the product amount along with the increase in the 5-azacytidine concentration was observed with *Hpa*II-treated DNAs, indicative of a decrease in the methylation level of some or all of the seven 'CCGG' sites in the 3·1 kb region.

(iii) *5-Azacytidine promotes excision of the* Tol2 *element*

On the chromosome of the i^b strain, the PCR primers comprising primer pair P(i^b) are separated by 5·1 kb, of which the inserted *Tol2* element occupies 4·7 kb (see Fig. 1). Therefore, excision of the element in part of the cells used for genome DNA preparation would be expected to lead to production of a 0·4 kb fragment in a PCR amplification with primer pair P(i^b). In addition, the product amount would reflect the excision frequency. Semiquantitative PCR analysis based on these was performed. The PCR conditions were [120 s at 94 °C], 40 cycles of [20 s at 94 °C, 20 s

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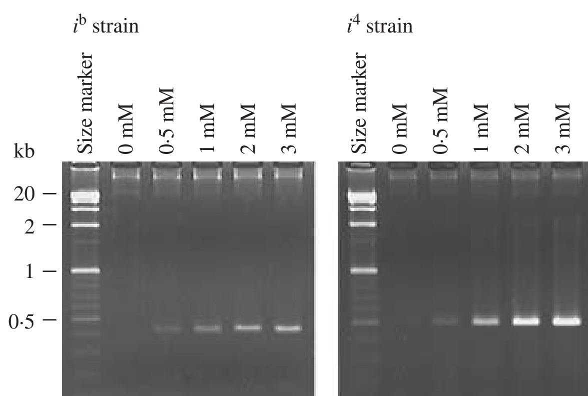

Fig. 3. PCR amplification of excision products. The PCR reaction mixtures were electrophoresed on 2·0% agarose gels.

at 64 °C, 20 s at 72 °C] and [180 s at 72 °C]. The incubation time for the extension step was set to be sufficient for a 0·4 kb fragment but not for a 5·1 kb fragment. The left-hand panel of Fig. 3 shows the results of gel electrophoresis of PCR reaction mixtures. A 0·4 kb fragment is seen even with no 5-azacytidine treatment (0 mM). This phenomenon has previously been observed and shown to be due to spontaneous excision of the *Tol2* element (Iida *et al.*, 2004*a*). Products of this size also appeared at other 5-azacytidine concentrations with a positive correlation. Equivalent results were obtained on PCR with the i^4 strain and primer pair P(i^4), the expected product size being 0·5 kb in this case. The results indicate that the excision frequency of the *Tol2* element becomes larger as the 5-azacytidine concentration increases, at least in the range examined.

(iv) *5-Azacytidine does not have a significant effect on the expression of the transposase gene*

There are various conceivable mechanisms by which 5-azacytidine could affect the excision frequency (see Section 4). One possibility, which can be examined with simple experiments, is to change the expression level of the *Tol2* transposase gene. We therefore carried out a semiquantitative RT-PCR analysis of the transposase mRNA.

Total RNA was extracted from 5-azacytidine-treated (0 to 3 mM) embryos, and reverse transcription with an oligo-dT primer was carried out, after adjustment of RNA samples for numbers of cells (see Section 2). Subsequent PCR amplification of a 1·1 kb region of the transposase mRNA (verified by a control experiment; Fig. 4*B*), delimited by primer pair P(*Tol*), was performed with a total reaction volume of 50 μl. The PCR conditions were [120 s at 94 °C], 30 cycles of [20 s at 94 °C, 20 s at 60 °C, 80 s at 72 °C] and [180 s at 94 °C]. For all five 5-azacytidine concentration classes, we prepared reactions containing

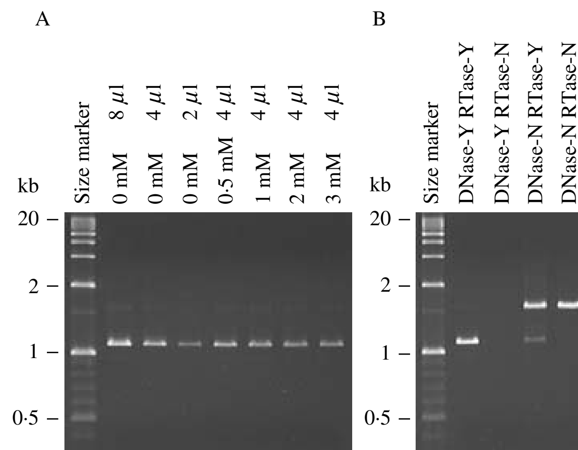

Fig. 4. RT-PCR analysis of the expression level of the transposase gene. (*A*) Gel electrophoresis of RT-PCR products. After reverse transcription with an oligo-dT primer, PCR amplification using primer pair P(*Tol*) was performed with a total reaction volume of 50 μl, in which the amount of reverse transcription reaction mixture indicated above the lanes was included. Final reaction solutions of 10 μl each were electrophoresed on a 1·2% agarose gel. (*B*) Control experiment to confirm the origin of the RT-PCR product. DNase and RTase indicate DNase I and reverse transcriptase, respectively. Y (yes) and N (no) indicate inclusion and exclusion, respectively, of these enzymes. Under the conditions shown above the lanes, RT-PCR amplification equivalent to that for the '0 mM, 4 μl' lane in (*A*) was carried out. The results indicate that the 1·1 kb fragment in (*A*) originates not from genomic DNA but from mRNA.

4 μl each of the reverse transcription reaction mixture, observing no clear difference in the product amount (Fig. 4*A*). We also prepared two other reactions containing a double (8 μl) and half (2 μl) volume of the reverse transcription reaction mixture of the 0 mM sample, revealing that differences in the amount of the transposase mRNA outside this range could be detected with respect to the product amount (Fig. 4*A*). These results indicate that the effect of 5-azacytidine treatment is with in this range at most. The results also provide evidence against a positive or negative correlation between the 5-azacytidine concentration and the amount of the transposase mRNA.

4. Discussion

We have shown that the excision frequency of the *Tol2* element in medaka fish somatic cells is positively correlated with reduction in the methylation level caused by exposure of embryos to 5-azacytidine. The effects were consistent across two methods applied and also with two strains.

Transposition of the *Tol2* element consists of two reactions: excision from the chromosome and reintegration into another location. In this study, we examined only the excision frequency because quantitative

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analysis of reintegration requires a much more complex measuring system. We are now targeting such improvements. However, it can be assumed that the excision frequency is correlated with the reintegration frequency. Therefore, our results suggest that methylation of genome DNA is a factor included in a putative mechanism to control the transposition frequency of the *Tol2* element.

Several findings support the existence of a mechanism to control the transposition frequency of the element in medaka fish. First, there is evidence of a rapid proliferation of the *Tol2* element in the genome and rapid expansion in natural populations (Koga *et al*., 2000). Nevertheless, its germline transposition in current fish is not sufficiently frequent to be detected over one generation with laboratory strains (Koga & Hori, 1999). Second, the copy number of the element per diploid genome is distributed in a narrow range. In 12 samples that represent the entire inhabitation area of this species, the minimum copy number was 10 and the maximum was 25 (Koga *et al*., 2000). The number of samples we have now examined has increased to 41, but all exhibited figures within this range (unpublished). Third, the *Tol2* element exhibits a high transposition frequency when artificially introduced into heterologous organisms, such as zebrafish (Kawakami, 2004) and the Indian medaka fish *O. melastigma* which is phylogenetically closely related to *O. latipes* but does not harbour this element (Koga & Hori, 2000). Thus, it is likely that the *Tol2* element has acquired, in its evolutionary process, some mechanism to control its transposition frequency, and the mechanism is interactive with its host species, the medaka fish.

Increase in the transposition frequency caused by reduction in the DNA methylation level has been reported with many DNA-based transposable elements of plants, including the maize *Activator* element (Scortecci *et al*., 1997) and the *Arabidopsis CACTA1* element (Miura *et al*., 2001). To our knowledge, there have hitherto been no such reports regarding vertebrates, reflecting the fact that active DNA-based elements are very few in vertebrates. Our results suggest that DNA methylation for control of the activity of DNA-based elements is a factor common to a wide range of organisms, including plants and vertebrates.

The purpose of the present study was to determine whether DNA methylation is a major factor controlling the *Tol2* transposition frequency. Having obtained supporting evidence, we are now interested in the mechanisms of regulation. The effect of 5-azacytidine may be direct or indirect. Possible direct effects are changes in the transcription level of the *Tol2* transposase gene (cf. Brutnell & Dellaporta, 1994), the efficiency of nuclear transport of the protein (cf. Iida *et al*., 2004*b*) and the accessibility of the transposase to the element that may be dependent on the element structure (cf. Martin *et al*., 1989; Ros & Kunze, 2001). Possible indirect effects are equivalent or other changes concerning host genes involved in the *Tol2* transposition, and DNA damage, such as nicks, resulting from the presence of 5-azacytidine. In the present study, we already examined the effect on the transcription level of the transposase gene by semiquantitative RT-PCR analysis, obtaining evidence against this possibility.

Many DNA-based transposable elements have been reported to involve DNA methylation in suppression of transposition, as listed earlier. However, there is one clear exception: transposition of the *Sleeping Beauty* element is enhanced by DNA methylation (Yusa *et al*., 2004). An important point that should be taken into account from the evolutionary viewpoint is that *Sleeping Beauty* is an element artificially reconstructed with sequence information of inactive copies in salmonid genomes (Ivics *et al*., 1997). Their copies were obviously active in the past because they were amplified in salmonid genomes, but the transposition activity was lost at some point. Failure of once-active copies of *Sleeping Beauty* to employ DNA methylation in their transposition control might have acted against their survival in their host genomes. The view that DNA methylation is a key component of the control mechanism of the transposition frequency for many 'live' DNA-based elements, and also RNA-mediated elements (cf. Hirochika *et al*., 2000; Roman-Gomez *et al*., 2005), is thus not inconsistent with the phenomenon observed with the *Sleeping Beauty* element.

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