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ORIGINAL ARTICLE

The *Tol1* element of medaka fish is transposed with only terminal regions and can deliver large DNA fragments into the chromosomes

Akihiko Koga · Ichizo Higashide · Hiroshi Hori · Yuko Wakamatsu · Yoriko Kyono-Hamaguchi · Satoshi Hamaguchi

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Abstract *Tol1* is an active DNA-based transposable element residing in the genome of the medaka fish *Oryzias latipes*. This element belongs to the *hAT* transposable element family, of which complete copies have relatively long sequences. In addition, we found that *Tol1* elements as long as 18 and 20 kb occur in the medaka fish genome. These facts suggest that *Tol1* is suitable for carrying large DNA fragments as a gene transfer vector. Focusing on this, we conducted two kinds of manipulations of the element. The first was to eliminate internal regions dispensable for transposition. It was revealed that a *Tol1* element consisting of 157-bp left- and 106-bp right-terminal regions could be transposed without a loss of transposition efficiency. Next, we prepared long *Tol1* elements by incorporating unrelated DNA fragments into this short *Tol1* clone and examined their transposition efficiencies. The transposition frequency decreased as the element size increased. The longest *Tol1* element we examined measured 22.1 kb, and its transposition frequency was approximately one fifth that of a 2.1-kb element. However, this frequency was still significantly higher than that of a random integration of DNA into the chromosomes. The element size of 22.1 kb is the longest ever reported for DNA-based elements currently used for mammals. Thus, *Tol1* is a superior gene-transfer vector with a large cargo capacity.

Keywords Transposable element · Gene-transfer vector · Cargo capacity

Introduction

DNA-based transposable elements, RNA-mediated elements, retroviruses, and adenoviruses have all been used as tools for genetic manipulation, such as gene transfer, mutagenesis, and gene/promoter/enhancer trapping. A great advantage of DNA-based elements is the simplicity and safety of their usage. Elements of this type currently used for mammals include the naturally occurring elements *Tol2* from the medaka fish (Koga et al. 2003; Koga et al. 2006) and *piggyBac* from a moth (Fraser et al. 1996; Wilson et al. 2007), as well as the resurrected element *Sleeping Beauty* from salmonid fishes (Ivics et al. 1997; Aronovich et al. 2007). We recently found another element, *Tol1* of the medaka fish, which has the potential for development as a genetic tool. The *Tol1* element with an embedded neomycin-resistance gene was efficiently transposed into the chromosomes of human and mouse cells, conferring neomycin resistance on the cells (Koga et al. 2007).

It is a general feature of transposable elements that transposition frequency decreases as element size increases. Thus, cargo capacity—the maximum length of a DNA fragment that can be delivered by the element—is an important factor to be considered in choosing an element.

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A. Koga (✉) · I. Higashide · H. Hori
Division of Biological Science, Graduate School of Science, Nagoya University, Nagoya 464-8602, Japan
e-mail: koga@bio.nagoya-u.ac.jp

Y. Wakamatsu
Bioscience and Biotechnology Center, Nagoya University, Nagoya 464-8601, Japan

Y. Kyono-Hamaguchi · S. Hamaguchi
Department of Environmental Science, Faculty of Science, Niigata University, Niigata 950-2181, Japan

The *Tol1* element is expected to have an advantage in this regard. First, the element belongs to the *hAT* (*hobo*/*Activator*/*Tam3*) transposable element family (Calvi et al. 1991). An important feature of this family is that their complete copies are long in comparison with those of other major families, such as the *mariner*/Tc1 family (cf. Koga et al. 2007). Second, our analysis of the medaka genome revealed the existence of *Tol1* copies that are 18 and 20 kb in length, as shown in the initial part of this study. From this observation, we made the inference that *Tol1* elements around these sizes can be transposed.

The aim of the present study was to develop the *Tol1* element into a gene-transfer vector that has the ability to deliver large DNA fragments into the chromosomes. For this purpose, we first removed internal regions dispensable for the transposition reaction from the original 1.9-kb element (*Tol1–tyr*, DDBJ accession number D42062), obtaining a basic vector as short as 0.3 kb. We then constructed *Tol1* elements of various lengths by inserting extra DNA fragments into the basic vector and measured their transposition frequencies. As previously described (Koga et al. 2007), the *Tol1* DNAs were introduced into cells by the lipofection method, and the transposition frequency was determined by counting surviving colonies after selective culturing with G418. Because the efficiency of DNA intake by lipofection is affected by DNA size, we took a measurement to compensate for the size effect: comparisons were made among plasmids of a defined total size that carried *Tol1* elements of different sizes. Our results indicated that a *Tol1* element of 22.1 kb in length can be efficiently transposed. This is the longest ever reported for DNA-based elements currently used for mammals.

Materials and methods

Genomic library

We prepared a genomic library of an albino medaka fish exhibiting partial melanin pigmentation in the skin and eyes (Koga et al. 2007). This library was used for the selection of *Tol1*-carrying genomic clones. The vector for the library was pCC1FOS (EPICENTRE Biotechnologies, Madison, WI, USA) and contained 33- to 48-kb mechanically sheared DNA fragments.

Plasmids

Two kinds of plasmids were used: donor plasmids and helper plasmids. In cells, the *Tol1* element was excised from the donor plasmid and integrated into the chromosomes by the catalysis of the transposase produced by the helper plasmid. The donor plasmids carried left- and right-terminal regions of the *Tol1–tyr* element in the plasmid backbone of pUC19. The lengths of these arms varied among experiments. In most cases, the donor plasmids also contained other DNA fragments, such as a marker gene and stuffer fragments. The helper plasmids were the same as those used in our previous study (Koga et al. 2007).

Transposition assays

Human HeLa and mouse NIH/3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and antibiotics in an incubator at 37°C with 5.0% CO_2. Aliquots of 2×10^5 cells were seeded in 12-well plates (22 mm in diameter) and incubated for 24 h. Mixtures of 100 ng of donor and 900 ng of helper plasmid DNAs per dish were then introduced into the cells using the Lipofectamine LTX reagent (Invitrogen Corp., Carlsbad, NM, USA). After 8 h of incubation, the cells were washed with phosphate-buffered saline (PBS) and incubated for another 24 h in fresh medium without plasmid DNA or transfection reagent. The cells were then trypsinized and suspended in 2.0 ml of medium, and 400-μl aliquots of cell suspension were transferred to dishes of different sizes (35, 60, and 90 mm) containing medium and 500 μg/ml G418. After 12 days under G418 selection, the cells were fixed with 20% formalin and stained with Giemsa's solution. Cell colonies were counted from dishes containing colony numbers closest to 100, and colony numbers per 10^5 seeded cells were determined. The transfection assays were carried out in triplicate.

Molecular methods

The molecular methods listed below followed standard procedures (Sambrook et al. 2001) and were also described previously (Koga et al. 2007): preparation of genomic DNA, polymerase chain reaction (PCR), cloning of PCR products, DNA sequencing, and colony hybridization. The PCR enzyme used in this study was LA Taq (Takara Bio Inc., Otsu, Japan). The PCR conditions are described for each case.

Results

Length variation of *Tol1* copies

The medaka genome contains 100–200 *Tol1* copies, which are not homogenous in length (Koga et al. 1995). To reveal

the degree of variation in length, we screened a genomic library for chromosomal fragments containing both terminal regions of *Tol1*, obtaining 130 such clones. We then conducted PCR to amplify the *Tol1* portion in each clone using primers representing the *Tol1* terminals. Production of fragments was observed with 114 of the 130 clones. Distribution of the element lengths was roughly unimodal, with a sharp peak at 1–2 kb (S1 of electronic supplementary material). One important finding was that *Tol1* elements of about 18 and 20 kb are present in the medaka genome.

Test for transposition activity of short *Tol1* clones

It is possible that the 1.9-kb *Tol1–tyr* element contains an internal region dispensable for transposition. We thus prepared several shorter versions by PCR and tested their transposition activity by introducing them into mouse culture cells together with either a functional or defective helper plasmid. Importantly, the transposition assays showed that a clone consisting of 157 bp left- and 106 bp right-terminal regions showed a transposition frequency comparable to that of the *Tol1–tyr* clone (Fig. 1). Further truncation of either the left or right arm to 26 bp led to a significant reduction or loss of transposition activity.

Based on the results obtained in the above experiments, we prepared pDon263Mcs containing 157-bp left and 106-bp right *Tol1* arms and a multiple cloning site (MCS) for six commonly used restriction endonucleases between the arms (Fig. 2).

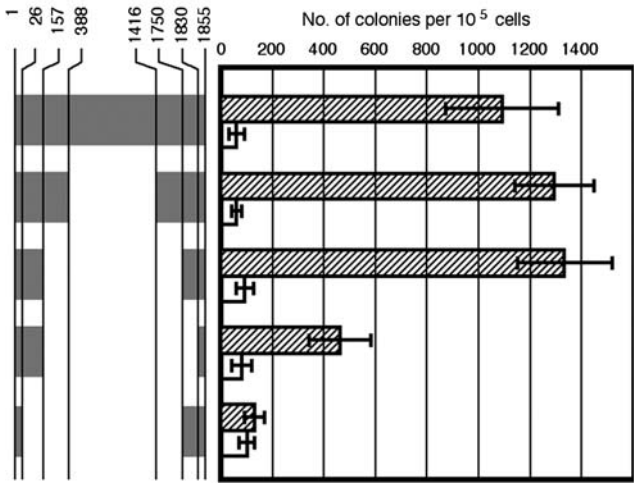

Fig. 1 Transposition frequencies of internally deleted *Tol1* clones. The tested *Tol1* clones are illustrated in the *left margin*. Only the portions of *Tol1* arms are shown, and their lengths are not to scale. Transposition assays were conducted either with the functional helper (*shaded bars*) or with the defective helper (*white bars*). The widths of the bars indicate the mean colony numbers from three independent assays, with standard errors of the mean indicated by *horizontal lines*

Comparison of transposition frequency of *Tol1* clones of different lengths

Using PCR, we amplified a DNA fragment of x kb in size ($x = 0,\ 5,\ 10,\ 15,\ 20$) and another fragment of y kb ($y = 20 - x$) as inner- and outer-stuffer fragments, respectively. These fragments were then inserted into the *Eco*RI (inside *Tol1*) and *Hin*dIII (outside *Tol1*) cutting sites of the vector pDon263McsNeo (Fig. 2). Each clone was called pDon263McsNeoE*x*H*y*. The distance between the

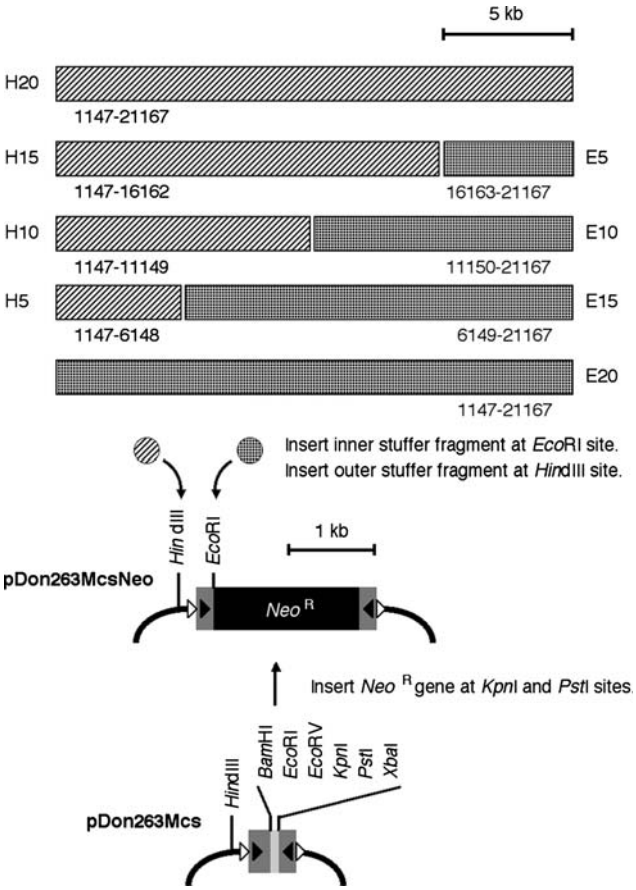

Fig. 2 Donor plasmids containing *Tol1* elements of different sizes. The pDon263Mcs shown at the *bottom* is the basic vehicle plasmid that carries a multiple cloning site. The pDon263McsNeo shown in the *middle* was prepared by inserting the *Neo*^{R} gene into pDon263Mcs. The *bars* shown at the *top* are inner and outer stuffer DNA fragments of various lengths prepared by amplifying parts of the DNA of bacteriophage λ (DDBJ J02459) with polymerase chain reaction (PCR). Nucleotide numbers of the amplified regions are shown *below the bars*. The PCR primers were designed so that they would carry a cutting site for *Eco*RI or *Hin*dIII at their 5′ ends. The stuffer fragments were digested with *Eco*RI or *Hin*dIII and inserted into pDon263McsNeo at their respective sites

left and right *Tol1* ends on pDon263McsNeoE*x*H*y* was (x + 2.1 kb), and the size of the entire plasmid was 24.8 kb, irrespective of x.

It is known that plasmid size is a factor that affects the efficiency of DNA intake in the lipofection method. The incorporation of outer-stuffer fragments in addition to inner-stuffer fragments served to eliminate the effects of the plasmid DNA size. This was done to allow an accurate comparison of the transposition frequency among different donor plasmids.

Transposition frequencies were determined for the five donor plasmids, each with the functional and defective helper plasmids (Fig. 3). In human and mouse cells alike, a negative correlation between element size and transposition frequency was observed. In the case of transfection with the functional helper plasmid, the ratio of frequency of the longest element (pDon263McsNeoE20) to that of the shortest element (pDon263McsNeoH20) was 0.21 and 0.28 in human and mouse cells, respectively. Transposition frequencies of the longest element transfected with the functional helper plasmid were eight and ten times higher than when transfected with the defective helper plasmid in human and mouse cells, respectively.

Demonstration of transposition

We went on to confirm that the *Tol1* element was integrated into the chromosomes by transposition. For this purpose, we first isolated two mouse-cell colonies produced in an assay with the longest element (pDon263McsNeoE20). These cell lines were propagated in different dishes, and their genome DNAs were extracted. Using these DNAs as templates, we amplified the *Tol1* terminals and their flanking chromosomal regions by inverse PCR and sequenced the fragments obtained. Alignment of the sequences revealed the creation of 8-bp target-site duplications in both cases (Fig. 4), indicating that the *Tol1* element of the donor plasmid was integrated into the recipient DNAs by means of transposition.

The results did not necessarily mean that the entire *Tol1* element, including the inner stuffer fragment, was integrated into the chromosomes without internal deletion or aberration. To confirm this, we cloned and analyzed the inserted *Tol1* element. Cloning was performed by PCR using primers that straddled the *Tol1*-terminal regions and their flanking chromosomal regions. The obtained PCR products were of the expected size, and their restriction enzyme digestion patterns were also as expected (S2 of ESM). These results indicate that the entire region of the 22.1 kb *Tol1* element was integrated into the chromosomes.

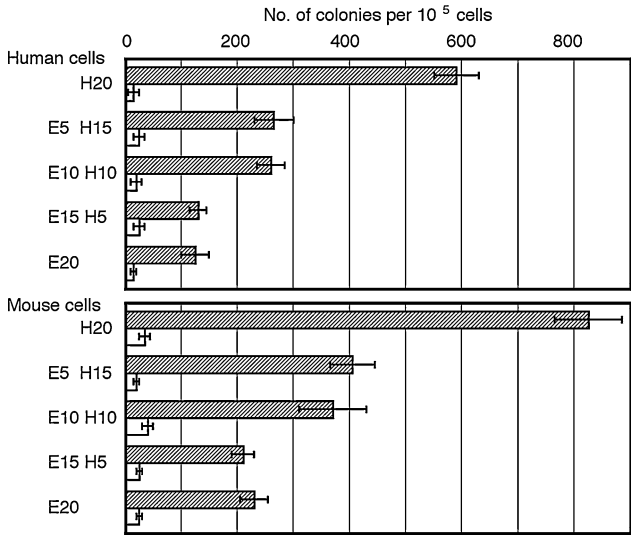

Fig. 3 Transposition frequencies of long donor plasmids. Transposition assays were conducted either with the functional helper plasmid (*shaded bars*) or with the defective helper plasmid (*white bars*). The donor plasmids used are shown in the form of E*x*H*y* in the *left margin*. *Bar width* indicates the mean colony numbers from three independent assays, with standard errors of the mean indicated by *horizontal lines*

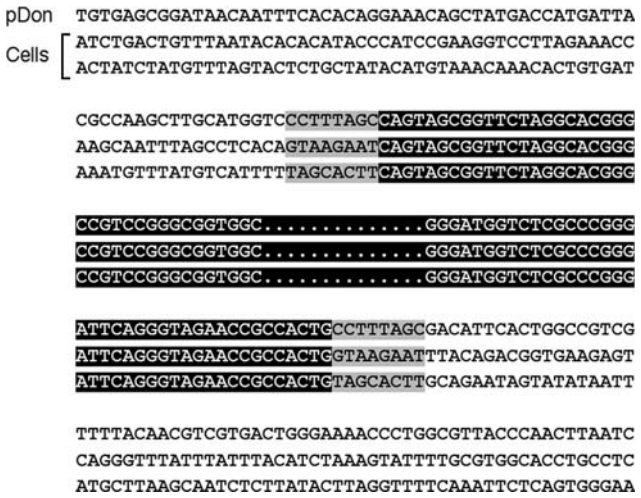

Fig. 4 Nucleotide sequences around insertion sites. Genomic DNA was extracted from two neomycin-resistant cell clones and digested with *Hin*dIII, whose cutting site is not present inside the *To1l* portion of the donor plasmid. DNA fragments of approximately 10–30 kb in size were isolated from electrophoresis gels and ligated with T4 DNA ligase. Inverse polymerase chain reaction (PCR) was performed on the ligated DNA using primers representing parts of the *Tol1* arms (nt 130–101 and nt 1758–1787 of D42062). PCR conditions were as follows: [94°C, 2 min], 36 × [94°C, 20 s; 64°C, 20 s; 72°C, 5 min], and [72°C, 5 min]. The PCR products were cloned into plasmids and sequenced. Nucleotide sequences around the insertion sites of these genomic clones are shown. The corresponding donor plasmid sequence is also shown at the top as reference. The *black*- and *gray*-shaded regions are *Tol1* arms and target side duplications (TSDs), respectively

Discussion

We constructed a basic *Tol1* vector consisting of 157 bp left- and 106 bp right-terminal regions, which is transposed as efficiently as the original 1,855-bp element. Thus, we were successful in removing an internal 1,592-bp region. This should be helpful in creating more room for cargo and in eliminating possible signals and signal-like sequences that might interact with cargo DNA or the host cell.

Although it might be possible to further shorten the arms of the vector by additional fine analyses, we planned to retain some extra arm regions in the vector because the regions could be used as a tag in DNA analyses after insertion. In many cases, the first step of these analyses is cloning of flanking chromosomal regions, and the principal method for this would be inverse PCR, in which parts of the arms are used for primer regions. Thus, we carefully left nucleotide blocks that can be used for PCR primers. Arm lengths (157 and 106 bp) were decided upon based on this reasoning.

We conducted transposition assays both with a functional helper plasmid and a defective helper plasmid. The numbers of surviving colonies with the defective helper were almost the same among the five donor plasmids. This result indicates that the effects of the plasmid size on the DNA intake efficiency of lipofection could be eliminated sufficiently in our assay system.

Our analysis of transformant cell colonies indicated that the entire *Tol1* element had been integrated into the chromosomes by transposition. The integration frequency was significantly higher than that by random integration, even in the case of the longest *Tol1* element (pDon263Mcs-NeoE20). The size of the *Tol1* portion of this donor plasmid was 22.1 kb, of which 0.3 kb was occupied by the *Tol1* arms. Thus, our basic plasmid pDon263Mcs can deliver a DNA fragment of up to 21.8 kb into the chromosomes, at least in the culture cells we used. It is also a significant finding that the DNA fragment delivered did not suffer internal deletion or aberration.

The *Sleeping Beauty* element is known to lose efficiency of transposition when the element size exceeds 9.1 kb (Karsi et al. 2001). The *piggyBac* element has been shown to serve as a gene-transfer vector when the entire size is 14.3 kb (Ding et al. 2005). In the case of the *Tol2* element, the maximum element size so far reported is 10.2 kb (Balciunas et al. 2006). It is possible that the latter two elements retain transposition activity when they are longer than the reported sizes. At present, the element size of 22.1 kb that we have shown with *Tol1* in this study is the largest ever demonstrated with DNA-based elements currently used for mammals. Furthermore, the length of the *Tol1* arms in our basic plasmid is as short as 0.3 kb. Thus, *Tol1* is a useful gene-transfer vector for delivering large DNA fragments into chromosomes of mammals.

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