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Homogeneity in the structure of the medaka fish transposable element *Tol2*

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Summary

The *hAT* family is a group of transposable elements of the terminal inverted repeat class, which includes *Ac* of maize, *hobo* of *Drosophila* and *Tam3* of *Antirrhinum* (snapdragon). All the members of this family so far examined are known to comprise complete and defective copies, with a good correspondence to autonomous and non-autonomous elements, respectively. Internal deletion is the most common cause of defective copies. *Tol2*, a transposable element of the medaka fish *Oryzias latipes*, is a member of the *hAT* family. We examined, mainly by the genomic Southern blot analysis, variation in the structure of copies of this element, and revealed that there are few or no internally deleted copies. This situation is unusual in a member of the *hAT* family. Possible causes of this anomaly are discussed.

1. Introduction

Transposable elements are repetitive sequences capable of changing their location in the genome of their host organism. The *hAT* family (Atkinson *et al*., 1993) is a group of transposable elements of the terminal inverted repeat class, which includes *Ac* of maize (McClintock, 1948; Fedoroff *et al*., 1983), *hobo* of *Drosophila* (McGinnis *et al*., 1983) and *Tam3* of *Antirrhinum* (snapdragon) (Sommer *et al*., 1985). On the basis of their amino acid sequence similarities these elements and other members of the family are proposed to have diverged from a common ancestor (Calvi *et al*., 1991; Atkinson *et al*., 1993; Warren *et al*., 1994). All the members of the *hAT* family for which several copies have been characterized at the molecular level are known to comprise complete and defective copies. Complete copies exhibit a specific length that is often called the 'full length', and defective copies vary in size but are usually shorter. Comparisons of nucleotide sequences between complete and defective copies have supported the view that most defective copies are generated by internal deletion of complete copies. There is a good correspondence of complete and defective copies to autonomous and non-autonomous elements, respectively (cf. Berg & Howe, 1989).

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We have recently identified the *Tol2* element of the medaka fish *Oryzias latipes* (Koga *et al*., 1996), present as 10–30 copies in its genome. The only *Tol2* copy for which the entire nucleotide sequence has been determined is *Tol2-tyr*, a copy inserted in the tyrosinase gene and consequently responsible for an albino mutation. *Tol2-tyr* is 4681 bp in length, carries terminal inverted repeats of 17 and 19 bp, and is flanked by an 8 bp target site duplication. It contains four open reading frames (ORFs), for 117, 352, 102 and 118 amino acids, which have amino acid sequence similarity with members of the *hAT* family. Footprinting analysis of *Tol2-tyr* showed that excision occurs, indicating that an autonomous member is present somewhere in the genome (Koga *et al*., 1996).

In an attempt to identify an autonomous *Tol2* element, we have studied structural variation of *Tol2* copies, in the expectation of finding a longer copy or confirming that *Tol2-tyr* is the longest. If *Tol2* copies of different lengths do exist, the longest copy should be taken as a candidate for an autonomous element. The results indicated that most, if not all, *Tol2* copies were identical in length and restriction map structure.

In this paper we describe the results of our genomic Southern blot analysis and DNA sequencing indicating a high homogeneity among *Tol2* copies that is unusual for a member of the *hAT* family.

2. Materials and methods

(i) *Medaka fish*

To provide random samples from a natural population, fish were collected in a suburb of Nagoya, Japan in 1997, and 12 males were randomly chosen. They were designated N-1 to N-12. The inbred strain used was HO5, a laboratory strain inbred for 70 generations (cf. Hyodo-Taguchi, 1996; *Medakafish* home page at http//biol1.bio.nagoya-u.ac.jp:8000/). Six males of this strain, denoted I-1 to I-6, were used.

The medaka fish demonstrates geographical variation, and according to data on isozyme frequencies (Sakaizumi, 1986) there are four regional populations: (1) Northern Japan, (2) Southern Japan, (3) Eastern Korea and (4) China and Western Korea. The random samples and the inbred strain described above both belong to the Southern Japan population.

(ii) *Clone of* Tol2-tyr

Tol2-tyr is a particular *Tol2* copy inserted in the tyrosinase gene of the i^4 albino mutant fish (Koga *et al*., 1996). This element is 4681 bp in length (GenBank accession no. D54109). p*Tol2-tyr* is a clone of a genomic DNA fragment containing the *Tol2-tyr* element and its flanking chromosomal regions. The chromosomal regions correspond to nucleotides 5720–7653 of the sequence of the tyrosinase gene (GenBank accession no. AB010101). The 8 bp target site duplication for *Tol2-tyr* comprises nucleotides 7589–7596.

(iii) *Analysis of genomic DNA*

Southern blotting and subsequent hybridization experiments were performed as described by Inagaki *et al*. (1994). Briefly, high-molecular-weight DNAs (5 μg for each gel slot) were digested with restriction enzymes, fractionated on agarose gels and transferred to nylon membranes. The membranes were then hybridized with ^{32}P-labelled probes. Hybridization conditions were 6× SSPE, 0·5% SDS, 50 μg/ml fragmented salmon sperm DNA and 5 ng/ml probe DNA at 65 °C for 16 h.

(iv) *Hybridization probes*

The entire *Tol2-tyr* element was amplified from the p*Tol2-tyr* clone by polymerase chain reaction (PCR) under the conditions described in Section 2(vi) below, and subsequently cloned into the pBluescript vector. Parts of the *Tol2-tyr* element generated by restriction enzyme digestion of the PCR product were also cloned into the vector. These clones were used as probes for hybridization experiments after removal of their vector portions. The regions and the designations of the probes are shown in Fig. 1.

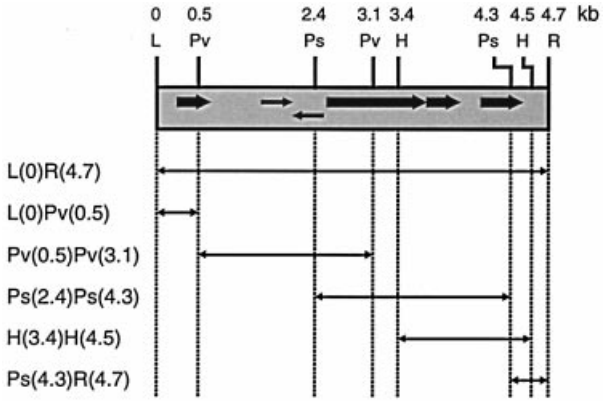

Fig. 1. Probes used for Southern blot analysis. The stippled box is *Tol2-tyr*. The thick arrows in the box indicate the spans and directions of the four open reading frames (ORFs). The thin arrows are internal inverted repeats of 302 bp and 303 bp. Restriction enzyme cutting sites (H, *Hin*dIII; Ps, *Pst*I; Pv, *Pvu*II) and the left and the right ends of the element (indicated by the letters 'L' and 'R') are shown with their locations in kilobase pairs. Each probe was given a designation that indicates its covered region. For example, probe Pv(0·5)Pv(3·1) is the 2·6 kb region between the *Pvu*II site at position 0·5 kb and the *Pvu*II site at position 3·1 kb.

(v) *Genomic library for cloning of* Tol2 *copies*

The medaka fish genomic library described by Koga *et al*. (1995) was used for cloning of *Tol2*-carrying phage clones. The library consists of recombinant phages containing partially *Sau*3AI-digested medaka fish genomic DNAs between the arms of the lambda DASH II phage. The fish used as the source of genomic DNA originated from the Southern Japan population.

(vi) *Cloning, sequencing and the PCR*

The library was screened for *Tol2*-carrying phage clones under the same conditions as those employed for the Southern blot analysis. Probe L(0)R(4·7) (see Fig. 1) was employed.

DNA sequencing was performed using the Model 4000 DNA sequencer of LI-COR. In most instances, both strands were sequenced. Ambiguity was excluded by sequencing with dGTP analogues.

PCR amplification was conducted with a pair of primers covering 28 nucleotides from the ends of *Tol2-tyr* (5′-CAGAGGTGTAAAGTACTTGAGTAATTTT-3′ and 5′-CAGAGGTGTAAAAAGTACTCAAAAATTT-3′). The conditions were: [2 min at 94 °C] an 25 cycles of [20 s at 94 °C, 20 s at 60 °C, 6 min at 72 °C].

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3. Results

(i) *Number of* Tol2 *copies*

Fig. 2 illustrates autodiagrams of Southern blots for estimation of the numbers of *Tol2* copies in single fishes. Genomic DNAs of fish of the inbred strain (I-1 to I-6) and random samples from a natural population (N-1 to N-12) were digested with *Pvu*II, and hybridized to probe L(0)Pv(0·5). As shown in Section 3(iv) below, all or most *Tol2* copies were found to carry a *Pvu*II site, corresponding to position 0·5 kb on the *Tol2-tyr* nucleotide sequence. Probe L(0)Pv(0·5) contains only the left-side region. Therefore, in this blot, each band represents a single 'heterozygous' *Tol2* copy or two 'homozygous' copies unless two copies located closely to each other are included in a single restriction fragment. 'Heterozygous' means that an element is present on one chromosome and is not present at the same location of its homologous chromosome. 'Homozygous' means that an element is also present at the same location of the homologous chromosome. All the fish of the inbred strain (I-1 to I-6) exhibited an identical restriction pattern consisting of 13 bands (Fig. 2*A*). Therefore, these fish contain a minimum of 13 pairs of homozygous *Tol2* copies at the same chromosomal locations. In the samples from the natural population (N-1 to N-12), the numbers and the sizes of bands differed among the fish (Fig. 2*B*), with numbers distributed between 11 and 30. It was impossible to determine which bands were homozygous and which were heterozygous. However, the fact that the band sizes differ from fish to fish, in sharp contrast to the results for the inbred strain, suggests that heterozygous bands are predominant and therefore the numbers of bands roughly represent the numbers of *Tol2* copies per diploid genome.

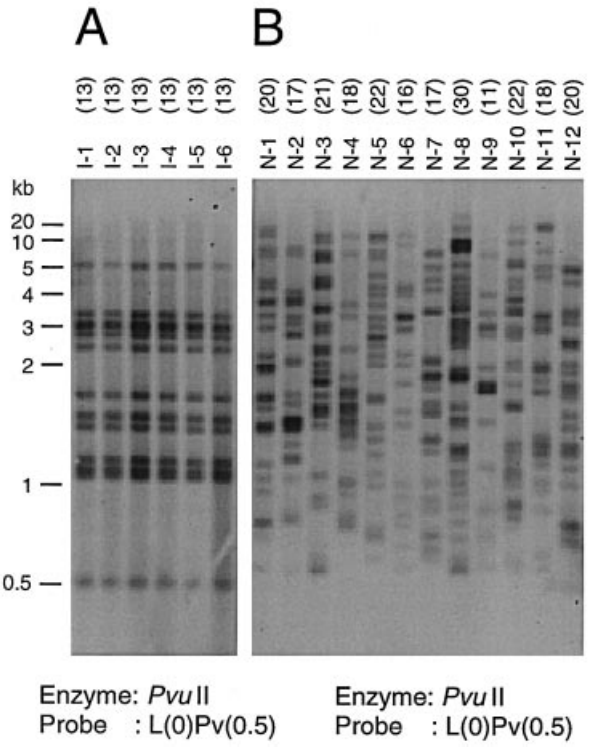

Fig. 2. Southern blot hybridization to examine the numbers of *Tol2* copies. Genomic DNAs were digested with *Pvu*II, electrophoresed on 1·0% agarose gels, transferred to nylon membranes and then hybridized with probe L(0)Pv(0·5). The numbers shown in parentheses are the numbers of bands obtained by scanning the hybridization membranes along the lanes with a radioactivity scanner and by counting radioactivity peaks. The sizes and mobilities of the size marker DNA fragments are indicated along the left margin. (*A*) Results of the six fish of the inbred strain (I-1 to I-6). (*B*) Results for the 12 fish collected from the natural population (N-1 to N-12).

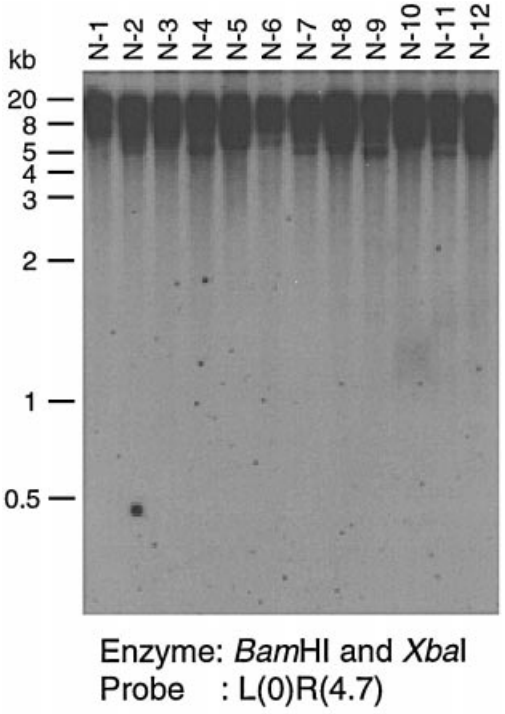

Fig. 3. Southern blot hybridization to examine the lengths of *Tol2* copies. Genomic DNAs of the 12 fish from the natural population (N-1 to N-12) were digested with *Bam*HI and *Xba*I, which do not have cutting sites in the *Tol2-tyr* sequence. The digested DNAs were electrophoresed on a 1·2% agarose gel, transferred to a nylon membrane and then hybridized with probe L(0)R(4·7).

(ii) *Length of* Tol2 *copies*

Probe L(0)R(4·7) represents the entire, 4·7 kb *Tol2-tyr* element. This probe was used for hybridization to genomic DNAs of the 12 fish from the natural population (Fig. 3). Agarose gels of a relatively high concentration (1·2%) were used to make the analysis sensitive for small-sized (less than 4·7 kb) bands.

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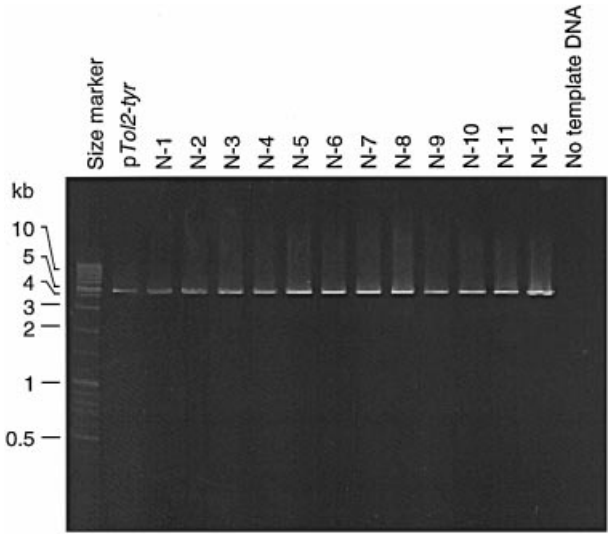

Fig. 4. PCR products from the *Tol2-tyr* clone and genomic DNAs. The template DNAs, indicated above the lanes, were: 10 pg of the p*Tol2-tyr* clone, 100 ng of fish genomic DNAs (N-1 to N-12), and no template DNA. Reaction mixtures were electrophoresed on a 1·0% agarose gel and stained with ethidium bromide.

Genomic DNAs were digested with *Bam*HI and *Xba*I, which do not have cutting sites in the *Tol2-tyr* sequence. As shown in the autodiagram, all fish exhibited multiple hybridization bands and, although the bands could not be distinguished from one another, they were all more than 4·7 kb in size. This result suggests that there are no *Tol2* copies with sizes less than 4·7 kb.

PCR amplification of *Tol2* copies from genomic DNAs was performed using a pair of primers that represent the termini of *Tol2-tyr* (Fig. 4). Products from the fish DNAs (N-1 to N-12) gave single bands of 4·7 kb. This result provides further evidence against *Tol2* copies of sizes other than that of *Tol2-tyr* (4·7 kb), especially shorter copies.

(iii) *Organization of* Tol2 *copies*

Genomic DNAs digested with *Bam*HI and *Xba*I were electrophoresed on gels of a lower concentration (0·7%) to magnify the areas for restriction fragments larger than 4·7 kb. Three membranes were prepared and hybridized separately to three probes: L(0)Pv(0·5), Ps(2·4)Ps(4·3) and Ps(4·3)R(4·7). The hybridization band patterns obtained (Fig. 5) were found to be similar. No difference in band patterns was detected by naked eye comparison, indicating that most, and possibly all, *Tol2* copies carried by the 12 fish of the natural population (N-1 to N-12) contain all the three probed regions.

(iv) *Restriction map variation among* Tol2 *copies*

Probe Pv(0·5)Pv(3·1) was used for hybridization to *Pvu*II-digested genomic DNAs of the 12 fish N-1 to N-12 (Fig. 6*A*). All the fish showed single bands of 2·6 kb and no bands of different sizes, indicating that every *Tol2* copy which generates a hybridization signal carries two *Pvu*II sites and, in addition, that the distance between the two sites is 2·6 kb. When probe Ps(2·4)Ps(4·3) was used for hybridization to *Pst*I-digested DNAs (Fig. 6*B*), single bands of 1·9 kb were observed for all the fish. Probe H(3·4)H(4·5) was hybridized to *Hin*dIII-digested DNAs (Fig. 6*C*), resulting in single bands, all 1·1 kb in size, for each fish.

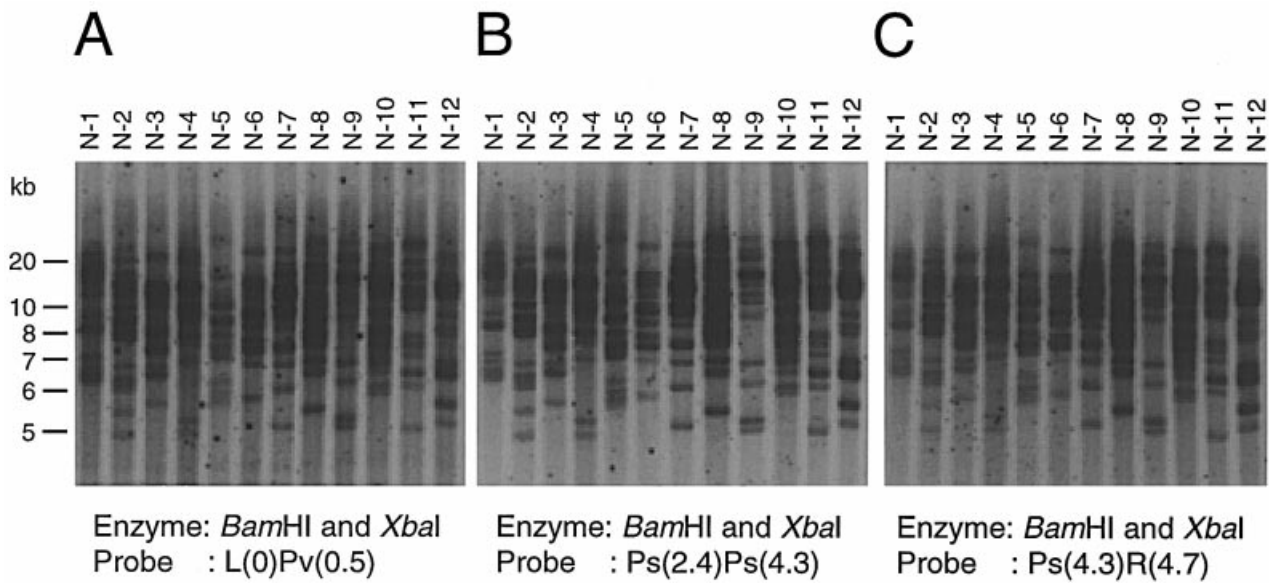

Fig. 5.(*A*)–(*C*). Southern blot hybridization to examine the organization of *Tol2* copies. Genomic DNAs from fish N-1 to N-12 were digested with *Bam*HI and *Xba*I, electrophoresed on 0·7% agarose gels, transferred to nylon membranes and then hybridized with the probes indicated under each panel.

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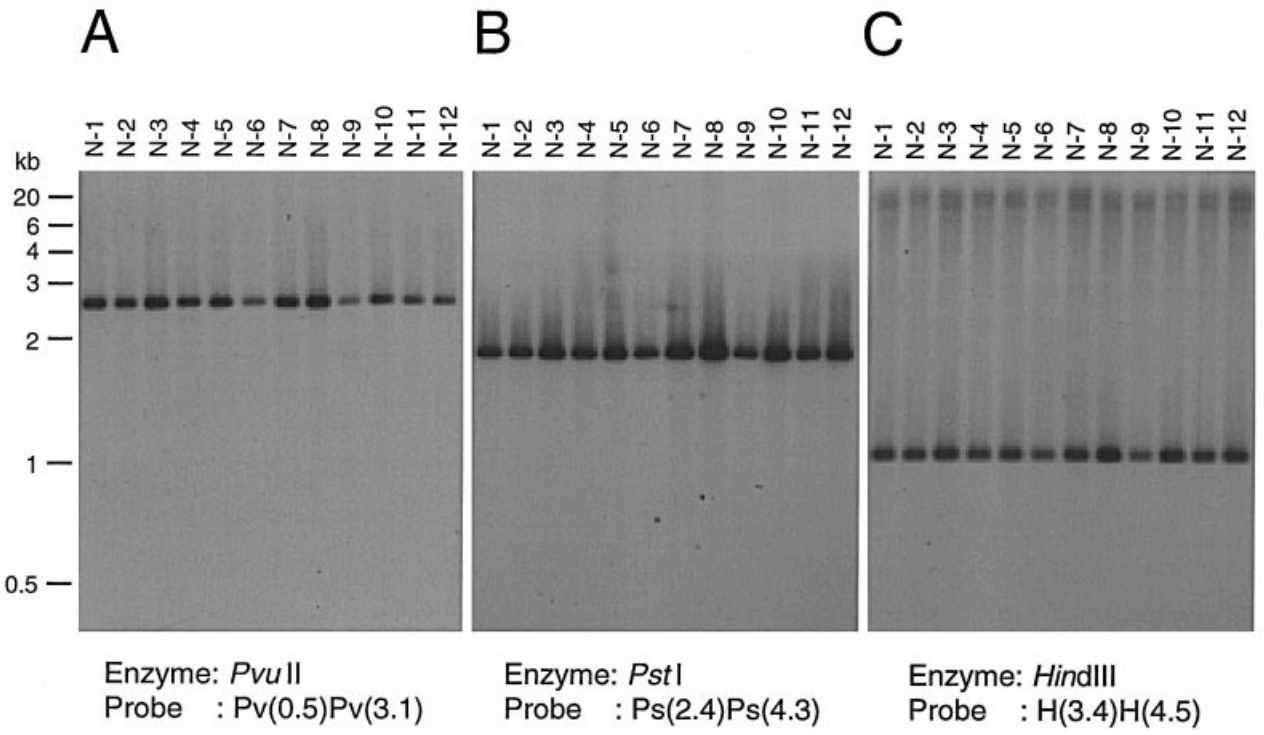

Fig. 6.(*A*)–(*C*). Southern blot hybridization for examination of restriction patterns of *Tol2* copies in the genomic DNAs from fish N-1 to N-12. Agarose gels of a 1·0% concentration were used. Restriction enzymes and probes are indicated under each panel.

As mentioned above, the bands of a single size demonstrated with probe Pv(0·5)Pv(3·1) indicate that every *Tol2* copy which generates a hybridization band carries two *Pvu*II sites, separated by 2·6 kb. However, it could not be ruled out that there are copies in which the probed region is totally deleted. Such a deletion, if present, would produce a band of different size in blots with probe Ps(2·4)Ps(4·3) unless this is also spanned by the deletion, the two probes themselves overlapping. With the same logic, the deletion also includes the region for probe H(3·4)H(4·5), because probe Ps(2·4)Ps(4·3) overlaps with probe H(3·4)H(4·5). Thus, deletions consistent with the results of the three blots (Fig. 6) would have to include all the probed regions (position 0·5 kb to position 4·5 kb on the *Tol2-tyr* sequence). Such deletions would be expected to be infrequent because all, or most, *Tol2* copies demonstrated the *Tol2* terminal regions [positive with probes L(0)Pv(0·5) and Ps(4·3)R(4·7)] together with the internal region [probe Ps(2·4)Ps(4·3)] (see Section 3(iii) above).

(v) *Nucleotide sequence variation among* Tol2 *copies*

The results of genomic Southern blot analysis and PCR analysis revealed a homogeneity in the structure of the *Tol2* copies. We carried out further analysis of the structure of *Tol2* by examining *Tol2* copies cloned from a genomic library. Four clones (*Tol2*-Or1, -5, -9 and -10) were randomly chosen from 16 obtained by screening the library, and sequenced for their entire *Tol2* elements. All four clones exhibited 4681 bp sequences identical to the *Tol2-tyr* sequence (Fig. 7).

(vi) *Sequences of other regions*

Nucleotide sequences were also determined for at least 400 bp beyond the points corresponding to the *Tol2-tyr* ends (Fig. 7). Comparison by dot-matrix in all the possible combinations revealed no obvious lines (data not shown). It is likely that these regions originate not from *Tol2* elements but from medaka fish chromosomes.

Tol2-tyr is a *Tol2* copy inserted in the tyrosinase gene of an albino mutant fish. A target site duplication of 8 bp was revealed by comparing the sequence of the *Tol2*-carrying tyrosinase gene with the sequence of the wild-type tyrosinase gene (Koga *et al.*, 1996). At exactly the same position as this duplication, there exists an 8 bp segment duplicated in each *Tol2*-carrying clone (Fig. 7). This finding, taken together with the observation that sequences differ from one another in and beyond these regions, indicates that each clone contains a 4681 bp *Tol2* element and its flanking chromosomal regions.

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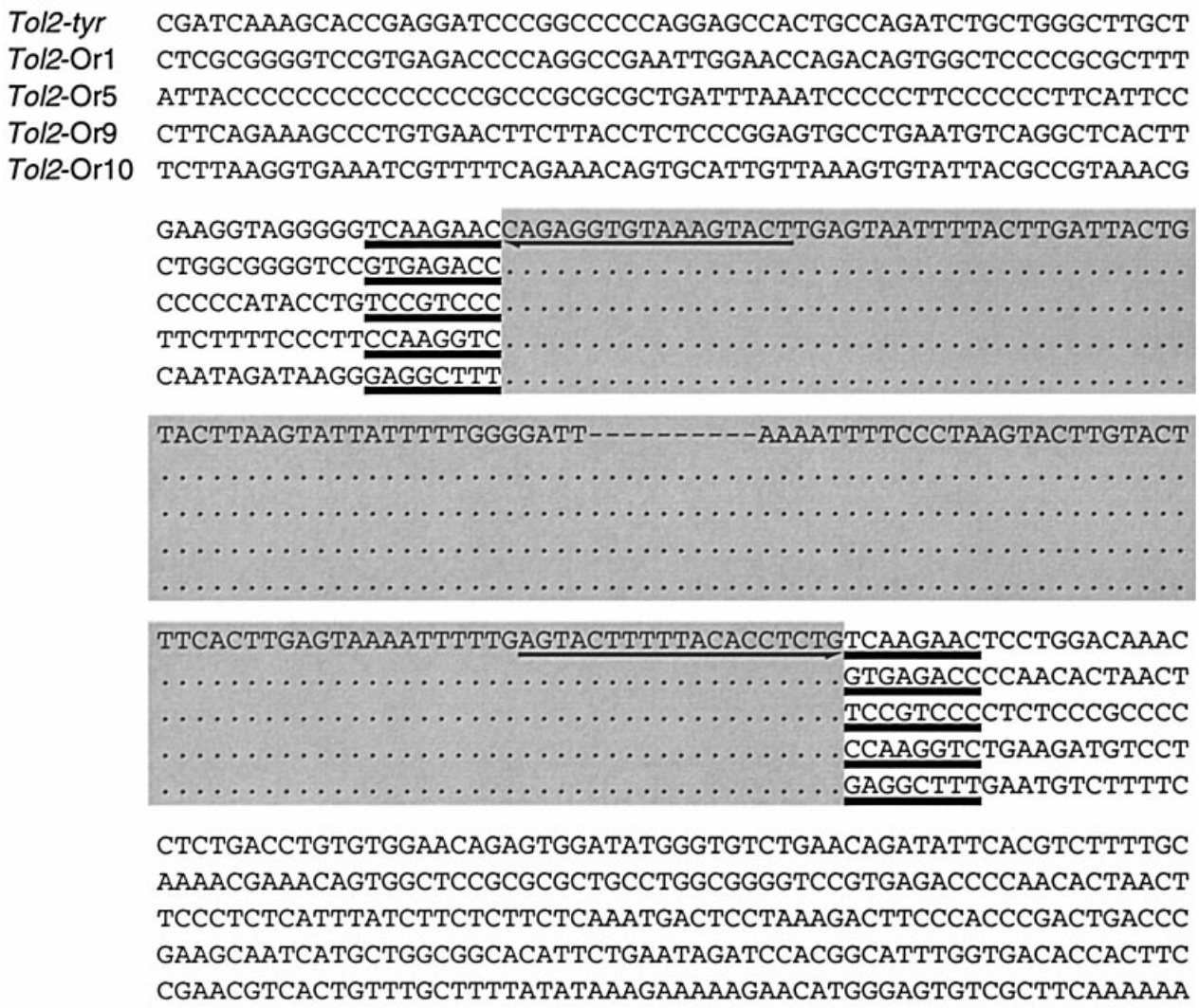

Fig. 7. Comparison of nucleotide sequences of *Tol2* clones. Four *Tol2*-carrying genomic clones (*Tol2*-Or1, -5, -9 and -10) were sequenced for their entire *Tol2* elements and *Tol2*-flanking chromosomal regions. The sequences were compared with that of the *Tol2-tyr* clone. Regions inside the *Tol2* copies are indicated by shading. The dashed line is for the internal region of *Tol2-tyr*. The arrows indicate the terminal inverted repeats of 17 bp and 19 bp. The separate thickly underlined sequences are the 8 bp target site duplications. The dots stand for nucleotide residues that are identical to those in *Tol2-tyr*.

4. Discussion

(i) *Structure of* Tol2 *copies*

The present examination of the structures of more than 200 *Tol2* copies carried by 12 fish randomly chosen from a natural population revealed identity in all cases. Thus the genomic Southern blot results indicate that few, if any, of the *Tol2* copies have an internal deletion. Further support for this was provided by the PCR finding of no *Tol2* copies of different lengths. However, it cannot be excluded that copies longer than *Tol2-tyr*, with additional sequences beyond the regions corresponding to the *Tol2-tyr* termini, might exist. Similarly, copies that lack their ends would not be detected. Such elements could only be infrequent, however, because the sequencing analysis demonstrated all the cloned *Tol2* copies to have ends at the same positions as for *Tol2-tyr*.

(ii) *Heterogeneity in size is common in* hAT *family elements*

Transposable elements of the *hAT* family are widespread in plants, fungi and insects. In members of the family for which several copies have been characterized at the molecular level, heterogeneity in size is common. It is mainly due to the co-presence of defective copies of smaller lengths. For example, Fedoroff *et al*. (1983) cloned and characterized three *Ac* elements inserted separately in the *Wx* locus of maize and showed that they are 4·3 kb, 4·1 kb and 2·0 kb, the last two being internally deleted copies of

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the first. The authors also showed that sequences with homology to the central portion of the longest copy are much less prevalent in the maize genomes they analyzed than are sequences homologous to the ends of the element. Streck *et al*. (1986) analyzed nine clones of the *Drosophila hobo* element, showing three to be full length (3·0 kb) and others to lack internal regions. Warren *et al*. (1994) reported, after an analysis equivalent to that performed for Fig. 4 in the present study, the presence of defective copies together with full-length (2·7 kg) copies of the *Hermes* element of the house fly. Besides members of the *hAT* family, the *P* element of *Drosophila* has been extensively studied for variation in size and structure. Nitasaka & Yamazaki (1994) cloned and restriction mapped a total of 99 copies of the *P* element. Of the clones, 21 were full-length (2·9 kb) elements and the rest were shorter elements due to internal deletions.

(iii) *Inferences from the homogeneity of* Tol2

Tol2 is highly homogeneous in structure, which is unusual for a member of the *hAT* family. We would like to propose the following three hypotheses regarding the cause of this situation. They are not mutually exclusive.

The first is that *Tol2* lacks factors that are involved, in other *hAT* family members, in the generation of internal deletions. For example, Streck *et al*. (1986) proposed a model for internal deletion of a *hobo* element. As the first step in their model, one *hobo* element suffered insertion of another *hobo* element at a target sequence present in the first element. In the second step, homologous recombination between an internal region of the first element and a terminal region of the second element removed the entire second element and part of the first element, generating an internally deleted copy of the first element. In this model, the target sequence and the homologous regions between the two elements are factors necessary for completion of the internal deletion. Some such factors are also required in other models, such as short direct repeats inside the *P* element in the model proposed by Engels (1989).

The second hypothesis is that internal deletion occurs but its products are less efficiently amplified in the genome. Non-autonomous elements can transpose as long as they retain all the factors on which autonomous elements or their products act. In the cases of *Ac* and *hobo*, such factors appear to be present in their terminal regions because elements lacking internal regions can transpose (Fedoroff *et al*., 1983; O'Brochta *et al*., 1994). *Tol2* may carry such factors throughout the element.

The last hypothesis rests on a viewpoint of evolution. It may be that the time span after the *Tol2* invasion of the medaka fish is not sufficiently long for internally deleted copies to have become prevalent. Lohe *et al*. (1995) studied the evolutionary process of the *mariner* element in insect species and proposed that mutational inactivation plays an important role: autonomous elements are inactivated by mutation and transformed into non-autonomous elements, which are relatively favoured by natural selection because of their less harmful effects on the host. In the *hAT* family members, internal deletion may be the predominant form of mutational inactivation and the prevalence of non-autonomous elements may be a result of natural selection. It should be noted here that this kind of natural selection is a response of the host to deleterious effects of transposition events and, therefore, it should occur with a delay. The homogeneity in the structure of *Tol2* elements described in this paper may thus reflect a relatively short interval between *Tol2* invasion of the medaka fish and the appearance of evolutionary change.

(iv) *Autonomy of* Tol2

The original purpose of our survey was to obtain a candidate for an autonomous element. If there exist *Tol2* copies of different lengths, the longest copy can be taken as a candidate. From the results indicating that the *Tol2* element is highly homogeneous, an inference about the autonomy of the *Tol2* element is possible: all, or most, of the *Tol2* copies are autonomous. We have already obtained evidence supporting this view. We identified and sequenced a *Tol2*-specific cDNA, and found it to consist of four exons, which represent the four ORFs in the *Tol2-tyr* sequence (M. Suzuki, A. Koga & H. Hori, unpublished data). This result suggests that the *Tol2-tyr* copy, as well as other copies, carries a functional gene that is interrupted by introns and not by in-frame translational stop codons or frameshifting mutations. The autonomy of the *Tol2-tyr* copy may be demonstrated by introducing its clone into *Tol2*-free fish genomes and detecting a new excision or insertion event. Unfortunately, all the samples from the four medaka fish regional populations (see Section 2(i)) that we have checked contained the *Tol2* element in similar copy numbers (unpublished data). However, *O. luzonensis* (a species closely related to *O. latipes*) and the zebrafish *Danio rerio* do not contain *Tol2*-hybridizing sequences (unpublished data). These species may thus be useful as recipients for the *Tol2-tyr* clone.

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