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Frequent transpositions of *Drosophila melanogaster* HeT-A transposable elements to receding chromosome ends

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HeT-A elements are a new family of transposable elements in *Drosophila* that are found exclusively in telomeric regions and in the pericentric heterochromatin. Transposition of these elements onto broken chromosome ends has been implicated in chromosome healing. To monitor the fate of HeT-A elements that had attached to broken ends of the X chromosome, we examined individual X chromosomes from a defined population over a period of 17 generations. The ends of the X chromosomes with new HeT-A additions receded at the same rate as the broken ends before the HeT-A elements attached. In addition, some chromosomes, ~1% per generation, had acquired new HeT-A sequences of an average of 6 kb at their ends with oligo(A) tails at the junctions. Thus, the rate of addition of new material per generation matches the observed rate of terminal loss (70-75 bp) caused by incomplete replication at the end of the DNA molecule. One such recently transposed HeT-A element which is at least 12 kb in length has been examined in detail. It contains a single open reading frame of 2.8 kb which codes for a gag-like protein.

Key words: chromosome healing/*Drosophila*/receding chromosome ends/telomere/transposon

Introduction

Telomeres are structures at the ends of linear eukaryotic chromosomes that serve a number of cellular functions, at least two of which are vital. The vital functions are to protect the chromosome ends from fusion and degradation (McClintock, 1938, 1939; Muller, 1940; Muller and Herskowitz, 1954), and to compensate for the inability of DNA polymerases to replicate the end of a linear chromosome completely (Watson, 1972). In *Drosophila* it has been possible to separate these two functions. A mutator has been identified that interferes with repair of radiation-induced chromosome breaks and thus potentiates the recovery of broken chromosomes that are missing a telomeric region (Mason *et al.*, 1984; J.M.Mason and L.E.Champion, unpublished). These broken chromosomes can be maintained for many generations, indicating that they are protected from fusion and degradation, but the chromosome ends recede at a rate consistent with the loss due to incomplete replication (Biessmann and Mason, 1988; Biessmann *et al.*, 1990a). Similar results were obtained with a chromosome break in the telomeric region of chromosome 3R induced by P element transposition (Levis, 1989).

Chromosomes in several eukaryotic phyla terminate in arrays of short tandem repeats (reviewed by Zakian, 1989; Biessmann and Mason, 1992). These terminal repeats have been implicated both in the stability of chromosomes (Lundblad and Szostak, 1988; Yu *et al.*, 1990) and in compensating for the loss due to incomplete replication (Blackburn, 1991). To date, however, such telomeric repeats have not been identified in *Drosophila*. Hybridization experiments with terminal repeats from other organisms that usually exhibit inter-species cross-reactivity or with various oligonucleotide probes have failed to provide evidence for such telomeric repeats in *Drosophila* (Richards and Ausubel, 1988; Meyne *et al.*, 1989). These negative results can be explained in two ways: either *Drosophila* has a very different telomeric structure, suggesting a different mechanism of telomeric formation and maintenance; or the difference between *Drosophila* and other organisms is quantitative rather than qualitative; i.e. *Drosophila* simply may have fewer telomeric repeats and/or these repeats may be more heterogeneous in sequence.

We have recently identified a new family of transposable elements in *Drosophila*, the HeT-A elements, which resemble polyadenylated, non-LTR retroposons (Biessmann *et al.*, 1990b, 1992) and which occur exclusively in the telomeric regions of all chromosomes and in the pericentric heterochromatin (Young *et al.*, 1983). However, the cytological restriction of HeT-A elements to heterochromatic regions is not absolute because, under special circumstances, they can also become attached to broken chromosome ends in a euchromatic region, e.g. to the *yellow* (*y*) gene, as occurred in the stocks *Df(1)RT394* and *Df(1)RT473* (Biessmann *et al.*, 1990b), or to the newly generated ends of a spontaneously opened ring-X chromosome (Traverse and Pardue, 1988). It is conceivable that the attachment of a HeT-A element to a broken chromosome end is one step in generating a new telomeric structure because, once a HeT-A element is present at the chromosome end, it may undergo secondary reactions such as homologous recombination with other telomeric HeT-A elements or bind specific proteins which are usually associated with HeT-A elements in telomeric positions.

We report here on the fate of previously transposed HeT-A elements at the tip of the X chromosome and describe the high frequency of new HeT-A element transpositions to receding chromosome ends. Sequence data of a newly transposed member of the HeT-A family indicate that this element encodes a 918 amino acid gag-like protein with three zinc finger motifs, but not a reverse transcriptase. The results suggest that HeT-A element transposition to receding chromosome ends may play an important role in healing of broken chromosome ends and in normal *Drosophila* telomere maintenance and elongation.

Results

Progressive shortening of terminal DNA fragments

In order to study the fate of HeT-A elements which had attached previously to the broken end of the X chromosome at the *y* gene in the stocks *Df(1)RT394* (transposition occurred between 1983 and 1984) and *Df(1)RT473* (transposition occurred between 1984 and 1986), we designed a mating scheme (Figure 1) to monitor individual X chromosomes with their attached HeT-A elements in a defined population over a period of 17 generations (34 weeks). The rationale for doing this was based on an earlier observation that the terminal HeT-A element in *RT473* had undergone frequent changes in length in a population (Biessmann *et al.*, 1990b). To establish the extent of heterogeneity in the populations at the beginning of the experiment, 15 males were taken from the *RT394* and *RT473* stocks, mated to *Df(1)y ac* females, and their daughters were collected for DNA analysis. At the same time, four individual males were taken from the same stock to establish new sublines (termed A, B, C and D), each with a defined but distinct length of HeT-A element sequence at the end of the X chromosome. Males in each line were mated to attached-X females, and every fourth generation six single males were picked at random from each of the sublines and individually mated to a harem of *Df(1)y ac* virgin females for 3–7 days. Female offspring were collected for DNA preparation and further analysis.

Previous restriction enzyme analysis of the original HeT-A elements in *RT394* and *RT473* showed that the HeT-A DNA (~3 kb) present at the end of these X chromosomes in 1987 is not cleaved with *Nru*I (Biessmann *et al.*, 1990b). Using the 4.5 kb *Eco*RI fragment 14a3B from the wild type *y* gene region (Biessmann, 1985) as a hybridization probe which does not hybridize with the *Df(1)y ac* chromosome, the

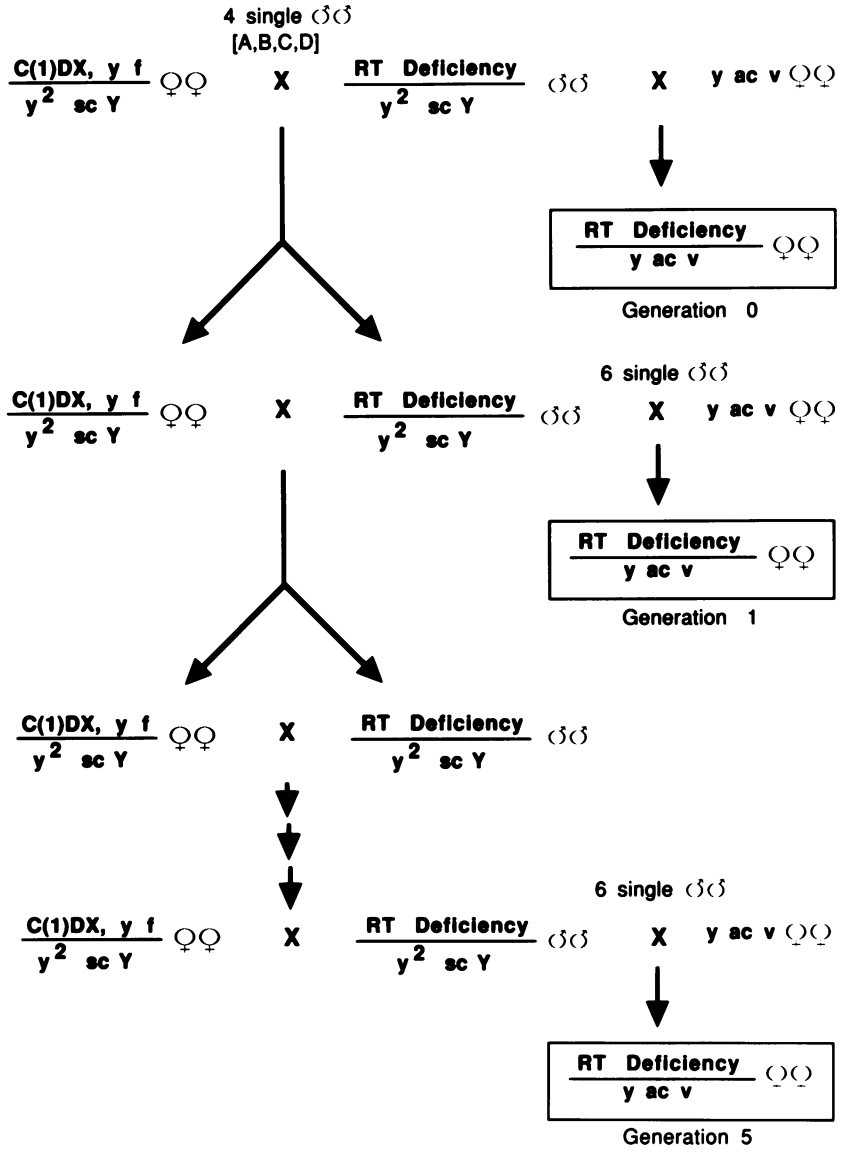

Fig. 1. Mating scheme used to maintain RT chromosome lines and sample the HeT-A elements on the X chromosomes in those lines over 17 generations. Two different chromosomes (*RT394* and *RT473*) were maintained independently. For each chromosome, four RT/y^2 *sc* Y males were mated individually with C(1)DX, *y* fly^2 *sc* Y females to establish four independent lines. At the same time, 15 males were mated *en masse* with homozygous Df(1)*y ac v* females to establish the extent of heterogeneity in the initial population. In subsequent generations the lines were maintained by mass transfers of adults to fresh food. In generations 1, 5, 9, 13 and 17, six males were picked at random and mated individually with Df(1)*y ac v* females for up to 7 days, and RT/Df(1)*y ac v* daughters were collected and frozen for DNA preparation.

length of the terminal DNA fragment in the RT chromosomes can easily be determined in genomic Southern blots. As shown in Figure 2, the probe will hybridize to an internal 2.1 kb *Nru*I fragment that originates from within the *y* gene, and also to the terminal fragment of the RT chromosome. By sequencing the HeT-A element−*y* gene junctions we recently established (Biessmann *et al.*, 1990b) that the element in *RT394* is attached with its oligo(A) tail at position +80 of the *y* gene, and that the oligo(A) tail of the HeT-A element in *RT473* is attached at position +183 of the *y* gene. This was confirmed by sequence analysis presented below. Measuring the sizes of terminal fragments in the four sublines of *RT394* and *RT473* at the beginning of the experiment (generation 1, Figure 3), we found that ~450 bp of the original HeT-A element of *RT394* and ~700 bp of the original HeT-A element of *RT473* were still present in January 1990. Genomic Southern blots of *Nru*I cut DNA from generation 0 revealed (Figure 3, lane M) that the stock population of *RT394* was heterogeneous with respect to the terminal band, exhibiting at least two major size classes. The same was true for the *RT473* stocks (data not shown).

Genomic Southern blots of *Nru*I digested DNAs prepared

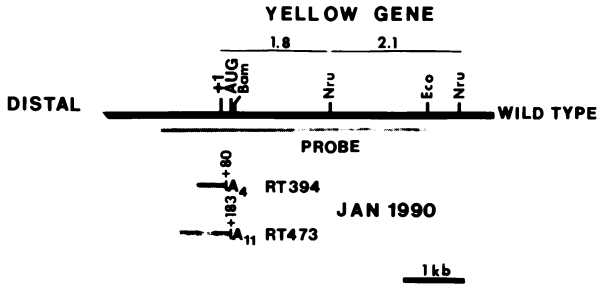

Fig. 2. Partial restriction map of the wild type *yellow* gene region and of the chromosomes *RT394* and *RT473* in which HeT-A elements have been attached to the ends of terminally deleted X chromosomes. As reference point we chose the beginning of transcription of the *y* gene (+1). Also indicated are the position of the AUG start codon (+172) and of the *Bam*HI site (+191) which resides in the first exon of the *y* gene. One *Nru*I site is at position +1835, and a second *Nru*I site at position +3933. In the *RT394* stock, a HeT-A element is attached with an oligo(A)$_4$ tail to position +80, and in *RT473* a different member of the HeT-A family is attached with an oligo(A)$_{11}$ tail to position +183. At the beginning of the experiment in January 1990, the HeT-A elements remaining at the tip of the X chromosome had lost all but the 3′-most sequences of ~450 bp (*RT394*) and ~700 bp (*RT473*). This makes the terminal *Nru*I fragment in the stocks appear 2.2 kb (*RT394*) and 2.3 kb (*RT473*) long.

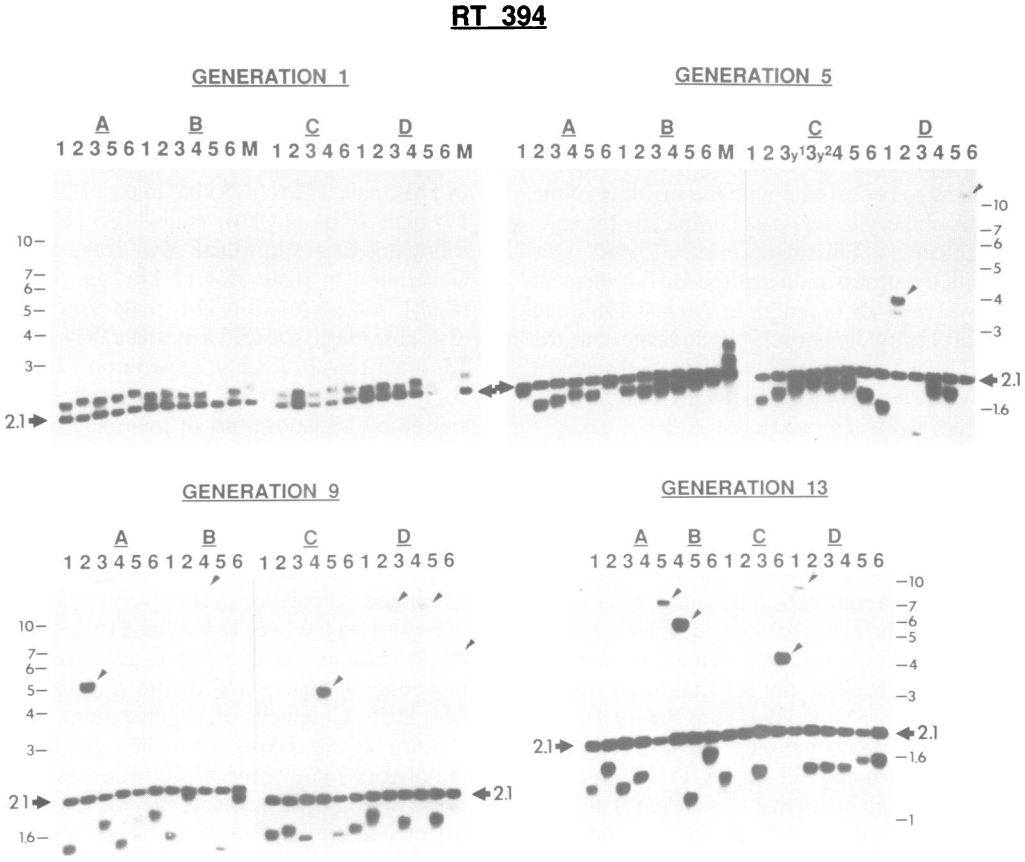

Fig. 3. Molecular events at the tip of the *RT394* X chromosome as monitored by genomic Southern blots. In order to study individual X chromosomes, each of the sublines (A, B, C and D) was started from a single male and kept through subsequent generations by crossing to attached-X females (see Figure 1 for the origin of the single males). In generations 1, 5, 9, 13 and 17 single males were taken from the sublines, crossed to *Df(1) y ac* females (this X chromosome does not hybridize to our DNA probe), and the daughters were collected for DNA preparation. Each lane represents DNA from daughters of a single male. DNA was cut to completion with *Nru*I, loaded on agarose gels, transferred and hybridized to the single copy *yellow* gene fragment 14a3B (see Figure 2 for map of the region, 14a3B is indicated as 'probe'). The 2.1 kb internal *Nru*I fragment in the *yellow* gene (see Figure 2) is indicated by arrows in each of the gels. The other band in each lane represents the terminal DNA fragment of each of the different X chromosomes in the offspring of the single males. These terminal fragments vary in size between X chromosomes from different males even in the first generation after the sublines have been established from single males. This size variation becomes more pronounced in later generations. Overall, the terminal DNA fragments become progressively shorter with every generation. In some of the males (e.g. 5D2, 5D6, 9A2, etc.) the terminal *Nru*I band has become drastically elongated (arrowheads) reflecting the addition of DNA to the chromosome end. Male 5C3 produced both y^1 and y^2 offspring which were tested separately. M: DNA from daughters of a mass mating of 15 males with *Df(1) y ac* females to demonstrate the heterogeneity in the original stock before establishing the sublines.

from the daughters of single males (*RT394/Df(1)y ac*) and hybridized to the *y* probe are shown in Figure 3. The 2.1 kb internal *Nru*I fragment from the *y* gene (see Figure 2) is indicated by an arrow in each of the blots. At the beginning of the experiment (generation 1) all four sublines (A, B, C and D) exhibited terminal *Nru*I fragments of ~2.2–2.3 kb measured from the distal-most *Nru*I site in the *y* gene. Comparing the sizes of terminal fragments in the offspring of single males that were taken from the population of the subline at generation 1 (e.g. A1, A2, A3, A5, A6), a fairly homogeneous distribution is observed, although a slight size variation is apparent.

It is obvious that the terminal DNA fragments become shorter in the subsequent generations and exhibit more variation in length. When the sizes of terminal *Nru*I fragments were determined from these blots and plotted against time in generations, it became apparent that the terminal ends of the remaining HeT-A elements in the *RT394* stock are receding at an average rate of 73 bp per generation. Terminal loss was determined separately for each of the four sublines. A, 84 bp/generation; B, 75 bp/generation; C, 75 bp/generation; D, 56 bp/generation (data not shown). The same was observed in *RT473* (not shown). This rate is the same as previously determined for receding chromosome ends of terminally deleted X chromosomes without HeT-A element attachments (Biessmann and Mason, 1988; Biessmann *et al.*, 1990a), and of a terminally deleted chromosome 3R (Levis, 1989). With the average number of rounds of DNA replication in the male germline we have calculated that this rate is consistent with the inability of the DNA replication machinery to replicate completely the ends of linear DNA molecules (Biessmann *et al.*, 1990a). Our results together with the observation that the HeT-A element in *RT394* which was ~3 kb in length in August 1987 had shortened to ~450 bp by January 1990, indicate that the newly attached terminal HeT-A element is apparently subject to the same rate of loss as chromosome breaks without such an element attached.

The phenotype of all sublines in generations 0 with ~450 bp of HeT-A element remaining attached to the *y* gene was the same as the original *RT394* stock, namely y^2-like (black bristles, yellow body). At the rate of terminal shortening, the chromosome end should recede into the first exon of the *y* gene by generation 6, thus causing a y^1-like, null phenotype (golden brown bristles and yellow body). However, such change in phenotype cannot be directly observed in the sublines because the RT males, as well as the attached-X females, carry a y^2 allele (see Figure 1). The phenotype of the RT chromosome only becomes apparent in the offspring after crossing RT males to *Df(1)y ac v* females. All of the 22 single males that were taken at generation 1 exhibited the y^2 phenotype. When 24 individual males were tested genetically and molecularly in generation 5, 21 exhibited the y^1-like phenotype, except A6 which produced *y* variegated females, C3 which produced both y^2-like and y^1-like offspring and was probably mosaic in the germline, and D6 which had acquired a longer terminal DNA fragment. We will discuss 5D6 below. In subsequent generations, all single males were y^1, except for those that had acquired new DNA at the end of their RT chromosome (see below) before it could recede into the *y* gene.

Transposition of HeT-A elements to receding chromosome ends

In addition to the receding ends of the HeT-A element, we observed several cases in which the terminal *Nru*I fragment has become substantially longer (Figure 3). This elongation does not occur gradually but seems to happen in a single step. We had observed such changes before in the stock population of *RT473* between August 1987 and August 1989 (Biessmann *et al.*, 1990b) but since, in that case, DNA was prepared from F1 females of mass matings, these genomic Southern blots only reflected the complexity of the population. By contrast, we have now followed events that happen at the receding chromosome ends under much more defined population conditions, so that each lane in Figure 3 represents DNA derived from daughters of a single male. Since we observe very little, if any, heterogeneity of these larger terminal DNA fragments derived from daughters of any single male we conclude that the event that led to its increased size must have occurred early enough to exclude heterogeneity in his germline. With some possible exceptions, however, the sizes of the new large terminal fragments do vary from one male to another, indicating that the elongation events occurred independently. They also occurred at least once in each of the four sublines. Sizes of the larger terminal *Nru*I fragments in *RT394* were as follows. Generation 5: male 5D2 (4 kb), male 5D6 (14 kb); generation 9: male 9A2 (5 kb), male 9B4 (14 kb), male 9C4 (5 kb), male 9D2 (14 kb), male 9D4 (14 kb), male 9D6 (7 kb); generation 13: male 13A5 (7.2 kb), male 13B4 (6 kb), male 13C6 (4.5 kb), male 13D1 (9 kb); generation 17: male 17B1 (14 kb), male 17B3 (14 kb). In *RT473* the following larger terminal *Nru*I fragments were detected. Generation 5: male 5C1 (7 kb); generation 9: male 9A2 (4 kb), male 9A4 (3.6 kb), male 9B4 (14 kb), male 9B5 (2.8 kb), male 9C1 (5.5 kb), male 9C4 (5.8 kb); generation 13: male 13 A2 (3.3 kb); generation 17: male 17C3 (6 kb). As we will demonstrate below, these elongation events are caused by transpositions of members of the HeT-A transposable element family to the receding chromosome ends. Chromosome ends appear to be the preferred targets for HeT-A elements since these elements have never been detected by *in situ* hybridization in the euchromatic chromosome arms (Young *et al.*, 1983). Based on maximum likelihood calculations, the average frequency of HeT-A transposition has been determined as 2.9 transposition events per generation. With a population size of 250–300 flies, this suggests that ~1% of the X chromosomes receive a new HeT-A element each generation.

Since the distal *Nru*I site in the *y* gene lies 2 kb away from the oligo(A) tail of the newly transposed HeT-A elements (see Figure 2), we calculate that the longest HeT-A element detected is 12 kb. However, we do not know whether the sizes of the new terminal *Nru*I fragments reflect the total length of the DNA addition or whether the added DNA is actually longer but contains an internal *Nru*I site. The limited amount of DNA obtained from the mini preparations does not allow us to do extensive mapping by genomic Southern blots as we did before (Biessmann and Mason, 1988). Therefore, the sizes of the terminal *Nru*I fragments have to be considered minimal lengths of the HeT-A DNA additions.

There are at least two potential mechanisms by which a

receding HeT-A sequence can acquire new DNA fragments resulting in the sudden elongation of the terminal fragment observed here: homologous recombination with other telomeric HeT-A sequences [which will elongate existing HeT-A elements without generating intervening oligo(A) tails] or transposition of another DNA element, possibly a HeT-A element, to the receding end of the chromosome. The latter would be similar to the transposition events that led to the attachment of the HeT-A elements to the end of the X chromosomes in the original stocks of *RT394* and *RT473* (Biessmann *et al.*, 1990b) and result in tandem arrays of HeT-A elements with their oligo(A) tails intact. In order to distinguish between these two possibilities, we cloned DNA fragments from the proximal region of six of the long terminal additions containing the junction between the old receding HeT-A element (which was only ~450 bp in generation 1 at the beginning of the experiment) and the newly added DNA sequences. We used a *Hin*dIII site positioned 8 kb proximal to the *Bam*HI site (position +191) in the *y* gene (see Figure 2) and a *Hin*dIII site present in the newly added DNA fragments of DNA from daughters of males 5D6, 9B4, 9C4, 9D2, 9D4 and 13B4 to construct genomic phage libraries. The libraries were screened with the *y* fragment 14a3B (see Figure 2). The desired shorter DNA fragments containing both the junction of the old HeT-A element of *RT394* at position +80 of the *y* gene (Biessmann *et al.*, 1990b), and the junction with the new DNA additions were obtained by cutting the phage DNA with *Bam*HI (in the *y* gene) and *Hin*dIII (in the new DNA addition) and subcloning into Bluescript plasmids. Sequence analyses yielded the following results. All six longer terminal fragments were caused by the attachment of a new HeT-A element to the remaining sequences of the old HeT-A element at the end of the *RT394* chromosome. The attachment of six new HeT-A elements occurred in four different positions: 5D6, 9D2 and 9D4 at 422 bp, 9B4 at 427 bp, 9C4 at 375 bp and 13B4 at 262 bp distal to the oligo(A) tail of the old HeT-A element in *RT394* (Figure 4).

The HeT-A element in the males 5D6, 9D2 and 9D4 has attached to the same position, and when the proximal ends of these elements were sequenced it became apparent that the three elements were identical. This was obvious because these elements contained the same 'double tail' at their proximal ends, consisting of two tandem 3′ HeT-A regions. This unusual structure will be discussed below. We conclude that the males 9D2 and 9D4 from generation 9 and the male 5D6 from generation 5 had the same ancestor and represent three isolates (four generations apart) of the same original transposition event that had occurred before generation 5 in the subline D.

All new HeT-A elements were attached with oligo(A) tails of different lengths to their target sites. Sequence comparison of the junctions of the new HeT-A element transpositions and their target sites in the old *RT394* HeT-A element (in Figure 5) does not reveal a common sequence motif at the target sites. Rather, it appears that the HeT-A elements can transpose to any broken end regardless of its sequence. The new elements are clearly members of the HeT-A family as defined by sequence comparison (Biessmann *et al.*, 1990b, 1992). They all exhibit the invariant sequence regions that are characteristic of the proximal 3′ end of all HeT-A

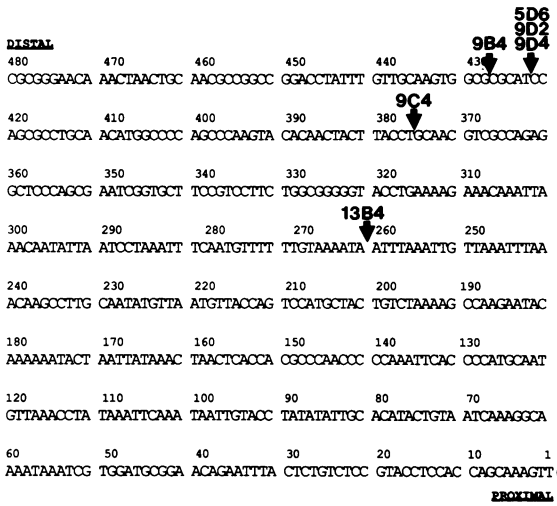

Fig. 4. Attachment points of HeT-A elements in the males 5D6, 9B4, 9C4, 9D2, 9D4 and 13B4. The 3′ (proximal) end of the *RT394* HeT-A element is shown from distal to proximal, where it is attached via an oligo(A)$_4$ tail to the *yellow* gene (see Biessmann *et al.*, 1990b). Numbering of nucleotides within the *RT394* HeT-A element is from proximal to distal, with the nucleotide adjacent to the oligo(A) tail as #1. It is not known whether the positions shown represent insertion of the 'new' HeT-A element into the *RT394* element, or attachment onto the exposed end of the chromosome.

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RT394:  TAATTGTACCTATATATTGCACATACTGTAATCAAAGGCAAAATAAATCGTGGATGCGGAACAG
RT473:   T               A  A A T                    T
9B4:
13B4:
9C4:     T               A  A A T                    T
5D6A:    ----------------------------------------
5D6B:                        C                        A

RT394:  AATTTACTCTGTCTCCGTACCTCCACCAGCAAAGTT (A)4 ctgaacatatataaacaaatata
RT473:      C                                (A)11aaagggtggatccttgtgaccct
9B4:                                       A (A)1 cgcatccagcgcctgcaacatgg
13B4:         T                             (A)17taatttaaattgttaaatgtaaa
9C4:        C                               (A)4 gcaacgtcgccagaggctcccag
5D6A:                                       (A)4 ccagcgcctgcaacatggcccca
5D6B:         T                             (A)10taaatcgtggatgcggaacagaat
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Fig. 5. Sequences at the proximal junctions between newly transposed HeT-A elements showing the degree of sequence homology. All sequences are compared with the published *RT394* sequence (Biessmann *et al.*, 1990b), and only nucleotide differences are indicated. All HeT-A elements terminate in an oligo(A) tail of various length, and no sequence similarities of the flanking regions, representing the target sites, can be detected. The elements *RT394* and *RT473* have transposed to the *yellow* gene region, elements 9B4, 13B4, 9C4 and 5D6 have attached during the course of the present experiment to sites in the *RT394* HeT-A element. For the exact positions of the attachment sites within the *RT394* HeT-A element, see Figure 4. The HeT-A element 5D6 has an unusual structure consisting of 'double tail'. This element became attached with its oligo(A)$_4$ tail (designated 5D6A) to position 422 of the old HeT-A element in *RT394*. Distal to this oligo(A)$_4$ tail extends a normal 3′ end of a HeT-A element for 57 bp. At this point another 3′ end of a HeT-A element (indicated by a dashed line) begins with an oligo(A)$_{10}$ tail (designated 5D6B), continuing distally.

elements sequenced to date, and distally they show the same sequence conservation of ~80% identity that is characteristic of all other members of the family.

The HeT-A element isolated from daughters of the males 5D6, 9D2 and 9D4 has an unusual structure consisting of

a 'double tail' (see Figure 5). It is possible that this double tail structure originated in our experiment by two successive independent HeT-A transpositions, the first one being a very truncated 57 bp long element, but we feel that it is more likely that the element already contained the double tail when it transposed, using the second, most proximal oligo$(A)_4$ to attach to the chromosome end. A similar duplication in the 3′ non-coding region has been observed in another *Drosophila* retroposon, the jockey element (Priimägi *et al.*, 1988).

A newly transposed HeT-A retroposon encodes a gag-like protein

The HeT-A addition from the male 9D4 contains at least 12 kb of DNA distal to its oligo(A) tail. In an effort to clone this addition, several unamplified phage libraries containing *Sau*3A partially digested DNA from the daughters of the male 9D4 were screened with the *y* gene probe 14a3B, yielding a total of 19 positive phages. As expected, the restriction maps at their proximal ends matched the *y* gene region and at the distal end reflected the map of the newly attached HeT-A element (Figure 6). However, even though there are at least 12 kb of new HeT-A element DNA distal to the 14a3B probe, and the recovered phages have an average insert size of 15 kb, none of them extended >5.7 kb distal to the probe. The insert distribution was clearly biased towards the proximal side, suggesting that the distal-most 6.3 kb of the new HeT-A element are not easily clonable in lambda phage. We also performed a differential screen of an unamplified phage library with the DNA fragment 14a3C which lies 5.5 kb proximal to 14a3B (see Figure 6), hoping to obtain phages that covered more distal parts of the 12 kb HeT-A element in 9D4. From a total of 24 positive phages, six hybridized to both probes, 16 to the proximal probe 14a3C alone, and only two to the distal probe 14a3B alone, confirming a bias for the proximal side. However, when we attempted to plaque purify these two phages, their hybridization signals decreased and the phages were eventually lost. A similar behavior was observed with two phages from the original set of 19 described above. We interpret these observations to indicate that sequences in the distal 6 kb region of the 12 kb HeT-A element render the recombinant phage unstable, possibly because they contain highly recombinogenic sequence arrangements that become lost when the phage is propagated. We are currently testing this hypothesis.

The phage 9D4Sau # 3 contains 5.7 kb of the proximal end of the 12 kb HeT-A element that transposed to the terminus of the receding HeT-A element in *RT394*. When the junction between the two HeT-A elements was sequenced it became apparent that the attachment site as well as the characteristic double-tail structure of the new HeT-A element were identical to 5D6 and 9D2 (see Figures 4 and 5), indicating that all three elements are descended from the same original transposition event. The remaining 5.7 kb of the proximal end of the HeT-A element were subcloned and sequenced. Since we know that transposition of the 12 kb DNA fragment must have occurred between the start of the subline from a single male and the 5th generation, and since the DNA was isolated in the 9th generation, the arrangement of fragments in the phage 9D4Sau # 3 very likely represents a typical organization of an active member of the HeT-A transposon family. The proximal 2.4 kb have no open

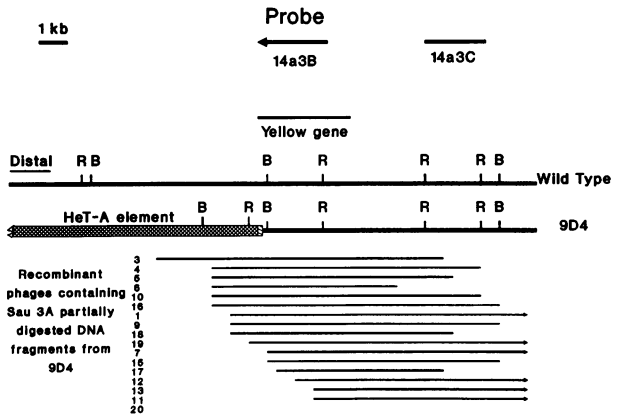

Fig. 6. Partial restriction map of the wild type *yellow* gene region, indicating the position of probes 14a3B and 14a3C, which were used to screen recombinant lambda phage libraries containing *Sau*3A partially digested DNA fragments from the 9D4 chromosome. This X chromosome, which carried at least a 12 kb HeT-A transposable element addition at its distal end, was isolated from the single male 9D4 by crossing him to *Df(1) y ac* females and harvesting his daughters for DNA preparation. The DNA organization at the tip of the 9D4 chromosome is depicted below the wild type map of the *yellow* gene region. It shows at the proximal end identity with the *yellow* gene region, followed by 422 bp of HeT-A element sequences (hatched) that remained of the receding HeT-A element *RT394* and that served as target for new HeT-A transposition. Distal to it are at least 12 kb of a newly transposed HeT-A element (cross-hatched) with a 4 bp oligo(A) tail at the proximal junction. Also indicated are the positions and extents of 17 recombinant phage inserts derived from the partial *Sau*3A library of 9D4 by screening with the 14a3B probe. Note the obvious bias of the clones in the proximal direction. The phage 9D4Sau3A # 3 extends the farthest distally and contains 5.7 kb of the newly transposed HeT-A element.

reading frame and exhibit the usual sequence homologies with all other members of the HeT-A family available to date which will be described in another publication (Biessmann *et al.*, 1992). From 2.4 to 5.2 kb distal to the oligo(A) tail, we find an open reading frame of 2.8 kb in length, reading from distal to proximal, which encodes a 918 amino acid (counted from the first methionine) gag-like polypeptide with three complete zinc finger nucleic acid binding motifs (Figure 7). The adjacent 0.6 kb distal (upstream) of the ORF to the end of the clone 9D4Sau # 3 do not encode a protein in any reading frame. Rather, this region contains two 144 and 156 bp imperfect direct repeats, separated by a 186 bp A/T-rich spacer.

FASTA (Pearson and Lipman, 1988) and BLAST (Altschul *et al.*, 1990) searches of the databases with the derived amino acid sequence of the 9D4 HeT-A element ORF showed the highest homology with the gag protein encoded by the *Drosophila* retroposon jockey (Priimägi *et al.*, 1988). Significant homology was also found with the gag-encoding ORF1 of the *Drosophila* retroposons I (Fawcett *et al.*, 1986), Doc (O'Hare *et al.*, 1991) and F (Di Nocera and Casari, 1987), and to a lesser degree with the gag proteins of retroviruses. In all cases, the homology region includes the three zinc fingers (Figure 8) and their immediate vicinity; only in the jockey element does the homology extend over a region of ~170 amino acids upstream of the zinc finger domain, and 95 amino acids downstream (Figure 9).

There is a significant difference in the size of the encoded polypeptides. While the gag proteins in jockey and Doc are

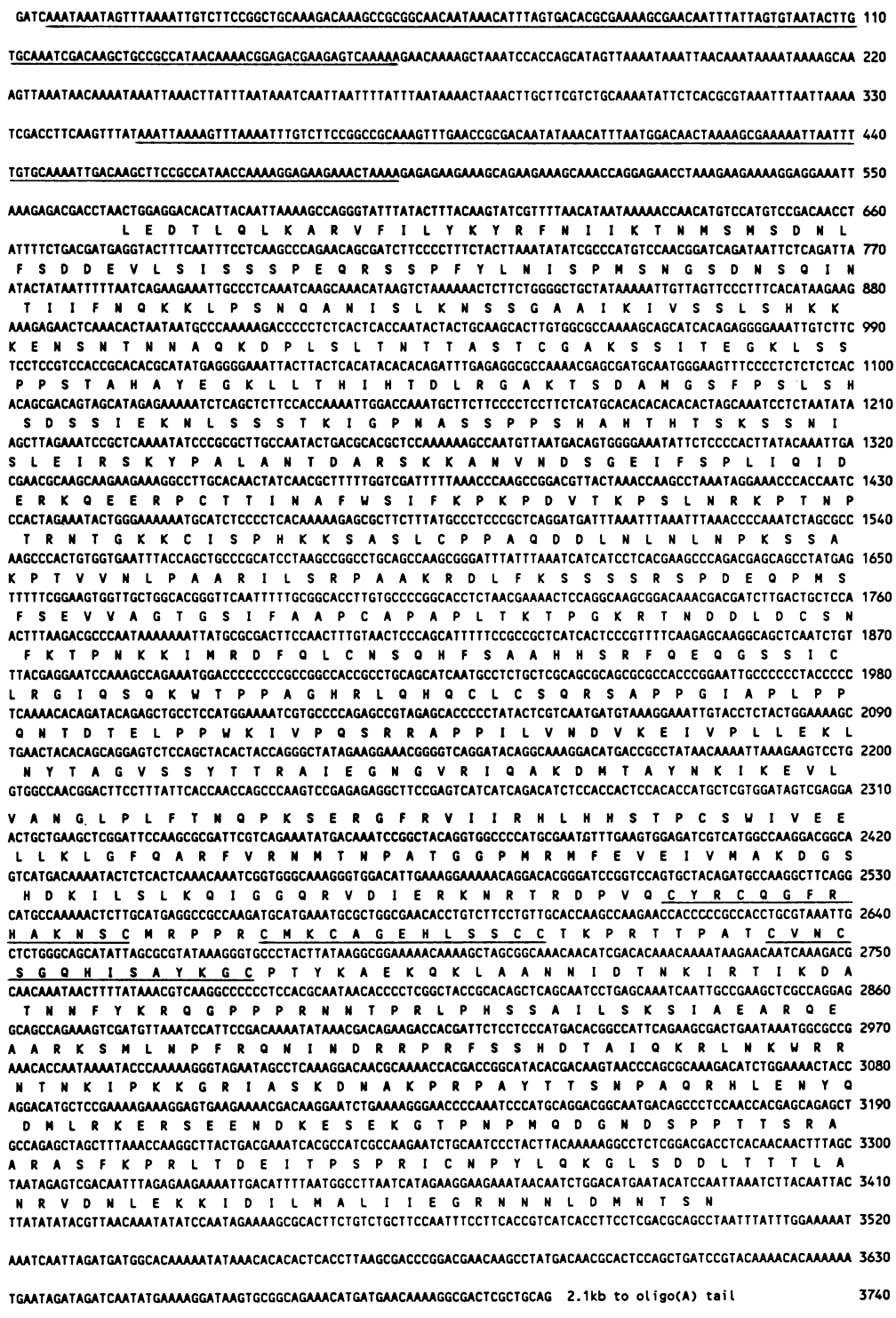

Fig. 7. Nucleotide sequence of the distal end of the phage clone 9D4Sau3A#3, comprising the region between 5.7 and 2.1 kb distal from the oligo(A) tail of the HeT-A element 9D4. The 2.5 kb region between the end of the ORF and the oligo(A) tail is of similar length and highly homologous to the corresponding region of four other HeT-A elements published recently (Biessmann *et al.*, 1992). The derived amino acid sequence of the 918 amino acid gag-like polypeptide encoded in the single 2.8 kb ORF is indicated. Amino acids forming the three zinc finger motifs are underlined. At the distal-most end of the sequence, the two 150 bp imperfect repeats, separated by a 186 bp AT-rich spacer are also underlined.

comprised of 568 and 580 amino acids respectively, the open reading frame in the 9D4 HeT-A element is capable of encoding a 918 amino acid long gag-like protein. If it is aligned with the jockey polypeptide at the zinc finger region, the HeT-A protein extends for additional 234 amino acids at the N-terminus, and for 116 amino acids at the C-terminus.

Discussion

Transposition of HeT-A elements to receding chromosome ends

In this paper we demonstrate that *Drosophila* HeT-A elements can frequently transpose to the ends of terminally

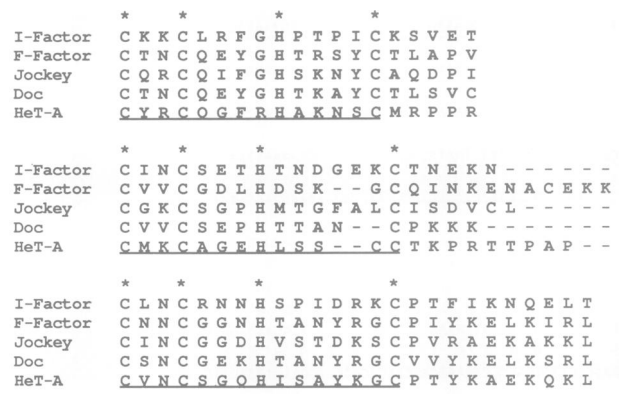

Fig. 8. Comparison of the amino acid sequences of the three zinc finger motifs found in the gag-like protein encoded by the *Drosophila* retroposons I, F, jockey, Doc and HeT-A. Asterisks indicate the highly conserved cysteine and histidine residues of the individual zinc finger domains.

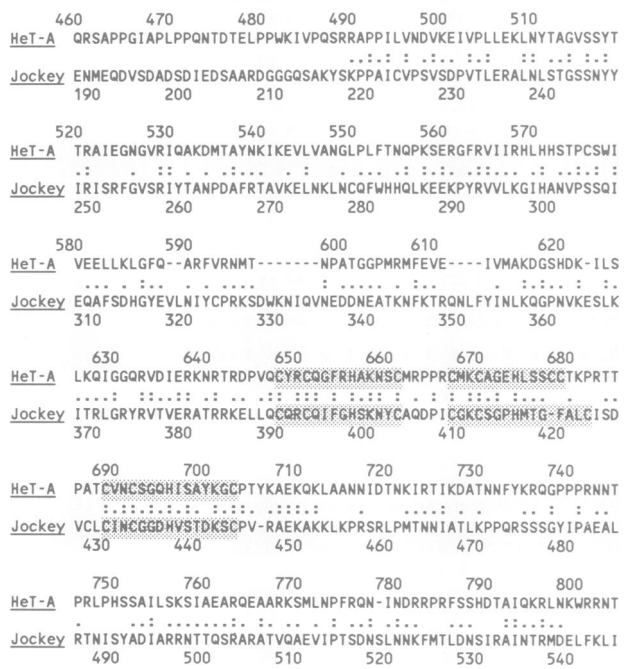

Fig. 9. Result of a FASTA search with the amino acid sequence of the HeT-A gag-like polypeptide and its alignment with the gag-like protein encoded by the *Drosophila* retroposon jockey which exhibits the highest degree of homology. The positions of the three zinc finger motifs are indicated.

deficient X chromosomes. The targets are the distal ends of other HeT-A elements which themselves had attached in the 5′ non-coding region of the *y* gene on the receding X chromosome tips several years earlier (Biessmann *et al.*, 1990b). Sequence comparison of the target sites indicated that HeT-A element transposition does not require homologous sequences nor does it show any target site sequence specificity.

The distal ends of the HeT-A elements in *RT394* and *RT473* themselves are receding by 70−80 bp per generation, confirming earlier observations on other receding chromosome tips (Biessmann and Mason, 1988; Levis, 1989; Biessmann *et al.*, 1990a). From the original HeT-A elements, which measured ~3 kb in length in August 1987, only 450 and 700 bp respectively were still present in January 1990, and they were still receding during the time of this investigation. This suggests that terminal attachment of HeT-A sequences *per se* does not prevent continuous loss of nucleotides caused by incomplete DNA replication, but that the apparent phenotypic stabilization that was observed previously (Biessmann and Mason, 1988; Biessmann *et al.*, 1990b) in the *RT394* stock (preservation of the y^2-like phenotype rather than conversion to the more extreme y^1-like null phenotype) can be explained by the extra 'buffer' DNA present at the chromosome ends. This is obvious in the original *RT394* stock in which a 3−4 kb HeT-A element had become attached to a minority of X chromosomes in 1984, or before. The chromosomes with the HeT-A attachment retained their y^2-like phenotype until early 1990, while the vast majority lost *y* activity in 1984 when the chromosome end receded into the first exon of the *y* gene. Thus, after the transposition of a HeT-A element to the end of a chromosome, material is lost from the distal end of the HeT-A element at a rate of 70−80 bp per generation, and the DNA sequence on the distal ends of HeT-A elements that reside at the extreme ends of the chromosomes is always changing. HeT-A transposition occurs in the germline. Our assay system does not allow us to detect somatic events because their contribution to the total DNA from 25−50 flies would be insufficient. Thus, we cannot comment on their occurrence. Since heterogeneity is not detected in the female offspring of single males, and chromosomes that have acquired a HeT-A element are inheritable, we conclude that HeT-A transposition must occur in the germline. The precise time of transposition, whether pre-meiotically or post-meiotically, has not been determined.

The HeT-A element is an unusual transposable element

We do not know the size of the full length HeT-A element, but the longest DNA addition that we have determined is ~12 kb. The HeT-A element may be even longer if it contains an internal *Nru*I site. To date, we have only characterized 5.7 kb from the 3′ end of such a HeT-A element termed 9D4.

The transposition mechanism and the transposition intermediate of this unusual element will be studied in the future, but our results presented here suggest that HeT-A elements may represent a novel family of non-LTR, oligo(A)-type retroposons. We have shown here that they are highly mobile and encode a 918 amino acid gag-like protein with three zinc finger nucleic acid binding motifs. They all terminate in a stretch of oligo(A) defining the proximal (3′) end of the unit in cases where the orientation is known, and a polyadenylation signal precedes the oligo(A) tail by 53 bp, although we realize that this distance is greater than the usual 9−19 bp found in other *Drosophila* retroposons (Di Nocera *et al.*, 1986; Di Nocera and Casari, 1987; Di Nocera, 1988; Priimägi *et al.*, 1988; O'Hare *et al.*, 1991). Moreover, the elements appear to be frequently truncated at their 5′ ends.

The 2.8 kb ORF encoding a gag-like protein is the only ORF found in the proximal 5.7 kb of the 9D4 HeT-A element. We sequenced 0.6 kb upstream and 2.4 kb downstream and detected no significant ORF. This result indicates an unusual structure of the HeT-A transposable element. Typically, retroposons contain two, often overlapping ORFs. The more 5′ ORF encodes a gag-like protein (ORF 1), while the more 3′, longer ORF 2 encodes a reverse transcriptase

polypeptide (Rogers, 1985; Weiner *et al.*, 1986; Boeke and Corces, 1989).

There are at least four possible explanations for our inability to find an ORF encoding a reverse transcriptase-like polypeptide. First, the reverse transcriptase may be encoded in an ORF upstream of the gag-encoding ORF on the element 9D4. Because of the arrangement of the gag and reverse transcriptase genes in other retroposons, we consider this unlikely, but we have not been able to test this hypothesis because of our inability to clone this upstream region in lambda phages (see Figure 6). The instability of lambda phages containing DNA sequences from the 5′ 6.3 kb of the 9D4 HeT-A element suggests that this region may be highly recombinogenic, perhaps due to an internally repetitive sequence organization of the kind that is evident at the distal-most end of the sequence reported here upstream of the ORF. If this region contained an ORF we would not have encountered any difficulties in cloning it in lambda phages. Second, the 9D4 HeT-A element may be defective and may have lost its reverse transcriptase-encoding ORF. Considering that rearrangements are found in other LINEs (Scott *et al.*, 1987) this seems, at first, to be a reasonable explanation. However, a reverse transcriptase-encoding ORF downstream of the gag-encoding ORF is absent from all of the five HeT-A elements in which this region has been sequenced (Biessmann *et al.*, 1992; this report). Therefore, we consider this idea also to be unlikely. Alternatively, other members of the HeT-A family may encode the reverse transcriptase upstream of the gag-like ORF, and the apparent repetitive sequence organization found in this region is peculiar to the 9D4 HeT-A element analyzed here. Again, this seems unlikely because a very similar sequence organization in the corresponding region, ~5 kb distal to the oligo(A) tail, is found in the HeT-A element TA1 present in λT-A (Valgeirsdottir *et al.*, 1990) and in several other isolates of HeT-A sequences (H.Biessmann, B.Kasravi, T.Bui, K.Ikenaga, L.E.Champion and J.M.Mason, unpublished). Third, all HeT-A elements analyzed to date may be incomplete, and only a few master elements exist that contain both ORFs encoding reverse transcriptase and the gag protein. Negative evidence does not constitute proof; thus this hypothesis would be difficult to test without actually identifying a master element. Fourth, 9D4 may be a typical member of the HeT-A family of retroposons, and these elements only contain the gag protein-encoding ORF. This protein may be responsible for the regulation of transcription and/or the recognition process by which the 3′ end of the postulated RNA intermediate becomes attached to the target sequence. The reverse transcriptase may be encoded elsewhere in the genome, not on a HeT-A element, and be provided *in trans*. This would be quite unusual but not inconceivable, considering the hypothesis, discussed below, that HeT-A elements may have a genuine cellular function in the elongation process of natural telomeres in *Drosophila*.

Some *Drosophila* non-LTR retroposons, such as I-elements (Finnegan, 1989), Doc (Schneuwly *et al.*, 1987; O'Hare *et al.*, 1991), jockey (Mizrokhi *et al.*, 1988) and F-elements (Di Nocera *et al.*, 1983), occur in both euchromatic and heterochromatic regions. Others seem to be confined to specific positions; these include G-elements (Di Nocera *et al.*, 1986) and the ribosomal DNA insertions types I and II (Jacubczak *et al.*, 1990), which occur primarily in the ribosomal DNA cluster. It has been suggested that restriction of these elements to specific chromosomal positions is due to the action of a sequence-specific endonuclease encoded by one of the retrotransposon genes (Xiong and Eickbush, 1988; Di Nocera, 1988). In transposition mechanisms commonly proposed for non-LTR, oligo(A) retroposons, the 3′ end of the polyadenylated RNA intermediate, complexed with one or more proteins (reverse transcriptase and possibly a gag-like protein) attaches to staggered nicks in genomic DNA (which will result in the observed target site duplication after successful integration), and reverse transcription is initiated using the free 3′ hydroxyl group of the genomic DNA stand as primer (Rogers, 1985; Weiner *et al.*, 1986; Schwarz-Sommer *et al.*, 1987; Pritchard *et al.*, 1988; Boeke and Corces, 1989; Bucheton, 1990). HeT-A elements are normally found only in the telomeric regions of all chromosomes and in the pericentric heterochromatin. However, this restriction to heterochromatic regions is not absolute because, under special circumstances, HeT-A elements can also move to broken chromosome ends in euchromatic regions (Traverse and Pardue, 1988; Biessmann *et al.*, 1990b; this paper). It is conceivable that the hypothetical RNA−protein complex of the HeT-A element recognizes as a target double strand breaks, short single stranded overhangs, or DNA−primer RNA hybrids which remain at the ends of linear DNA molecules after replication. To the extent that the end of a double stranded molecule with a single strand overhang resembles a molecule with staggered nicks, the mechanism of HeT-A attachment may resemble the mechanism of retroposon insertion. All analyzed HeT-A transpositions are highly polar, and the elements are always oriented so that their proximal oligo(A) tails form the junction to sequences at the end of the chromosome.

A novel Drosophila telomere elongation mechanism

We have recently shown that no telomeric repeats are present at broken chromosome ends which could act as primers for elongation by a hypothetical telomerase (Biessmann *et al.*, 1990a), and to our knowledge, no telomerase activity has yet been found in *Drosophila*. The inability of several laboratories to detect any telomeric repeats with oligonucleotide probes in *Drosophila* (Richards and Ausubel, 1988; Meyne *et al.*, 1989) can be explained in two ways. Either the difference between *Drosophila* and other organisms is quantitative rather than qualitative, and *Drosophila* simply has fewer and/or more complex telomeric repeats, or *Drosophila* has a different telomere mechanism. We favor the latter possibility and propose here that the two aspects which we have observed at broken chromosome ends in the RT stocks, namely progressive loss of sequences due to incomplete DNA replication of the ends, and frequent transpositions of HeT-A elements to these ends, may also apply to natural chromosome ends. Since ~1% of the chromosomes in the population receive a new HeT-A element each generation in a stochastic process, and the average estimated minimal size of HeT-A elements is 6 kb, ~60 bp would be added per generation. Thus the rate of HeT-A element transposition would be adequate to balance the loss of 70−80 bp per generation. A long enough zone of middle repetitive sequences in the subtelomeric region would provide a 'buffer' which would prevent loss of single copy genes from the receding ends before elongation occurs again by transposition of a new HeT-A element to the end.

If true, this would represent a telomere elongation mechanism which is drastically different from the one based on simple telomeric repeats and a telomerase adding more such repeats to the ends. This model makes several predictions which we are currently testing. First, *Drosophila* chromosome ends would not be defined by short tandem telomeric repeats but, at any given time, different sequences would be exposed at different chromosome ends due to terminal loss of nucleotides. Second, the vast majority of terminal fragments would originate from HeT-A elements, i.e. sequences from any region within the complete HeT-A element would almost always be at the very end of a natural chromosome. Third, transposition of new HeT-A elements to an old terminus would result in tandem arrays of these elements. Finally, because the chromosome ends are always receding, the HeT-A elements in a tandem array would be truncated at different places on their distal ends, while their proximal ends would be very similar.

This model can explain the high frequency of *l(2)lgl* mutants found in natural populations. The *l(2)gl* gene is the most distal locus on the left arm of chromosome 2. Golubovski (Golubovsky and Sokolova, 1973; Golubovsky, 1978) found that 1−2% of second chromosomes in nature carried a heterozygous *l(2)lgl* mutation. Cloning and molecular analysis of the locus (Mechler *et al.*, 1985) revealed that the vast majority of *l(2)lgl* mutations are associated with terminal chromosome deficiencies resulting in 3′ truncations of the gene, which is transcribed from proximal to distal. If continual loss of material from the ends of the chromosomes is counterbalanced by stochastic addition of HeT-A elements to the ends, then one would expect the occasional loss of all HeT-A material from a chromosome end and concomitant loss of distal portions of distal genes.

If our model for the maintenance of telomeres in *Drosophila* is correct, does it follow that *Drosophila* is unique in not having short tandem repeats at chromosome ends replicated by a telomerase with an internal RNA template (Blackburn, 1991)? Although data from several eukaryotic phyla are consistent with the telomerase model, data are less compelling in others (reviewed by Biessmann and Mason, 1992). Telomeric repeats have not been found in arthropods, echinoderms or molluscs, for example. In yeasts the telomeric DNA sequences resemble telomeric sequences in protozoa and vertebrates in having a G-rich strand, but there is no obvious repeat unit (see Zakian, 1989). It is difficult to reconcile the relatively complex telomeric DNA sequences found in yeasts with the telomerase model of telomere maintenance. Although telomeric repeats with a variable number of Cs may arise by template slipping of a telomerase (Yu and Blackburn, 1991), Zakian (Zakian *et al.*, 1990; Wang and Zakian, 1990) has presented evidence that telomeres in yeast can be acquired and maintained by a mechanism similar to gene conversion. Thus, it is conceivable that there are at least three, and possibly more, mechanisms for telomere maintenance.

Materials and methods

Special chromosomes

Most of the genetic markers used are described by Lindsley and Grell (1968). The *C(1)DX, y f* chromosome is an attached-X chromosome that is missing the rDNA and thus requires a Y chromosome to survive. The *y ac v* chromosome, *Df(1)y ac*, is deleted for the *y* and *ac* genes but not for vital genes, and thus provides an opportunity to examine the *y* gene region on the RT chromosomes in the absence of homologous *y* sequences on the homolog. The RT chromosomes carry one-break deficiencies for the tip of the X chromosome (Mason *et al.*, 1984; Biessmann and Mason, 1988) and initially lacked telomere-specific DNA sequences at their tips (Biessmann *et al.*, 1990a). The *RT394* and *RT473* chromosomes have since acquired HeT-A element DNA sequences at the broken end (Biessmann *et al.*, 1990b). The y^2 *sc* Y chromosomes is a fully functional Y chromosome that carries a duplication of X chromosome material, including virtually all of cytological region 1. This duplication is marked genetically with the y^2 and sc^1 mutations and is used to cover the RT deficiencies.

Collection of flies

Stocks of RT mutants were maintained by crossing RT/y^2 *sc* Y males to C(1)DX, *y f*/y^2 *sc* Y females. Individual lines were initiated with a single male and maintained by mass transfer of flies to fresh bottles every 14 days. To obtain females for analysis males were taken from a stock bottle at the time of transfer and mated to *y ac v* females. In generation 0, 15 males were mated *en masse* with 20 females. In later generations six males were mated individually with 20 females each. After 3 days the parents were transferred to fresh bottles for 4 days, then discarded. RT/*y ac v* females were collected from the first and possibly the second bottle up to a few hundred.

DNA preparation

Genomic DNA was prepared by a small scale procedure adapted from Dan Hartl's laboratory. Fifty *RT/Df(1)y ac* females were homogenized in a glass homogenizer for 5 s in 1.2 ml of 0.2 M sucrose, 0.1 M Tris−HCl, pH 9.2, 50 mM EDTA, 0.5% SDS, 4 μl diethyl pyrocarbonate. The homogenate was incubated at 65°C for 30 min, then transferred to Eppendorf tubes, precipitated with 150 μl of 3 M potassium acetate, pH 4.5, and placed on ice for 15 min. After centrifugation for 5 min at room temperature in a microfuge, the supernatant was transferred to a fresh Eppendorf tube, incubated on ice for another 15 min and centrifuged again at 4°C for 5 min. Nucleic acids were precipitated from the supernatant with 2 vol of ethanol at −20°C for 1 h and collected by centrifugation at 4°C for 10 min. The pellet was dissolved in 400 μl TE (100 mM Tris−HCl, pH 7.5, 1 mM EDTA), and after addition of 8 μl of 5 M NaCl the solution was carefully extracted with an equal volume of phenol/chloroform followed by two chloroform extractions. DNA was precipitated with an equal volume of isopropanol and collected by centrifugation for 5 min at room temperature. The pellet was dissolved in 250 μl TE, 5 μl of 5 M NaCl, and DNA was re-precipitated with 550 μl ethanol overnight at −20°C. DNA was again pelleted, dried and dissolved in 40 μl TE. This DNA preparation was sufficiently clean to be cut completely by restriction enzymes. It was used in all genomic Southern blot hybridizations and for constructing genomic libraries.

Genomic Southern blots and hybridizations

Routinely, 5 μl of genomic DNA from the small scale preparations were cleaved to completion by incubation with 15 U *Nru*I (Stratagene or Boehringer) for 3 h, and loaded on a 1% agarose gel. After electrophoresis in TAE, the DNA was denatured and transferred to nylon membranes (Hybond N^+, Amersham) for 36 h and immobilized by UV crosslinking (UV Stratalinker, Stratagene). Hybridizations were done at 42°C for 36 h in 50% deionized formamide, 5 ×SSC, 50 mM Tris−HCl (pH 7.6), 10 × Denhardt's solution (0.2% each of Ficoll, polyvinyl pyrrolidone and bovine serum albumin), 0.1% SDS, 2.5 mM EDTA and 0.1 mg/ml sonicated salmon sperm DNA. Filters were washed at 50°C in 0.1% SSC, 0.1% SDS for 1.5 h, dried and exposed on Kodak X-Omat AR film with Quanta III intensifying screen at −70°C (DuPont).

The probe used in all experiments was the plasmid 14a3B, as indicated in Figure 2, which carries a 4.5 kb fragment from the *y* gene, beginning at the *Eco*RI site at position 38.0 in the second exon and extending distally to position 33.5 in the wild type *y* map (Biessmann, 1985). The 4.5 kb insert was purified through low-melt agarose electrophoresis and labeled with [^{32}P]dCTP (3000 Ci/mmol, NEN) with the random priming reaction according to the manufacturer's suggestions (Prime-It kit, Stratagene).

Genomic lambda phage libraries and subcloning

In order to isolate the junctions of the newly extended terminal fragments of the X chromosome of the males 5D6, 9B4, 9C4, 9D2 and 13B4, DNA was cut to completion with *Hin*dIII, since the expected fragments, as determined by genomic Southern blots, had the appropriate sizes for cloning into lambda phage, and ligated into *Hin*dIII cut λ2001 phage arms (Promega). For isolation of the 12 kb HeT-A element, 5 μl of genomic DNA from the daughters of male D4 of the 9th generation were partially cut to yield fragments in the 10−20 kb range by incubation in 50 μl with 0.5 U of *Sau*3A

for 4 min. After phenol/chloroform extractions a partial fill-in reaction was done with Klenow fragment of *Escherichia coli* DNA polymerase and dGTP and dATP. These partially filled *Sau*3A fragments were ligated into partially filled *Xho*I arms of λGEM12 phage (Promega). Phage were packaged with Gigapack Gold (Stratagene), plated on *E.coli* strain NM538, and the libraries were screened with the random-primed insert of 14a3B described above. All subcloning was done in Bluescript plasmids (Stratagene).

Sequencing

Sequencing was done from double-stranded Bluescript vectors by dideoxynucleotide termination, using T4 DNA polymerase (Pharmacia), according to the sequenase manual of United States Biochemical Co. GC-rich regions were also sequenced with Taq polymerase in a Perkin Elmer Cetus Thermal Cycler using ^{32}P-kinased primers. All sequences were done on both strands and across all restriction sites.

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