Coevolution of the Telomeric Retrotransposons Across Drosophila Species

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ABSTRACT

As in other eukaryotes, telomeres in *Drosophila melanogaster* are composed of long arrays of repeated DNA sequences. Remarkably, in *D. melanogaster* these repeats are produced, not by telomerase, but by successive transpositions of two telomere-specific retrotransposons, *HeT-A* and *TART*. These are the only transposable elements known to be completely dedicated to a role in chromosomes, a finding that provides an opportunity for investigating questions about the evolution of telomeres, telomerase, and the transposable elements themselves. Recent studies of *D. yakuba* revealed the presence of *HeT-A* elements with precisely the same unusual characteristics as *HeT-A*^{mel} although they had only 55% nucleotide sequence identity. We now report that the second element, *TART*, is also a telomere component in *D. yakuba*; thus, these two elements have been evolving together since before the separation of the *melanogaster* and *yakuba* species complexes. Like *HeT-A*^{yak}, *TART*^{yak} is undergoing concerted sequence evolution, yet they retain the unusual features *TART*^{mel} shares with *HeT-A*^{mel}. There are at least two subfamilies of *TART*^{yak} with significantly different sequence and expression. Surprisingly, one subfamily of *TART*^{yak} has >95% sequence identity with a subfamily of *TART*^{mel} and shows similar transcription patterns. As in *D. melanogaster*, other retrotransposons are excluded from the *D. yakuba* terminal arrays studied to date.

STUDIES of insect telomeres have provided the first exceptions to the general mechanism of telomere formation (reviewed in Pardue and DeBaryshe 1999). In most animals, plants, and single-celled eukaryotes an enzyme, telomerase, produces long arrays of simple DNA sequences on the ends of chromosomes. Some insects share this mechanism. For example, in the silkworm, *Bombyx mori,* telomeres consist of long arrays of the sequence, TTAGG, only one nucleotide different from the human repeat, TTAGGG (Okazaki *et al.* 1993; Sahara *et al.* 1999). The first exception to the general telomere mechanism was found in *Drosophila melanogaster*, whose telomeres are polarized head-to-tail arrays of DNA repeats generated by successive transpositions of the two telomere-specific non-LTR retrotransposons, *HeT-A* and *TART* (see Figure 1). A second exception to the general telomerase mechanism has been described in three species of Chironomus (Rosen and Edstrom 2000). Chironomus telomeres are also composed of head-to-tail arrays of repeats but the repeats are an order of magnitude longer than those known to be made by telomerase. These arrays undergo rapid concerted evolution, apparently by gene conversion (Kamnert *et al.* 1998), but it is not clear whether this process also results in the net DNA synthesis needed to counteract telomere recession or whether the repeats are generated by another mechanism.

Both telomerase and the Drosophila retrotransposons extend telomeres by copying RNA sequences, and it is possible that Chironomus telomere extension is also a variation of this basic theme. Understanding how these different telomere types are related would help us to understand how telomeres evolved. In addition, comparison of variant telomeres in different species could identify features that are universally important for telomere structure and function. The information needed will come only from the study of several branches of the phylogenetic tree, with the distance between the branches carefully chosen to maximize our understanding.

Identifying alternative Drosophila telomere types is not easy, even in species closely related to *D. melanogaster*, because of the rapid change of telomere sequences within each species. This difficulty was seen in using cross-hybridization to find and characterize *HeT-A* in *D. yakuba* (Danilevskaya *et al.* 1998a). It has been estimated that the separation between *D. yakuba* and *D. melanogaster* occurred 5–15 million years ago (Lachaise *et al.* 1988). Studies of *HeT-A* elements from these two species showed that *HeT-A* forms a multicopy family in each. These multicopy families have maintained sequence similarity within each species while diverging significantly from the elements in the other species, a clear example of concerted evolution. *HeT-A*^{mel} and *HeT-A*^{yak} have only 55% nucleotide sequence identity but the conserved features show that these are homologous elements.

The rapid sequence divergence for *HeT-A* was not

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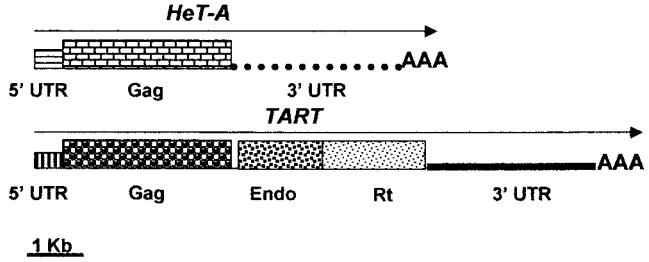

FIGURE 1.—Diagrams of the telomere elements from *D. melanogaster*. The elements are shown as the sense strand of their transposition intermediates and are drawn approximately to scale. Functional divisions are indicated under the diagrams. UTR, untranslated region; Gag, ORF 1; Endo, endonuclease domain of ORF 2; Rt, reverse transcriptase domain of ORF 2; AAA indicates the 3′ oligo(A) that characterizes non-LTR retrotransposons.

unexpected. The element encodes a Gag protein, and retroelement Gag protein sequences diverge more rapidly than the Pol sequences (MCCLURE *et al.* 1988). The remainder of the *HeT-A* sequence is noncoding DNA [mostly in the 3′ untranslated region (3′ UTR)]. Noncoding DNA is also expected to evolve rapidly because it is not under the constraints that affect coding sequences. Nonetheless, the *HeT-A* noncoding sequences show a conserved pattern—a regular spacing of A-rich regions. Because the *HeT-A* 3′ UTR is abundant in heterochromatic regions, both at telomeres and in the Y chromosome, we have suggested that these regions might be involved in protein binding to form heterochromatin (DANILEVSKAYA *et al.* 1998b).

Sequence differences between *HeT-A*^{mel} and *HeT-A*^{yak} are distributed fairly evenly over the element with 65% nucleotide identity in the coding region and 50% identity in the 3′ UTR. Despite these extensive sequence changes throughout the element, the features that characterize *HeT-A*^{mel} are conserved in *HeT-A*^{yak} (DANILEVSKAYA *et al.* 1998a). (For example, both elements transpose only to telomeres where they form long head-to-tail arrays; both have long 3′ UTRs; and neither codes for its own reverse transcriptase.)

The conservation of these distinctive features argues that the features are important for *HeT-A* to function as telomeres. Thus, the rapid sequence divergence of these *HeT-A* elements suggests that the 5–15 million years separating *D. melanogaster* from *D. yakuba* may give meaningful information about the evolution of other aspects of telomere biology. If so, this will be very useful because much of the telomerase-mediated telomere biology has been stable over long evolutionary periods.

One of the unusual characteristics of the *D. melanogaster* telomere is its complexity when compared to the homogeneous simple repeats generated by telomerase. Drosophila telomeres contain mixed arrays of the two non-LTR retrotransposons. Despite their invariant association in *D. melanogaster* telomeres, *HeT-A* and *TART* are now thought to belong to different lineages of non-LTR retrotransposons and to have acquired their telomeric roles as the result of convergent evolution (DANILEVSKAYA *et al.* 1999). The two elements are present in mixed arrays in every *D. melanogaster* stock that has been studied. This observation leads one to ask if both elements are present in telomeres because they cooperate, because they compete, or simply by chance. The interspersion of *HeT-A* and *TART* in telomere arrays has not allowed us to eliminate either element by genetic crosses in *D. melanogaster* so these questions cannot be answered directly.

Non-LTR retrotransposons are reverse transcribed onto the chromosome, primed by a 3′ hydroxyl of the target DNA (LUAN *et al.* 1993). This mechanism for integration of retroelements is called target-primed reverse transcription. In principle, the 3′ hydroxyl primer could be either on the end of a chromosome or exposed by a nick within a DNA molecule. In practice, it appears that *HeT-A* and *TART* prime their first-strand synthesis only off the chromosome end and extend the chromosome by a mechanism very similar to that used by telomerase. In contrast, all other known non-LTR retrotransposons add only to nicks within the chromosome, although the priming mechanism appears similar. It is of interest to know whether these nonoverlapping transposition patterns are conserved in other species. Do *HeT-A* and *TART* transpose specifically to chromosome ends in other Drosophila species? Have other non-LTR retrotransposons moved into telomeres of other species?

One way to approach these questions is to study the phylogenetic distribution and conserved features of the telomeric elements. Therefore, we have extended our study of *D. yakuba* telomeres to examine sequences that associate with *HeT-A*^{yak} at chromosome ends. We find that *TART* is a telomeric element in *D. yakuba* and, other than *HeT-A*, is the only element found in its telomere arrays. As with *HeT-A*^{yak}, at least one subfamily of *TART*^{yak} displays significant sequence difference from the *D. melanogaster* lineage; nevertheless, the entire family conserves the unusual structural and localization characteristics of *TART* in *D. melanogaster*.

MATERIALS AND METHODS

Fly stocks: We analyzed four stocks of *D. yakuba*: Y-1, a stock of unknown provenance used in our earlier studies (DANILEVSKAYA *et al.* 1998a); U-S180, from the Ivory Coast, and U-S181, from Kenya (both obtained from the Umea Stock Center, Sweden); and S15, from Cameroon (obtained from M. Ashburner). All four stocks have both subfamilies of *TART*^{yak} elements. The *D. melanogaster* stock was Oregon R.

Southern blot hybridization: For each sample, 20 μg of genomic DNA was digested with restriction enzymes, fractionated in a 0.7% agarose gel, and transferred to Hybond-N membrane (Amersham Pharmacia Biotech). Moderate-stringency hybridization was overnight at 60° in 4× SET (1× SET: 0.15 M NaCl, 0.03 M Tris pH 7.4, 2 mM EDTA), 5× Denhardt's solution, 0.5% SDS, and 50 μg/ml salmon sperm DNA. Washes were at 60°, 2 × 20 min in 2× SSC, 0.5% SDS, and 2 × 20 min in 1×

SSC, 0.5% SDS. Low-stringency hybridization was overnight at 55° in the same hybridization solution followed by 4 × 20-min washes at 55° with 2× SSC, 0.5% SDS. After the initial exposure of low-stringency hybridization, the filters were washed 2 × 20 min at 55° with 1× SSC and reexposed. The filters were then washed 2 × 20 min at 65° with 0.5× SSC and exposed for a final time. DNA probes were labeled with [^{32}P]dATP by random primer labeling (Feinberg and Vogelstein 1983).

Library screening: A *D. yakuba* genomic library in Lambda Fix II (Stratagene, La Jolla, CA) was obtained from Michael Griswold (North Carolina State University). The library was screened as described in the instruction manual for Lambda Fix II, using the moderate-stringency conditions described above.

Cloning and sequencing of library clones: Inserts were mapped at high resolution by restriction digestion and hybridization with *D. yakuba* cloned *HeT-A* and *TART* sequences. All *TART* elements were sequenced completely and all junctions between elements were sequenced through. *HeT-A* elements were sequenced from both ends and the sizes of the regions between those ends were determined to ensure that only a single element was present at each site; however, not all of the central regions of *HeT-A* elements have been sequenced. Sequences are deposited in GenBank as AF468023–AF468026.

Northern hybridization: RNA extraction was as described by Danilevskaya *et al.* (1999). A total of 20 μg of total RNA per lane was treated with glyoxal, separated on a 0.7% agarose gel overnight at 35 V, and transferred to Hybond-N membrane. Hybridization was overnight at 65° in 4× SET, 5× Denhardt's solution, 0.5% SDS, and 50 μg/ml salmon sperm DNA. Filters were washed three times at 65° with 1× SSC and 0.5% SDS and then treated at 37° for 1 hr with 100 units/ml RNAseT1 (Boehringer Mannheim, Indianapolis) in buffer (10 mm Tris-HCl pH 7.5, 5 mm EDTA, 300 mm NaCl). After a rinse with 1× SSC, 0.5% SDS, filters were exposed for autoradiography. ^{32}P-labeled RNA probes were transcribed *in vitro* from DNA fragments inserted into pBluescript II SK (Stratagene), according to the manufacturer's protocol.

Probes: Rt1 is nucleotide (nt) 1665–3804 of GenBank no. AF468023. Rt2 is nt 4173–5991 of GenBank no. AF468026. FIBE is nt 1–1804 of GenBank no. AF468023.

Sequence analyses: Sequences were analyzed by Blast searches of FlyBase and GenBank. Identity percentages were calculated using the LAlign program available at the Genestream Network (IGH, Montpellier, France). Phylogenetic analyses were performed with CLUSTALW (Thompson *et al.* 1994) and the Mega software version 2.1 (Kumar *et al.* 2001). The trees were constructed on the basis of the number of differences determined by CLUSTALW alignment of the sequences, using both the neighbor-joining and the UPGMA algorithms. Bootstrap tests were performed with 500 replications and a cutoff value of 50% for the consensus tree. DotPlot (Maizel and Lenk 1981) analyses were performed using a window of 25 and a stringency of 15.

***In situ* hybridization:** *D. yakuba* polytene chromosomes were hybridized as in Pardue (2000) except that probe [5 ng DNA in 10 μl hybridization buffer (50% formamide, 3× SSC, 10% dextran sulfate)] was added to each preparation before chromosomes and probe were denatured by heating slides at 95° for 2 min. Hybridization was overnight at 37°. DNA probes were labeled with digoxygenin-dUTP using the High-Prime kit (Roche Diagnostics, Indianapolis). Probes were detected by the enzymatic activity of antidigoxygenin-alkaline phosphatase conjugate.

PCR: Primer sequences: The Y chromosome-specific FIBE-reverse transcriptase junction was amplified with the primers F1BP (5′ GGAACCTAAAGAACGCCGTT 3′) and YRT1 (5′ GTG TCGCTTTCGTAGGTAGG 3′). These sequences were taken from GenBank sequence AF468023. The positive control sequence found in both sexes was amplified with primers POL2D (5′ GTCACCGAAACTAGACTAGA 3′) and POL2R (5′ GACCC TTGATCTTCACATTC 3′). These sequences were taken from GenBank sequence AF468026. Amplification was with Taq polymerase and reaction buffer (Roche Diagnostics) for 35 cycles. The program for F1BP and YRT1 primers was 95° for 30 sec, 58° for 30 sec, and 72° for 90 sec. The program for primers POL2D and POL2R was 95° for 30 sec, 50° for 30 sec, and 72° for 120 sec. For both primers the final extension was increased to 7 min.

RESULTS

***D. yakuba* has non-LTR retrotransposons related to *TART*^{mel}:** Reverse transcriptase coding sequences are the least rapidly evolving sequences in retroelements (McClure *et al.* 1988). Therefore, we initiated our search for *TART* elements in *D. yakuba* by probing Southern blots of *D. yakuba* DNA with a fragment of reverse transcriptase coding sequence from *TART*^{mel} (nt 434–2683 of GenBank accession no. U02279). When hybridization was carried out at moderate stringency, the *D. melanogaster* probe bound to multiple restriction fragments of *D. yakuba* DNA. Two of these fragments were cloned and sequenced. The sequences of the two clones were distinctly different but both were very similar to that of *TART*^{mel} with Blast scores between 3e-54 and 1e-29, depending on the *TART*^{yak} subfamily sequence used as query. No other transposable element was retrieved by the Blast search.

The cloned *D. yakuba TART* sequences were used to probe a library of λ phage carrying *D. yakuba* DNA. Eight clones were recovered. Duplicate clones were identified by restriction mapping and discarded, leaving three cloned sequences. Each of these clones was composed of a mixed array of *TART*^{yak} and *HeT-A*^{yak} elements (Figure 2). Two of these three clones have *HeT-A* and *TART* elements in arrays like those found in *D. melanogaster* telomeres. The third clone closely resembles a class of nontelomeric repeats found in the heterochromatic *D. melanogaster* Y chromosome that was originally identified because its members contained fragments of *HeT-A* (Danilevskaya *et al.* 1993). Experiments described below show that this *D. yakuba* clone belongs to this class.

The *D. yakuba TART* sequences are not found in euchromatic regions: *HeT-A*^{mel} and *TART*^{mel} are remarkable because they never transpose into euchromatic gene-rich regions where most non-LTR retrotransposons can be found. An earlier study of *HeT-A*^{yak} (Danilevskaya *et al.* 1998a) showed the same limits to transposition seen in *D. melanogaster*; *HeT-A*^{yak} was never found in euchromatin. All of the *TART* clones analyzed here also contained *HeT-A*^{yak} elements and therefore could not have come from euchromatin. However, this does not eliminate the possibility that *D. yakuba* has some *TART* elements that transpose into euchromatin. This possibility can be investigated at high resolution by *in situ* hybridization to

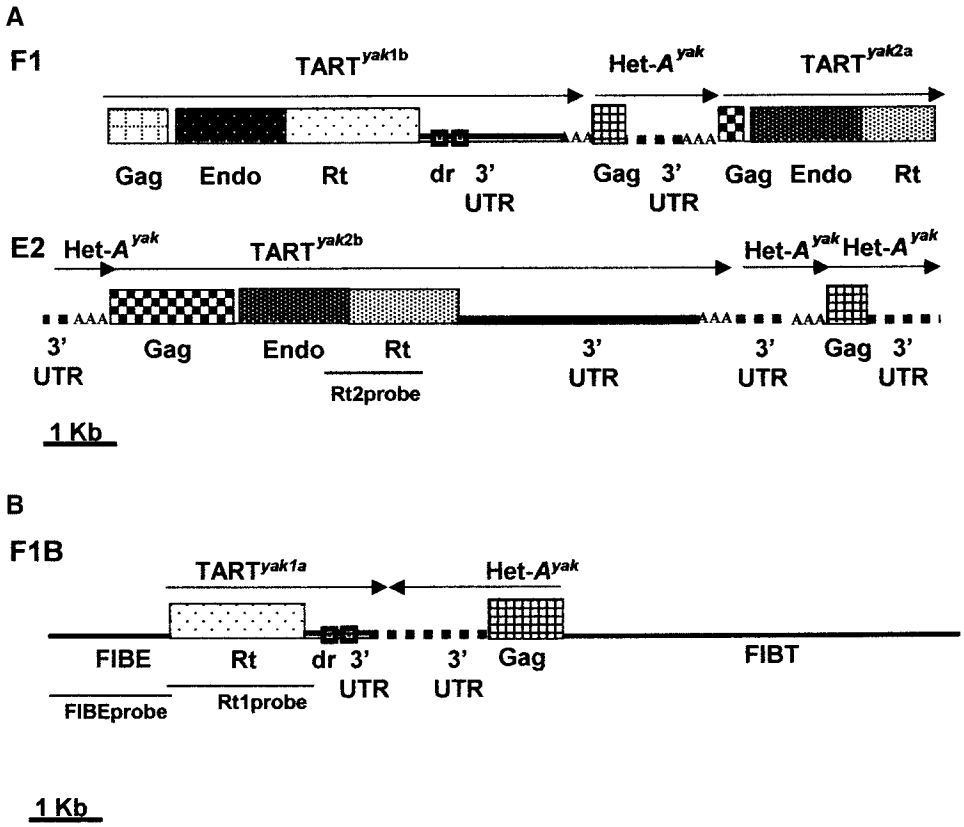

FIGURE 2.—Diagrams of the three *D. yakuba* phage clones studied. (A) Telomeric clones. (B) Y chromosome clone. Each retrotransposon is identified above the element; sequence regions are indicated below each element, as in Figure 1. Equivalent parts of each subfamily of elements have been represented with identical patterns. FIBE and FIBT are the only two sequences that are not part of *HeT-A*^{yak} or *TART*^{yak} elements. Probes used in this work are shown as solid bars. AAA indicates the 3′ oligo(A) that characterizes non-LTR retrotransposons. Arrows point 5′ → 3′ on the sense strand of each element. dr indicates the direct repeats in the *TART*^{yak1} 3′ UTR. Diagrams are approximately to scale. F1B, F1, and E2 are the names of the phage clones.

polytene chromosomes because polytenization provides amplification of euchromatic sequences, making it easy to detect single-copy genes. We studied the distribution of *TART*^{yak} in *D. yakuba*, using probes for the reverse transcriptase. These sequences show no hybridization in the banded chromosome arms. We conclude that there are no *TART* elements in euchromatic regions in *D. yakuba*.

The probes for *TART*^{yak} reverse transcriptase bind to the most terminal band on chromosome arms, as expected for a telomere sequence (Figure 3). Both probes also bind to regions of the heterochromatic chromocenter, as do *HeT-A*^{yak} probes. This contrasts with the situation in *D. melanogaster*; neither *HeT-A*^{mel} nor *TART*^{mel} hybridizes with the chromocenter in *D. melanogaster*. The chromocenter is fused and partially underreplicated centromeric heterochromatin. Although *HeT-A*- and *TART*-related sequences are in pericentric regions (TRAVERSE and PARDUE 1989; DANILEVSKAYA *et al.* 1998a; AGUDO *et al.* 1999; SIRIACO *et al.* 2002), under stringent hybridization conditions these are not detected in polytene chromocenters. The chromocenter must also contain the tiny short arms of chromosomes X and 4 although it is not known whether these are polytenized in either or both species. The structure of the chromocenter is amorphous, with no landmarks to distinguish the regions where *HeT-A* and *TART* bind. Thus, the chromocentral hybridization in *D. yakuba* may be due to telomeres on the short arms of chromosomes X and 4. However, there is also a class of sequences that we designate *HeT-TART*-related mosaic sequences. These sequences, found in nontelomeric heterochromatin, contain fragments of *HeT-A* and *TART* mixed with other sequences. We discuss below a clone of these mosaic sequences from the *D. yakuba* Y chromosome (see last section of RESULTS). Y chromosomes are not polytenized and therefore Y chromosome sequences would not be responsible for the chromocentral hybrid; however, it is possible that other members of this class reside in pericentric heterochromatin and are responsible for the chromocentral hybridization.

***D. yakuba* telomeres contain mixed head-to-tail arrays of *HeT-A* and *TART*:** The two larger cloned sequences consist of mixtures of *HeT-A*^{yak} and *TART*^{yak} elements with no other sequences interspersed (see Figure 2A). As in *D. melanogaster* telomeres, all elements are oriented in the same direction. Such polar arrays are presumably generated by successive target-primed transpositions onto the end of the chromosome. The elements in these clones are truncated by varying amounts at their 5′ ends; similar 5′ truncations are seen in *D. melanogaster* telomere arrays and are common for non-LTR elements generally. This truncation is thought to be due to failure to complete reverse transcription. For *HeT-A* and *TART*, truncation could also result from end erosion of the chromosome before the addition of the next retrotransposon.

Neither of these two clones contains any additional sequence interrupting the *HeT-A* and *TART* arrays. As in the telomere arrays of *D. melanogaster*, *HeT-A*^{yak} and *TART*^{yak} elements associate only with each other.

***TART*^{yak} elements form subfamilies that differ in se-**

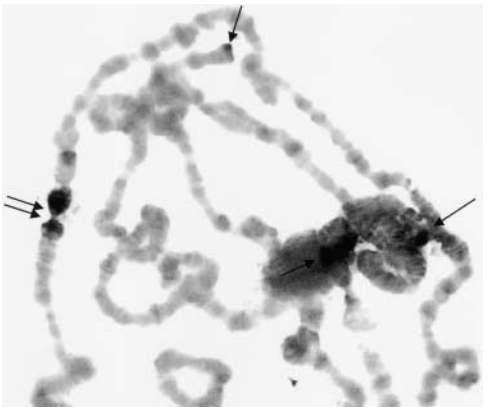

FIGURE 3.—*TART*^{yak} probes bind to telomeres and part of the chromocenter in *D. yakuba* polytene chromosomes. Part of a salivary gland nucleus probed with Rt1 DNA is shown (*TART*^{yak1} reverse transcriptase sequence, see Figure 2). The four telomeres visible have bound probe (arrows), including two ectopically paired telomeres (double arrows). As with *D. melanogaster*, there are different amounts of hybrid over different chromosome ends. Amounts of hybridizing material tend to be chromosome specific within a given stock. There is no hybridization over the banded chromosome regions but a discrete region of the heterochromatic chromocenter is labeled (arrow on chromocenter). The equivalent probe from *TART*^{yak2} hybridizes to these same sites. Chromosomes are stained with Giemsa.

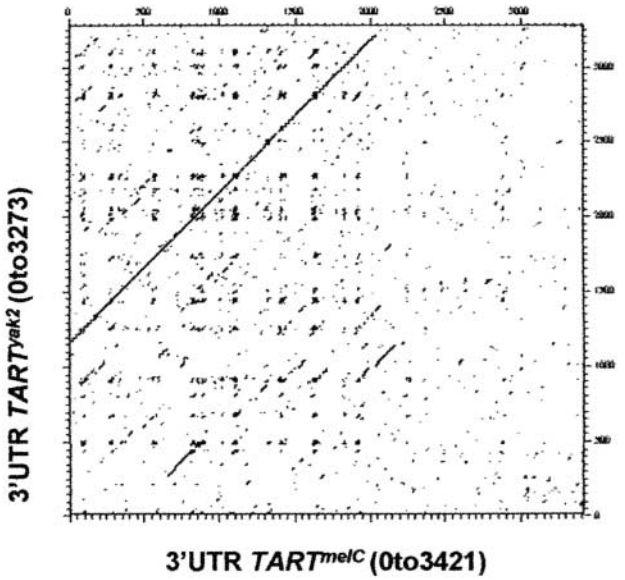

FIGURE 4.—Dot matrix comparisons showing the nucleotide similarity between the 3′ UTR sequence of *TART*^{yak2} and *TART*^{melC}. The comparison was performed with a base window of 25 and stringency of 15. The percentage of nucleotide identity of the two sequences in the region of the dot matrix diagonal is 95% (calculated by LAlign; see MATERIALS AND METHODS). The only available sequence of *TART*^{melC} is a 5′-truncated 3′ UTR. Both the *TART*^{yak} and the *TART*^{mel} sequences appear to have complete 3′ ends, finishing in a typical oligo(A) sequence.

quence and in degree of similarity to *TART*^{mel} subfamilies: The cloned sequences contain four *TART*^{yak} elements (Figure 2). The three elements in telomere arrays are partial, truncated at the 5′ end by attachment of another element or at the 3′ end by the cloning vector. All junctions of the telomeric *TART*^{yak} elements are with *HeT-A*^{yak}. The Y chromosome *TART*^{yak1} (see last section of RESULTS) is truncated on both ends.

The *TART*^{yak} elements can easily be divided into two subfamilies, 1 and 2, on the basis of the sequence of the 3′ UTR. These sequences are so different that it is not possible to do a meaningful alignment of the 3′ UTRs to compare the subfamilies. Precisely the same situation is seen with the *TART* elements in *D. melanogaster*, where three subfamilies, A, B, and C, have been identified on the basis of significant differences in the sequence of their 3′ UTRs. Although these differences are too great to allow alignment of 3′ UTR sequences of the *TART*^{mel} subfamilies, dot matrix comparisons reveal some sequence similarity in the 3′-most kilobase of the element in all of the *D. melanogaster* subfamilies (data not shown). No region of similarity is seen in dot matrix comparisons of the *TART*^{yak} subfamilies.

Because of so little evidence of sequence conservation of the 3′ UTR among elements within either species, it was a surprise to find that the 3′ UTR of *TART*^{yak} subfamily 2 (*TART*^{yak2}) is highly similar to that of the C subfamily of *TART*^{mel}. The two 3′ UTRs have 95% nucleotide identity over 2040 bp (Figure 4). The comparison is limited on the 5′ end because the only available *TART*^{melC} sequence is truncated. On the 3′ end the *TART*^{mel} sequence extends for 1381 bp beyond the end of *TART*^{yak2a}. It does not appear that the *TART*^{yak2} is truncated because it has the typical 3′ oligo(A) stretch. The lack of a *TART*^{yak} counterpart of the 3′-most terminal region of *TART*^{mel} is puzzling because the terminal region is the one showing some similarity between the *TART*^{mel} subfamilies.

As expected from the *D. melanogaster* data, the *TART*^{yak} subfamilies have much more sequence similarity in the coding regions than in the 3′ UTRs. Nevertheless, analyses of both DNA and protein sequences show the same pattern of subfamily divergence that we see in the 3′ UTR sequence (Figure 5). To analyze the relationships of the *TART*^{yak} subfamilies, coding sequences from all available *TART*^{yak} and *TART*^{mel} subfamilies were compared. *TART*^{melC} was not included because the only available sequence is a portion of its 3′ UTR. Two mechanisms that can facilitate concerted evolution are recombination and gene conversion. These mechanisms might be expected in telomeres because the *TART*^{yak} subfamilies are mixed in these arrays, and both they and their neighboring *HeT-A* elements have sufficient sequence similarity to encourage both recombination and gene conversion events involving *TART* elements. We looked for interchange between the *TART*^{yak} subfamilies by analyzing three regions of the coding sequence independently. We divided the open reading frame (ORF) 2 region into the endonuclease domain and the reverse transcriptase domain. We used only the 3′ end of ORF 1

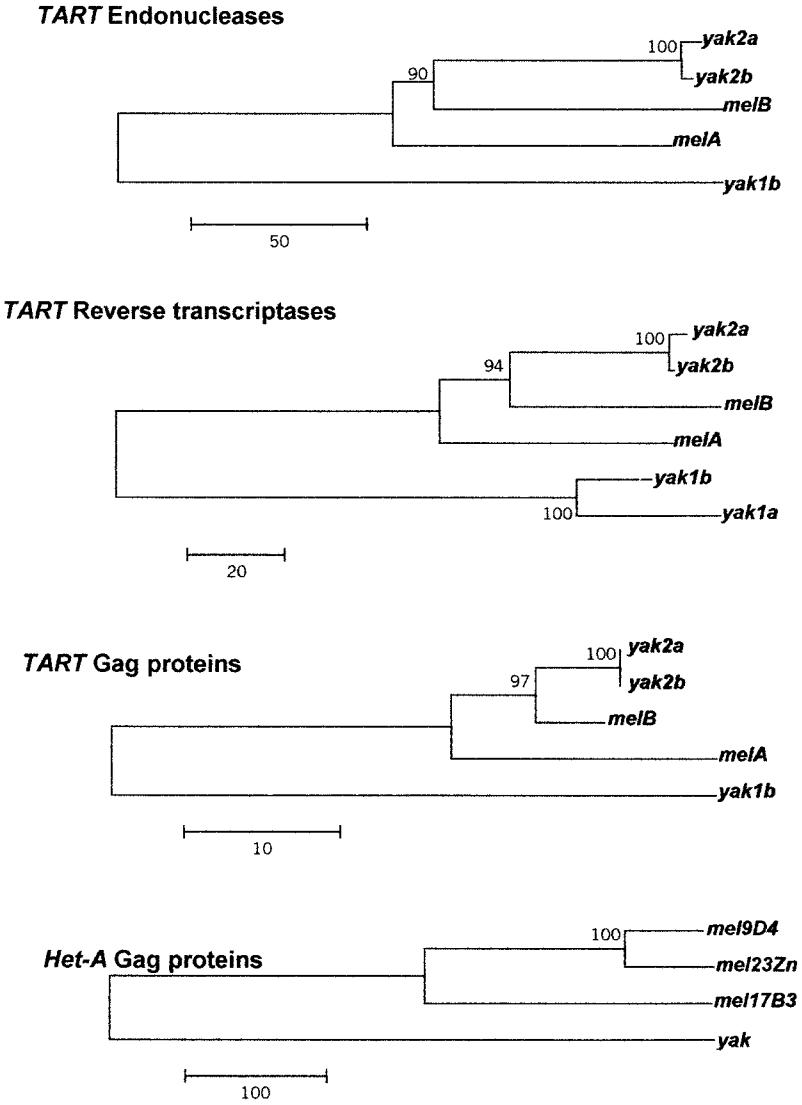

FIGURE 5.—Phylogenetic relationships among *TART* and *HeT-A* coding regions in *D. melanogaster* and *D. yakuba*. Nucleotide and amino acid sequences of all available elements were aligned using CLUSTALW and then analyzed in MEGA2.1 software. Neighbor-joining trees for the nucleotide sequences are shown. (The UPGMA trees yield the same relationships, as do the amino acid trees.) Bootstrap tests were performed with 500 replications and a cutoff value of 50% for the consensus tree. Numbers indicate bootstrap values >50% in the corresponding node. When comparing coding regions that were not complete, the smallest one was used to trim the others. Additional analyses were performed without the shortest sequence to see whether it was biasing results; however, relationships did not change. Bars indicate the number of changes between the sequences. The only sequence from a Y chromosome mosaic repeat is the RT from $TART^{yak1a}$. Note that the only available *TART* Gag sequences were from the more conserved 3′ part of the coding region. The *HeT-A* Gag sequences, added for comparison, are all from complete coding regions.

because all of the cloned elements were truncated at the 5′ end.

The coding regions were compared as both nucleotide and amino acid sequences using neighbor-joining and UPGMA algorithms. Only the neighbor-joining trees of nucleotide sequence analyses are shown (Figure 5), but all analyses yield the same conclusions: All three regions of $TART^{yak2}$ elements are more similar to the corresponding $TART^{mel}$ regions than to those of $TART^{yak1}$ even though the three regions of the ORFs diverge at different rates, as is found for other retroelements (McCLURE *et al.* 1988). Therefore, at least in our limited sample, the $TART^{yak}$ subfamilies appear to be maintained as intact units without sequence exchanges.

Although there is evidence that recombination and/or gene conversion can occur during the repair of short, recently healed *D. melanogaster* telomeres (KAHN *et al.* 2000), our observation that *TART* subfamilies appear to be evolving as units suggests that in the normal course of events, replacement of elements by new transposition, rather than by recombination or gene conversion between elements, may be the predominant force in sequence change. This suggestion is similar to the one made by PEREZ-GONZALEZ and EICKBUSH (2001) to explain the evolution of multiple lineages of R1 and R2 elements within the rDNA locus.

The relative abundance of the two *D. yakuba* subfamilies can be evaluated by Southern blot analysis. When the hybridization is performed at medium stringency, bands corresponding to both subfamilies cross-hybridize (Figure 6) but the relative strength of the signal depends on the subfamily member used as a probe (compare bands marked with an asterisk in Figure 6). Higher-stringency washes eliminate most of the cross-hybridization (not shown). These analyses show that the $TART^{yak1}$ subfamily is more abundant in *D. yakuba* than the $TART^{yak2}$ family.

The two subfamilies of $TART^{yak}$ differ somewhat in expression and possibly in translation: The *D. yakuba* TART elements produce both sense and antisense transcripts, as do the *TART* elements in *D. melanogaster*.

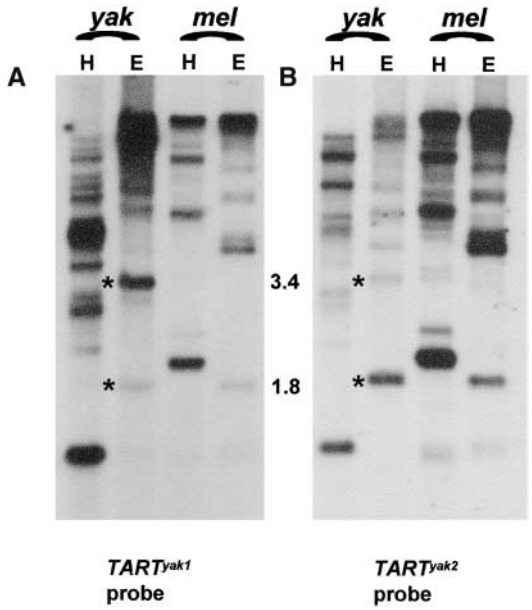

FIGURE 6.—Southern blot hybridizations comparing the hybridization of *TART*^{yak1} and *TART*^{yak2} probes to DNA from *D. yakuba* (*yak*) and *D. melanogaster* (*mel*). Genomic DNA was digested with *Hin*dIII (lanes H) and with *Eco*RI (lanes E). (A) Filter probed with sequence coding for the reverse transcriptase from *TART*^{yak1}. (B) Filter probed with sequence coding for the reverse transcriptase of *TART*^{yak2}. The probes hybridize with different efficiency to different bands in *D. yakuba* DNA, showing that two subfamilies are in this DNA. Note, for example, the bands marked with an asterisk (*). In contrast, the *TART*^{yak1} probe shows less hybridization than the *TART*^{yak2} probe to all bands in *D. melanogaster* DNA. Hybridization was at medium stringency. After higher-stringency washes, cross-hybridizing bands are no longer detected. (For details of the DNA fragments used as a probe, see Figure 2.)

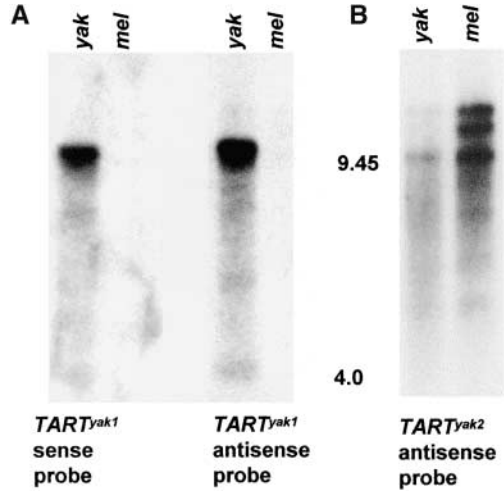

FIGURE 7.—Northern blot hybridizations showing the different expression patterns of *TART*^{yak1} and *TART*^{yak2}. Total RNA from *D. yakuba* (*yak*) and *D. melanogaster* (*mel*) was probed to detect both sense and antisense sequences from the reverse transcriptase coding region of (A) *TART*^{yak1} and (B) *TART*^{yak2}. RNA probes were transcribed from the same sequences used for the probes in Figure 6. All exposures were overnight and the difference in intensity shows that *TART*^{yak1} is much more strongly expressed than *TART*^{yak2}. The *TART*^{yak1} probes detect equal amounts of both sense and antisense transcripts of 9.45 kb in *D. yakuba* RNA but do not cross-hybridize significantly with *D. melanogaster* RNA. The *TART*^{yak2} probes detect three antisense transcripts of 9.45 kb and greater in *D. melanogaster* RNA and, after longer exposures, in *D. yakuba* RNA. As in *D. melanogaster* RNA, sense-strand transcripts of *TART*^{yak2} elements are difficult to detect in *D. yakuba* RNA (data not shown). It appears that *TART*^{yak2} elements produce many times more antisense transcripts in both *D. yakuba* and *D. melanogaster*.

TART^{mel} elements yield many more antisense transcripts than sense-strand transcripts (DANILEVSKAYA *et al.* 1999). Probes for the *TART*^{yak2} subfamily, the subfamily most like *TART*^{mel} in sequence, also detect a large excess of antisense RNA (Figure 7). [The sense transcripts of this family are much less abundant and blots show much background due to the long exposure needed (data not shown)]. In contrast, we find approximately equal amounts of sense and antisense RNA from *TART*^{yak1}. Although our clones do not have any complete *TART*^{yak} elements, the sizes of the major bands in the Northern blots are comparable to the sizes of *TART*^{mel} elements. We presume that these large transcripts come from full-length elements not present in our clones.

Non-LTR retrotransposons should require only sense-strand RNA because this strand serves as both mRNA and the transposition template. As expected, *HeT-A* yields only sense transcripts. In contrast, *TART* and a few elements in other organisms make both sense and antisense transcripts. The function of the *TART* antisense RNA is unknown, but *TART* shows structural similarity (DANILEVSKAYA *et al.* 1999) to the Dictyostelium element, *DRE*, which requires both strands of RNA for replication (SCHUMANN *et al.* 1994). Although the ratios of the two strands differ in the *D. yakuba* subfamilies, the ability to yield both strands has been conserved.

A second characteristic of *TART*^{mel} sequence that is conserved in *TART*^{yak2}, but not *TART*^{yak1}, is the sequence joining the *gag* coding region (ORF 1) to the *pol* coding region (ORF 2). Retroelements tend to translate ORF 2 as part of a polyprotein linked to the product of ORF 1 either by a frameshift or by readthrough of a leaky stop codon (JACKS 1990). Nevertheless at least one RNA virus, hepatitis C virus, has been shown to translate ORF 2 independently of ORF 1 (BROWN *et al.* 1992), and other retroelements, *e.g.*, human LINE-1 (MCMILLAN and SINGER 1993) and the I element of *D. melanogaster* (BOUHIDEL *et al.* 1994), have been shown to be capable of internal initiation to translate ORF 2. *TART*^{mel} sequences suggest that *TART* also uses internal initiation to translate ORF 2 because the arrangement of stop codons in the three frames between the ORFs would require complex ribosome movements to link the translation products (Figure 8). Both of the *TART*^{yak2} sequences reported here show the same arrangement of stop codons seen in *TART*^{mel}, although there are differences in

A

```
                1                                29
mel13-05        TAA-ACGCTCTTGCTAGTAGCATCAGAA-AGTGACGTATCTTATG 43
mel14101        TAA-ATGCTCTTTCTAGTAACATCAGAA-AGTGACGTTTCCTATG 43
yak2a           TAA-ATGCTCTTTCTAGTAACATCAGAA-AGTGACGTCTCCTATG 43
yak2b           TAA-ATGCTCTTTCTAGTAACATCAGAA-AGTGACGTCTCCTATG 43
yak1            TAAAATTCTCCCTCTACACATACCGGAATAGTGACATCTAGGATG 45
```

B

Translation of the above sequences starting after stop codon of ORF1 (frame1)

```
mel14101    *MLFLVTSESDVSY
mel13-05    *TLLLVASESDVSY
yak2a       *MLFLVTSESDVSY
yak2b       *MLFLVTSESDVSY
yak1        *NSPSTHTGIVTSRM
```

Translation of the above sequences starting one nucleotide downstream of frame1

```
mel14101    KCSF**HQKVTFPM
mel13-05    KRSC**HQKVTYLM
yak2a       KCSF**HQKVTSPM
yak2b       KCSF**HQKVTSPM
yak1        KILPLHIPE**HLG
```

FIGURE 8.—Alignment of the nucleotide sequences linking *TART* ORF 1 and ORF 2 in elements from both *D. yakuba* and *D. melanogaster*. (A) For each of the elements, the nucleotide sequence begins at the stop codon of ORF 1 (TAA in boldface type) and continues to the start codon of ORF 2 (ATG in boldface type). Gaps in the alignment are indicated by a dash (–). (B) Translation of the above sequences in the two relevant frames. Stop codons are represented by an asterisk (*). The first amino acid (M) in ORF 2 is indicated in boldface type for each element. In frame 1, all sequences are shown from the final stop codon of ORF 1 but only the element $TART^{yak1}$ begins translation of ORF 2 in this same frame. (This element has two extra nucleotides that change the frame relative to the other sequences.) The other sequences all have at least one more stop in the nine codons beyond those shown here for frame 1. All elements except $TART^{yak1}$ begin translation of ORF 2 in the second frame but have two stop codons between the stop in frame 1 and the ATG in frame 2.

the nucleotide sequences. Again, the conservation of the positions of the stop codons suggests that this small region has a role in directing translation, although direct proof of the method of translation must await development of appropriate antibodies. Because *HeT-A* does not encode reverse transcriptase, it is possible that the *TART* ORF 2 product provides this function *in trans*. This possibility makes the translational regulation of *TART* ORF 2 of special interest. The sequence between the two ORFs for all $TART^{mel}$ and $TART^{yak2}$ elements would require readthrough of at least one stop codon followed by a frameshift to link the ORF 2 product to the ORF 1 protein (Figure 8). In contrast, the $TART^{yak1}$ sequence has two differences that could facilitate translational linkage. In this element, two additional nucleotides eliminate the need to frameshift into the ORF 2 frame and eliminate the two intervening stop codons present in that frame in other elements. It seems likely that $TART^{mel}$ and $TART^{yak2}$ produce an independent ORF 2 product. If so, it will be interesting to see whether sequence changes in $TART^{yak1}$ have an effect on translation.

Fragments of *TART* and *HeT-A* are scrambled in Y-associated repeats in *D. yakuba* resembling the Y chromosome *HeT-TART*-related mosaic repeats of *D. melanogaster*: The third *D. yakuba* clone differs markedly from the other two in sequence arrangement: It has a partial $TART^{yak1}$ element in one orientation and a partial $HeT\text{-}A^{yak}$ element in the opposite orientation (Figure 2B). Furthermore, each of these elements is truncated near its 3′ end where it attaches to the other element. Neither tail-to-tail attachments nor 3′ truncations are found in telomeric regions. Tail-to-tail attachments should not be found in telomeres because telomere extension is by target-primed reverse transcription onto the chromosome end and priming from the chromosome end dictates uniform polarity. Truncation at the 3′ end is not expected because this transposition mechanism requires the extreme 3′ end sequences of the element (LUAN *et al.* 1993).

In addition to the 3′ truncations, both elements in this third clone are truncated at the 5′ end by attachment of unrelated sequences. The 5′ end of the $TART^{yak}$ element is associated with a novel 1.8-kb sequence (FIBE) with no protein-coding regions or similarity to known transposable elements. The 5′ end of the $HeT\text{-}A^{yak}$ element is associated with ~5 kb of a second novel sequence (FIBT) that makes up the rest of the clone. We have sequenced the 1.8-kb FIBE sequence. It has no ORFs but has significant similarity to a scaffold sequence of unknown function in the euchromatin of chromosome 3 of *D. melanogaster*. Sequences of several fragments from both ends and the center of the 5-kb FIBT fragment adjacent to the *HeT-A* element all show high similarity to the same scaffold sequence located in the euchromatin of chromosome 2 of *D. melanogaster*. The region of the *D. melanogaster* scaffold with similarity to the FIBT sequence has no ORFs. The atypical features of this third clone suggested that it is derived from a class of nontelomeric sequence mosaics that we initially identified in *D. melanogaster*. In that study (DANILEVSKAYA *et al.* 1993), these mosaics were found in several families

of tandem repeats. In each family the unit repeat contained scrambled fragments of *HeT-A* and other unrelated sequences, some known and some unknown; we therefore called them *HeT-A*-related repeats. We now know that some families of these repeats also contain fragments of *TART*; thus, we prefer the term *HeT-TART*-related mosaic repeats. Repeats from two of the families on the *D. melanogaster* Y chromosome have been sequenced (Danilevskaya *et al.* 1993). One, the 356 repeat, has two fragments of *HeT-A* 3′ UTR joined to a fragment of *TART* 3′ UTR. The other, the 665 repeat, contains sequence from the *HeT-A* 3′ UTR with fragments of *Stellate*, a gene located in the euchromatic polytene region 12E, and the transposable element *Copia*. These repeats are present in the nontelomeric heterochromatin of the Y chromosome, with possibly the largest array being pericentric (Agudo *et al.* 1999). *In situ* hybridization experiments also suggest that similar repeats are present in the pericentric heterochromatin of the autosomes but these sequences have not been characterized (Traverse and Pardue 1989). The hypothesis that the third *D. yakuba* clone derives from a mosaic repeat on the Y chromosome leads to three testable predictions. First, individual sequences in the clone, although present at other sites, should be more abundant in male DNA because they are repeated on the Y chromosome. Second, the junctions between the *HeT-A* or *TART* fragments and the nontelomeric components of the repeat will be found only in male DNA because this mosaic is only on the Y chromosome. Third, mosaic sequences other than the *HeT-A* and *TART* fragments will be found only in nontelomeric regions by *in situ* hybridization to polytene chromosomes. All three of these predictions are satisfied by the *D. yakuba* clone.

The abundance of the repeat sequence in DNA from males and females was measured by hybridizing Southern blots with a probe for the FIBE sequence because this sequence is less repeated in the genome than are *HeT-A* and *TART* and thus easier to measure on the Southern blot. The probe hybridized with DNA from both sexes but there was a significant excess of hybridizing sequence in DNA from males (Figure 9A), as expected for a sequence repeated on the Y chromosome but also present elsewhere in the genome.

If FIBE and *TART* are associated only in the mosaic fragment on the Y chromosome, the junction between these two sequences should be found only in DNA from males. As predicted, analytical PCR experiments with male DNA amplified a fragment spanning the FIBE-*TART* junction, whereas a control fragment was amplified equally from DNA of both sexes (Figure 9B). The junction PCR primers were chosen to amplify a 1.6-kb fragment extending from one primer in FIBE to a second primer in the *TART* sequence (Figure 9, B and C). Even in overloaded gel lanes, this 1.6-kb fragment was not found in PCR products of female DNA. As a control to test the ability of the female DNA to serve as a template for PCR we used primers from within a telomeric *TART* that should be present in DNA from the two sexes.

In situ hybridization to polytene chromosomes cannot be used to map sequences on the Y chromosome because the Y does not polytenize and is therefore undetectable in these nuclei. However, *in situ* hybridization can find other sites occupied by the fragments that make up the mosaic. Southern hybridization had shown that some of the FIBE sequence was present in females. On polytene chromosomes, the FIBE probe hybridized to only one site, a large band in a euchromatic region of one chromosome. There was no hybridization to any telomeric site (data not shown). Thus, FIBE is not associated with *HeT-A* or *TART* in any of the sites detected in polytene chromosomes; the association is found only on the Y chromosome. *In situ* hybridization to a second *D. yakuba* stock (not shown) shows the same chromosomal site, supporting our conclusion that it is not a mobile element. As discussed above, there is precedent for finding euchromatic sequence in *HeT-TART*-related mosaics; fragments of the *Stellate* gene are found in the *D. melanogaster* 665 repeat family.

DISCUSSION

***HeT-A* and *TART* occupy the same niche in the genomes of *D. yakuba* and *D. melanogaster*:** The *D. melanogaster* genome contains a number of families of non-LTR retrotransposons, including *HeT-A* and *TART*. These elements have several well-conserved features atypical of non-LTR retrotransposons; these features are presumably related to their exclusive association to telomeres and heterochromatin. Target-primed reverse transcription, by which non-LTR elements insert into new sites, explains the ability of *HeT-A* and *TART* to add to chromosome ends where they form arrays in which the 3′ ends are oriented toward the centromere. Other non-LTR retrotransposons use the same mechanism to transpose into many sites in euchromatic regions of the chromosomes but are never found in telomeric arrays. *HeT-A* and *TART* are the only elements found in telomere arrays and they are never found in euchromatin.

The only *D. yakuba* telomeric element previously characterized is *HeT-A*, which shows the same pattern of telomere-specific transposition seen in *D. melanogaster*. The *TART* clones characterized here show that *HeT-A* also shares its telomeric sites with *TART* in *D. yakuba*. Neither of these telomeric elements is found in euchromatin in *D. yakuba*, nor do we detect new retrotransposons that have acquired telomeric specificity.

Long runs of sequence that do not code for proteins needed for transposition are rare in retrotransposons, yet both *HeT-A* and *TART* elements have large regions of noncoding DNA. This DNA is undergoing concerted evolution but there are underlying patterns of sequence conservation. The conservation suggests function, al-

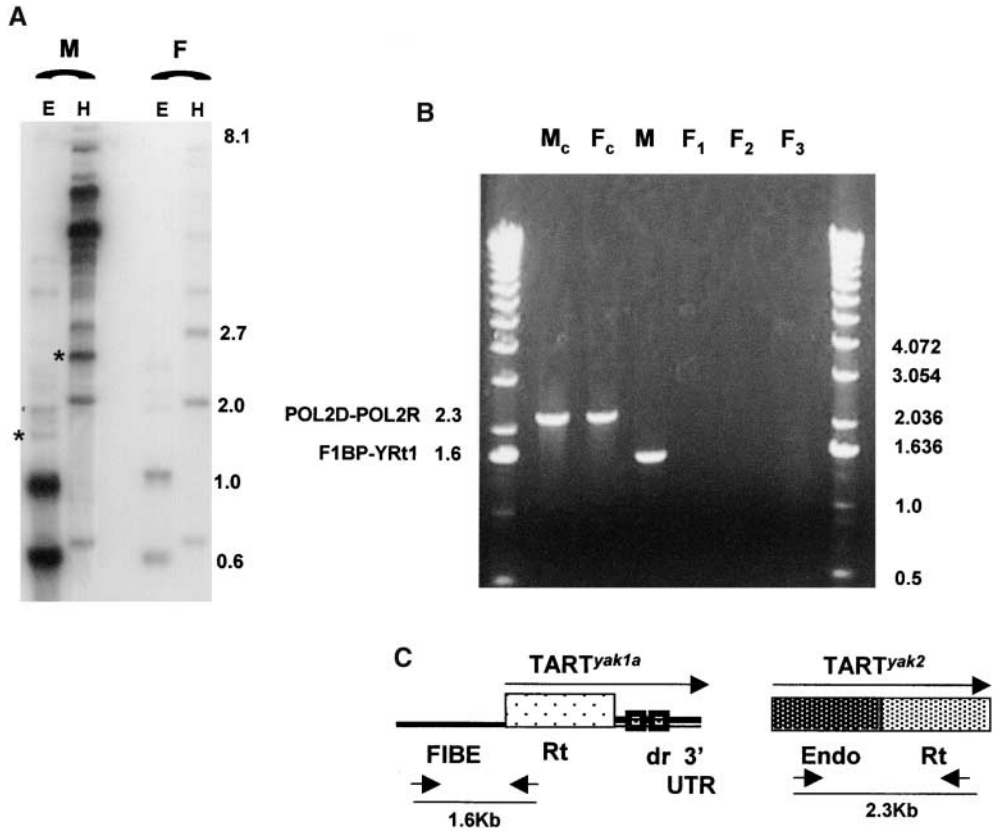

FIGURE 9.—Evidence that the sequence in clone F1B is derived from the Y chromosome. (A) Southern blot hybridization showing that DNA from *D. yakuba* males has more FIBE sequence than DNA from females. Lanes were loaded with 20 ug of DNA from males (M) or females (F). Lanes E, DNA digested with *Eco*RI; lanes H, DNA digested with *Hin*dIII. The probe was the ^{32}P-labeled FIBE sequence (see Figure 2). Some male-specific bands are highlighted with an asterisk (*). (B) Evidence that the junction between the FIBE sequence and the *TART*^{yak1} RT sequence is present only in DNA from males. Primers that amplify a 1.6-kb fragment spanning the junction were used for PCR amplification of DNA from *D. yakuba* males (M) and females (F_1, F_2, and F_3). Only male DNA gave the expected product. No product of these primers was detected from female DNA even when gel lanes were loaded with three times (lane F_2) and five times (lane F_3) the material loaded in M and F_1. To show that the female DNA was suitable for PCR, primers for a telomere sequence expected to be present in both male and female DNA were used (lanes Mc and Fc). These primers amplified DNA equally well from the two sexes. The same male or female DNA samples were used for both PCR reactions. (C) Diagrams showing the positions of the primers used in B. The sequences used to design primers were taken from the clones shown in Figure 2.

though function other than protein coding is not easily deciphered from sequence. An illustrative example of marked change in a noncoding sequence of defined function is seen in the gene for the RNA template used by telomerase to extend telomeres. Telomerase RNAs from different organisms vary greatly in both size and sequence but some sequence conservation preserves the folding pattern of the transcript (CHEN *et al.* 2000). Presumably, this folding pattern is important for interactions between the RNA and protein components of telomerase. It is possible that the conserved sequences in the 3′ UTRs of the telomeric retrotransposons play a similar role in the transposon RNA, that they are involved in heterochromatization of the telomere, or that they are needed for protein binding after incorporation into the heterochromatic DNA of the telomere. In any case, these conserved sequences are likely to be important for interactions with other molecules.

***D. yakuba TART* elements are undergoing concerted evolution in at least two subfamilies:** One somewhat unexpected finding of this study is that the two subfamilies of *TART*^{yak} are evolving separately. A surprising finding is the high similarity between the sequence of *TART*^{yak2} and *TART*^{melC}.

Such high sequence conservation, especially in a noncoding region, raises the possibility of horizontal transmission. Transmission of a *TART*^{melC} element from *D. melanogaster* could explain the high similarity between *TART*^{yak2} and *TART*^{melC}. It is known that transposable elements can transpose horizontally between species (KIDWELL 1992). Transmission between species appears to be more frequent for DNA transposons (CLARK and KIDWELL 1997), but has also been described for RNA transposons (KIDWELL 1992; JORDAN *et al.* 1999). The presence of the non-LTR retroelement *jockey* in *D. melanogaster* and *D. funebris*, but not in the intervening species, has led to a proposal of horizontal transfer (MIZROKHI and MAZO 1990); however, the possibility that *jockey* has been lost in the intermediate species cannot be ruled out (MALIK *et al.* 1999). Invasion of *D. melanogaster* by *P* elements has been demonstrated because this element is not present in stocks placed in laboratories before ~1950 (ASHBURNER 1989). We have analyzed the four available *D. yakuba* stocks and found that all have significant numbers of *TART*^{yak2} elements and that these elements are transcribed. If this element has invaded *D. yakuba*, the element has now spread through populations in the geographical regions sampled (see MATERIALS AND METHODS for origin of stocks).

Although we cannot rule out horizontal transmission

of *TART*^{yak2}, there is a well-documented case of maintenance of two lineages of a non-LTR retrotransposon through a long evolution (Gentile *et al.* 2001). The R1 element, found throughout the arthropods, has two major lineages: One is present in all of the 35 Drosophila species sampled and the other is found in 11 species but has been lost multiple times. R1 elements transpose into a defined site in the genes for ribosomal RNA. The rRNA genes, like the telomere arrays, would seem to be regions where sequence exchange would drive convergent evolution of the retrotransposon families, yet the two R1 lineages are maintained separately. Our preliminary evidence that *TART*^{yak2} sequences, rather than *TART*^{yak1} sequences, cross-hybridize with more distant species of Drosophila suggests that characterization of these species may reveal a pattern of evolution similar to that of R1.

Fragments of *HeT-A* and *TART* sequences are present in Y chromosome mosaic repeats: One of the intriguing problems of eukaryotic chromosome structure is the evolution of heterochromatic sex chromosomes. Drosophila has contributed insight into this because in one species, *D. miranda*, an ancestral autosome has become attached to the Y chromosome. This neo-Y chromosome arm is becoming heterochromatic while its former homolog (now called X2) is acquiring the ability to dosage compensate its genes, as does the ancestral X chromosome. The neo-Y chromosome now contains much complex repeated DNA, including transposable elements found elsewhere in the genome (Steinemann and Steinemann 2000). Surprisingly, the homolog is also accumulating repeated DNA, but of a very different kind. The repeats on the X2 are simple mono- and dinucleotide repeats (Lowenhaupt *et al.* 1989).

The *HeT-TART*-related mosaic repeats in *D. melanogaster* identified a new class of sequences in Y chromosomes. The F1B clone described here shows that this class of sequences is also present in *D. yakuba*. Neither the mosaic repeats in *D. melanogaster* nor those in *D. yakuba* appear to have been formed by target-primed reverse transcription. An understanding of the origin of these repeats would give insight into the formation of Y chromosomes and other heterochromatic regions.

In conclusion: Comparison of the mechanisms used by telomerase and the reverse transcriptases of non-LTR retrotransposons suggests that little, if any, modification would be necessary to enable retrotransposons to extend telomeres (Pardue *et al.* 1996). Evidence that the two telomeric transposons in *D. melanogaster* have different origins is consistent with this supposition. However, acquisition of the ability to extend telomeres must be a rare event because *HeT-A* and *TART* are the only known telomeric retrotransposons. These studies of *D. yakuba* show that the telomere specificity of both these elements must have developed before the separation of the *D. melanogaster* and *D. yakuba* species complexes. Since that time, none of the other non-LTR elements now active in Drosophila genomes has joined the telomere, and despite much sequence change, the basic features of the Drosophila telomere have been conserved.

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