Telomere elongation (*Tel*), a New Mutation in *Drosophila melanogaster* That Produces Long Telomeres

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ABSTRACT

In most eukaryotes telomeres are extended by telomerase. *Drosophila melanogaster*, however, lacks telomerase, and telomere-specific non-LTR retrotransposons, *HeT-A* and *TART*, transpose specifically to chromosome ends. A Drosophila strain, Gaiano, that has long telomeres has been identified. We extracted the major Gaiano chromosomes into an Oregon-R genetic background and examined the resulting stocks after 60 generations. *In situ* hybridization using *HeT-A* and *TART* sequences showed that, in stocks carrying either the *X* or the second chromosome from Gaiano, only the Gaiano-derived chromosomes display long telomeres. However, in stocks carrying the Gaiano third chromosome, all telomeres are substantially elongated, indicating that the Gaiano chromosome *3* carries a factor that increases *HeT-A* and *TART* addition to the telomeres. We show that this factor, termed *Telomere elongation* (*Tel*), is dominant and localizes as a single unit to 69 on the genetic map. The long telomeres tend to associate with each other in both polytene and mitotic cells. These associations depend on telomere length rather than the presence of *Tel*. Associations between metaphase chromosomes are resolved during anaphase, suggesting that they are mediated by either proteinaceous links or DNA hydrogen bonding, rather than covalent DNA-DNA bonds.

TO maintain stable telomere length, all eukaryotes must regulate the addition of new telomeric sequences to counterbalance the loss of terminal sequences occurring during DNA replication. In most organisms telomere length depends on proper activity of telomerase and on telomere structure. For example, telomere length in yeast is regulated by Rap1p, which recruits other proteins to telomeric DNA. When yeast telomeres are bound by a high number of Rap1 polypeptides, they assume a conformation that renders them inaccessible to telomerase. When the number of Rap1 proteins decreases below a certain threshold, yeast telomeres become accessible for elongation by telomerase (Marcand *et al.* 1997). A similar mechanism for regulation of telomere length has been proposed for human cells, in which the switching mechanism is governed by the TRF1 and TRF2 proteins (van Steensel and de Lange 1997; Smogorzewska *et al.* 2000). It has been suggested that a high local concentration of these telomere-binding proteins promotes the looping of telomeric DNA, making it inaccessible to telomerase (Griffith *et al.* 1999; Smogorzewska *et al.* 2000).

Telomere maintenance in *Drosophila melanogaster*, however, does not depend on the action of telomerase. Instead, two families of non-LTR retrotransposable elements, *HeT-A* and *TART*, maintain telomere length by transposing specifically to chromosome ends (Biessmann *et al.* 1990; Levis *et al.* 1993; Mason and Biessmann 1993, 1995). *HeT-A* elements have been shown to transpose to a single chromosome end with frequencies ranging from 10^{-1} to $<10^{-4}$ (Biessmann *et al.* 1992; Kahn *et al.* 2000; Golubovsky *et al.* 2001), although nothing is known about the control of transposition. Telomeric arrays in Drosophila may also accumulate transposable elements via gene conversion under some circumstances (Kahn *et al.* 2000). The relative importance of transposition and conversion in telomere length maintenance is not known.

Here, we provide the first evidence for genetic regulation of telomere length in Drosophila. We have characterized a *D. melanogaster* strain, Gaiano, which has unusually long telomeres. This strain contains a dominant genetic factor, *Telomere elongation* (*Tel*), that maps to ~69 on chromosome *3* and causes addition of both *HeT-A* and *TART* elements to all Drosophila telomeres.

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The chromosome ends with long telomeres associate with each other in both polytene and mitotic cells, but the end-to-end associations between metaphase chromosomes are resolved during anaphase.

MATERIALS AND METHODS

Drosophila stocks and genetic crosses: All genetic markers and special chromosomes are described by Lindsley and Zimm (1992). The *T(2;3)ap^{Xa}/CyO; TM6* stock was a generous gift of I. Boussy. Oregon-R is a standard laboratory wild-type strain. The multiply marked stocks *ru h th st cu sr e^s ca/TM3* and *ru h th st cu sr e^s Pr ca/TM6B* were provided by the Bowling Green stock center and have been in the lab for many years. The stocks containing *C(1)DX, y f, Sco/SM1*, or *Sb/TM6* were generated in crosses to Oregon-R to replace the unmarked chromosomes in these stocks with chromosomes from Oregon-R. The original Gaiano (Gaiano-O) was a generous gift of J. Lim. Stocks labeled Gaiano-I, Gaiano-II, or Gaiano-III were generated as shown in Figure 1. To examine females carrying two Gaiano-I *X* chromosomes, the *X* chromosome from Gaiano-I was reextracted into a homozygous stock.

All stocks were maintained and crosses were made on standard Drosophila medium at 25°. Stocks were kept on a 2-week cycle, 26 transfers per year.

The position of *Tel* along chromosome *3* was determined by allowing free recombination in *Tel/ru h th st cu sr e^s ca* females. Recombinant chromosomes were collected in males over *ru h th st cu sr e^s Pr ca* and placed into stock by crossing these males to *Sb/TM6, Ubx* females and interbreeding Ubx Pr$^+$ sibling progeny. Presence of *Tel* on the recombinant chromosomes was determined after 50 generations by backcrossing to *Sb/TM6* and examining the *2*L tips in salivary gland cells for uneven telomere length between the homologs.

Cytological procedures: Colchicine-treated metaphase chromosome preparations, stained with either aceto-orcein or Hoechst 33258, and aceto-orcein-stained anaphase figures were obtained from larval brains, as described previously (Gatti and Goldberg 1991; Gatti *et al.* 1994). In Gaiano-II telomere-telomere associations (TAs) were examined in Hoechst-stained metaphases, where the second chromosome can be easily identified. Other cytological analyses of mitotic TAs were performed on orcein-stained brain squashes. Polytene chromosomes were stained with aceto-orcein or prepared for fluorescent *in situ* hybridization (FISH) according to Mason *et al.* (1984) and Engels *et al.* (1989), respectively. Mitotic chromosome preparations for FISH were obtained according to Gatti *et al.* (1994).

Fluorescent *in situ* hybridization: The FISH procedure used is described by Gatti *et al.* (1994). The probe used for *HeT-A* hybridization was a mixture of the 2-kb *Apa*I fragment of the 3′ untranslated region (UTR) and the 23Zn-1 fragment containing the open reading frame (ORF; Biessmann *et al.* 1994; Danilevskaya *et al.* 1994), while the 2.25-kb *Sst*I fragment of the *TART*-A1 element was used for TART hybridization.

Chromosome preparations were analyzed using a Zeiss (Oberkochen, Germany) Axioplan epifluorescence microscope equipped with a cooled CCD camera (Photometrics, Woburn, MA). The fluorescent signals, recorded separately as gray-scale digital images by IP Lab Spectrum software, were pseudocolored and merged using Adobe Photoshop 3.0.

To estimate telomere length in Oregon-R, Gaiano-O, and Gaiano-III chromosomes we examined polytene chromosome preparations after *in situ* hybridization with *HeT-A*. Telomere length was quantified by measuring the fluorescence intensity of the *HeT-A* telomeric signals, using Adobe Photoshop. Measurements were taken from Oregon-R/Gaiano-O and Oregon-R/Gaiano-III heterozygous female polytene preparations, where the signals on the homologous telomeres were separate. For both Oregon-R/Gaiano-O and Oregon-R/Gaiano-III the values obtained were normalized with respect to the Oregon-R *2*L telomeric signal, which was chosen as the telomere length unit. At least 10 chromosome ends were examined for each telomere. The differences in telomere length obtained between Gaiano-O and Gaiano-III were confirmed by measuring telomeric signals in Gaiano-O/Gaiano-III heterozygous female polytene preparations.

Immunostaining with HP1: Polytene chromosome preparation and the immunostaining technique for HP1 are described in Fanti *et al.* (1998). The polytene chromosomes were incubated overnight with the anti-HP1 C1A9 monoclonal antibody (James *et al.* 1989) diluted 1:50 in PBS. This primary antibody was detected by a 2-hr incubation with fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse Ig (Jackson Laboratories, Bar Harbor, ME), diluted 1:20 in PBS.

Genomic DNA isolation, Southern blotting, and hybridizations: Genomic DNA was isolated from adults according to standard procedures (Lis *et al.* 1983), as were DNA transfers from agarose gels to nylon membranes and hybridization with nonradioactive probes (Sambrook *et al.* 1989). The nonradioactive DNA probes from *HeT-A, TART*, and *jockey* elements were prepared by random priming, using a digoxygenin (DIG) DNA labeling and detection kit (Boehringer Mannheim, Indianapolis). Because the pericentromeric heterochromatin contains tandem arrays of the *HeT-A* 3′ UTR (Young *et al.* 1983; Traverse and Pardue 1989; Danilevskaya *et al.* 1993), only the *HeT-A* ORF was used as a probe to minimize detection of *HeT-A* heterochromatic sequences. The pol ORF was used as a TART probe and a complete element was used as jockey probe. Blots were stripped and reprobed with the *Eco*RI/*Sac*I fragment of the mini-*w* gene. Southern blots were optically scanned and the density of hybridization signal in each lane or individual band was determined using NIH Image 1.61 software. Comparisons between strains were made after normalization to the *white* hybridization signal in the same lane.

RESULTS

The Gaiano-O stock was caught in the wild, probably near Endine Gaiano, a small town in the Bergamo province in northern Italy. The flies must have been collected on or before 1946, as a brief report mentioning this strain was published in November 1946 by Barigozzi and Trapani (1946). The stock was subsequently kept at the Istituto di Genetica, Universita' di Milano for many years. It was "exported" to the United States in the 1970s and was propagated among several laboratories (S. Beckendorf, personal communication). The Gaiano flies are phenotypically indistinguishable from Oregon-R or Canton-S wild-type flies. They also exhibit normal viability and fertility and have the same life span as Oregon-R flies (our unpublished observations).

Our interest in Gaiano arose as a result of J. Lim's observation that Gaiano polytene chromosomes display long telomeric regions that are often associated with each other. We, therefore, analyzed Gaiano telomeres by addressing three basic questions: (1) Does the Gaiano strain contain one or more genetic factors responsible

for the increase in telomere length?, (2) are the telomeres in this strain particularly enriched in *HeT-A* and *TART* sequences?, and (3) what is the basis for TAs that are seen in the Gaiano stock?

The Gaiano stock contains a genetic factor(s) that increases telomere length: To identify and map any genetic factors that might be responsible for generating long telomeres in Gaiano, the major Gaiano chromosomes were extracted into an Oregon-R genetic background (Figure 1), generating three stocks, each containing a different Gaiano chromosome. Because of its small size, chromosome *4* was ignored in these crosses and in the following discussion. The stocks were examined after 60 and 130 generations for telomere length by *in situ* hybridization, using a *HeT-A* probe. In stocks carrying either the *X* chromosome (Gaiano-I) or chromosome *2* (Gaiano-II) from Gaiano, the length of the telomeres reflected their origins; chromosomes that originated from Gaiano had long telomeres, while chromosomes from Oregon-R had short telomeres (Figure 3 and data not shown). In the stock carrying chromosome *3* from Gaiano (Gaiano-III), however, all the major chromosomes had long telomeres that hybridized strongly with *HeT- A* (Figure 2). These findings indicate that exposure to chromosome *3* from Gaiano for several generations results in a dramatic growth in Oregon-R-derived telomeres. This suggests that there is a genetic factor(s) on the Gaiano chromosome *3* that induces *HeT-A* addition to chromosome ends at high frequency.

Cytological characterization of Gaiano telomeres: To assess relative degrees of telomere extension after 130 generations, we crossed Oregon-R females to either Gaiano-O or Gaiano-III males. The polytene chromosomes in the female progeny of these crosses contain an Oregon-R chromosome tightly synapsed with its Gaiano homolog, allowing direct comparison between telomeric regions. We also compared Gaiano-O and Gaiano-III telomeres by examining the polytene chromosomes of Gaiano-O/Gaiano-III females. These polytene chromosomes were examined using FISH with a *HeT-A* probe and counterstained with 4′,6-diamidino-2-phenylindole (DAPI) to visualize the chromosomes. This procedure allowed unambiguous recognition of the telomeric regions enriched in *HeT-A* and a reliable estimation of their length.

Analysis of the Oregon-R/Gaiano-O polytene chromosomes revealed that all Gaiano-O telomeres are longer than their Oregon-R counterparts (Figure 2, B and I). However, the Gaiano-O chromosomes differ from each other in telomere length. In Gaiano-O the telomeres of the left arm of the *X* chromosome (*X*L) and the right arm of chromosome *3* (*3*R) are substantially longer than those of the other chromosome arms (Figure 2I). The telomeres of the Gaiano-III chromosomes are longer than both their Oregon-R and Gaiano-O counterparts. Recall that the Gaiano-III strain contains the

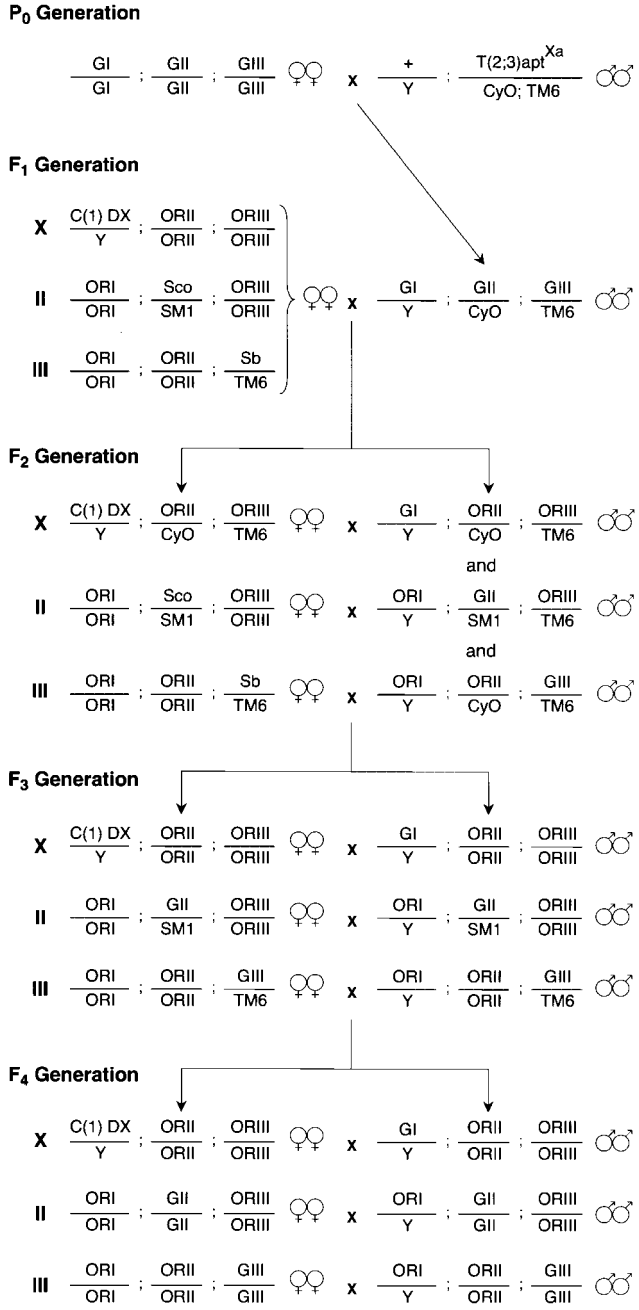

FIGURE 1.—Genetic crosses made to generate Gaiano-I, Gaiano-II, and Gaiano-III stocks. Many of the symbols and special chromosomes are described by LINDSLEY and ZIMM (1992), with the exceptions that G represents Gaiano; OR represents Oregon-R; and I, II, and III represent chromosomes *X*, *2*, and *3*, respectively.

X and second chromosomes from Oregon-R and the third chromosome from Gaiano-O (Figure 1). Thus, in these experiments the Oregon-R *X*L, *2*R, and *2*L telomeres display a dramatic increase in their length after 130 generations' exposure to the Gaiano third chromosome. Interestingly, the Gaiano-III *3*L and *3*R telomeres are also longer than their Gaiano-O counterparts (Figure 2, E and F). The lengths of all the Gaiano-

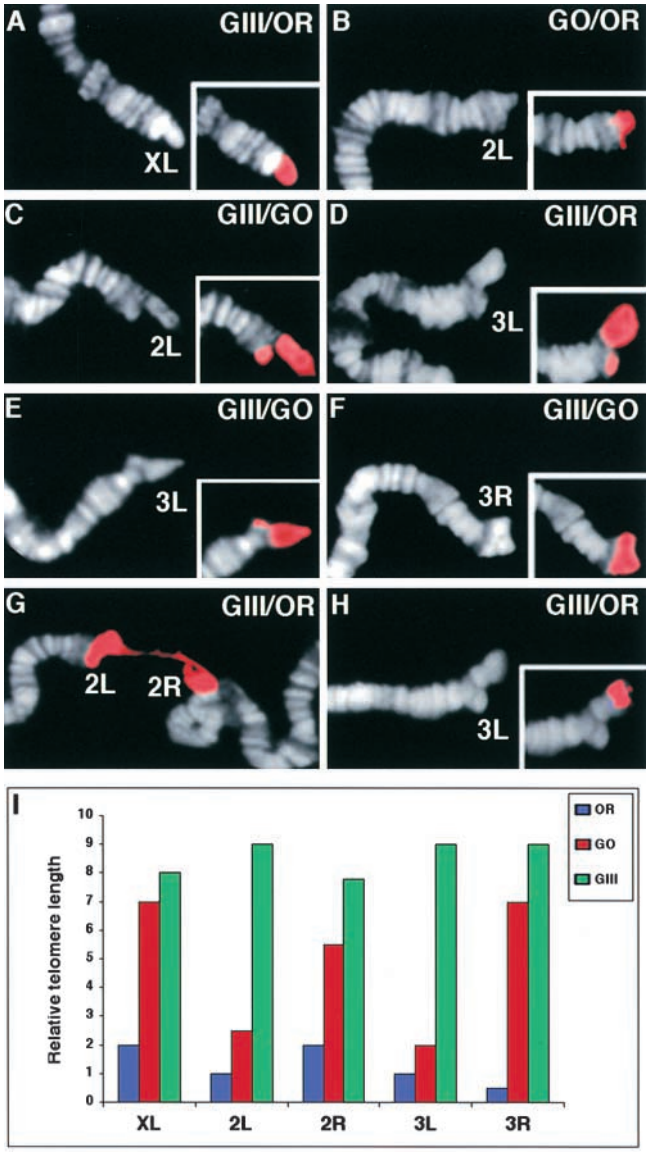

FIGURE 2.—Quantification of telomere length to polytene chromosomes by FISH. (A–H) Polytene chromosomes of GO/OR, GIII/OR, or GO/GIII females hybridized with either *HeT-A* (A–G) or *TART* (H) probes. (G) The *2*L and *2*R telomeres are connected by a thread of *HeT-A*-containing material. (I) The relative lengths of the Oregon-R, Gaiano-O, and Gaiano-III telomeres, estimated by measuring the intensities of the *HeT-A* telomeric signals. Abbreviations are as in Figure 1.

III telomeres are comparable, regardless of their lengths at the time Gaiano-III was constructed (Figure 2I). The finding that Gaiano-O telomeres differ in length suggests that these flies may carry additional elements that regulate telomere extension, thus limiting telomere growth. These elements may also be present in Gaiano-III, as the amount of *HeT-A*, measured by Southerns, is similar after 85 and 130 generations (see below).

Hybridization of polytene chromosomes from Oregon-R/Gaiano-III females with a probe for *TART*, the other telomere-specific transposon, revealed that all the telomeres of Gaiano-III chromosomes are also enriched in *TART* sequences (Figure 2H). The intensities of the fluorescent signals generated by *TART* hybridization, however, were much lower than those observed after hybridization with *HeT-A*. This suggests that *TART* addition contributed to telomere elongation in Gaiano-III to a lesser extent than *HeT-A* addition.

Although *HeT-A* and *TART* are localized primarily at the telomeres, weak hybridization signals were also observed in the chromocenter, while the euchromatic arms were completely devoid of signals (data not shown). Because the chromocenter of Drosophila polytene chromosomes contains heterochromatin, which is underreplicated during polytenization (RUDKIN 1969; ZHIMULEV 1998), it is possible that the hybridization signals observed in the chromocenter of Gaiano-O and Gaiano-III polytene chromosomes reflect significant accumulations of *HeT-A* and *TART* elements in heterochromatic regions. Previous work has shown that *HeT-A* sequences (YOUNG *et al.* 1983; TRAVERSE and PARDUE 1989; DANILEVSKAYA *et al.* 1993), like other transposable elements (PIMPINELLI *et al.* 1995), are located in discrete clusters in both centric heterochromatin and in the heterochromatic *Y* chromosome.

To ask whether the Gaiano factor(s) that increases *HeT-A* addition to telomeres also affects the amount of *HeT-A* in heterochromatin, we hybridized *HeT-A* to mitotic chromosomes of the Oregon-R, Gaiano-I, Gaiano-II, and Gaiano-III stocks (Figure 3). These studies first established that the Gaiano factor also increases telomere length in mitotic chromosomes. In Oregon-R, weak telomeric signals are observed only at the *2*R and *X*L telomeres, and these signals are detectable only under particularly favorable conditions (Figure 3A). Gaiano-O chromosomes exhibit clear signals at all but the *3*L telomere, which is usually unlabeled (Figure 3B). In Gaiano-I and Gaiano-II, clearly visible telomeric *HeT-A* signals are restricted to the *X* chromosome and second chromosome, respectively (Figure 3, C and D). This is consistent with the origin of the chromosomes, as these are the only Gaiano-derived chromosomes in each stock. In Gaiano-III, however, all the telomeres are always clearly labeled after 130 generations (Figure 3, E and F).

In contrast, the Gaiano factor(s) has little effect on heterochromatic *HeT-A* accumulations. In Oregon-R, the *Y* chromosome displays a signal on the tip of the short arm (*Y*S), two weaker signals in the interior of the chromosome, and a weak subtelomeric signal on the tip of the long arm (*Y*L; Figure 3A, inset). *Y* chromosomes in Gaiano-O, Gaiano-I, Gaiano-II, and Gaiano-III exhibit the same signals in location and intensity observed in their Oregon-R counterpart. The Gaiano-O and Gaiano-III *Y* chromosomes, however, show an additional signal at the tip of *Y*L (Figure 3, B and F, insets). While the signals observed at the *Y*S ends of all the *Y* chromosomes may correspond to either subtelomeric *HeT-A* clusters or real *HeT-A*-enriched telomeres, the

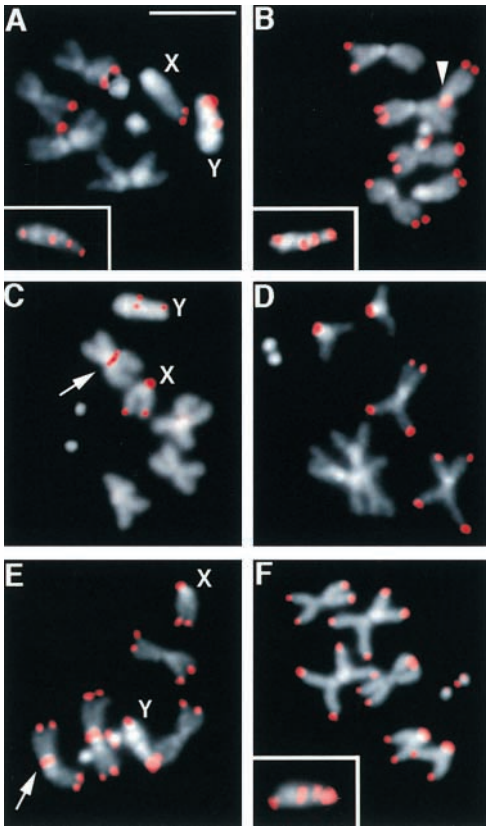

Figure 3.—Fluorescent *in situ* hybridization with a *HeT-A* probe (red) to mitotic chromosomes. Chromosomes were obtained from colchicine-arrested brain cells of Oregon-R (A), Gaiano-O (B), Gaiano-I (C), Gaiano-II (D), and Gaiano-III (E and F) larvae. The inserts in A, B, and F show *Y* chromosomes from Oregon-R, Gaiano-O, and Gaiano-III stocks, respectively. Note that the labeling pattern of all these *Y* chromosomes is similar, except for the tip of *Y*L, which is labeled in Gaiano-O and Gaiano-III but not in Oregon-R, where the fluorescent signal is subterminal. Also note the polymorphic *Het-A* clusters in *X*R (arrowhead) and in the third chromosome pericentric heterochromatin (arrows). See text for further explanations. Bar, 5 mm.

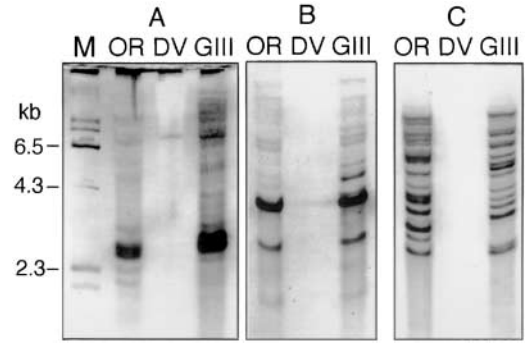

Figure 4.—Genomic abundance of *HeT-A*, *TART*, and *jockey* in Oregon-R and Gaiano-III. Southern blots of genomic DNAs extracted from *D. melanogaster* stocks Oregon-R (OR) and Gaiano-III (GIII) and from *D. virilis* (DV), a species without *HeT-A* (Biessmann *et al.* 2000), were probed with the *HeT-A* ORF (A) or the pol ORF of *TART* (B) or *jockey* (C). DNA bands of different sizes are detected in A and B, which reflect a highly polymorphic *HeT-A/TART* terminal array. In A, a strong band at ~3.0 kb is identified in both Oregon-R and Gaiano-III. This suggests that there is another *Bam*HI site in the extreme end of the 3′ UTR, which was not identified in the sequenced *HeT-A* clone (Biessmann *et al.* 1992). In B, two major bands at ~3.4 and 4.2 kb are detected in both Oregon-R and Gaiano-III, with an additional intense band at ~4.4 kb identified in Gaiano-III. The 3.4- and 4.2-kb bands were expected from the *TART* sequence (no. U14101), which predicts a restriction map with three *Bam*HI sites. (C) DNAs from both Oregon-R and Gaiano-III exhibit a similar pattern of *jockey* element distribution. M shows the *Hin*dIII marker bands. The marker band profile in B and C is the same. To quantitate the relative amounts of *HeT-A* and *TART* DNA, the blots were scanned and the relative intensities of different lanes were determined and normalized to a *white* hybridization in the same lane. Abbreviations are as in Figure 1.

signal at the tip of the *Y*L in Gaiano-O and Gaiano-III is likely to represent a genuine telomeric accumulation of *HeT-A* sequences.

The Oregon-R and Gaiano-O stocks are both polymorphic for a *HeT-A* cluster located in the short arm (*X*R) of the acrocentric *X* chromosome (Figure 3B). This polymorphism is also present in Gaiano-I and Gaiano-II, but not in Gaiano-III, where all individuals examined ($n = 20$) showed a clear signal at the *X*R tip. Thus, it is quite likely that in Gaiano-III the *X*R telomeres have acquired newly transposed *HeT-A* sequences that mask the polymorphism.

Finally, Oregon-R, Gaiano-O, and the derivative strains are polymorphic for a *HeT-A* cluster located near the centromere of chromosome *3* (Figure 3). The intensities of the signals associated with these clusters are comparable in all stocks, suggesting that the Gaiano factor had no effect on the *HeT-A* copy number in these heterochromatic regions.

In summary, the results obtained by FISH on mitotic chromosomes are fully consistent with the observations of *HeT-A* labeling at polytene chromosomes and provide two additional pieces of information. First, the Gaiano factor(s) causes the extension not only of euchromatic telomeres, but also of heterochromatic telomeres such as those on *X*R and *Y*L. Second, this genetic factor(s) does not cause *HeT-A* accumulation in centric heterochromatin. We, therefore, conclude that the Gaiano factor specifically increases the rate of *HeT-A* addition to both euchromatic and heterochromatic telomeres.

Gaiano exhibits an increase in *HeT-A* and *TART* copy number: To quantify the relative amount of *HeT-A* and *TART* sequences in Oregon-R and Gaiano-III, DNA from these stocks was blotted and probed with either the *HeT-A* ORF or the pol ORF of the *TART* element. Southern blotting experiments performed after 85 and 130 generations, following the Gaiano-III stock construction, gave similar results. These Southern blots showed a fourfold increase in *HeT-A* copy number and a twofold increase in *TART* copy number in Gaiano-III relative to Oregon-R (Figure 4). The relative increase

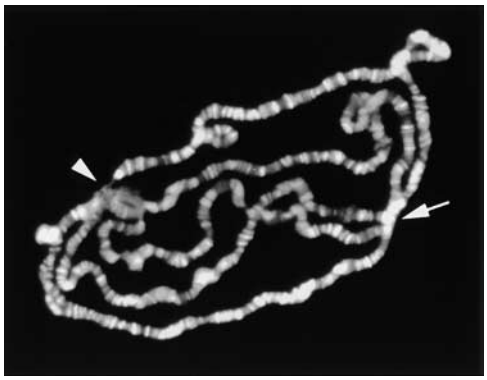

FIGURE 5.—Polytene chromosomes from the Gaiano-III stock stained with DAPI. Note that all the telomeres are fused (arrow), forming a structure reminiscent of a chromocenter (arrowhead).

in *HeT-A* copy number revealed by Southern blotting does not fully agree with the *in situ* hybridization data, which suggest that the Gaiano-III telomeres are approximately sevenfold longer than Oregon-R telomeres (Figure 2I). However, we believe that this discrepancy does not depend on the method used for *HeT-A* quantification, but rather on the presence of heterochromatic *HeT-A* sequences. We have shown that Oregon-R and Gaiano-III are both polymorphic for *HeT-A* heterochromatic clusters. If some of the *HeT-A* copies in these clusters were detected by Southern blotting, they would mask the difference in abundance of telomeric *HeT-A* sequences between the two stocks.

We also performed Southern blot analysis and *in situ* hybridization experiments using *jockey*, a *D. melanogaster* retrotransposon very similar in structure to *HeT-A* and *TART* (BIESSMANN *et al.* 1992; LEVIS *et al.* 1993). These experiments did not detect significant differences between Oregon-R and Gaiano-III in total *jockey* DNA (Figure 4C) or in the number of *jockey* insertions on polytene chromosomes (data not shown), suggesting that the Gaiano factor(s) is specifically involved in the control of *HeT-A* and *TART* copy number.

Telomere-telomere associations in Gaiano: Examination of polytene chromosomes from Gaiano-III larvae revealed very frequent TAs. Analysis of 50 polytene nuclei hybridized with *HeT-A* showed that all of them exhibited at least one TA. In the majority of these nuclei most if not all of the telomeres were associated with other telomeres, thus giving rise to a chromocenter-like structure (Figure 5). In addition, in several cases telomeres were connected by thread-like structures that hybridized with the *HeT-A* probe (Figure 2G), as seen occasionally in wild-type larvae (RUBIN 1978). We believe that these *HeT-A*-positive threads result from the stretching of telomeric material caused by the squashing procedure used to obtain polytene chromosome preparations.

The frequent TAs seen in Gaiano-III might be a direct

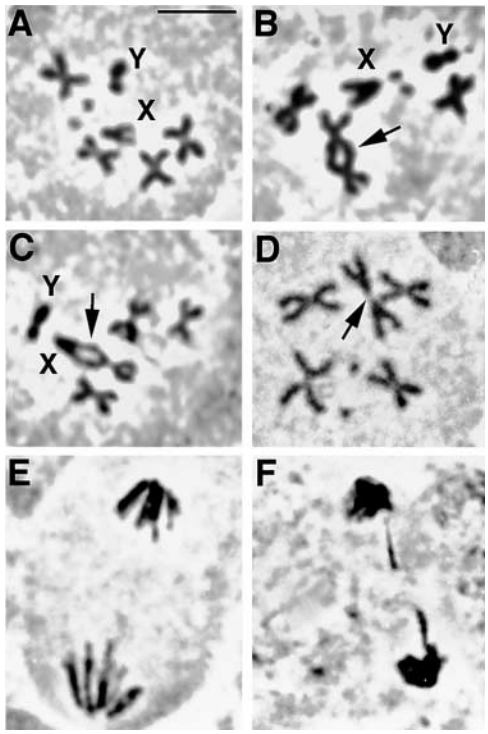

FIGURE 6.—Telomere-telomere associations in larval brain cells from Gaiano stocks. (A–D) Colchicine-arrested metaphases. (E and F) Anaphases. (A) Normal male metaphase. (B) Male metaphase with a DTA involving two metacentric autosomes (arrow). (C) Male metaphase with a DTA involving a metacentric autosome and the long arm of the acrocentric *X* chromosome (arrow). (D) Female metaphase with a DTA between the *X*R telomeres (arrow). (E) A normal anaphase. (F) An anaphase showing a resolved chromosome bridge. Bar, 5 μm.

result of the activity of the Gaiano factor(s), or they could be a consequence of the extraordinary length of Gaiano-III telomeres. To discriminate between these two possibilities, we examined the polytene chromosomes of the Gaiano-II stock, which contains second chromosomes with long telomeres but which lacks the genetic factor(s) on the Gaiano third chromosome. Of 50 Gaiano-II polytene nuclei hybridized with *HeT-A*, 26 displayed TAs, all involving second chromosome ends. This strongly suggests that long *HeT-A*-containing telomeric regions tend to fuse to each other, even in the absence of a putative genetic factor(s) that enhances *HeT-A* addition.

We next asked whether the long telomeres associate with each other in mitotic cells. Analysis of colchicine-arrested metaphases from larval brain cells revealed that the Gaiano-O and Gaiano-III stocks both display a significant frequency of TAs, reminiscent of those described in *UbcD1* mutants (CENCI *et al.* 1997; Figure 6). Chromosome end associations are normally classified as either double telomere associations (DTAs), in which one pair of sister chromatids interacts with another pair, or single telomere associations, where the two sister chromatids behave independently (CENCI *et al.* 1997).

TABLE 1

Frequencies of telomere-telomere attachments in Gaiano and Oregon-R stocks

Stock	No. of cells scored	Frequency of DTAs (%)
Gaiano-O	1023	2.2
Gaiano-I	1069	1.0
Gaiano-II	968	0.9
Gaiano-III	491	14.6
Oregon-R	1000	0.2

In Oregon-R, Gaiano-O, Gaiano-II, and Gaiano-III, scoring was carried out in both male and female brains. In Gaiano-I, only females were scored.

Here we consider only DTAs, because they are cytologically unambiguous and almost completely absent in wild type. As shown in Table 1, metaphases of cells with Gaiano chromosomes exhibit 4.5–28 times the frequency of DTAs observed in the Oregon-R control. In addition, 8 of the 9 DTAs observed in Gaiano-II involved at least one of the two second chromosomes, while 7 of the 11 DTAs found in Gaiano-I females involved at least one of the *X* chromosomes. These data clearly indicate that long *HeT-A*-containing telomeres also tend to associate with each other in mitotic cells. Again, telomere length seems to be an important factor for the formation of TAs.

We also investigated the fate of TAs observed in Gaiano-III. Examination of non-colchicine-treated Gaiano-III brain cells revealed that the TAs seen in metaphase generate chromatin bridges in anaphase. However, most if not all of these bridges are resolved, as no lagging acentric fragments in anaphase figures were observed. In *UbcD1* mutants TAs are similarly resolved in mitotic anaphases but are not resolved during male meiosis, giving rise to extensive chromosome breakage (Cenci *et al.* 1997). In contrast, we found no evidence for chromosome breakage during meiotic anaphase I or II in Gaiano-III males (data not shown).

HP1 localization in Gaiano telomeres: Mutations in the *Su(var)205* gene, which encodes heterochromatic protein 1 (HP1), cause extensive TAs that fail to be resolved during anaphase (Fanti *et al.* 1998). HP1 binds the chromocenter, the telomeres, and multiple euchromatic sites on all Drosophila chromosomes (James *et al.* 1989; Kellum *et al.* 1995; Fanti *et al.* 1998). In addition, HP1 binds the ends of terminally deleted chromosomes that lack *HeT-A* and *TART* sequences, indicating that HP1 telomere binding is independent of *HeT-A* and *TART* (Fanti *et al.* 1998).

To examine HP1 binding to the long Gaiano telomeres we immunostained polytene chromosomes of Gaiano-O/Gaiano-III heterozygous females. This analysis showed that HP1 binds both the Oregon-R and the Gaiano-III telomeres. However, while the Oregon-R telomeres usually exhibit a compact HP1 staining, the

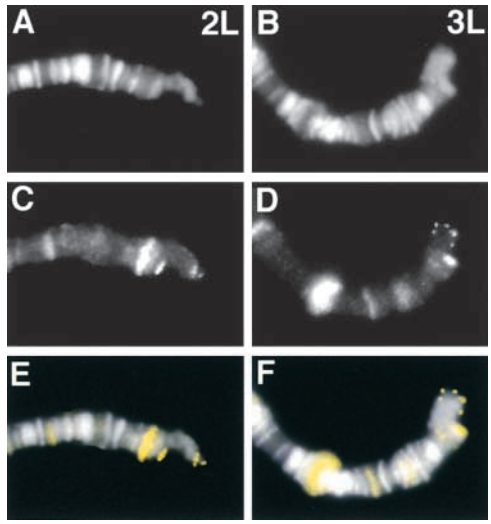

Figure 7.—HP1 immunostaining of polytene chromosomes from Oregon-R/Gaiano-III hybrids. (A and B) DAPI staining. (C and D) HP1 immunostaining. (E and F) Merged images; the HP1 signal is yellow. Note the discontinuous HP1 staining on the long Gaiano-III telomeres.

Gaiano-III telomeres often display a discontinuous HP1 labeling (Figure 7). Nonetheless, the amount of HP1 associated with the Gaiano-III telomeres is similar to, or only slightly lower than, that seen on Oregon-R telomeres. The reason for the discontinuous HP1 labeling at the Gaiano-III telomeres is unclear. This staining pattern may reflect either a squashing artifact or a peculiar structure of the Gaiano-III telomeres. For example, an uneven termination of the DNA strands at the Gaiano-III telomeres may result in a punctate and discontinuous HP1 localization.

Genetic mapping of the Gaiano factor: To ask whether the Gaiano factor maps as a single genetic unit, we crossed the Gaiano-III stock to a strain carrying the markers *ru h th st cu sr* e^s *ca*, which span chromosome *3*. After allowing free recombination in the heterozygous progeny females, 20 recombinant third chromosomes were recovered and placed in stock with non-Gaiano genetic backgrounds. As controls, two nonrecombinant chromosomes were also recovered, one with all of the markers from the multiply marked chromosome, the other with none of these markers. The former is identified as recombinant *16*; the latter was lost. After 50 generations these stocks were outcrossed to the *Sb/TM6, Ubx* stock used in the last step in their construction, and the *2*L telomeres of the heterozygous offspring were examined in orcein-stained polytene chromosomes (as shown in Figure 2, differences in telomere length can be clearly seen even in the absence of *in situ* hybridization with *HeT-A*). Length differences between homologous telomeres were evident in some recombinant lines, but not in others (Table 2). For most recombinants, the telomere phenotype is consistent with a single telomere-elongating genetic factor mapping between *sr* and *e*, at

TABLE 2

Recombinants between chromosome *3* of Gaiano-III and a *ru h th st cu sr e^s ca* multiply marked chromosome

Recombinant no.	Visible markers	Telomere elongation
1	*th st cu sr e^s ca*	No
2	*sr e^s ca*	No
3	*ru h th st cu*	Yes
4	*th st cu sr*	Yes
5	*ca*	Yes
6	*ru e^s ca*	No
7	*th st cu*	No
8	*th st cu sr e^s ca*	No
9	*th st cu sr*	Yes
10	*ru h th st cu*	Yes
11	*sr e^s*	No
12	*cu sr e^s*	No
13	*ru sr e^s ca*	No
14	*th st cu sr e^s*	No
15	*cu sr e^s ca*	No
16	*ru h th st cu sr e^s ca*	No
17	*e^s ca*	No
18	*th st cu*	Yes
19	*ru h th st cu ca*	Yes
20	*ru h th e^s ca*	Yes
21	*cu sr e^s*	No

~69. One recombinant line, however, does not fit the pattern. According to its genetic markers, recombinant *7* should carry the telomere-elongating factor from Gaiano-III. Nonetheless, it does not exhibit long terminal arrays on chromosome *2*. In addition, although the right arm of this recombinant chromosome should derive from the Gaiano-III parental chromosome, it does not display a long *3*R telomere. It is possible that recombinant *7* was generated by multiple recombination events, including double crossing over between the *sr* and *e^s* markers. Alternatively, the telomeres of this recombinant line may be short for reasons independent of the presence of the telomere-elongating factor.

The *ru h th st cu sr e^s ca* chromosome has been kept in heterozygous condition for many years and has accumulated lethals and other deleterious mutations. In particular, we mapped a lethal mutation segregating on this chromosome very close to *cu*. Thus, several recombinant chromosomes, including *4*, *10*, *18*, and *20*, were homozygous lethal and were maintained over the *TM6* balancer. Most of the other recombinant chromosomes, while not strictly lethal, required the *TM6* balancer to maintain a healthy stock. As telomeres grew in these heterozygous lines, we conclude that the genetic factor responsible for telomere elongation is dominant. Taken together, our mapping data suggest that the process of telomere elongation observed in Gaiano-III is due to a dominant mutation in a single genetic unit, and we propose the name *Telomere elongation* (*Tel*) for this new gene.

DISCUSSION

Regulation of *HeT-A* and *TART* addition: This article includes the first description of the genetic control of telomere length in Drosophila. We have found a mutation, *Tel*, at ~69 on chromosome *3* that is correlated with increased addition of *HeT-A* and *TART* elements to chromosome ends and telomere elongation.

The mechanism by which *Tel* mediates *HeT-A* and *TART* addition to chromosome ends is not known. One possibility is that *Tel* increases the rate of *HeT-A* and *TART* transposition to the telomeres. Previous studies have clearly shown that these elements can specifically transpose to chromosome ends. Starting with a terminal deficiency chromosome broken in the *yellow* (*y*) gene, Biessmann *et al.* (1990) found that *HeT-A* elements transposed to the tip of the *X* chromosome and attached to the *y* sequence by their oligo(A) tails. New *HeT-A* elements then attached to the first in a head-to-toe fashion (Biessmann *et al.* 1992).

Various *HeT-A* elements have either a single ORF (Biessmann *et al.* 1994) or two overlapping ORFs (Danilevskaya *et al.* 1994) that may encode RNA-binding proteins. These retroelements, however, do not encode a reverse transcriptase (RT), and it has been proposed that an RT is provided *in trans*, possibly by the host, as a means of controlling telomere length (Biessmann *et al.* 1994). *Tel* may increase, directly or indirectly, the activity of an RT that could promote both *HeT-A* and *TART* transposition, leading to telomere elongation. RT-like proteins also regulate telomere length in other eukaryotes. In these organisms telomere extension is modulated by levels of telomerase catalytic subunits (Lingner *et al.* 1997; Bodnar *et al.* 1998), which contain conserved domains common to all known reverse transcriptases, including those encoded by non-LTR retrotransposons, such as *HeT-A* and *TART* (Eickbush 1997). Alternatively, *Tel* may encode a product that increases the accessibility of an RT to both *HeT-A* and *TART* RNAs or a transposition intermediate to chromosome ends. At present, however, we cannot distinguish among these alternatives, nor can we establish whether a putative *Tel* product is encoded by the host genome *per se* or by another transposable element.

Alternatively, *Tel* might increase *HeT-A* and *TART* addition to telomeres by promoting recombination/gene conversion events at chromosome ends. Telomere recombination may be the primary mechanism for maintaining chromosome length in some organisms that lack telomerase, for example, in other dipteran insects, such as the mosquito Anopheles (Roth *et al.* 1997) and the midge Chironomus (Lopez *et al.* 1996). Even in organisms that normally rely on telomerase to extend chromosome ends, telomeres may be elongated by recombination when telomerase is defective or missing. In the yeasts Saccharomyces (Lundblad and Blackburn 1993), Schizosaccharomyces (Nakamura *et al.* 1997), and Kluy-

veromyces (McEachern and Hicks 1993) most cells lacking a component of telomerase senesce and die. Survivors, however, arise relatively frequently in all three organisms, and in both Saccharomyces and Kluyveromyces survival requires a RAD52-dependent recombination mechanism. In Saccharomyces, two classes of survivors are found. Type I survivors carry tandem duplications of the subtelomeric Y′ elements, possibly generated by gene conversion (Lundblad and Blackburn 1993). Kluyveromyces (McEachern and Blackburn 1996) and Saccharomyces (Teng and Zakian 1999) type II survivors carry very long terminal repeat arrays that resemble the long terminal arrays found in human tumors (Bryan *et al.* 1997) and immortalized cultured cells (Bryan *et al.* 1995) that lack telomerase. These immortalized telomerase-negative human cells use an alternative mechanism to lengthen telomeres (ALT) and carry distinctive nuclear bodies that contain telomeric DNA, telomere-binding proteins TRF1 and TRF2, as well as recombination proteins RAD51 and RAD52 (Yeager *et al.* 1999), consistent with the idea that human telomeres are elongated by recombination in the absence of telomerase. Direct evidence has been found for the transfer of a plasmid DNA tag from one terminal repeat array to another in ALT cells, as expected for a recombination or gene conversion mechanism (Dunham *et al.* 2000).

Recent work has suggested that telomere elongation in Drosophila can also occur via a recombination/gene conversion pathway. It has been shown that broken *X* chromosomes that terminate in the *y* gene (Mikhailovsky *et al.* 1999), or in a *HeT-A* element directly attached to the *y* gene (Kahn *et al.* 2000), can also be extended by a gene conversion mechanism that adds *y* sequences or *HeT-A* sequences to the terminus, respectively. Moreover, P. Georgiev (personal communication) found that chromosome *3* from Gaiano carries a dominant genetic factor(s) that increases the rate of gene conversion. However, it remains to be seen whether this factor(s) is *Tel*.

The basis for telomere-telomere associations: Both the polytene and mitotic nuclei of the Gaiano stocks display frequent TAs. These associations preferentially involve long telomeres and occur in the absence of *Tel*. Thus, the formation of TAs in Gaiano stocks depends primarily on telomere length rather than the genetic activity of *Tel*.

Telomeric associations have been observed both in mammals and Drosophila. In mammals frequent TAs are present in cells with short telomeres, such as senescent human fibroblasts (Benn 1976), tumor cells (de Lange 1994), and fibroblasts from mice lacking functional telomerase (Hande *et al.* 1999a). TAs are also found in human cells overexpressing a dominant negative allele of the telomere-binding protein TRF2 (van Steensel *et al.* 1998). It has, thus, been suggested that short telomeres become fusigenic because they cannot recruit a sufficient amount of TRF2 (van Steensel *et al.* 1998). The TAs induced by inhibition of TRF2 are due to covalent fusions of telomeric DNA and are not resolved during anaphase, thus leading to chromosome breakage (van Steensel *et al.* 1998).

Frequent TAs have also been found in mouse cells carrying mutations in components of the DNA-protein kinase (PK) complex (Slijepcevic *et al.* 1997; Bailey *et al.* 1999; Hande *et al.* 1999b; Difilippantonio *et al.* 2000; Samper *et al.* 2000). This complex consists of a catalytic subunit (PKcs) and a heterodimeric regulatory factor, Ku, containing the Ku70 and Ku80 proteins (Jeggo 1997). Interestingly, the TAs observed in mice deficient for either PKcs or Ku80 do not result from telomere shortening, as these mice have longer telomeres than their wild-type counterparts (Slijepcevic *et al.* 1997; Hande *et al.* 1999b; Samper *et al.* 2000). It is unclear whether these TAs are resolved during anaphase.

In Drosophila frequent TAs have been observed in *UbcD1* and *Su(var)205* mutants (Cenci *et al.* 1997; Fanti *et al.* 1998). The *UbcD1* gene encodes a ubiquitin-conjugating (E2) enzyme (Cenci *et al.* 1997), while *Su(var)205* encodes HP1, a protein enriched in both centric heterochromatin and telomeric regions (Eissenberg 1989; Fanti *et al.* 1998). In larval brain cells of *Su(var)205* mutants, TAs result in anaphase bridges leading to extensive chromosome rearrangements and may, therefore, involve covalent fusions of telomeric DNA (Fanti *et al.* 1998). In contrast, TAs between mitotic chromosomes of *UbcD1* mutants are resolved during anaphase without causing chromosome breakage or nondisjunction (Cenci *et al.* 1997), suggesting that these TAs are due to proteinaceous linkages between telomeres that result when one or more telomere-associated proteins fail to be degraded via ubiquitin-mediated proteolysis (Cenci *et al.* 1997).

Because they can be resolved, the TAs observed in Gaiano brain cells are similar in strength to those elicited by the *UbcD1* mutations. The basic mechanisms that give rise to telomeric associations may, nonetheless, differ. In Gaiano stocks those chromosomes that exhibit an increase in *HeT-A* and *TART* at chromosome ends preferentially form TAs. This suggests that an increase in length, and more specifically acquisition of *HeT-A* and *TART*, make a Gaiano telomere sticky. In contrast, telomeres in *UbcD1* mutants, even those devoid of *HeT-A* and *TART* sequences, participate in telomeric associations with high frequency, suggesting that the presumptive UbcD1 substrates bind chromosome ends independently of DNA sequence (G. Cenci, G. M. Siriaco and M. Gatti, unpublished data). It is, thus, unlikely that Gaiano telomeres are sticky because these UbcD1 substrates accumulate on them. It is also unlikely that HP1 plays a role in mediating telomere associations in Gaiano, as HP1 binds chromosome ends independently of the presence of *HeT-A* and *TART* sequences (Fanti *et*

al. 1998). Accordingly, the amount of HP1 localized to the long Gaiano telomeres is comparable to that associated with the Oregon-R telomeres. Finally, TAs in HP1 mutants are not resolved in anaphase, indicating that the mechanism of fusion is not the same as in Gaiano. Thus, it appears that the mechanism of TA formation in Gaiano is distinct from those involving either UbcD1 or HP1. Given that the Gaiano TAs are weak associations, it is likely that they are mediated by a *HeT-A* and/or *TART* binding protein(s) or by DNA hydrogen bonding rather than covalent DNA-DNA bonds.

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