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High incidence of rapid telomere loss in telomerase-deficient *Caenorhabditis elegans*

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ABSTRACT

Telomerase is essential to maintain telomere length in most eukaryotes. Other functions for telomerase have been proposed but molecular mechanisms remain unclear. We studied *Caenorhabditis elegans* with a mutation in the *trt-1* telomerase reverse transcriptase gene. Mutant animals showed a progressive decrease in brood size and typically failed to reproduce after five generations. Using PCR analysis to measure the length of individual telomere repeat tracks on the left arm of chromosome V we observed that *trt-1* mutants lost ~125bp of telomeric DNA per generation. Chromosome fusions involving complex recombination reactions were observed in late generations. Strikingly, *trt-1* mutant animals displayed a high frequency of telomeres with many fewer repeats than average. Such outlying short telomeres were not observed in *mrt-2* mutants displaying progressive telomere loss very similar to *trt-1* mutants. We speculate that, apart from maintaining the average telomere length, telomerase is required to prevent or repair sporadic telomere truncations that are unrelated to the typical 'end-replication' problems.

INTRODUCTION

A minimum number of repeats are required at every chromosome end in order to form a proper telomere structure that prevents activation of a DNA damage response (1,2). In order to maintain telomere repeats most eukaryotes require the enzyme telomerase, which minimally consists of a RNA template and a reverse transcriptase. It has been proposed that telomerase has functions other than telomere length maintenance, such as telomere end protection and regulation of DNA damage responses (1,3–6). However, details of the molecular mechanisms involved in such functions are lacking.

Various model organisms have been used to study the role of telomerase. In *Saccharomyces cerevisiae*, null mutations in any of the five genes, *EST1*, *EST2*, *EST3*, *CDC13* and *TLC1*, result in progressive telomere shortening followed by senescence (7–11). Late generations of the telomerase-deficient *est1*Δ strain showed elevated mutation rates and frequent chromosomal rearrangements and end-to-end fusions (12). Telomerase null mutants in multicellular organisms also display progressive telomere shortening and genetic instability. In the *Arabidopsis* telomerase mutant (*AtTERT*$^{-/-}$) developmental abnormalities and chromosomal instability became evident after five generations (13–15). Molecular characterization revealed that most fusions in *AtTERT*$^{-/-}$ involve a telomeric end and a subtelomeric end (15). In addition, Ku-dependent non-homologous end joining (NHEJ) appears to be the major mechanism by which these fusions form.

In mice, homozygous disruption in either the telomerase RNA template gene (*mTERC*) or the reverse transcriptase gene (m*TERT*) results in progressive telomere shortening and chromosomal instability in late generations (16–19). Recent studies have linked mutations in one allele of the *hTERC* or *hTERT* gene to bone marrow failure in humans (20–23). Of note, telomerase activity is typically readily detectable in the cultured lymphocytes from such patients, yet the telomere length in such cells is typically (very) short. These observations indicate that even modest (e.g. 2-fold) reductions in telomerase levels are poorly tolerated in human cells (24). Compared with the absence of a disease phenotype in mice that are haplo-insufficient for either *mTERC* (25) or *mTERT* (26), the marrow failure in patients with comparable genetic defects is remarkable. One possibility is that telomerase in humans has a role outside telomere length maintenance (6). Alternatively, the large differences in average telomere length between inbred mice (~50 kb) and man (~5 kb) could be important for these observed phenotypic

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differences. In order to clarify these possibilities further studies on the role of telomerase in multicellular organisms are needed.

The putative reverse transcriptase component of *Caenorhabditis elegans* telomerase is encoded by *trt-1* (27). Telomeric DNA in *C.elegans* consists of TTAGGC repeats and has been shown to span between 4 and 9 kb in the wild-type strain N2 (28). We have adapted previously the PCR-based technique STELA (single telomere length analysis) (29) to telomere length measurement in *C.elegans* (30). Using STELA, we show here that besides progressive telomere shortening and telomere fusions, disruption of telomerase in *C.elegans* leads to a high frequency of short outlying telomeres, suggesting that telomerase is required to prevent or repair large-scale truncations of telomeric DNA.

MATERIALS AND METHODS

Strains

Worms were handled as described by Brenner (31) but were grown at room temperature (19–23°C) unless stated otherwise. The strains used in this study included N2, *glp-4(bn2ts)* that has been outcrossed to N2 10 times (KR4138 and KR4139), and *trt-1(ok410)* that has been outcrossed to N2 for at least 10 times (KR4050). The deletion allele *ok410* was generated by Robert Barstead Laboratory (Oklahoma Medical Research Foundation, OK) and characterized by the Vancouver Gene Knockout Facility, which is part of the *C.elegans* Gene Knockout Consortium (for more information of the allele see www.wormbase.org).

Single telomere length analysis

Measurement of VL telomere length by STELA was carried out as described in Cheung *et al.* (30). Briefly, an oligonucleotide was first ligated to the 5′ end of telomeres. PCR was then carried out using a primer against the oligonucleotide and a primer against a unique sequence in the subtelomeric region of Chromosome VL, followed by Southern blotting using a probe that hybridizes to the subtelomeric region. Telomere length was calculated by subtracting 1.1 kb (the distance between the primer recognition site in the subtelomeric region and the start of the telomeric sequence) from the size of the band amplified.

Analysis of STELA data

From a gel file generated by PhosphorImager, intensity of signals was measured (in 0.1 mm intervals) along a lane by ImageQuant 5.0 software. Data were imported into Microsoft Excel for size analysis. From a size marker lane, a plot of size (kb) against distance (mm) was generated and fitted by the power function ($y = ax^b$), which works best for bands <5 kb. The formula was then used to calculate the size, and 1.095 kb was subtracted to obtain telomere length for each measured intensity value. To calculate the rate of VL telomere shortening in *trt-1* mutants, plots of intensity against telomere length were generated for each generation in each line from Figure 3. In most cases where a major cluster of bands were amplified, telomere length for the generation was determined to be the peak of the plot. In cases where bands were spread out, telomere length was arbitrarily determined to be the middle of the spread. Telomere length determined this way was plotted against generation and the plot was fitted by linear regression. The rate of VL telomere shortening is the slope of the linear curve.

Characterization of telomere fusions

Genomic DNA was extracted from single worms using phenol:chloroform:isoamyl alcohol as described in Cheung *et al.* (30), except that DNA pellet was resuspended in 10 mM Tris–HCl (pH 8.5), and used directly as template in nested PCR. Primary PCR was carried out in a 20 μl reaction containing half or the whole of the genomic DNA extracted from a single worm, 1× PCR buffer IV (ABgene), 2 mM $MgCl_2$, 0.1 μM of primer 798 (5′-GGGATGCGCAGCTAACTATAGGAC-3′), 0.3 mM of each dNTP (Amersham) and 1.5 U Extensor Hi-Fidelity PCR Enzyme Mix (ABgene). Thermal cycling conditions were the following: initial denaturation at 94°C for 3 min, 25 cycles of 94°C for 20 s, 70°C for 8 min, followed by final elongation at 70°C for 10 min. Primary PCR products (0.2 μl) were used as templates in nested PCRs, which contained the same concentrations of buffer, $MgCl_2$, dNTPs and Enzyme Mix as in primary PCRs, with 0.1 μM of primer 797 (5′-AAATGACAGTACTTATGGGTTTCGTTC-3′). Thermal cycling conditions were the same as in primary PCR, except that 30 cycles were carried out instead of 25. PCR products were purified, A-tailed, and cloned into pGEM®-T Vector (Promega), which was then transformed into DH5α™ competent cells (Invitrogen). Inserts were sequenced at the Nucleic Acid Protein Service Unit at the University of British Columbia (Vancouver, BC).

RESULTS AND DISCUSSION

We analyzed the length of telomeric DNA at the left end of chromosome V (VL) in *C.elegans* using STELA (30) in different generations of the wild-type strain N2. Marked fluctuations in telomere length over multiple generations were observed (Figure 1A), consistent with the results generated by Southern hybridization reported previously (32). Despite these fluctuations, VL telomeres were maintained within a range of ∼1 kb in all generations examined (Figure 1A). This range reflects the sum of telomere length heterogeneity in all (five) individuals analyzed from each generation. To examine variations in the distribution of telomere length among individuals, a hermaphrodite parent and 10 of its progeny were analyzed by STELA (Figure 1B). Individuals with clearly distinct telomere length distribution were apparent (progeny 3 and 9, Figure 1B). Hence, considerable telomere length variation occurs within one generation and may explain the telomere length fluctuations shown in Figure 1A. Examination of the temperature sensitive mutant *glp-4(bn2ts)*, which becomes essentially germline-less when grown at the restrictive temperature (33) suggested that the germline may be an important source of the observed telomere length diversity (Supplementary Figure S1).

The putative reverse transcriptase component of *C.elegans* telomerase is encoded by *trt-1* (27). The *trt-1* allele *ok410* carries a deletion spanning the region that encodes for three of the seven conserved reverse transcriptase motifs as

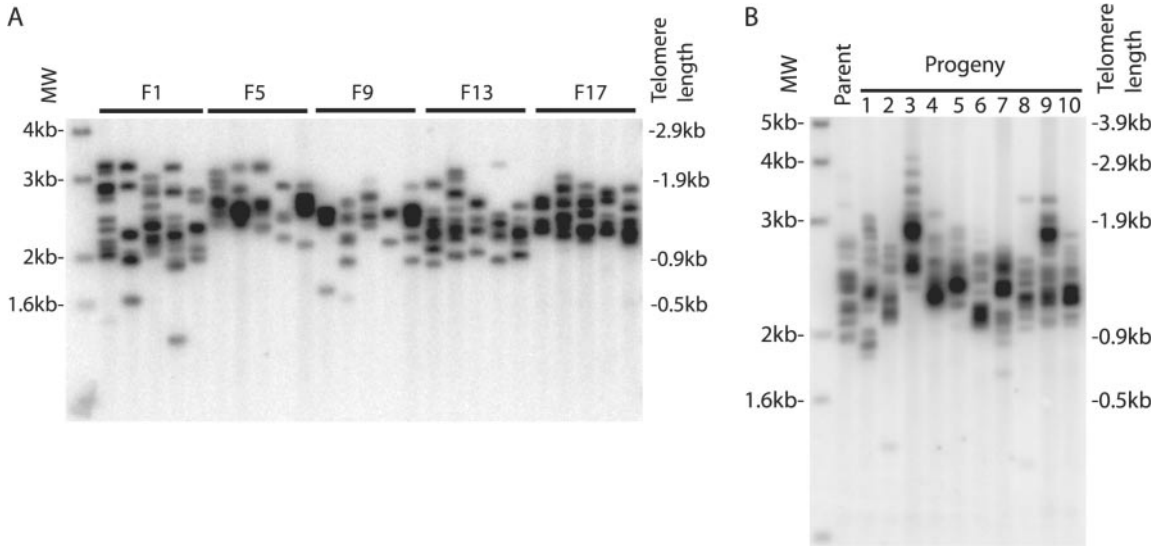


Figure 1. Telomere length heterogeneity in wild-type *C.elegans*. (**A**) Telomere length fluctuations in the wild-type strain N2. Telomere length of VL was measured by STELA at F1, F5, F9, F13 and F17. DNA was extracted from five reproductive-stage adult sampled at each of the generations. DNA was ligated to the telorette and 0.1 worm equivalent was used in each PCR. Marker lane is shown on the left and the corresponding telomere length is indicated on the right. Actual telomere length was 1.1 kb shorter than the size of the PCR product because 1.1 kb of subtelomeric sequences were also amplified. (**B**) Telomere length heterogeneity in a clonal population. A N2 parent and 10 of its progeny were analyzed by STELA. DNA was extracted from each single worm, ligated to telorette, and the entire DNA sample was used as template in PCR. Each lane represents a single worm.

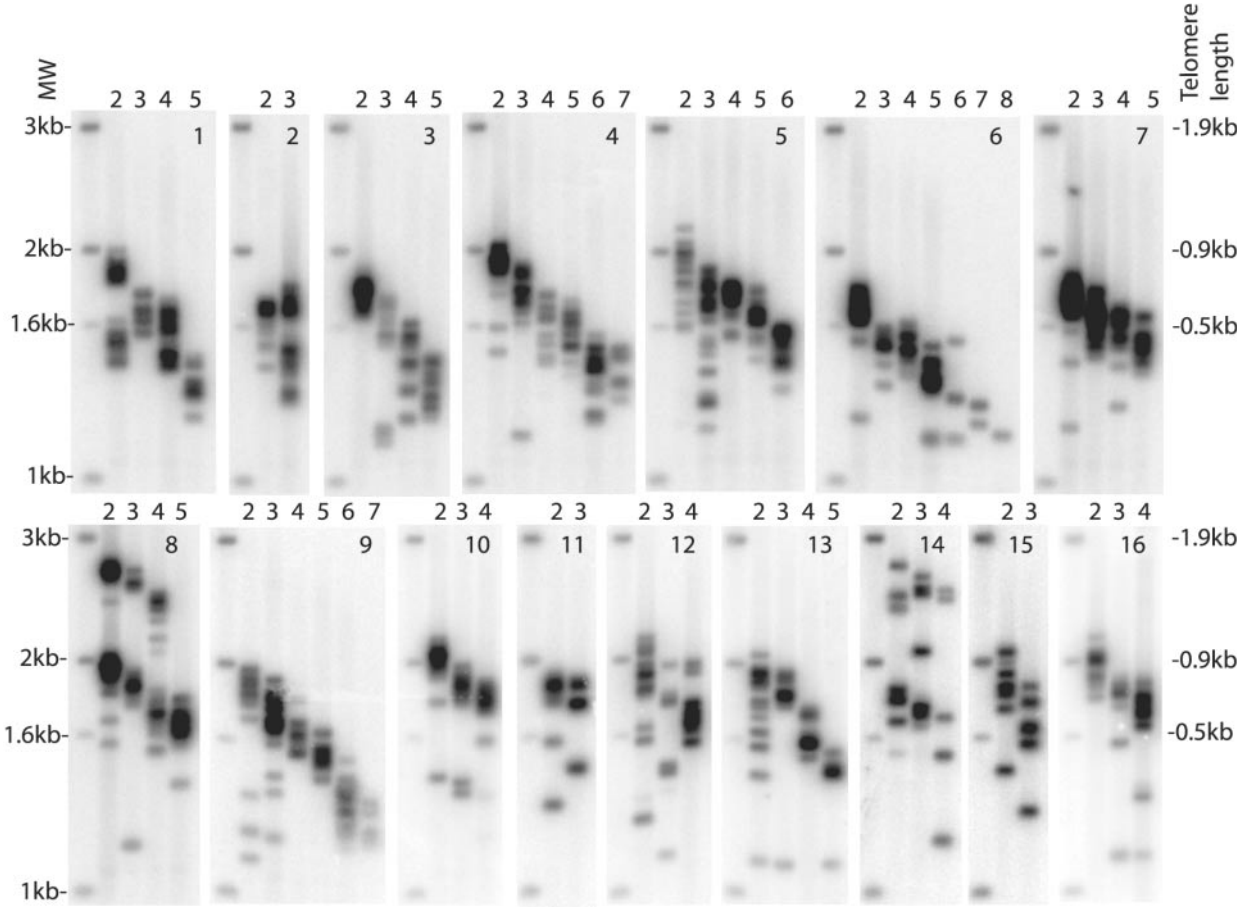


Figure 2. Progressive telomere shortening in *trt-1*. *Trt-1* was outcrossed to N2 males. From 2 heterozygous parents, 16 homozygous *trt-1* lines were set up separately. For each generation, the parent (post-reproductive stage) was analyzed by STELA, starting from F2 and ending at the generation that became sterile. Generation numbers are indicated on top of each panel; the heterozygous parent is considered as Po. A number was assigned for each line (1–16) and it is shown at the top right corner of each panel.

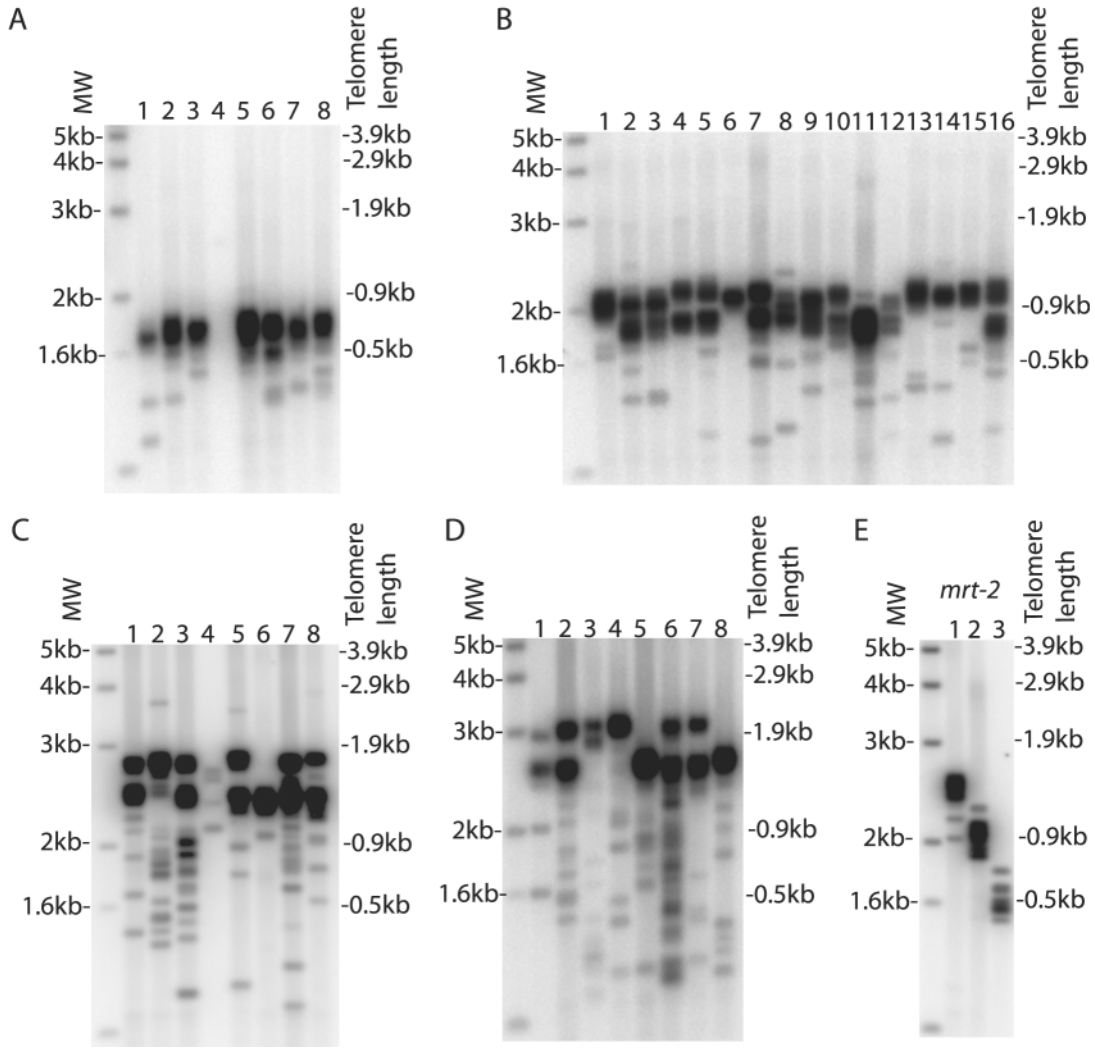


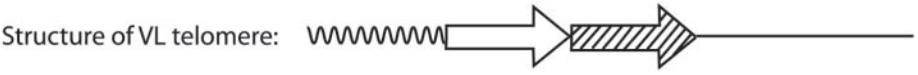
Figure 3. Short outlying telomeres are readily observed in the DNA from single *trt-1* mutants but not in the DNA from five *mrt-2* mutants. (**A**–**D**) Eight to sixteen reproductive-stage individual progeny from four separate *trt-1* parents were analyzed by STELA. Each lane represents a single worm. The two clusters of bands in most worms likely represent the two alleles of VL. In order to maintain the strain, *trt-1* has to be routinely outcrossed. The longer VL telomere could be derived from the wild-type parent and the shorter VL telomere from the *trt-1* parent. (**E**) DNA was extracted from five worms from the same *mrt-2* parent in each of F2 (lane 1), F5 (lane 2) and F9 (lane 3) and subsequently used in STELA. A DNA equivalent of one worm was used as the template in each PCR.

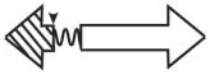
predicted by Malik *et al*. (27) (Figure S1). Sixteen lines of *trt-1* were serially propagated until the line became sterile. For each generation, the number of adult progeny was counted and the parent was analyzed by STELA after all eggs were laid. In the early generations, most adult worms appeared healthy, although *trt-1* had variably reduced progeny number (Supplementary Table S1). In later generations, the worms became sluggish and early death was frequent (data not shown). Therefore, similar to telomerase mutants in other organisms (13,16), *trt-1* has decreased general fitness. Consistent with TRT-1 being a component of telomerase, all 16 lines of *trt-1* displayed progressive shortening of telomeres (Figure 2). In most lines, the VL telomere shortened by between 100 and 150 bp per generation (Supplementary Table S2). With an estimation of 10–15 cell divisions per generation (34), we calculated that *C.elegans* loses ~10 bp of telomeric DNA per cell division. Although this rate was measured from only one chromosome end, results from different chromosome ends are expected to be similar. This rate of telomere shortening is more similar to *S.cerevisiae* (~4 bp per cell division) than to mammals (100–150 bp per cell division) (10,35), suggesting that the processing of telomeric ends after replication in *C.elegans* may be more closely related to yeast than to mammals. In a number of instances, VL telomere did not appear to shorten between generations (e.g. F3 to F4 in line 6 and F2 to F3 in line 11; Figure 2). It is possible that in those cases, rare longer telomeres in the population of germ cells were inherited, resulting in no apparent telomere shortening between those generations.

Telomere length heterogeneity in *trt-1* was studied by STELA on individual progeny from a single parent (Figure 3A–D). In contrast to wild-type (Figure 1B), telomere length was highly uniform among *trt-1* progeny (the two clusters of bands in most worms shown in Figure 3A–D likely represent the two alleles of VL), supporting the notion that telomerase is involved in the generation of telomere length diversity observed in wild-type animals (Figure S2). In addition, *trt-1* lacks the abundance of long telomeres seen

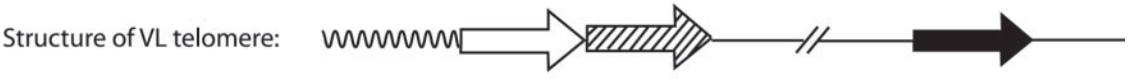
in wild type. The lack of long telomeres in *trt-1* is compatible with telomerase being important in telomere elongation in wild type. However, a high frequency of short outlying telomeres relative to wild type was also observed in *trt-1* (compare Figure 1B and Figure 3A–D). Although not present in every individual, a ladder of short outlying bands was amplified in most *trt-1* animals. These short outliers were more apparent in mutants with longer telomeres (compare Figure 3A and B with

I. Simple end-to-end fusions



	Structure of fusion	Microhomology	Insertion	telomeric repeats
(1)		TA	no	0
(2)		T	no	0
(3)		no	C	25

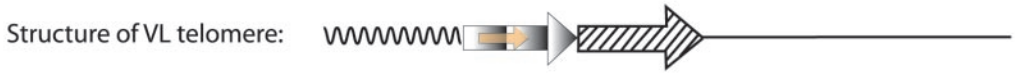
II. Complicated rearrangement involving VL internal sequences



	Microhomology Left	Microhomology Right	Insertion Left	Insertion Right	telomeric repeats	length of ➡
(4)	ATTTT	CTAAG	no	no	18	3.8kb
(5)	T	CTAAGCCTAA	no	no	30	0.47kb
(6)	ATTT	CCTAAG	no	no	11	0.73kb

	Microhomology Left	Microhomology Right	Insertion Left	Insertion Right	telomeric repeats	length of ➡
(7)	no	no	GCCTAAAGCCCAAGCGTAAG	G	46	50bp

III. Complicated rearrangement involving VL subtelomeric sequences



	Microhomology Left	Microhomology Right	Insertion Left	Insertion Right	telomeric repeats	length of ➡
(8)	A	ATTTTTTG	no	no	0	0.18kb

Figure 3C and D). Because telomerase adds telomeric repeats to only a fraction of telomeres in each cell cycle (36), these short telomeres could be explained if they escaped telomerase elongation through all cellular divisions. However, *trt-1* lost on average between 100 and 150 bp per generation (Figure 2 and Figure S1), and the short outlying telomeres were almost always more than a few hundred basepairs shorter than the majority (Figure 3A–D). Therefore, the short outlier telomeres were probably not generated by the typical loss predicted to occur during telomere replication (37).

It is interesting to note that in mutants of *mrt-2*, which is a highly conserved DNA damage checkpoint gene homologous to *S.cerevisiae RAD17* and *Schizosaccharomyces pombe rad1*$^{+}$, telomeres are shortened at a similar rate as observed in *trt-1* (~125 bp per generation) (34), suggesting that telomere shortening in *mrt-2* could be caused by lack of elongation by telomerase. Ahmed and Hodgkin (34) speculated that MRT-2, in its role as a checkpoint protein, could recognize telomeres as a form of DNA damage during replication, when telomeres are expected to adapt an open structure, revealing a structure that resembles a double-stranded break (1). MRT-2 could contribute to telomerase recruitment, possibly by coordinating the temporal and spatial association of telomerase. One major noticeable difference in telomere phenotype between *mrt-2* and *trt-1* is the lack of short outlying telomeres in *mrt-2* (30). Figure 3E shows telomere length measured from F2, F5 and F9 *mrt-2* mutants. In each generation, DNA extracted from five worms was used in STELA. While *mrt-2* also displayed reduced telomere length heterogeneity as in *trt-1*, an increased frequency of short outlying telomeres was not observed even when telomere length was relatively long (F2 in Figure 3E). Perhaps telomerase in *mrt-2* is defective in elongating telomeres after replication but still functions in an *mrt-2*-independent manner to prevent rapid telomere loss.

End-to-end fusions have been observed in telomerase mutants in different organisms (12,13,16,19). To study if telomerase deficiency leads to telomere fusions in *C.elegans*, single primer nested PCR was carried out. By including a single primer that recognizes the VL subtelomeric region in PCRs, we reasoned that only fusions between two VL telomeres would be amplified. In support of this notion, PCR products could indeed be detected in some *trt-1* worms but not in N2 (data not shown). Cloning and sequencing of eight of the amplified products revealed different types of telomere fusions: simple end-to-end fusions (category I), and more complicated rearrangements that involved either insertion of a sequence normally found in an internal VL chromosomal location (category II) or duplication within the VL subtelomeric region (category III) (Figure 4). We note that simple end-to-end fusions with the subtelomeric–telomeric and the telomeric–telomeric configurations (Figure 4) could be underrepresented because PCR amplification through long repeat sequences is generally difficult. Therefore, the relative frequency of the different types of fusions could be skewed. All eight clones characterized in this study display either microhomology or insertion at the fusion junctions, similar to the observations in *Arabidopsis* lacking telomerase (15). Two characteristics of the fusions are noted. First, in all eight fusions, at least one telomere end involved in the fusion was truncated. Truncation occurred within the same 10 bp fragment in five out of the eight clones (clones 1–3, 7 and 8). Second, in all four cases of category II fusions, the site where the inserted internal VL fragment joined the telomeric repeats of a VL end contained multiple imperfect telomeric repeats. The imperfect telomeric repeats were always in the same direction as the telomeric repeats to which they joined. A correlation between the direction of the imperfect repeats and the orientation of insertion is illustrated by clone 7. The entire 50 bp-inserted sequence in clone 7 was interspersed with imperfect telomeric repeats. By inserting in an opposite orientation as clones 4–6, the direction of the imperfect repeats relative to the telomeric repeats was maintained. We speculate that the complicated fusions were generated by recombination, while the end-to-end fusions were likely to have resulted from NHEJ.

In this study, we have demonstrated that mutation in telomerase leads to progressive telomere shortening, telomere fusions, reduced telomere length heterogeneity and increased frequency of short outlying telomeres. It appears that the germline may be an important source of telomere length heterogeneity (Supplementary Figure S2). We have shown in this study that *trt-1* contributes to telomere length heterogeneity both within an animal and within a clonal population: VL telomere length distribution within an individual is narrower, and variations among siblings are much smaller in *trt-1* mutants (Figure 3A–D). Since significant levels of expression of *trt-1* have only been observed in isolated oocytes, reproductive-stage adults and certain stages of embryogenesis (38,39), we propose that telomerase activity in the embryo and in the germ cells is responsible for the observed telomere length heterogeneity within individuals and within clonal populations respectively. It is likely that telomere length

Figure 4. Structure of telomere fusions isolated from *trt-1*. Eight clones of telomere fusions amplified from nested PCR were sequenced and classified into three categories. Category I consists of simple end-to-end fusions. White block arrow denotes the sequence between the inner primer and the beginning of telomeric DNA (represented by the wiggly line). Hatched block arrow denotes sequence between the inner primer and the outer primer. The presence of microhomology or insertion at the fusion junction (shown by an arrow head) is also indicated. In clone 3, 25 telomeric repeats were present at the fusion junction. Category II consists of complicated rearrangements in which a fragment from an internal VL site (denoted by a black block arrow) was inserted between the two ends. In clone 4, the black block arrow represents a 3.8 kb fragment found within the cosmid Y39D8B (~360 kb from the VL telomere). In clone 5, the black block arrow represents a 0.47 kb fragment found within the cosmid B0348 (~16 kb from the VL telomere). In clone 6, it represents a 0.73 kb fragment from cosmid B0348 that overlaps with the 0.47 kb fragment inserted in clone 5. The inserted fragments in clones 4–6 all contained imperfect telomeric repeats around the region where it joined the telomeric repeats (represented by wiggly line). In clone 7, the black block arrow represents a 50 bp fragment found in the cosmid C39F7 (1.2 Mb from the VL telomere). This fragment is interspersed with imperfect telomeric repeats. It was inserted in an opposite orientation as the other clones in this category. Category III consists of a clone which involved complicated rearrangement of the VL subtelomeric sequence. Within the fragment between the inner primer and the beginning of telomeric DNA, a 172 bp sequence is replicated 2.5 times (indicated by the division of the block arrow into three parts in gradient) but the replicates carry several mismatches. In clone 8, a sequence (denoted by the small orange block arrow) that overlaps with two of the replicates was inserted between two chromosome ends, one of which was truncated within the 10 bp fragment as in clones 1–3 in category I. Although only the nested primer was used in secondary PCRs, most clones (1–3, 7 and 8) were flanked by the outer primer sequence and the nested primer sequence, indicating that the low concentration of the outer primer in the secondary PCRs was enough to prime amplification with the more abundant nested primer.

heterogeneity in wild-type *C.elegans* results mostly from direct elongation by telomerase. However, indirect mechanism(s) may also play a role. It has been proposed that telomere repeat addition by telomerase is required for recombinational repair of nicks in telomeric DNA by break-induced replication (37). Recombination reactions could generate the unusually long telomeres observed occasionally in wild-type animals (e.g. progeny 3 in Figure 1B).

The generation of extremely short telomeres has been inferred from the high frequency of fusions between induced double-stranded breaks and short tracts of telomeric repeats in *S.cerevisiae* telomerase mutants (40). In this study we have provided direct evidence for the presence of such extremely short telomeres in *C.elegans* in the absence of functional telomerase. We previously described that in all of the wild-type *C.elegans* strains characterized, occasional short outlying telomeres, indicative of processes other than end-replication losses and telomerase-mediated lengthening could be observed (30). The model put forward initially by Lustig and coworkers (41–43) to explain telomere rapid deletion in *rap1*^{t} yeast mutants, and later by Wang *et al*. (44) to account for the presence of T-loop-sized, telomeric repeat-containing circular DNA induced by TRF$^{\Delta B}$, involves intrachromatid recombination at telomeres. Recently, it has been suggested that sporadic telomere loss events could also result from (oxidative) damage to telomeric DNA or from failure to resolve higher order structures of G-rich DNA (37). Similar processes could also generate the short outlying telomeres observed in different strains of wild-type worms and in human clonal fibroblast cultures (29,30). In addition to generating heterogeneous telomere length, our data suggest that a major function of telomerase could be related to the repair of sporadic telomeric DNA loss events.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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