Telomere Shortening Is Proportional to the Size of the G-rich Telomeric 3′-Overhang*

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Most normal diploid human cells do not express telomerase activity and are unable to maintain telomere length with ongoing cell divisions. We show that the length of the single-stranded G-rich telomeric 3′-overhang is proportional to the rate of shortening in four human cell types that exhibit different rates of telomere shortening in culture. These results provide direct evidence that the size of the G-rich overhang is not fixed but subject to regulation. The potential ability to manipulate this rate has profound implications both for slowing the rate of replicative aging in normal cells and for accelerating the rate of telomere loss in cancer cells in combination with anti-telomerase therapies.

Telomerase is not expressed in most normal tissues but is present in 85–90% of all human tumors (1), and there is considerable interest in the potential oncologic use of telomerase inhibitors. One concern is that such inhibitors would not directly kill tumor cells but only initiate telomere shortening, and thus it might take many cell divisions before a therapeutic effect occurred. Cultured human cells exhibit different rates of telomere shortening (2–5), implying that this rate is not fixed but might be subject to manipulation. Agents that accelerate the rate of shortening might greatly augment the efficacy of anti-telomerase treatments. However, virtually nothing is known about what controls the rate of telomere shortening in normal telomerase-negative human cells.

Telomeres of eukaryotic cells contain G-rich single-stranded 3′-overhangs, which extend beyond the double-stranded region. While the exact structure of these overhangs varies between species, the presence of overhangs is both conserved and believed to be essential for the maintenance of chromosome end structure and function. Studies in ciliates and yeast indicate that the length of these overhangs is extensive or that the final RNA primer for lagging strand synthesis is not placed at the very end of the G-rich strand. However, these studies did not determine whether this length varied in cells whose telomeres shortened at different rates. Variable rates of telomere loss could be due to processing events that affected both C- and G-rich strands, and that did not change the size of this overhang, to different rates of sustaining oxidative damage that caused the loss of large segments of telomeric DNA (21, 22) without affecting the size of the overhang on the remaining telomeres, or to mechanisms that regulate the size of the single-stranded overhang. To distinguish between these possibilities, we examined the length of the single-stranded telomeric 3′-overhang in hypotrichous Saccharomyces cerevisiae (12), supporting the concept that this fill-in activity is carried out by the conventional lagging strand synthetic machinery (13). The 12–14-nucleotide single-stranded G-rich 3′-overhang in hypotrichous ciliate telomeres (14) and the identification of a primase activity that can initiate DNA synthesis at the very 3′-end of the G-rich strand (15, 16) have led to the concept that the overhang is produced following digestion of a terminally positioned RNA primer. Telomeres of yeast mutants lacking telomerase shorten by only 3–5 bp per division (17), showing that even in the absence of telomerase yeast end-processing activities are able to replicate all but a few nucleotides at the end of the telomere.

In contrast, rates of telomere shortening in human cells lacking telomerase can vary from 30 to several hundred bp per division (2–5).

The length of the single-stranded G-rich telomeric overhang in some human cells has been shown to be 150–200 nucleotides (18–20), suggesting that either nuclease processing of the C-rich strand is extensive or that the final RNA primer for lagging strand synthesis is not placed at the very end of the G-rich strand. However, these studies did not determine whether this length varied in cells whose telomeres shortened at different rates. Variable rates of telomere loss could be due to processing events that affected both C- and G-rich strands, and that did not change the size of this overhang, to different rates of sustaining oxidative damage that caused the loss of large segments of telomeric DNA (21, 22) without affecting the size of the overhang on the remaining telomeres, or to mechanisms that regulate the size of the single-stranded overhang. To distinguish between these possibilities, we examined the length of the single-stranded telomeric 3′-overhang in human fibroblast, breast epithelial, and vascular endothelial cell strains. We show that the size of the overhang is directly proportional to the rate of telomere shortening, varying from about 300 nt in cells that lose 100 bp per division to a telomeric 3′-overhang of 150 nt in cells that lose 50 bp per division. The size of the overhang is thus an important correlate of the rate of telomere shortening (e.g. cells with long overhangs lose more telomeric repeats with each cell division). Possible mechanisms regulating overhang length are discussed.

EXPERIMENTAL PROCEDURES

DNA from cultured cells was prepared using modifications that permit the rapid processing of large numbers of samples (23), digested with a mixture of six different restriction enzymes with 4-base recognition sites, and analyzed on agarose gels. The size of the telomere restriction fragments was determined from PhosphorImager scans (Molecular Dynamics), using weighted mean calculations that normalize the signal intensity relative to the size of each digestion product (2).

Telomeres were purified by annealing a biotinylated C-rich oligonucleotide to the G-rich telomeric overhangs (19). The overhangs were

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1 The abbreviations used are: bp, base pair(s); nt, nucleotide(s).
coated with T4 gp32 single-stranded binding protein, and the length of the overhang measured as described previously (19). The measured lengths were converted to nt by reference to the measured length of decorated plasmid DNAs containing single-stranded gaps or overhangs of known lengths.

RESULTS

Normal diploid human cells were cultured, and throughout their proliferative lifespan multiple DNA samples were analyzed. Fig. 1 shows the progressive decrease in telomere restriction fragment length that occurred as a function of the number of population doublings. The rate of telomere shortening varied from 49 ± 5 to 101 ± 8 bp per division. Telomeres were then purified from these four cell types early in their lifespan, and the G-rich, single-stranded overhangs were coated with T4 gene-32 single-stranded binding protein (gp32). The length of the overhang can be determined by comparing the measured length of the protein-coated region to a series of standards of known sizes using electron microscopy (19). Fig. 2 shows the distribution of overhang lengths observed for these cells. Fig. 3 demonstrates a very strong linear relationship between the rate of telomere shortening and the average size of the telomeric overhang, with a slope of 0.31 ± 0.03.

DISCUSSION

These observations show that cell strains in which telomeres shorten twice as fast (e.g. umbilical vein endothelial cells lose 101 ± 8 bp per division, whereas BJ fibroblasts lose 49 ± 5 bp per division: Fig. 1) have overhangs that are twice as long (e.g. umbilical vein endothelial cells have 322 ± 14-nt overhangs whereas BJ fibroblasts have 156 ± 7-nt overhangs: Fig. 2). This suggests that the size of the overhang may be important in determining the rate of shortening. These results imply that alternate hypotheses for telomere shortening that would not affect the length of the overhang, such as oxidative damage to telomeric DNA producing single and double-strand breaks (21), are unlikely to contribute significantly to the rate of telomere shortening, at least in human cells cultured under normal conditions.

The demonstration that the size of the overhang is directly proportional to the rate of shortening and the average size of the telomeric overhang, with a slope of 0.31 ± 0.03, greatly increases the need to understand the detailed mechanisms regulating this process. An explanation of
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Fig. 4. Model for telomere shortening. The quantitative relationship between the size of the overhang and rates of shortening are shown for one possible model of the factors that might produce single-stranded overhangs. The model is shown for telomerase-negative normal human cells, so the addition of telomeric sequence by telomerase is not considered. X represents the final size of the overhang on the daughter molecule produced by lapping strand synthesis. As drawn, it represents the distance between the last RNA priming event of lapping-strand synthesis and the end of the chromosome, but it could also be produced by nuclease digestion without altering the calculations. Y represents the size of the overhang produced by nuclease processing (6–10) of the parental C-rich strand that templated leading strand synthesis. We have not included possible contributions of nuclease digestion of the G-rich 3'-overhang in this model. If G-strand-specific nuclease activity were a prominent factor, it would result in the failure factor of the rate of shortening to be proportional to the size of the overhang. The formula for the rate of shortening from the above model is 0.25(X + Y). The average overhang length is the average of the overhang in both daughter strands, which is (X + Y)/2. Since 0.25X + Y = 0.5(X+Y)/2, the rate of shortening should be one-half the average overhang length. The slope of the line in Fig. 3 demonstrates that the observed rate of shortening is one-third of the size of the average measured overhang. Within the context of this model, this implies that the average measured overhang does not reflect the true average of all X and Y overhangs. The average measured overhang length may differ from the true average for the following reasons: 1) Telomeres were purified by annealing a biotinylated (CCCTAA)4–6 to restriction-digested double-stranded DNA and then retrieving telomeres in which the G-rich 3'-overhang had hybridized to the probe. Only 20–50% of the telomeres are recovered using this procedure (19). 2) Telomeres with very short overhangs, overhangs annealed within T-loop structures (25) and overhangs inaccessible to hybridization due to non-canonical structures such as G-quartets might all contribute to the failure to retrieve all of the telomeres. 3) Although we can purify 5-kilo-base molecules containing telomeric overhangs as small as 12 nt (19), we estimate the limits of detection of T4 gp32-decorated overhangs by electron microscopy to be about 50 nt using our procedure. One possibility is that the slope of 0.31 is obtained because most of the X overhangs are near or below the 50 nt limit of T4 gp32 coated single-stranded regions that we are able to detect. Most of these ends may thus be excluded from the calculation of the measured average overhang. This is roughly consistent with our ability to observe overhangs on only about 70% of the purified telomeres (19).

APPENDIX

The following is an explanation of why the measured overhang is likely to be a result of the failure to detect overhangs that are less than 50 nt in size.

Let X and Y be the lengths of lapping- and leading-strand overhangs, respectively (Fig. 4). The rate of shortening, r, is given as follows (Equation 1).

\[ r = \frac{X + Y}{4} \]  
(Eq. 1)

We find from the linear relationship between the observed rate of shortening and the measured overhang length, \( L_{app} \), that \( r \sim L_{app}^3/3 \) (Fig. 3).

If the measured overhang length is correct, then the average measured overhang length will be weighted by the proportion of lapping- and leading-strand overhangs recovered. Let the ratio of lapping- to leading-strand overhangs be denoted by a. \( L_{app} \) is then given as follows (Equation 2).

\[ L_{app} = \frac{Y + aX}{1 + a} \]  
(Eq. 2)

Combining Equations 1 and 2, we obtain the following (Equation 3).

\[ \frac{X + Y}{4} = \frac{Y + aX}{3(1 + a)} \]  
(Eq. 3)

Solving for a gives the following (Equation 4).

\[ a = \frac{3X - Y}{X - 3Y} \]  
(Eq. 4)

Since a is a positive quantity, a value 3Y < X is consistent with the observation that longer overhangs are recovered more efficiently than very short overhangs.
Larger values of $Y$ require stronger biases for the recovery of lagging-strand over leading-strand overhangs than those we observe. For example, taking $Y = X/4$ gives a value of 11 for $a$, which is significantly greater than the ratio of 2 to 3 that we have observed by using bromodeoxyuridine labeling (12). Relative sizes of $X$ and $Y$ that are roughly compatible with the 2–3-fold enrichment of lagging- over leading-strand overhangs are inconsistent with the observed distributions of overhang lengths. For example, $Y = X/10$ gives $a = 4.1$, but creates other problems. From Fig. 4, $r = 0.25 (X + Y)$ and if $Y = X/10$, then $r = 0.28X$. In BJ cells, $r$ is 49 bp per division, thus $X$ is 175 nt and $Y$ is 18 nt. However, no overhangs this short were detected (see Fig. 2), because we believe the limits of detection using T4 gp32 coating are about 50 nt.

Using the value $Y = X/4$ gave an unreasonable value for $a$ as described above. However, if this value is used to calculate $X$ for BJ cells, we obtain $r = 1.25X$, and hence $X = 39.2$ nt. This size is still below our estimated limit of detection. Thus, we conclude that the reason the apparent average overhang length is not equivalent to actual average is that $Y$ is likely to be below the limits of detection.

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