Abnormal Micronuclear Telomeres Lead to an Unusual Cell Cycle Checkpoint and Defects in Tetrahymena Oral Morphogenesis

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Telomere mutants have been well studied with respect to telomerase and the role of telomere binding proteins, but they have not been used to explore how a downstream morphogenic event is related to the mutated telomeric DNA. We report that alterations at the telomeres can have profound consequences on organellar morphogenesis. Specifically, a telomerase RNA mutation termed ter1-43AA results in the loss of germ line micronuclear telomeres in the binucleate protozoan Tetrahymena thermophila. These cells also display a micronuclear mitotic arrest, characterized by an extreme delay in anaphase with an elongated, condensed chromatin and a mitotic spindle apparatus. This anaphase defect suggests telomere fusions and consequently a spindle rather than a DNA damage checkpoint. Most surprisingly, these mutants exhibit unique, dramatic defects in the formation of the cell’s oral apparatus. We suggest that micronuclear telomere loss leads to a “dynamic pause” in the program of cortical development, which may reveal an unusual cell cycle checkpoint.

Telomeres have been implicated in a number of diverse cellular processes. Normal telomeres serve as the means by which chromosomes can be replicated completely, and they function as a cap, thereby protecting the natural ends from inappropriate fusion. Telomeric mutants can be generated by making predictable changes in the RNA template of the enzyme telomerase, which synthesizes telomeres, such that the corresponding complementary mutation is made at the telomeres. Although these types of telomeric DNA mutants in complex eukaryotes are poorly studied, particularly with respect to the downstream developmental consequences, those in simplified yeasts and the ciliated protozoa are better understood.

Ciliated protozoa, such as Tetrahymena thermophila, provide excellent model systems for studying telomerase, telomeric DNA mutants, and the downstream developmental consequences to changing the telomeric repeat sequence. This is because the components of telomeres and telomerase have been exceptionally well documented (5) and the cells are large enough (roughly 60 to 70 μm) to allow a glimpse of their unique developmental processes.

Interestingly, T. thermophila, like other ciliates, bears two nuclei distinct in function and mode of division (reviewed in reference 25). The micronucleus represents the germ line nucleus and is transcriptionally silent, whereas the macronucleus serves as the vegetative nucleus of the cell. The micronucleus contains five pairs of chromosomes, which are passed on through mitosis and meiosis. In contrast, the macronucleus contains many subchromosomal fragments, each of which is amplified to ~45 copies, excluding the ribosomal DNA, which has thousands of copies. The macronuclear chromosomes are not faithfully inherited, as the macronucleus divides amitotically.

The telomeres of the two T. thermophila nuclei are dramatically different. The macronuclear telomeres, present at a copy number of roughly 40,000 per cell, contain about 0.3 to 0.5 kb of G4T2 repeats (3). Micronuclear telomeres, in sharp contrast, are present at a copy number of 20 per cell and have much longer telomeric tracts of ~2.5 kb that contain an inner tract of a variant G4T3 repeat sequence (26). This variant repeat tract is not present in the macronucleus, presumably as it is eliminated with the rest of the micronuclear telomeres during development of the new macronucleus upon conjugation.

A number of telomere mutants have been constructed in T. thermophila, for example, by mutating the telomerase RNA and consequently the telomeric DNA itself (40) or by depleting a protein that binds the telomeres (24). The telomeric DNA is mutated in ter1-43AA cells by altering the telomerase RNA template such that it synthesizes a combination of G4T3 repeats, which are harmless, and G4T4 repeats, which are toxic (40). Even with this sequence alteration, the macronuclear telomere length is only subtly altered. Nonetheless, a dramatic arrest appears in micronuclear mitosis, where the chromatin appears elongated during anaphase (27, 40), which takes about 30 times longer than wild-type (WT) anaphase. In a different type of telomere mutant, where the G-strand telomeric binding protein POT1a is depleted, the cells undergo a growth arrest where the macronucleus is enlarged and the macronuclear telomeres become extremely lengthened (24). Thus, there may be more than one kind of cell cycle arrest involved due to telomere defects within the two different nuclei.

Not only is T. thermophila binucleate, but it exhibits a wealth of unusual developmental landmarks as it progresses through the program of vegetative growth and cell division. It has a remarkable cell cortex, with an oral apparatus (mouth) that
takes in bacteria and nutrients and a cytoprotect (anus) that eliminates waste. Oral development alone is a rich landscape of beautifully choreographed developmental events (12). At a precise location on the cell’s cortex, a field of basal bodies proliferates, forming an “anarchic field.” Waves of morphogenetic activity then organize this field into a set of uniquely sculpted membranelles, creating the cell’s oral apparatus. There appear to be four parallel programs involving the nuclei and oral apparatus that are coordinated as cells divide: (i) they replicate their micronucleus by mitosis (28, 29), (ii) they replicate their macronucleus by amitotic fission (14, 15, 36), (iii) they synthesize and assemble a gallery of cortical organelles associated with the oral apparatus (9, 10), and (iv) they undergo cytokinesis in a fashion coordinated with the completion of the other three programs (2, 11, 13, 35, 37).

In this study, we have used the T. thermophila telomerase RNA template mutant terl-43AA to assess the molecular status of the micronucleus and to correlate its mitotic events with cortical events associated with formation of the oral apparatus. Although it was shown previously that the sequences of the macronuclear telomeres in this mutant are altered (40), the micronuclear telomeres had not been examined. We show here that the micronuclear telomeres are dramatically shortened. Furthermore, many aspects of the blocked micronuclei are consistent with end-to-end fusion during anaphase, specifically the persistence of condensed chromatin and an intact mitotic spindle apparatus. Finally, we demonstrate that the midanlage population, or single cells were isolated.

MATERIALS AND METHODS

Cell lines and culture conditions. Cells were of the CU428 and SB1969 strains, kindly provided by Peter J. Bruns and Eduardo Orias, respectively. Cells were prepared for mating by starving the cells at 31°C (23). After 12 to 18 h of starvation, equal numbers of cells were mixed in 96-well plates at dilutions such that clonal lines were obtained from single transformed cells by selection with 120 μg/ml paromomycin (Sigma). Control cells were transformed by electroporation as described in references 16 and 17. Transformed cultures were plated in 96-well plates at dilutions such that clonal lines were obtained from single transformed cells by selection with 120 μg/ml paromomycin (Sigma). Control cells were transformed by electroporation with the WT telomerase RNA gene (prTer1 [46]). Beginning at 4 days after electroporation, terl-43AA transformant cell lines were analyzed for micronuclear telomere length and cytology from the densest wells were diluted, and the isolation was repeated.

RESULTS

Micronuclei have lost the majority of telomeric DNA. In order to determine if a telomerase RNA template mutant affects telomere length in both T. thermophila nuclei, we first tested whether the micronuclear telomeres were altered in the terl-43AA mutant. Although the macronuclear telomeres in this mutant are known to comprise G4T3 and G4T4 mutant repeats in addition to the WT G4T2 repeats (40) (Fig. 1A), the telomeric repeat composition of micronuclear telomeres had not been addressed previously. Therefore, we could not ascribe aspects of the phenotype to changes in the micronuclear telomeres. We investigated whether there were changes in the lengths of micronuclear and macronuclear telomeric tracts by using Southern blot analysis.

Micronuclear telomeres are difficult to detect due to their relative paucity in the cell. The ratio of micronuclear to macronuclear telomeres is approximately 1:2,000. Thus, we relied on two previous findings regarding WT telomeres (26). First, the micronuclear telomeres are dramatically longer than those of the macronucleus (−2.5 kb versus −0.5 kb, respectively).
Second, the micronuclear telomeres have an inner, homogeneous tract of variant G4T3 repeats not present in the macronuclear telomeres yet constituting roughly the inner one-third of the telomeric tract. We used several oligonucleotide probes in our Southern blot assay to assess the lengths and sequences of the telomeres of both nuclei: a WT probe (G4T2) that recognizes both macronuclear and micronuclear telomeres, a probe (G4T3) unique to micronuclear telomeres, and a probe unique to mutated telomeres (G4T4).

When we probed total DNA that had been digested with a frequently cutting restriction enzyme, we detected macronuclear telomeres (~0.5 kb) in both the WT and the mutant, using a G4T2 probe (Fig. 2A, lanes WT and 43AA). When the identical blot was reprobed with G4T3 or G4T4 sequences, the results corroborated previous findings that both types of repeat are incorporated into \textit{ter1-43AA} macronuclear telomeres (40) (Fig. 2B and C, lanes 43AA) but not into those of the WT (Fig. 2B and C, lanes WT). The macronuclear telomeres in the \textit{ter1-43AA} mutant cells are slightly shorter than those in the WT (Fig. 2A) by roughly 150 bp. As was shown previously, this macronuclear telomere shortening is not responsible for the mutant phenotype (40).

Micronuclear telomeres are not evident in the blot probed with the G4T2 sequence (Fig. 2A), due to the background signal from the macronuclear telomeres. Nonetheless, when the blot was probed with the G4T3 sequence, which recognizes an ~0.8-kb stretch of the innermost telomere repeats of the micronucleus (Fig. 2B, lane WT), there was clear visualization of micronuclear telomeres in the 3- to 4-kb range of WT cells.
Surprisingly, we did not observe a larger, more intense signal, due to de novo G4T3 synthesis in the mutant (Fig. 2B, lane 43AA) but instead saw substantially less signal intensity, indicative of loss of the micronuclear telomere sequence.

The mutant micronuclear telomere signal was shortened by ~0.6 kb. This amount of shortening should not affect the inner G4T4 sequence that is being probed, as this sequence is confined to the inner one-third (~0.8 kb) of a ~2.5-kb telomere, as described above. The G4T3 signal intensity would start to diminish only when the telomeres are shortened by more than ~1.7 kb. Thus, the loss of signal intensity in this size range, coupled with an absence of noticeable G4T3 signal elsewhere in the lane, strongly suggests some loss of the inner G4T3 repeats. Thus, the loss of signal here most likely corresponds to a loss of most or all of the micronuclear telomeric DNA from a fraction of the telomeres.

We checked for the loading of DNA by probing the same blot with an α-tubulin DNA control. The DNA in the ter1-43AA mutant lane appears underloaded, but after determining with gel quantification software that the DNA in this lane represents about 60% of the WT level (Fig. 2E) and normalizing it to this amount, we determined that roughly 70% of the total telomeric DNA signal is lost from the mutant lane. Therefore, we conclude that a great deal of telomere sequence from the micronucleus has been lost.

We next asked whether in the ter1-43AA mutant, the remaining, largely intact micronuclear telomeres that were ~0.6 kb shorter, contained de novo mutant repeats without suffering complete degradation. Alternatively, this signal is due to WT telomeres that were slightly shortened but perhaps had not yet been acted on by the mutant telomerase. If the former is the case, then we should be able to detect the action of mutant telomerase by using the G4T4 probe. The results indicate that there is no obvious G4T4 signal at the mutant micronuclear telomeres around 3 to 4 kb (Fig. 2C, lane 43AA). It is possible that a very low number of repeats are added to the micronuclear telomeres and hence are undetectable in this assay, but this seems unlikely given the strong mutant signal at macro-nuclear telomeres. We also tested whether both strands of the micronuclear telomeres in ter1-43AA cells are absent by probing with a C4A3 probe to the opposite telomeric strand. The absence of signal indicates that the G-rich strand is also lost (Fig. 2D, lane 43AA). Finally, loss of the micronuclear telomeres is not due to a general DNA degradation mechanism, since an internal α-tubulin probe remained intact (Fig. 2E).

We conclude from these results that the majority of micronuclear telomeres have been lost in transformed cells expressing ter1-43AA. Furthermore, the small percentage of nearly intact, remaining micronuclear telomeres is most likely WT.

ter1-43AA cells have intact spindles and condensed chromatin in anaphase bridges. Tetrahymena thermophila strains that carry ter1-43AA telomerase mutations exhibit a profound cell cycle arrest characterized by enlarged cells and elongate micronuclei characteristic of anaphase. Since this phenotype occurs in a cell that expresses both mutant and WT telomerase activities, the mutation clearly behaves in a dominant manner. We used immunofluorescence to examine three markers of cell cycle progression in these mutants to more fully characterize their phenotype. First, we examined whether or not the micronuclear histone H3 is in its phosphorylated state. If H3 is phosphorylated, this indicates that cells have indeed entered mitosis and undergone chromatin condensation. Second, we used acetylated α-tubulin antibody to visualize whether or not the mitotic spindle was assembled in these arrested mutants. Finally, we employed antibodies against basal-body epitopes to visualize the pattern of cortical ciliation that accompanies oral development during cell division. This marker allows a precise evaluation of when and where in the cell cycle our telomerase mutant was arrested.

The antibody raised specifically against phosphorylated histone H3 decorates micronuclear chromatin when it is in its condensed, mitotic configuration (44). Since the micronuclear chromatin does not stain with this antibody, the micronuclei are more clearly visible in the ter1-43AA mutant cells as they are not obscured by the large DAPI-stained macronucleus. We were able to identify a much greater proportion of micronuclei that were elongated and stained with this antibody in mutant cells than we had previously detected with DAPI alone (40). In WT log-phase cultures of Tetrahymena (n = 1,345), typically 20% of the cells exhibit positive micronuclear labeling, indicating mitosis. In contrast, 77% of ter1-43AA transformants (n = 254) exhibited positive labeling, indicating that they had entered mitosis but failed to exit.

Figure 3A to C show condensed micronuclei at various stages of mitosis in WT cells. The condensed state persists through late anaphase (Fig. 3C). Examples of ter1-43AA cells are shown in Fig. 3D to I. About 56% of the cells in the ter1-43AA population have elongated micronuclei that are accompanied by an apparently intact, α-tubulin-stained spindle apparatus, compared to 17% for the WT population. This apparatus (20.2 ± 2.3 μm) is roughly twice as long (as determined using a measurement tool in MetaMorph) as that observed for WT cells (11.6 ± 0.8 μm). The persistence of the spindle apparatus and the lengths of the microtubules from pole to pole strongly suggest that the micronuclear chromosomes are stuck or intertwined and unable to separate.

Micronuclei from these arrested mutants are shown enlarged in Fig. 3G to I. In most cases, the chromatins stretch continuously from one pole to the other, consistent with the hypothesis that bridges have formed between chromosomes via end joining (Fig. 3H). In rare cases, chromatins form two discrete masses at either end of the mitotic spindle, suggesting that anaphase has segregated two masses of chromatin (Fig. 3I). In this case, there appears to be unequal chromatin segregation on each side of a conspicuous gap.

In summary, there are roughly three times the number of ter1-43AA cells with condensed micronuclear chromatin as WT cells, and this chromatin is almost always joined from one pole to the other. In the rare cases in which chromatin has disjoined, it often appears aneuploid. Furthermore, the mitotic micronuclear spindle apparatus is nearly doubled in length from pole to pole, and the number of cells with mitotic micronuclear spindles exceeds the WT number by threefold. These factors support the hypothesis that there is chromosome end joining in this telomeric DNA mutant.

Most cells do not divide after single-cell isolation. Since the micronuclear phenotype was observed within a population, it was difficult to determine the percentage of cells that continued through a cell division or the stage in the cell cycle at which they were blocked. To assess these characteristics of the ter1-
FIG. 3. Cells with micronuclear telomere mutations are blocked in midanaphase with chromosome bridges and intact spindle microtubules. Immunofluorescence microscopy of WT and mutant *Tetrahymena* cells showing acetylated tubulin (green), phosphorylated micronucleus-limited histone H3 (red), and DAPI-stained chromatin (blue). WT cells showing early micronuclear anaphase (A), midanaphase (B), and late anaphase (C) configurations. Mutant ter1-43AA cells showing an elongated micronucleus with bridging chromatin (D) (with the nucleus enlarged in panel G), a mutant cell showing an elongated micronucleus (E) with a more limited region of bridging chromatin material (enlarged in panel H, with an arrow indicating bridging material), and an example mid-late anaphase (F) showing unequal segregation of chromatin (enlarged in panel I, with an arrow indicating a gap). Bar, 10 μm.
short telomeres and developmental defects

43AA mutant, we performed a type of longitudinal study. As soon as cell populations had divided enough in selective media after transformation, two transformed wells at high density, having coordinates C9 and F2, were arbitrarily chosen. From these wells, single cells were isolated into a drop of medium. This was repeated for a total of 48 isolated cells, which were then allowed to grow for 24 h (passage 1) (A) or were taken from the densest wells in passage 1 and allowed to grow for 24 h (passage 2) (B). Cell numbers were quantified and placed into the following categories: category 0, representing 0 or 1 cells; category 1, representing 2 to 10 cells; category 2, representing 11 to 100 cells; and category 3, representing >100 cells.

The results showed that after passage 1, about 40% of the isolated cells failed to divide at all, and about 35% went through only approximately three cell divisions (Fig. 4). The remainder of the cells had more vigorous growth, but all grew through very few cell divisions. Even the cells that do go through a number of cell divisions ultimately block with a pronounced cellular phenotype. Thus, even the strongly dividing cells give rise to progeny that do not divide.

Telomere mutant pauses during oral development. We took advantage of this severe block in micronuclear mitosis to assess the developmental consequences on cortical development. To our knowledge, there have been no studies correlating mutant telomeres with developmental programs, such as stomatogenesis, in an organism like T. thermophila. When Tetrahymena cells divide, they must not only replicate their nuclear contents but also undergo a dramatic proliferation and patterning of their cortical architecture, resulting in the formation of a new oral apparatus in the posterior division product. The details of stomatogenesis are well documented (12).

Using an antibody that recognizes basal bodies, we followed the progression of basal-body proliferation and assembly into oral membranelles from WT cells and noted the correspondence to stages of micronuclear anaphase from the onset, were allowed to grow for an additional 24 h, and the cell number was quantified as before. This was referred to as passage 2 (Fig. 4B). In this analysis, the cells from F2 showed a dramatic increase in the proportion that was blocked. Nearly 75% of these cells did not go through another division. Furthermore, only 18% of the cells went through roughly three divisions in the second 24-hour period (passage 2), and only 8% divided close to normally. This finding was verified by observing cells from passage 1 that had continued to grow for a second 24-hour period. Here, most cells did not increase in numbers, indicating that division was still blocked. Surprisingly, the cells that stayed in category 1, having divided fewer than three times, continued to show abnormal swimming patterns. Thus, these cells went through as few as three divisions and survived for 2 days. These results are consistent with findings that mutant cell populations stay in anaphase, on average, 30 times longer than WT populations (27).

Our results show that the majority of ter1-43AA mutants go through very few cell divisions. Even the cells that do go through a number of cell divisions ultimately block with a pronounced cellular phenotype. Thus, even the strongly dividing cells give rise to progeny that do not divide.
and elongates (Fig. 5A and B). After promembranelles have formed, basal-body triplet formation is initiated and the undulating membrane (UM) begins to emerge. The elongate micronucleus is no longer associated with the macronucleus (Fig. 5C and D). The triplet membranelles begin to straighten, and the UM becomes distinct, yet anarchic basal bodies persist at the posterior of the field. At this point, the micronucleus elongates into a midanaphase configuration (Fig. 5E and F).
As the UM is completed, the anarchic basal-body field is nearly all incorporated (or resorbed). At this point, the micronucleus is in the late stage of anaphase, where spindle elongation produces two distinct micronuclei (Fig. 5G and H). The UM is completed, and membranelle “sculpturing” takes place. Micronuclei now appear round, the micronucleus has begun to elongate, and the cytokinetic furrow is just noticeable (Fig. 5I and J). Cytokinesis and macronuclear division are well under way. Development of the oral primordium is complete (Fig. 5K and L).

*T. thermophila* cells expressing the ter1-43AA telomerase RNA template mutation reveal an extraordinary cortical phenotype associated with stomatogenesis (Fig. 6). We found that cells arrested in the cell cycle exhibited profoundly elongate oral primordia. Ciliary rows show buckling due to continued basal-body proliferation without cell division (Fig. 6B). The basal bodies within the oral primordia showed one of three configurations, corresponding to early, middle, and late stages of oral development. The early-stage oral primordia (Fig. 6A to C) showed basal bodies proliferating, and at the anterior end of the basal-body field, basal-body doublets appeared to be forming (for example, see basal bodies between arrows in Fig. 6C). Each basal body appears to be nucleating the formation of its basal-body partner. This is particularly clear in Fig. 6C. Here, each doublet appears as a single brightly staining basal body coupled with a faintly staining and diminutive partner (Fig. 6C, insert).

Other ter1-43AA telomerase mutants appeared to progress a bit further. Figure 6D to F show basal-body doublets that are just beginning to organize into promembranelles (Fig. 6F). Two other features appear in these preparations. First, there appears to be a gradient of development, and to variable extents, these elongate fields appear broken into multiple discrete oral primordia. The earliest stage (mere basal-body proliferation) occurs in the posterior-most fields and corresponds to stage 1 (Fig. 6E, bottom bracket). More-anterior basal bodies become tightly packed, corresponding to stage 2 (Fig. 6E, middle bracket). Even-more-anterior basal-body doublets have formed (Fig. 6E, top bracket), and in the most-anterior fields of some cells, doublets have assembled into promembranelles (linear assemblages of aligned basal-body doublets [Fig. 6F and G to I]). Within the most advanced oral primordia, we could see discrete breaks forming in the abnormally long promembranelles (Fig. 6H and I).

Two events that were conspicuously rare (or nonexistent) were the formation of a flanking UM and the addition of the third row of basal bodies that transform promembranelles into mature membranelles. This allows us to pinpoint the stage in oral development at which these cells become arrested. We have drawn a schematic diagram to indicate this stage (Fig. 7) and to further clarify the stages of oral development linked to nuclear division. The UM first makes its appearance at the same stage that the third row of basal bodies is assembled, namely, stage 4B (Fig. 7). Cells expressing the ter1-43AA mutant telomerase become arrested at or before stage 4A in oral development, a stage at which the micronucleus shows an elongated football form yet has not begun to undergo spindle elongation. The cortical phenotype suggests that while these mutants are prevented from advancing through the cell cycle, the dynamics of cortical development may be said to be arrested in an active, proliferative state.

In summary, the ter1-43AA telomerase template mutation undergoes a “dynamic pause” in oral development. During cell cycle arrest, basal bodies continue to proliferate nucleating synthesis of their doublet partners, and the resulting doublets align into promembranelles, yet no UM is formed, and promembranelles fail to assemble a third row characteristic of a mature membranelle.

**DISCUSSION**

Here, we have shown that by forcing *Tetrahymena* cells to express a mutant telomerase RNA gene (ter1-43AA), it is the micronuclear telomeres, and not the macronuclear telomeres, which undergo a substantial loss of telomeric DNA. This finding is consistent with phenotypic evidence (25, 40; this study), where it was demonstrated that a block in micronuclear anaphase is the first defect observed. Although it is still possible that the macronuclear telomeres may cause a defect that goes undetected in these mutant cells, the initial cause is most likely due to the shortening of the micronuclear telomeres.

Many characteristics of the micronucleus not seen previously (40) are consistent with end joining at the telomeres. Specifically, over three-quarters of the cells displayed condensed chromatin, most of which remained joined from one pole to another. Most of the micronuclei in anaphase also had an intact mitotic spindle apparatus. In addition, single-cell isolation indicated that most cells were unable to undergo even one cell division after 48 h. However, a small percentage of cells divided normally many times but were ultimately blocked. Finally, we have identified an aspect of cortical development that manifests a profound “dynamic pause” during oral development.

**Telomeres and cell division checkpoints in anaphase.** According to one hypothesis (Fig. 8), the mutated ter1-43AA template must initially create micronuclear telomere tracks with altered sequences, which are prone to degradation. These shortened telomeres fail to interact properly with end-protective proteins. As the ends erode due to endogenous exonuclease activity, they associate with the DNA repair mechanism, and chromosome end joining is promoted. The resulting chromosome bridges may trigger a midanaphase checkpoint due to tension induced during centromere fission and chromosome progression. It is possible that the mutated macronuclear telomeres may have some effect of their own, for example, leading to a high proportion of cells with an elongated macronucleus that is blocked in amitosis, but this has not been observed.

In a related study (24), the telomere protection protein POT1a was depleted in live *Tetrahymena* cells. POT1a was shown to localize over macronuclei, but not micronuclei, and depletion resulted in lengthened macronuclear telomeres. A cell cycle arrest was associated with these mutants as well, suggesting another type of mitotic checkpoint. This cell cycle arrest appears to be in macronuclear S phase (macronuclear G2), in that cells continue growing and their macronuclear ploidy increases, yet there are no signs of cortical development indicating entry into mitosis. It would seem that extreme lengthening of macronuclear telomeres may be associated with
FIG. 6. Fluorescence microscopy of basal bodies shows dramatic defect in oral morphogenesis in the ter-434A mutant. Mutant cells arrested with early-stage oral primordia (A to C). Persistent basal-body proliferation is under way, and at the anterior end of the basal-body field, basal-body doublets are forming (note insert). Oral primordia arrested at a later stage: basal-body doublets are organizing into promembranelles (D to F; see arrows in panel F). The bottom bracket in panel E shows the posterior-most field with the earliest stage of development (basal-body proliferation). The middle bracket (E) shows tight packing of the “anarchic field.” The top bracket (E) shows the most anterior fields of some cells, with doublets arranged into promembranelles. The most advanced arrested oral primordia appear in panels G to I. Multiple rows of basal-body doublets appear (I).
an interphase arrest, whereas shortening of micronuclear chromosomes results in a midanaphase mitotic arrest.

One question raised by our analysis is how shortened telomeres lead to a cell cycle arrest. In tumor cells of vertebrates, telomeres are progressively shortened (6) and are subject to instability. Telomere shortening can result in chromosome end joining, leading to the formation of anaphase bridges (20, 21, 33). Indeed, many cancers are characterized by a destructive cycle of chromosome bridging, fusion, and breakage (6, 42). It has been shown that the frequency of anaphase bridges is directly dependent upon the level of telomerase activity and the subsequent telomere length (41). It is possible that as telomeres shorten to some critical length, the chromosome ends are recognized as “broken ends,” thereby activating the DNA repair machinery, and it is this that results in end-to-end recombination, anaphase bridges, and possibly activation of a cell cycle checkpoint arrest (1, 18, 19).

Several laboratories have explored telomere-associated checkpoints in Saccharomyces cerevisiae by analyzing telomerase-defective cell lines. In the telomerase RNA mutant tlcΔ of S. cerevisiae, telomeres gradually shorten, and cells become arrested somewhere near the end of stomatogenic stage 4A. Basal bodies continue to proliferate. The oral morphogenetic field extends down the postoral ciliary row, recruiting ever-more-posterior basal bodies to initiate replication. Basal bodies within the growing anarchic field nucleate formation of their doublet partners in more-anterior regions of the oral primordium, and doublets align with one another, forming linear arrays or “promembranelles” in the most anterior regions of the oral primordium.

FIG. 7. A “Dynamic pause” occurs in anaphase-arrested cells compared to WT cells. (A) WT cortical development (stages of basal-body proliferation and stomatogenesis) is linked with both micronuclear and macronuclear events and cytokinesis. Based on data from reference 12. (B) The ter1-43AA mutant undergoes a “dynamic pause.” Three processes continue to operate, although cortical development is arrested somewhere near the end of stomatogenic stage 4A. Basal bodies continue to proliferate. The oral morphogenetic field extends down the postoral ciliary row, recruiting ever-more-posterior basal bodies to initiate replication. Basal bodies within the growing anarchic field nucleate formation of their doublet partners in more-anterior regions of the oral primordium, and doublets align with one another, forming linear arrays or “promembranelles” in the most anterior regions of the oral primordium.
arrests in Saccharomyces (30), depending on when DNA damage is induced, at G1/S, intra-S, or G2/M. None of these studies point to a midanaphase arrest, and in fact, most lack the detailed cellular resolution necessary to distinguish a midanaphase arrest from an earlier metaphase/anaphase arrest.

One exception is where researchers demonstrated the existence of a midanaphase checkpoint in yeast that is dependent upon Rad9, part of the DNA damage checkpoint surveillance machinery (45). This could be caused by a chromosome bridge triggered by a dicentric chromosome or, in our case (Fig. 8), by chromosome end joining. Supporting this possibility is a recent finding that mammalian Rad9 has in fact been implicated in telomere stability and its loss associated with end joining (38, 39).

**One anaphase checkpoint or two?** Little work has been done to characterize the DNA damage/repair mechanisms in Tetrahymena cells, yet one study is provocative. It was demonstrated that RAD51, which participates in double-stranded-DNA repair, is necessary for vegetative maintenance of the micronucleus (31). Cells bearing the RAD51-null mutation exhibit a mitotic defect in which the micronucleus is arrested in early anaphase while the cell proceeds through cytokinesis (32). This result in segregation of the entire (arrested) anaphase spindle to one daughter cell or the other. This suggests that Tetrahymena cells exhibit two distinct types of anaphase arrest.

In the telomerase mutant phenotype, end-joining bridges may delay chromosome segregation, leading to an arrest blocking the cell’s progression into cytokinesis and macronuclear fission. We attempted to determine if the telomerases of the micronucleus had fused to each other by PCR, but we were unable to detect a fusion product (unpublished observations). In Rad51-null mutants, DNA damage is presumably incurred during synthesis and left unrepaired, and this triggers a checkpoint arrest early during anaphase elongation without blocking the cell’s progression into cytokinesis. It is tempting to propose that the Rad51 defect-induced anaphase arrest occurs prior to spindle elongation and is restricted to the micronuclear division program. In contrast, the telomere defect-induced anaphase arrest permits the middle stages of anaphase elongation, while blocking progress into the final stages of anaphase elongation and subsequent cytokinesis. It is curious that the program leading to cytokinesis and macronuclear division should be so tightly coupled with the midstage anaphase checkpoint (that triggered by telomerase defect and putative telomere cross-bridges), while being uncoupled from the earlier anaphase checkpoint (that induced by the Rad51 defect and putative unrepaired DNA damage).

**Telomeres affect development of cell cortex.** The telomerase phenotype sheds light on the events that accompany normal cell division in Tetrahymena strains. In particular, we have gained several new insights into the events that build an oral primordium during the process of cortical stomatogenesis. First, it has not been perfectly clear whether basal-body doublets form by aggregation of existing basal bodies or whether existing basal bodies nucleate de novo synthesis of their partners-to-be. The anti-centrin antibody appears to discriminate between two sets of basal bodies: large, brightly staining basal bodies (such as one sees in the mature ciliary row [Fig. 6C]) and more-faintly staining, smaller basal bodies (such as one sees anterior to and the viewer’s right of the mature, ciliary row basal bodies). The latter clearly represent newly formed structures. If doublets formed through aggregation or recruitment of already mature basal bodies, then one would expect each partner in a doublet to stain equally brightly. The most sensible interpretation of these images is that each mature basal body has nucleated the synthesis of a partner that appears dim and underdeveloped at this stage. Most previous studies on WT oral development have used silver staining or scanning electron microscopy images or an antibody to an uncharacterized basal-body epitope. It is likely that this phenomenon that we observed escaped earlier notice due to differences in imaging techniques and their more exaggerated manifestations unique to this phenotype.

Second, there appears to be a process whereby long promembranelles are severed into shorter segments. In WT cells, this probably occurs only once per membranelet and might easily be missed. In the overelongated membranelles of our mutants, multiple clipping sites can be seen (Fig. 6H and I). Promembranelle severing appears to occur in stage 4A of oral development, prior to triplet assembly. Here, we see severing only after doublet promembranelles are well formed and prior to third-row basal-body addition. This restricts the possibility to early stage 4. Third, given earlier results demonstrating that these telomerase mutants are arrested in midanaphase and our current results demonstrating that such cells are arrested in cortical stage 4A, it appears that we can infer that cortical stage 4A corresponds to midanaphase, whereas stage 4B may stand at the gateway to late anaphase and the spindle elongation that occurs as one enters stage 5A.

Finally, it is interesting to note that a cell cycle arrest can be viewed as a “dynamic pause.” That is to say, while progress through the cell division cycle has been arrested, the cortical “subroutines” associated with a given developmental stage continue to operate. Though our telomerase mutants cannot initiate the final stages of spindle elongation associated with late anaphase, nor can they initiate formation of the UM or the proliferation of third-row basal bodies, processes active during midanaphase continue: basal bodies continue to replicate, these then continue to nucleate doublet assembly, and these doublets are organized into ever longer promembranelles, which may then be severed at appropriate lengths. The result is the continuing growth of an exaggerated oral primordium and the continued maturation and patterning of its members up to a set point in development.

This novel study correlates the complexities of telomere-induced nuclear division properties with downstream developmental events in Tetrahymena thermophila. Future studies with T. thermophila could shed light on the development and morphogenesis of human cancer cells with altered telomerases.

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