Effects of reconstitution of telomerase activity on telomere maintenance by the alternative lengthening of telomeres (ALT) pathway

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Received May 8, 2001; Revised and Accepted June 24, 2001

Telomere length maintenance is essential for cellular immortalization, and thus tumorigenesis. Most human tumors and immortal cell lines maintain their telomeric DNA via the activity of a specialized reverse transcriptase, telomerase. Stabilization of telomeric repeat tracts may also be achieved through a telomerase-independent mechanism, referred to as alternative lengthening of telomeres (ALT). ALT cells are telomerase negative and are characterized by extremely long and heterogeneously sized telomeres and novel multienzyme structures called ALT-associated PML nuclear bodies which are unique to ALT cells. To determine if reconstitution of telomerase activity suppressed ALT and restored wild-type telomere lengths, we introduced the catalytic subunit of telomerase into two ALT cell lines. Initially, two clonal lines exhibited enrichment of shorter telomeres while maintaining a population of ultra-long telomeres similar to that observed in the parental line, suggesting that telomerase is stabilizing the shorter telomeres in the population. Telomere length in the third clonal line was not detectably different from that in the parental cell line. One clonal line with a phenotype of shorter telomeres maintained this pattern over time in culture while the second gradually reverted to the parental ALT telomere length pattern, concurrent with reduction of telomerase activity. All clones continued to maintain ALT-associated PML nuclear bodies regardless of whether telomerase was present. The data suggest that introduction of telomerase activity alone is not sufficient to completely repress ALT, that telomerase acts preferentially on the shortest telomeres in the culture and that the ALT and telomerase pathways may be present concurrently in mammalian cells.

INTRODUCTION

Telomeres are specialized structures that confer stability to the ends of linear chromosomes. In most eukaryotes, telomeres are composed of tandem repeats of short G-rich sequences complexed with telomere specific binding proteins (reviewed in 1). In somatic cells with a finite proliferative potential, telomeric sequences are lost at each cell division (2,3). In contrast, maintenance of telomeric DNA permits continued proliferation of eukaryotic cells (4–7) linking cellular immortalization to the maintenance of telomeric DNA repeats.

In most tumors and immortalized cell lines, telomere loss is bypassed through the action of telomerase (reviewed in 8), a reverse transcriptase that utilizes an RNA moiety as the template for the addition of telomeric repeats onto the 3′ ends of linear DNA molecules (reviewed in 9). However, a subset of tumors and immortal cell lines utilize a telomerase-independent mechanism, termed alternative lengthening of telomeres (ALT), to maintain telomeric DNA (10,11) and thereby circumvent the telomere length-dependent limitation on proliferation. Recent evidence suggests that telomeres in cells that utilize ALT may be maintained via homologous recombination and copy switching between telomeric tracts (12). Cells that utilize the ALT pathway have two characteristics in addition to being telomerase negative: they contain ultra-long, heterogeneously sized telomeric arrays (10) and have novel multienzyme structures, called ALT-associated PML nuclear bodies (APBs), in which telomeric proteins and DNA co-localize with the PML nuclear body (13). APBs appear in cells coordinately with the appearance of the ultra-long telomeric restriction fragments, linking these structures to telomere maintenance by the ALT pathway.

Mutation of telomerase in the yeast Saccharomyces cerevisiae results in telomere loss and eventual culture senescence (14). Rare survivors do arise for these populations and fall into two classes, termed Type I and Type II; this survival is dependent upon RAD52 (15). These survivors also fall into two classes with respect to telomere structure (15,16). Type I survivors display short telomere tracts interspersed between subtelomeric Y′ elements, indicating that recombination between Y′ elements is the underlying mechanism for telomere stabilization. Type II survivors have long stretches of telomeric repeats which are probably maintained via non-reciprocated recombination between two telomeres. Interestingly, reintroduction of telomerase into telomerase-deficient yeast strains results in restoration of wild-type telomeres (16). This occurs rapidly in the

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Type I survivors and with slower dynamics in Type II survivors where restoration of wild-type telomere length requires several hundred population doublings. These data indicate that generation of either class of survivors did not require additional mutation of the proteins that usually regulate telomere length and indicate that telomerase is the preferred mode of telomere maintenance in yeast.

Repression of the ALT pathway has also been observed in mammalian cells (17,18). In these experiments somatic cell hybrids were created between ALT cell lines and telomerase-positive immortal cell lines or primary mortal cell lines. The result was suppression of ALT in the hybrids, suggesting that inter-telomere recombination is normally either suppressed or less efficient than telomerase at maintaining telomeres. It has been demonstrated previously that telomerase activity can be reconstituted in ALT cell lines by exogenous expression of components of the telomerase holoenzyme (19). However, the fate of telomeres and the ALT pathway was not investigated in these experiments. To determine whether telomerase alone was sufficient to suppress ALT and restore wild-type telomere lengths, we introduced the catalytic subunit of telomerase, hTERT, into two ALT cell lines. Similar experiments have been carried out by others (18,20,21). In two cases, reconstitution of telomerase activity did not alter characteristics of the ALT pathway (18,21), whereas in the third report, ALT was suppressed in two of the derived cell lines (20). In the experiments described here, two cloning lines initially exhibited enrichment of shorter telomeres while maintaining a population of ultra-long telomeres similar to that observed in the parental line. Telomere length in the third cloning line was not detectably different from that in the parental cell line. In the cloning lines exhibiting shorter telomeres, one maintained this pattern over time in culture while the second gradually lost the shorter telomeres and reverted to the parental pattern of ultra-long telomeres concurrent with reduction of telomerase activity. All clones maintained APBs over the course of these experiments. The data suggest that telomerase activity alone is not sufficient to repress ALT in these cell lines and are consistent with telomerase acting preferentially on the shortest telomeres in the culture.

RESULTS

Identification of telomerase positive clones

The catalytic subunit of telomerase, hTERT, was introduced into two cell lines derived from human ovarian surface epithelium (HOSE), HIO118 and HIO107, by transfection to induce telomerase activity. These cell lines have previously been characterized and demonstrated to use the ALT pathway for telomere maintenance (22). Transient transfection with the hTERT construct resulted in readily detectable telomerase activity, indicating that these lines express the RNA subunit of telomerase, hTER (data not shown). Following selection and isolation of clonal cell lines, the TRAP assay was used to identify those clones that contained in vitro detectable telomerase activity. Three cell lines, HIO118A5, HIO118D6 and HIO107C6, were identified which contained telomerase activity (Fig. 1A, lanes 1–12 and data not shown). As expected, inclusion of RNase in the reaction inhibited formation of TRAP assay products. Two cell lines, HIO118A6 and HIO107B5, which were hygromycin resistant but did not exhibit detectable telomerase activity were also isolated (Fig. 1, lanes 13–21 and data not shown). Extract from the telomerase-positive HeLa cell line was included to demonstrate that the lack of detectable telomerase activity in the HIO118A6 and HIO107B5 cell lines was not due to a diffusible inhibitor (Fig. 1A, lanes 14, 15, 17, 18, 20 and 21). The lack of telomerase activity in these cell lines is probably a result of the integration of the construct such that hTERT is not expressed. These lines provided internal controls for alterations observed in the telomerase positive clones isolated from the same transfection.

The level of telomerase activity present in the HIO118A5, HIO118D6 and HIO107C6 cell lines was quantitated relative to the levels of telomerase activity in HeLa cells. It has been demonstrated previously that the TRAP assay is inhibited at higher levels of protein extract (23). For our HeLa cell extracts, this occurred at a level between 0.5 and 1 µg of protein extract (Fig. 1B). Likewise, titrations were carried out for the protein extracts from each cloning line to establish that the assay was conducted at levels that exhibited increasing product with increasing amount of extract used (data not shown). Based on these results, quantitation of telomerase activity was carried out using 0.1 µg of each extract and was compared to that present in 0.1 µg of HeLa cell extract (Fig. 1C). The HIO118A5 and HIO107C6 cell lines had relatively stable levels of telomerase activity over the time course of these experiments. For the HIO118A5 cell line telomerase activity ranged from 1.2 to 1.5 times that present in HeLa cell extract, whereas for the HIO107C6 cell line, telomerase activity ranged from 0.5 to 0.8 times that present in HeLa cell extract. In contrast, the HIO118D6 cell line initially had almost 1.8 times the level of telomerase activity as is present in HeLa cells; this activity gradually decreased over time in culture until it was completely absent by 80 population doublings (PDs).

Telomere length analysis

Telomere length in each cloning line was analyzed by Southern blot analysis at various times during culture (Fig. 2). Both the HIO118 and HIO107 parental cell lines exhibit the ultra-long telomeres characteristic of cells that utilize the ALT pathway for telomere maintenance (Fig. 2) (10). At the earliest possible point for analysis, 16 PDs, telomere length in both HIO118 telomerase positive subclones, HIO118A5 and HIO118D6, had altered from that in the parental HIO118 cell line exhibiting an increased hybridization intensity of lower molecular weight DNA fragments (Fig. 2A, bracket). These data indicate that the presence of telomerase activity alters the telomere size distribution in ALT cells.

To determine if the telomere array size changes observed at early PD were retained, telomeric DNA was analyzed in the HIO118 derived subclones over ~70 PD. In the HIO118A5 clone, shorter telomeres persisted in the population for the length of these experiments (Fig. 2A). In contrast, in the HIO118D6 cell line telomeres gradually lengthened with increased time in culture and eventually reverted to a pattern similar to that in the parental HIO118 cell line (Fig. 2). Importantly, neither cell line exhibited complete loss of the parental ALT type terminal restriction fragment pattern, with
Figure 1. Introduction of in vitro telomerase activity in ALT cells. (A) An example of a TRAP assay used to detect telomerase activity in whole-cell extracts prepared from the HIO118 clones A5, D6 and A6 at the indicated PDs. The assay was carried out using 0.5 µg of whole-cell extract in each reaction. Inclusion of RNase in the reaction inhibits formation of telomerase products by destroying the RNA template molecule, hTER (lanes 2, 4, 6, 8, 10, 12, 15, 18, 21, 23). Mixing 0.5 µg of telomerase-positive HeLa cell extract with 0.5 µg of extract from the telomerase-negative HIO118A6 did not affect the level of HeLa cell telomerase activity in the reaction (compare lanes 14, 17 and 20 with lane 22), demonstrating the absence of a diffusible inhibitor. (B) Quantitation of telomerase activity in HeLa cell extracts. The TRAP assay was carried using the indicated amounts of protein extract in the absence (–) or presence (+) of RNase. An example of a TRAP gel is shown in the left panel and quantitation of that gel is shown in the right panel. The total amount of reaction products generated under each condition is expressed as a percentage of the maximum amount obtained. Note that at higher protein levels the formation of TRAP assay products is inhibited. (C) Quantitation of the level of telomerase activity present in the HIO118A5, HIO118D6 and HIO107C6 cell lines at the indicated PDs relative to the amount present in HeLa cell extracts. The assays were carried out using 0.1 µg of protein extract, established previously by titration experiments to be below the level of protein that inhibits formation of TRAP assay products.
significant hybridization at the limit of mobility of conventional agarose gels over the course of these experiments. The size distribution of telomeric restriction fragments was also characterized in the telomerase negative HIO118A6 clone. As expected based on absence of telomerase activity, the distribution of telomeric fragments appeared unchanged relative to the parental line (Fig. 2A).

To further characterize the size changes suggested by conventional gel electrophoresis in the HIO118 and HIO107 cell lines, telomere length was analyzed by pulsed field gel electrophoresis (PFGE) (Fig. 2B). This analysis indicated gradual shortening of the longest telomeric fragments in the HIO118A5 cell line and gradual lengthening of the telomeric fragments in the HIO118D6 cell line. Telomere attrition of the longest telomeres in the HIO118A5 occurs despite levels of telomerase activity greater than that present in HeLa cells.

To establish the degree of length diversity that accompanies clonal variation, telomere length was analyzed in three additional clonal cell lines that were independently derived from the parental HIO118 cell line following transfection with an unrelated vector that did not contain the hTERT open reading frame. Telomere length was similar to that in the parental HIO118 cell line in all three clonal lines as assessed by Southern blot analysis following PFGE (data not shown). These results suggest that the changes in telomere length observed in HIO118A5 and HIO118D6 at early PDs are due to the presence of biologically active hTERT rather than to clonal variation.

Telomere length was also determined in the telomerase-positive HIO107C6 and telomerase-negative HIO107B5 clonal cell lines. In contrast to what was observed with the HIO118 cell lines, no change in terminal restriction fragment lengths was observed for the HIO107C6 cell line, despite the presence of in vitro telomerase activity (Fig. 2A). As expected and similar to the results obtained in the HIO118 background, the telomerase-negative HIO118B5 and three independently derived cell lines exhibited no detectable change in the distribution of terminal restriction fragments (Fig. 2A and data not shown).

**Telomerase activity does not affect the frequency of APBs**

Cell lines that utilize the ALT pathway for telomere maintenance contain large multiprotein complexes in which telomeric proteins and DNA co-localize with the PML nuclear body, called APBs (13). To determine whether forced expression of telomerase in ALT cell lines resulted in inhibition of this marker of the ALT pathway, we carried out indirect immunofluorescence and determined the frequency of these structures in logarithmically growing cells. All of the clonal cell lines generated here contained APBs, irrespective of whether or not they had detectable telomerase activity (Fig. 3; Table 1). The frequency of APBs varied both among the clonal lines and within each cell line at different PDs after clonal isolation. To confirm that this variation was within a range consistent with that due to clonal variation, we determined the frequency of APB-positive cells in five independently derived HIO118 clonal cell lines and eight independently derived HIO107 clonal cell lines following transfection with a vector that did
not contain the hTERT open reading frame. In both back-grounds, the frequency of APB-positive cells varied from <10% of the cells in the population up to ∼30% of the cells in the population (data not shown). Thus, the variation in the frequency of APB-positive cells observed in the hTERT-derived cell lines is within that accompanying clonal variation.

We, and others, have shown previously that the frequency of APB-positive cells in the population is increased in cultures enriched for cells in the G2 phase of the cell cycle (22,24). To determine whether this aspect of APB regulation was altered by the presence of telomerase, the telomerase-positive HIO118A5 and HIO118D6 and the telomerase-negative HIO118A6 cell lines were arrested in G2/M by exposure to the microtubule poison, nocodazole. FACS analysis was carried out to confirm the enrichment of cells with a G2 DNA content in the nocodazole-treated cultures (Fig. 4A). The frequency of APB-positive cells was determined in the arrested population and compared to that in parallel untreated cultures. The frequency of APB-positive cells was increased to a similar level in the G2/M arrested cellular populations regardless of whether the cell line exhibited detectable telomerase activity (Fig. 4B). The extent of enrichment, ∼3-fold, is similar to that reported previously for the parental cell lines (22). These results indicate that telomerase activity does not affect the presence or cell cycle regulation of APBs.

Table 1. Frequency of cells in logarithmically growing cultures of each cell line which contained APBs at the indicated PD

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>PD</th>
<th>Telomerase</th>
<th>APB-positive/Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIO118</td>
<td>241</td>
<td>Negative</td>
<td>22/108 (20.0)</td>
</tr>
<tr>
<td>HIO118A5</td>
<td>6</td>
<td>Positive</td>
<td>2/64 (3.1)</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td></td>
<td>5/57 (8.8)</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td></td>
<td>9/79 (11.4)</td>
</tr>
<tr>
<td>HIO118D6</td>
<td>6</td>
<td>Positive</td>
<td>3/81 (3.7)</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td></td>
<td>1/68 (1.5)</td>
</tr>
<tr>
<td></td>
<td>68</td>
<td></td>
<td>5/68 (7.4)</td>
</tr>
<tr>
<td>HIO118A6</td>
<td>6</td>
<td>Negative</td>
<td>3/58 (10.3)</td>
</tr>
<tr>
<td></td>
<td>46</td>
<td></td>
<td>4/60 (6.7)</td>
</tr>
<tr>
<td></td>
<td>68</td>
<td></td>
<td>14/72 (19.4)</td>
</tr>
<tr>
<td>HIO107</td>
<td>244</td>
<td>Negative</td>
<td>27/109 (24.8)</td>
</tr>
<tr>
<td>HIO107C6</td>
<td>4</td>
<td>Positive</td>
<td>9/69 (13.0)</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td></td>
<td>18/64 (28.1)</td>
</tr>
<tr>
<td>HIO107B5</td>
<td>12</td>
<td>Negative</td>
<td>9/116 (7.8)</td>
</tr>
</tbody>
</table>

Analysis of end-protection function

Telomeres serve the essential function of providing stability to ends of linear chromosomes. One phenotype exhibited by cells that have lost telomere end-protection function is the end-to-end fusion of chromosomes (25,26). During anaphase, these fusions are manifested as bridges of unresolved DNA between the separating daughter nuclei. An increase in the frequency of anaphase bridges is seen in primary cultures as they approach crisis and is correlated with extremely short telomeres (25). When the fate of a single marked telomere in ALT cells was characterized it exhibited gradual shortening over time in culture, punctuated by rapid increases in length and resumption of telomere attrition (27). This cyclical behavior suggests that ALT acts preferentially on telomeres that reach a critically short length and that a steady state level of cells exhibiting compromised telomere function might be a feature of cultures that utilize ALT for telomere maintenance.

To determine whether expression of telomerase resulted in increased telomere stability, we analyzed the frequency of anaphase bridges in parental HIO118 and HIO107 cell lines, and independently derived cell lines, HIO114, which activated telomerase spontaneously upon immortalization (Table 2). The parental ALT cell lines have a higher frequency of anaphase bridges than does HIO114, consistent with there being a higher level of telomere malfunction in these cell lines. The frequency of anaphase bridges in the clonal lines analyzed here was similar regardless of the presence of telomerase activity. The lowest frequency of bridges (reduced by almost 2-fold from that in the parental cells) was present in the HIO118A5 cell line, which maintained the highest consistent level of telomerase activity during the course of these experiments. However, the HIO118A5 cell line still had a higher frequency of anaphase bridges than did the telomerase-positive HIO114 cell line. In the HIO118D6 cell line, anaphase bridges occurred at a frequency similar to that in the parental cell line, although this cell line also had relatively high levels of telomerase.
activity (0.8 times that present in HeLa cells at the time of analysis). The HIO107C6 cell line did not exhibit an alteration in the frequency of anaphase bridges relative to the HIO107 parental cell line or to the telomerase-negative HIO107B5 cell line.

**DISCUSSION**

Maintenance of telomere repeat arrays is essential for cellular immortality. To date, two mechanisms which may be utilized by human cells to circumvent telomere length-dependent limitations on proliferation have been characterized, telomerase activation and ALT. Recent evidence suggests telomere stabilization by way of the ALT pathway may occur through non-reciprocal recombination of telomeres (12). The genes regulating ALT remain elusive, but based on results from somatic cell hybrids (17) and from chromosome transfer experiments (28), the existence of one or more repressors of ALT has been suggested. In *S. cerevisiae*, it has been noted that reintroduction of telomerase into telomerase-deficient strains that contain long telomeres results in reversion to wild-type telomere lengths (16). These data suggest that in yeast the transition to recombination-based telomere maintenance did not require mutations in constitutive components of the telomere complex. While these data do not directly address whether the survivor pathway is repressed in *S. cerevisiae*, they are consistent with telomerase being a more efficient mode of telomere replication. To determine whether telomerase is able to restore wild-type telomere length regulation and/or suppress ALT in human cells, we introduced the catalytic subunit of telomerase, hTERT, into two ALT cell lines. The resulting telomerase-positive clones exhibited alterations in telomere length while maintaining characteristics of the ALT pathway.

The discovery of unique multiprotein complexes within ALT cells has provided a valuable tool to characterize the presence of this pathway in cell lines. The presence of APBs within a culture is tightly linked to activation of ALT, as they are detected with the simultaneous appearance of the ultra-long telomeres characteristic of ALT (13). The frequency of cells in cultures that contain APBs is variable between cultures. Furthermore, cells containing APBs are more common when the population is enriched in the G2-phase of the cell cycle (22,24). All the clonal cell lines derived here retained APBs and exhibited a G2-dependent increase in the frequency of APB-positive cells regardless of whether or not telomerase was present. Similar results have been observed in additional ALT cell lines that have been modified through exogenous addition of telomerase components to force the expression of telomerase (18,21), suggesting that ALT and telomerase may be present concurrently in human cells. In contrast, it has recently been reported that forced expression of telomerase may occasionally result in clonal cell lines that do not retain characteristics of the ALT pathway (20). This apparent contradiction, together with the differences observed in the lines derived here with respect to telomere length dynamics, suggests that modifiers are present which dictate the susceptibility of a given cell line to telomerase or ALT.

Telomeres are essential for chromosome end protection and loss of this function is manifested as end-to-end fusion of chromosomes (25,26). In *S. cerevisiae*, it has been suggested that short telomeres are the initiating substrate for telomere elongation through the recombination based survivor pathway and that telomerase is able to suppress this pathway by elongating these critically short telomeres (16). We analyzed the frequency of anaphase bridges, a manifestation of end-to-end fusions, in the parental cell lines, the clonal lines derived here and a cell line derived from the same tissue, human ovarian surface epithelium, that spontaneously activated telomerase upon immortalization. All the cell lines analyzed had a greater frequency of anaphase bridges than was present in the telomerase-positive HIO114 cell line. This outcome is in contrast to what would be expected if telomerase is rescuing short telomeres resulting in increased genome stability. However, it is also possible that the bridges observed here are occurring through some mechanism other than telomere malfunction, e.g. these cell lines may have a high frequency of spontaneously occurring double-stranded DNA breaks leading to chromosome fusions and subsequent bridge formation in anaphase that is independent of the ALT pathway.

Although the cell lines analyzed here retained characteristics of the ALT pathway, the presence of telomerase activity resulted in changes in telomere length in the HIO118 derived
subclones. However, no alterations in telomere length in the telomerase-positive HIO107C6 cell line were observed. This may reflect differences in the level of telomerase activity or in telomere length regulation in the HIO107 parent as compared to the HIO118 parent, e.g. by harboring different mutations in telomere-binding proteins. Alternatively, the lack of an effect of telomerase expression on telomere length in the HIO107C6 clone may reflect differences in the regulation of the ALT pathway in the two parental cell lines, perhaps through loss of different repressors that are differentially sensitive to ectopic telomerase expression. Finally, it is a possibility that the telomerase activity observed in vitro in the HIO107C6 cell line is unable to act on telomeres in vivo. Examples of alleles of hTERT that exhibit dissociation of in vitro telomerase activity and in vivo telomere maintenance have been described previously (29). However, this explanation is unlikely given previous demonstrations that the hTERT construct used in these experiments is able to stabilize the telomeres of a number of cell lines (29,30) and the changes in telomere length observed in the HIO118 subclones.

Both the HIO118A5 and HIO118D6 cell lines had apparently experienced a rescuing of the shortest telomeres in the culture during the early points of culture expansion, as evidenced by increased hybridization of lower molecular weight telomeres at the earliest analyzable timepoint. Alternatively, the increased hybridization of lower molecular weight telomeric fragments in the 5–23 kb size range in these cell lines might indicate gradual telomere shortening in these cultures. However, FISH analysis of telomerase-modified ALT cell lines carried out by two other laboratories indicates a preferential increase of telomeric signal on chromosome ends that had previously had very weak or undetectable hybridization signals (18,21). The simplest interpretation of these data would be that telomerase acts preferentially on short telomeres in the culture. In the HIO118A5 cell line, continual telomere attrition of the largest telomeric fragments was observed. Telomere shortening in the presence of telomerase activity may be forced through overexpression of telomere-binding proteins. Thus, it is conceivable that the long telomeres in ALT cell lines recruit sufficient protein to prevent telomerase from acting on them such that stabilization and equilibration of telomere arrays only occurs when an equilibrium is reached between the amount of telomere-binding proteins and the level of telomerase activity. Despite telomere shortening, the HIO118A5 cell line retained APBs, suggesting that ALT was not completely suppressed by exogenous expression of telomerase.

In contrast to what was observed in the HIO118A5 cell line, the HIO118D6 cell line exhibited gradual telomere elongation with continued time in culture and had a telomeric hybridization pattern consistent with ALT by 68 PDs. Although the HIO118D6 cell line eventually lost all detectable telomerase activity, the telomere lengthening observed in this cell line occurred in the presence of telomerase activity at levels equivalent to that present in HeLa cells. Because telomere length was analyzed at the population level it is not possible to determine whether the rate of telomere growth is consistent with continued action of telomerase or with rapid growth of a few telomeres in the population at each division. These data suggest that differences exist between the two HIO118 derived clonal lines with respect to telomere length regulation. This may be due to the difference in the level of telomerase activity between these two cell lines or to factors that affect the relative efficiency of telomerase versus ALT as a means of telomere length regulation. The alterations in telomere length observed in the HIO118 clones are unlikely to be due to clonal variation because we did not detect changes in telomere length in the HIO118A6 clone, or in three additional clones derived following transfection with a vector that did not contain the hTERT open reading frame.

Finally, similar to a previous observation (11), we have recently characterized an ALT cell line derived from a telomerase-positive tumor (A.Tosolini, A.De Rienzo, J.V.Grobelny, S.C.Jhanwar, D.Broccoli and J.R.Testa, manuscript in preparation) raising the possibility that telomerase and ALT may co-exist in some tumors. The importance of telomere maintenance in ensuring continued proliferation and prevalence of telomerase in human tumors has made this enzyme an attractive target for developing new drug strategies to combat cancer. If telomerase and ALT do, in fact, co-exist in some tumors, this would have important implications for the response of tumors to telomerase inhibitors.

**MATERIALS AND METHODS**

**Cell lines and culture conditions**

The HIO118 and HIO107 human ovarian surface epithelium cell lines were maintained in a 1:1 mixture of Media 199 and MCDB-105 media, supplemented with 4% fetal bovine serum and 0.2 IU/ml pork insulin (Novagen). The pBabehygro-hTERT construct, a gift from Dr R.Weinberg (29) was transfected into parental lines using the FUGENE6 reagent (Roche) following the manufacturer’s specifications. HIO118 clones were selected with 50 µg/ml hygromycin and the HIO107 clones were selected with 100 µg/ml hygromycin. Following 2–3 weeks of selection, colonies were picked and transferred to 24-well dishes. Upon reaching confluence, the clonal lines were seeded into 6-well dishes and this was assigned as population doubling 1. Cells were subcultured 1:4 for the remaining culture period. Cultures were arrested in G2/M phase by addition of nocodazole (Sigma) to 1.5 µg/ml for 24 h and harvested for analysis.

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**Table 2. Frequency of cells in each cell line which contained anaphase bridges at the indicated PD**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>PD</th>
<th>Bridge-positive/Total (%)</th>
</tr>
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<tbody>
<tr>
<td>HIO118</td>
<td>210</td>
<td>43/101 (42.6)</td>
</tr>
<tr>
<td>HIO118A5</td>
<td>72</td>
<td>7/27 (25.9)</td>
</tr>
<tr>
<td>HIO118D6</td>
<td>68</td>
<td>41/98 (41.8)</td>
</tr>
<tr>
<td>HIO118A6</td>
<td>68</td>
<td>37/106 (34.9)</td>
</tr>
<tr>
<td>HIO107</td>
<td>213</td>
<td>21/100 (21.0)</td>
</tr>
<tr>
<td>HIO107C6</td>
<td>28</td>
<td>20/100 (20.0)</td>
</tr>
<tr>
<td>HIO107B5</td>
<td>26</td>
<td>21/70 (30.0)</td>
</tr>
<tr>
<td>HIO114</td>
<td>229</td>
<td>12/111 (11.0)</td>
</tr>
</tbody>
</table>

*The HIO114 cell line activated telomerase spontaneously upon immortalization.*
Analysis of telomerase activity

Telomerase activity was assessed using the TRAP assay. Whole-cell protein extracts were prepared as described previously (31). Protein concentrations were determined using the Bradford assay (Bio-Rad). The TRAP assay was used to detect telomerase activity as described previously (23,32). All reactions were carried out using 0.1–5.0 µg of protein extract in duplicate without or with inclusion of 20 ng RNase. RNase sensitivity was used to ensure that reaction products were due to telomerase. In the case of the HIO118A6 extracts that lack telomerase activity, the reactions were carried out with the addition of 0.5 µg of telomerase-positive HeLa extract to confirm the absence of a diffusible inhibitor of telomerase activity.

Southern analysis

Genomic DNA was extracted following standard procedures. Restriction enzyme digestion of genomic DNA was carried out with HinfI and RsaI, the resulting fragments were resolved on 0.7% agarose gels and transferred to Hybond N membranes as described (31,33). Alternatively, genomic DNA was resolved by PFGE on 1% agarose/0.5× TBE gels using a CHEF DRII apparatus (Bio-Rad) at 6 V/cm for 18 h with a 5 s constant switch time. Telomeric restriction fragments were detected following hybridization with oligonucleotides complementary to the telomeric repeats, TTAGGG and AATCCC, as described previously (34). 100 ng of each oligonucleotide was labeled at its 5′ end using T4 polynucleotide kinase and [γ-32P]ATP.

Indirect immunofluorescence

Cells were seeded directly onto coverslips and immunofluorescence was carried out as described previously (35). Briefly, cells were fixed in 3.7% formaldehyde/1× PBS and permeabilized with 0.5% NP-40/1× PBS. For detection of anaphase bridges, DNA was stained with 0.2 µg/ml 4,6-diamino-2-phenylindole (DAPI) at this time. Detection of APBs was carried out using a Becton-Dickinson FacScan and CellQuest software.

FACS analysis

Cells were collected by trypsinization, washed twice with PBS/2 mM EDTA and fixed in cold 70% ethanol. Cells were stained with propidium iodide (50 mg/ml) and analyzed using a Becton-Dickinson FacScan and CellQuest software.

ACKNOWLEDGEMENTS

We thank R. Reddel and S. Bacchetti for sharing results prior to publication, and R. Weinberg for the pBabehygrohTERT construct. D.B. is an Ellison Medical Foundation Scholar and a V Foundation Scholar. M.K-M. was supported by a fellowship from the NIH (CA-09035-25). This work was supported by grant RPG-00-259-01-GMC (A.C.S., D.B.) and an appropriation from the Commonwealth of Pennsylvania (F.C.C.).

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