The Loss of a Single Telomere Can Result in Instability of Multiple Chromosomes in a Human Tumor Cell Line

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Abstract

Spontaneous telomere loss has been proposed as an important mechanism for initiating the chromosome instability commonly found in cancer cells. We have previously shown that spontaneous telomere loss in a human cancer cell line initiates breakage/fusion/bridge (B/F/B) cycles that continue for many cell generations, resulting in DNA amplification and translocations on the chromosome that lost its telomere. We have now extended these studies to determine the effect of the loss of a single telomere on the stability of other chromosomes. Our study showed that telomere acquisition during B/F/B cycles occurred mainly through translocations involving either the nonreciprocal transfer or duplication of the arms of other chromosomes. Telomere acquisition also occurred through small duplications involving the subtelomeric region of the other end of the same chromosome. Although all of these mechanisms stabilized the chromosome that lost its telomere, they differed in their consequences for the stability of the genome as a whole. Telomere acquisition involving nonreciprocal translocations resulted in the loss of a telomere on the donor chromosome, which consequently underwent additional translocations, isochromosome formation, or complete loss. In contrast, telomere acquisition involving duplications stabilized the genome, although the large duplications created substantial allelic imbalances. Thus, the loss of a single telomere can generate a variety of chromosome alterations commonly associated with human cancer, not only on a chromosome that loses its telomere but also on other chromosomes. Factors promoting telomere loss are therefore likely to have an important role in generating the karyotype evolution associated with human cancer. (Mol Cancer Res 2005;3(3):139–50)

Introduction

Telomeres are DNA-protein complexes that contain short repeat sequences added on to the ends of chromosomes by the enzyme telomerase (1). Telomeres serve multiple functions, including protecting the ends of chromosomes and preventing chromosome fusion (2). In humans, telomeres are thought to be maintained in germ line cells, but shorten with age in somatic cells due to the lack of sufficient telomerase activity to compensate for the loss of small amounts of telomeric repeat sequences with each cell division (2). Telomere shortening in somatic cells is a signal for cell senescence, which involves a permanent cell cycle arrest in G1 involving p53 (3, 4). Primary human fibroblasts that have lost the ability to senesce continue to show telomere shortening and eventually enter “crisis”, which involves increased chromosome fusion, aneuploidy, and cell death (5). Similarly, human epithelial cells that fail to senesce enter “agonesence”, which like crisis involves extensive chromosome fusion due to telomere loss (6). Rare cells that survive crisis are invariably those that have regained the ability to maintain their telomeres, either through activation of telomerase (5, 7) or through an alternative mechanism involving recombination (8, 9).

Chromosome instability can play an important role in producing the multiple genetic alterations associated with cancer (10). One mechanism for chromosome instability is through the loss of telomeres (1, 2). Chromosome instability resulting from telomere loss can occur through breakage/fusion/bridge (B/F/B) cycles, which were first described in maize by McClintock (11). B/F/B cycles are initiated when a chromosome without a telomere replicates and the sister chromatids fuse at their ends. The fused sister chromatids then break unequally when the centromeres are pulled in opposite directions during anaphase. Because the chromosome will again be without a telomere in the daughter cells, this process will be repeated in the next cell cycle. These B/F/B cycles can continue for many cell generations and result in extensive DNA amplification and complex chromosome rearrangements, and end when the chromosome eventually acquires a new telomere and again becomes stable (12, 13). The importance of telomere loss as a mechanism for chromosome instability in cancer has been emphasized by the demonstration that telomerase-deficient mice that are also deficient in p53 have a high incidence of humanlike cancers (14), and the fact that the tumor cells from these mice contain chromosome rearrangements typical of B/F/B cycles. In addition, chromosome rearrangements resulting from telomere loss in breast cancer have been shown to involve chromosome fusions that occur during crisis when telomeres...
become very short (15). This study also showed that despite the expression of telomerase, tumor cells surviving crisis can continue to show telomere-associated chromosome instability that can promote tumor cell progression (15). Consistent with this latter observation, early-passage human tumor cells (16) and cell lines (12, 13) show high rates of telomere loss and anaphase bridges leading to chromosome instability.

To learn more about the consequences of telomere loss for chromosome stability, we developed a system to study changes in individual chromosomes after the loss of a telomere. This system uses a herpes simplex virus thymidine kinase (HSV-tk) selectable marker gene located immediately adjacent to a telomere to identify cells in the population that have lost a telomere, and has been established in both a human tumor cell line and mouse embryonic stem cells (12, 13, 17). In some cells that had lost the HSV-tk gene, telomeres were added at a new site on the end of the marker chromosome. However, in most HSV-tk–deficient human tumor cells (>90%), telomere loss resulted in sister chromatid fusion, which was followed by prolonged B/F/B cycles, as shown by the absence of a telomere and the presence of anaphase bridges involving the marker chromosome more than 20 generations after the loss of the telomere. The B/F/B cycles eventually ended when the marker chromosome acquired a new telomere, as shown by the stability of the marker chromosome in subclones of these HSV-tk–deficient cells. Although these studies provide important insights into the consequences of telomere loss for an individual chromosome after the loss of its telomere, they do not provide information of how the loss of a telomere on one chromosome affects the stability of the genome as a whole. Therefore, we have now characterized a subclone of this human tumor cell line in which the marker chromosome is actively involved in B/F/B cycles to determine how the mechanisms of telomere acquisition after telomere loss on one chromosome influence the rearrangement of other chromosomes.

**Results**

**Identification of Cells with a Chromosome Actively Involved in B/F/B Cycles**

Mechanisms of telomere acquisition during B/F/B cycles were investigated using clone B3 of the EJ-30 human tumor cell line that contains a single copy of the pNCT-tel plasmid containing an HSV-tk selectable marker gene located adjacent to the telomere on the short arm of chromosome 16 (Fig. 1A). This telomere was created when the linearized plasmid with telomeric repeat sequences on one end “seeded” a new telomere 4 Mb from the original end of the chromosome (12), and is stably maintained with a length similar to that of other telomeres in the cell (17). The marker chromosome containing the plasmid is referred to as der16 due to the 4-Mb terminal deletion on the short “p” arm containing the plasmid sequences. Selection for the loss of the HSV-tk gene with ganciclovir showed that most HSV-tk–deficient subclones of clone B3 contain large inverted duplications on the end of the marker der16 chromosome, consistent with sister chromatid fusion (12, 13). Cytogenetic analysis of these cells revealed heterogeneous duplications on the end of the marker chromosome (12) and amplification of the DNA proximal to the telomeric plasmid (13), both of which are rearrangements typical of B/F/B cycles (Fig. 1B). A mechanism involving prolonged B/F/B cycles was confirmed by the absence of a telomere and the presence of anaphase bridges involving the marker chromosome in many cells in the population even after more than 20 cell generations after the loss of the telomere (17).

**Translocation as a Mechanism for Telomere Acquisition during B/F/B Cycles**

We previously observed that the marker der16 chromosome involved in B/F/B cycles can acquire a telomere and again become stable (12, 13). In one subclone undergoing B/F/B cycles, the marker chromosome acquired a new telomere, as shown by the stability of the marker chromosome in subclones of these HSV-tk–deficient cells. Although these studies provide important insights into the consequences of telomere loss for an individual chromosome after the loss of its telomere, they do not provide information of how the loss of a telomere on one chromosome affects the stability of the genome as a whole. Therefore, we have now characterized a subclone of this human tumor cell line in which the marker chromosome is actively involved in B/F/B cycles to determine how the mechanisms of telomere acquisition after telomere loss on one chromosome influence the rearrangement of other chromosomes.

**FIGURE 1.** The use of selectable marker genes to study chromosome instability resulting from telomere loss. A. Clone B3 contains a single copy of the pNCT-tel plasmid integrated on the end of the short arm of chromosome 16 (Chrom 16p). The telomere of the marker chromosome 16 (der16) therefore contains an HSV-tk gene for negative selection, a neo gene for positive selection, and plasmid sequences (amp/ori) immediately adjacent to the telomere. B. The B/F/B cycle is initiated when sister chromatids fuse after the loss of a telomere. The sister chromatids then form a bridge during anaphase when their centromeres are pulled in opposite directions. Breakage occurs at locations other than the site of fusion, resulting in amplification of sequences in one daughter cell and deletions in the other. The location of telomeres ( ), centromeres ( ), and orientation of the subtelomeric sequences ( horizontal arrows) are shown.
cycles, G71, the marker der16 chromosome had acquired a new telomere in ~80% of the cells in the population more than 20 cell generations after the initial selection for the loss of its telomere, most often due to translocations of the ends of other chromosome. The stability of the marker der16 chromosomes after telomere acquisition was evident by the absence of additional rearrangements in the marker der16 chromosome in many subclones of G71. We have taken advantage of these prolonged B/F/B cycles in G71 to perform an in-depth analysis of the mechanisms of telomere acquisition during B/F/B cycles, not only on the chromosome that lost its telomere but also on other chromosomes. These studies were done by sequential analysis of 297 metaphase spreads using multicolor fluorescence in situ hybridization (M-FISH), in which all 24 human chromosomes can be identified with a combination of five different fluorescent probes, as well as conventional FISH using telomere- and subtelomeric-specific probes. Similar to our earlier studies, we observed that the marker der16 chromosome had yet to acquire a telomere in 49 of the metaphase spreads (17%) after more than 20 generations (Table 1). These marker chromosomes lacking a telomere seem to be actively involved in B/F/B cycles, as shown by their rounded ends, indicating sister chromatid fusion, and the fact that the fraction of cells in which the marker chromosome is lacking a telomere is similar to the fraction in which it is involved in anaphase bridges (17). In the other 248 metaphase spreads, telomeres had been acquired by a variety of mechanisms (Table 1). In more than half of these metaphase spreads, the marker der16 chromosome acquired a telomere through translocations of portions of other chromosomes. The presence of a deletion and absence of a fragment of chromosome 16 on the chromosome donating the translocation showed that 60 of these translocations (24% of acquired telomeres) were nonreciprocal. In many instances, these nonreciprocal translocations (NRT) involved whole chromosome arms and resulted in the loss of the telomere from the donor chromosome. In the example shown (Fig. 2A), hybridization using M-FISH and subtelomeric probes for the ends of a chromosome 1 donating the translocation confirmed that the translocation was nonreciprocal by the loss of the subtelomeric DNA from the long "q" arm of chromosome 1 and its appearance on the end of the marker der16 chromosome, without the appearance of DNA from chromosome 16 on chromosome 1. Hybridization with a telomere-specific probe showed that the marker der16 chromosome acquired a telomere in the process, whereas the donor chromosome 1 lost the telomere on its q arm.

**Telomere Acquisition by Nonreciprocal Translocation Transfers Instability to Donor Chromosomes**

It is clear from the above results that NRTs are a common mechanism for telomere acquisition that can stabilize a chromosome that has lost its telomere. However, the absence of a telomere on the end of nearly half of the chromosomes donating NRTs in our study (Table 2) indicated that they may in turn become unstable due to the loss of their telomeres. To investigate this possibility, we analyzed subclone G71 by M-FISH to determine whether the chromosomes donating the NRTs can themselves acquire a telomere by translocation from a third chromosome. The results showed that half (52%) of the chromosomes donating NRTs contained translocations from other chromosomes (Table 2), demonstrating that the instability in the marker der16 chromosome had been passed on to these chromosomes. In 13 of the 31 chromosomes donating NRTs, these translocations were nonreciprocal (Table 2), indicating that the instability might again be transferred to a third chromosome.

To confirm that the acquisition of telomeres through NRTs is an ongoing process and that instability in the donor chromosome occurs subsequent to the initial NRT, we did M-FISH on a subclone of G71, G71i (Fig. 2B). In this subclone, 60% of the cells in the population had an initial NRT from chromosome 6 on the marker chromosome, whereas chromosome 6 lacked a telomere. As a result, chromosome 6 subsequently became unstable, as shown by the fact that it had secondary NRTs originating from other chromosomes in some cells in the population. In 10% of the metaphase spreads analyzed, chromosome 6 had a NRT from chromosome 3 (Fig. 2C, top). Whereas this translocation provided a telomere on the end of chromosome 6, chromosome 3 now lacked a telomere. In 3% of the metaphase spreads, chromosome 6 had a NRT from chromosome 8, whereas chromosome 8 lacked a telomere (Fig. 2C, bottom). Thus, a single telomere loss can initiate cascades of chromosome instability involving multiple chromosomes, resulting in clonal karyotypic evolution of tumor cells.

**Telomere Acquisition by Duplication Does Not Transfer Instability to the Donor Chromosome but Generates Allelic Imbalance**

In addition to NRTs, the translocations on the marker der16 chromosome also occurred through duplications of the arms of other chromosomes (Table 1). However, unlike NRTs, these duplications do not result in the transfer of instability because the donor chromosome does not lose its telomere in the process. In subclone G71, 60 of the translocations (24% of acquired telomeres) were found to involve duplications of the donor chromosome because both homologues of the donor

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**Table 1. Origin of Telomeres Acquired by Marker der16 Chromosome during B/F/B Cycles**

<table>
<thead>
<tr>
<th>Der16 telomere status</th>
<th>No. of metaphases (total of 297)</th>
<th>% of metaphases</th>
</tr>
</thead>
<tbody>
<tr>
<td>No telomere</td>
<td>49</td>
<td>17</td>
</tr>
<tr>
<td>Telomere acquired</td>
<td>248</td>
<td>83</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mechanism of telomere acquisition</th>
<th>No. of metaphases (total of 248)</th>
<th>% of der16 with telomere</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonreciprocal translocation</td>
<td>60</td>
<td>24</td>
</tr>
<tr>
<td>Duplication/translocation</td>
<td>60</td>
<td>24</td>
</tr>
<tr>
<td>Reciprocal translocation</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Translocation/donor lost</td>
<td>27</td>
<td>17</td>
</tr>
<tr>
<td>Dicentric</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>Small duplication 1q</td>
<td>20</td>
<td>8</td>
</tr>
<tr>
<td>No detectable</td>
<td>67</td>
<td>28</td>
</tr>
<tr>
<td>translocation/duplication</td>
<td></td>
<td></td>
</tr>
<tr>
<td>One telomere missing*</td>
<td>(38)</td>
<td>(16)</td>
</tr>
<tr>
<td>All telomeres present*</td>
<td>(29)</td>
<td>(12)</td>
</tr>
</tbody>
</table>

*Status of other chromosomes in the same metaphase spread.
chromosome appeared to be unrearranged. In the example shown (Fig. 3), hybridization with M-FISH and chromosome 1–specific subtelomeric probes shows the marker der16 chromosome has a translocation from the p arm of chromosome 1, whereas one homologue of chromosome 1 lacks its p arm and its telomere (pink arrow) and the other homologue of chromosome 1 seems intact (red arrow). Illustration depicting the NRT of the q arm of chromosome 1 (blue) to the marker der16 chromosome (green), resulting in the acquisition of a telomere (red) on the marker chromosome but loss of the telomere on chromosome 1. B. A metaphase spread of subclone of G71i, which has a NRT from chromosome 6 on the marker der16 chromosome (white arrow) in 60% of the cells in the population. One homologue of chromosome 6 is missing a telomere (pink arrow), whereas the other chromosome 6 homologue seems intact (red arrow). Illustration depicting the NRT from chromosome 6 (light blue) on to the marker der16 chromosome (green), resulting in the acquisition of a telomere (red) on the marker chromosome but loss of the telomere on chromosome 6. C. Two metaphase spreads of subclone G71i in which the marker der16 chromosome has a NRT from chromosome 6 (white arrow) and the donor chromosome has NRTs from a third chromosome. In one of these metaphase spreads (top), chromosome 6 has a NRT from chromosome 3 (blue arrow), whereas a homologue of chromosome 3 lacks a telomere (pink arrow). In the other metaphase spread (bottom), chromosome 6 has a NRT from chromosome 8 (blue arrow), whereas a homologue of chromosome 8 lacks a telomere (pink arrow). Illustrations depicting the secondary NRTs from either chromosome 3 (purple) or chromosome 8 (dark blue) on to chromosome 6 (light blue), resulting in the acquisition of a telomere (red) on chromosome 6 but loss of the telomere on the donor chromosomes.
Seven of the 31 chromosomes donating telomeres to the marker der16 chromosome by NRT were also found to contain large duplications of the arms of other chromosomes (Table 2). In one metaphase spread, the marker der16 chromosome had a NRT from chromosome 17, whereas chromosome 17 had a translocation from chromosome 20 (Fig. 4A). Because both homologues of chromosome 20 were intact, this translocation involved a duplication, consistent with the presence of telomeres on all of the chromosome ends. Thus, similar to the large translocations resulting from duplications on a chromosome that originally lost its telomere, large duplications of chromosome arms can also lead to stabilization of chromosomes donating NRTs.

**Telomere Acquisition by Duplication of the Other Arm of Chromosomes Donating NRTs Results in the Formation of Isochromosomes**

Seven of the 31 chromosomes donating NRTs to the marker der16 chromosome were also found to contain duplications of their other arm (Table 2). In the example shown (Fig. 4B), hybridization with painting and subtelomeric probes specific for the X chromosome revealed that the marker der16 chromosome contained a translocation from the p arm of the X chromosome. The absence of hybridization of chromosome 16–specific probes on the X chromosome showed that this was a NRT. The X chromosome in turn replaced its donated p arm with its q arm, and therefore has q arms on both ends, as shown by the 4',6-diamidino-2-phenylindole banding pattern and hybridization with painting and subtelomeric probes for the q arm (Fig. 4B). These results showed that duplication of the long arm had occurred, creating an “isochromosome” in the process. This formation of isochromosomes seems to occur primarily after the complete loss of a chromosome arm because when G71 was grown with G418 selection to maintain the telomeric plasmid sequences, the marker der16 chromosome was never observed to form isochromosomes. In contrast, when G71 subclones were grown without G418, we frequently observed the complete loss of the p arm from the marker der16 chromosome and the formation of isochromosomes after duplication of the q arm. Thus, chromosomes that lose an entire arm can acquire a telomere through the duplication of the remaining arm. Although this type of telomere acquisition would terminate B/F/B cycles and prevent the transfer of instability to other chromosomes, the loss of an arm and subsequent duplication would generate a further allelic imbalance.

**Stabilization of Chromosomes without Detectable Translocations**

Although translocations are clearly the most common mechanism for telomere acquisition during B/F/B cycles in EJ-30, they were not detected in many of the metaphase spreads in which the marker der16 chromosome acquired a telomere. Hybridization with the GS-240-G10 BAC clone specific for the subtelomeric region on the q arm of chromosome 16 showed their other arm (Table 2). In the example shown (Fig. 4B), hybridization with painting and subtelomeric probes specific for the X chromosome revealed that the marker der16 chromosome contained a translocation from the p arm of the X chromosome. The absence of hybridization of chromosome 16–specific probes on the X chromosome showed that this was a NRT. The X chromosome in turn replaced its donated p arm with its q arm, and therefore has q arms on both ends, as shown by the 4',6-diamidino-2-phenylindole banding pattern and hybridization with painting and subtelomeric probes for the q arm (Fig. 4B). These results showed that duplication of the long arm had occurred, creating an “isochromosome” in the process. This formation of isochromosomes seems to occur primarily after the complete loss of a chromosome arm because when G71 was grown with G418 selection to maintain the telomeric plasmid sequences, the marker der16 chromosome was never observed to form isochromosomes. In contrast, when G71 subclones were grown without G418, we frequently observed the complete loss of the p arm from the marker der16 chromosome and the formation of isochromosomes after duplication of the q arm. Thus, chromosomes that lose an entire arm can acquire a telomere through the duplication of the remaining arm. Although this type of telomere acquisition would terminate B/F/B cycles and prevent the transfer of instability to other chromosomes, the loss of an arm and subsequent duplication would generate a further allelic imbalance.

**Table 2. Origin of Telomeres Acquired by Chromosomes Donating NRTs**

<table>
<thead>
<tr>
<th>Donor telomere status</th>
<th>No. of metaphases (total of 60)</th>
<th>% of metaphases</th>
</tr>
</thead>
<tbody>
<tr>
<td>No telomere</td>
<td>29</td>
<td>48</td>
</tr>
<tr>
<td>Telomere acquired</td>
<td>31</td>
<td>52</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mechanism of telomere acquisition</th>
<th>No. of metaphases (total of 31)</th>
<th>% of donor chromosomes with telomere</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonreciprocal translocation</td>
<td>13</td>
<td>42</td>
</tr>
<tr>
<td>Duplication/translocation</td>
<td>14</td>
<td>45</td>
</tr>
<tr>
<td>From another chromosome</td>
<td>(7)</td>
<td>(22.5)</td>
</tr>
<tr>
<td>From other arm of donor</td>
<td>(7)</td>
<td>(22.5)</td>
</tr>
<tr>
<td>Translocation with donor lost</td>
<td>4</td>
<td>13</td>
</tr>
</tbody>
</table>

**FIGURE 3.** Acquisition of telomeres by duplication of the arms of other chromosomes. Analysis of a metaphase spread of subclone G71 using the same sequential hybridization as in Fig. 2A. **Top,** the marker der16 chromosome (white arrow) contains a translocation/duplication from chromosome 1, whereas both homologues of chromosome 1 seem intact (red arrows). **Center,** the translocation on the marker der16 chromosome contains the subtelomeric region of the p arm of chromosome 1 (green), whereas both homologues of chromosome 1 have the subtelomeric region of the p arm (green) on one end and the q arm (red) on the other. **Bottom,** the marker der16 chromosome and both homologues of chromosome 1 have telomeres on both ends (red). Illustration demonstrating the translocation on the marker der16 chromosome involving the duplication of the arm of chromosome 1, which results in the acquisition of a telomere (red) on the marker chromosome without the loss of the telomere from chromosome 1.
that in 20 of the 248 metaphase spreads in which the marker der16 chromosome acquired a telomere (8%), the new telomere on the p arm originated by small duplications of the end of its q arm. For a more in-depth analysis of these events, five different sets of cytogenetic probes were hybridized sequentially to the same metaphase spreads (Fig. 5). The example shows both the marker der16 chromosome and the normal chromosome 16 homologue from the same metaphase spread. The marker der16 chromosome (Fig. 5, bottom) can be identified by the fact that the RT99/317H7 cosmid probes that contain DNA adjacent to the integrated plasmid, now hybridize at an internal site and show increased intensity compared with the normal homologue (Fig. 5, top). This chromosome therefore contains a large duplication of the p arm and amplification of DNA proximal to the telomeric plasmid, typical of chromosomes involved in B/F/B cycles. Hybridization with a telomere-specific probe shows
that both the normal homologue and the marker chromosome contain telomeres on both ends. In addition, the GS-240-G10 BAC probe specific for the q arm hybridizes to both ends of the marker der16 chromosome, demonstrating the presence of a duplication of the q arm on the end of the p arm. Finally, a chromosome painting probe for the q arm of chromosome 16 that does not hybridize to subtelomeric DNA shows no detectable hybridization to the p arm, demonstrating that the size of the duplicated region is relatively small.

The above results suggest that the small duplications on the p arm of the marker der16 chromosome primarily originated from the q arm of chromosome 16. Consistent with this conclusion, an extended study found that only 1 in 26 of these small duplications involved the p arm. In addition, an in-depth analysis of 200 metaphase spreads with subtelomeric probes for each of the other 46 chromosomes failed to show small duplications of subtelomeric regions similar to those observed for the q arm of chromosome 16, although duplications of large chromosome fragments were observed, typical of the translocations observed by M-FISH. Thus, the small duplications resulting in telomere acquisition seem to primarily originate from the other end of the same chromosome or its homologue, strongly suggesting a mechanism distinct from that involved in the duplications responsible for the large translocations of the ends of other chromosomes.

Telomere Acquisition without Detectable Translocations or Duplications

In 67 of the 248 metaphase spreads in which the marker der16 chromosomes acquired a telomere (28%), no detectable NRTs, large duplications, or small duplications were evident. In 38 of these metaphase spreads (16%), another chromosome lacked a telomere, indicating that the telomeres could have been acquired by NRTs that were too small to be detected (Table 1). However, in the other 29 cells in this category (12%), all of the other chromosomes contained telomeres on both ends (Table 1). The marker chromosome in these cells could therefore have acquired a telomere by de novo addition by telomerase or by duplication of a very small region of the end of another chromosome that is undetectable with subtelomeric probes. Regardless of the mechanism, it is clear from the prolonged period of B/F/B cycles that de novo addition of telomeres by telomerase in the EJ-30 tumor cell line does not occur at a high enough rate to prevent this type of chromosome instability.

Of the remaining translocations observed in our study, 6 involved reciprocal translocations, and 27 could not be characterized as NRTs or duplications due to the loss of the donor chromosome (Table 1). This loss of the chromosomes donating NRTs is 15-fold more likely than the loss of other chromosomes in this cell line (0.09 versus 0.006 chromosomes lost per metaphase), demonstrating that NRT occurring during B/F/B cycles can also result in loss of heterozygosity. Finally, in eight other metaphase spreads (3%), the marker der16 chromosome appeared as a dicentric, and therefore had acquired a telomere by fusion with another chromosome. However, because dicentric chromosomes are highly unstable (18), it is likely that this chromosome will be lost or undergo subsequent rearrangements, as we have previously observed (12). Thus, in addition to NRTs and large duplications, telomere loss can also result in large deletions and complex chromosome rearrangements between chromosomes. The combined results of our study therefore show that the mechanism of telomere acquisition during B/F/B cycles has important consequences for the stability of the genome in human cancer cells.

Discussion

The results presented here show that in addition to amplification and deletion of DNA on the end of a chromosome, B/F/B cycles resulting from telomere loss on one chromosome can cause extensive rearrangement of other

![Figure 5](image)

**FIGURE 5.** Acquisition of telomeres on the marker der16 chromosome p arm through small duplications of the q arm. A marker der16 chromosome demonstrating telomere acquisition through a small duplication of its other end, as shown by the presence of subtelomeric DNA from the q arm on both ends of the chromosome, but no detectable duplication by hybridization using less sensitive painting probes specific for the p and q arms. Hybridization was done with Chr16, a chromosome 16–specific painting probe (green); RT99/317H7, the cosmid clones RT99 and 317H7 specific for the region adjacent to the plasmid integration site on 16p (yellow); Telo, a telomere-specific protein nucleic acid probe (red); Subtelo 16 p/q, the 16p- and 16q-specific subtelomeric BAC clones GS-240-G10 (red) and GS-121-I4 (green); and 16arms, painting probes specific for the p (green) and q (red) arms of chromosome 16. Chromosomes were counterstained with either propidium iodide (red) or 4,6-diamidino-2-phenylindole (blue). Illustration depicting a small duplication of the subtelomeric region (dark red circles) of the long arm (hatched green) on the short arm (solid green) of the marker der16 chromosome, resulting in the acquisition of a telomere (red circles). Due to having undergone B/F/B cycles, the marker der16 chromosome also has a large duplication of the short arm with DNA adjacent to the integrated plasmid at the junction (yellow circles).
chromosomes. Although the number of other chromosomes rearranged may be limited to one or a few chromosomes in any one cell, the cell population as a whole can experience a variety of rearrangements affecting many different chromosomes. Thus, the loss of a single telomere can have important consequences for the karyotypic evolution leading to cancer. Several important factors can affect the extent of chromosome instability resulting from telomere loss in cancer cells. The first is the rate at which telomeres are lost. The major effect of telomere loss in cancer has been proposed to occur at the time of crisis, when extensive chromosome fusion occurs as telomeres become very short due to the failure of cells to senesce (19). However, it is now clear that cancer cells often continue to have problems maintaining telomeres even after the expression of telomerase (15, 16), indicating a fundamental defect affecting telomere stability in cancer cells (20). In fact, this inability to maintain telomeres may be the most important mechanism driving chromosomal instability due to telomere loss because crisis may select for cells in the population that already express telomerase, and therefore cells that survive crisis will not have experienced extensive chromosome fusion during crisis.

A variety of mechanisms could lead to an increased rate of telomere loss in cancer cells. Telomere loss could result from an inability to maintain telomeres due to fluctuations in the expression of telomerase, as has been reported in some tumor cell lines (21, 22). However, in our studies, telomere loss seems to result through stochastic mechanisms that result in the rapid deletion of large portions of the telomere. These rapid deletions could occur as a result of problems in replication of telomeres due to alterations in proteins involved in telomere structure or replication. A fundamental change in telomere structure in cancer cells is indicated by the fact that most cancer cells have telomeres shorter than those found in normal cells despite the expression of telomerase (23, 24). This is not simply due to an inability to maintain telomeres because tumor cell lines with very short telomeres can maintain telomeres precisely at a specific length (25). These changes in telomere length can occur due to altered expression of telomere-associated proteins such as TRF1 (26) which can also pose problems for DNA replication (27). Alterations in proteins required for replication of telomeres can also affect telomere loss, as has been shown with the Rrm3 helicase in yeast (28) and the WRN helicase in human cells (29, 30). Rapid deletion of telomeres could also result from an increased rate of DNA damage in cancer cells because telomeres would be especially vulnerable due to their inefficient repair. Studies in yeast have shown that regions near telomeres are deficient in nonhomologous end joining (31), whereas mammalian telomeres have been reported to be deficient in repair of UV light–induced damage and single-strand breaks (32, 33). This lack of proper repair within telomeric regions may also contribute to chromosome instability by promoting sister chromatid fusion. The junctions involved in sister chromatid fusion have short regions of similarity (13) and occur in the absence of nonhomologous end joining (34), suggesting that they result from an as yet undefined mechanism of double-strand break repair.

Another factor influencing chromosome instability resulting from telomere loss is the length of B/F/B cycles. Our results show that with subclone G71, the marker der16 chromosome still lacks a telomere in 17% of the cells in the population even more than 20 generations after the loss of a telomere. Similar prolonged B/F/B cycles were seen in subclones of G71 (Fig. 2B and C) or other subclones of clone B3 that had undergone sister chromatid fusion after the loss of a telomere (12). Thus, this tumor cell line is relatively inefficient in reestablishing lost telomeres, which allows the expansion of the population containing the chromosome involved in B/F/B cycles (Fig. 6). As a result, even if only a limited number of other chromosomes are affected in each cell, the population as a whole will experience a large variety of karyotypic changes.

The ability to regulate the cell cycle in response to DNA damage can also influence the consequences of telomere loss for the stability of the genome. Cells involved in B/F/B cycles enter the next cell cycle with a chromosome lacking a telomere, which would normally initiate a cell cycle arrest. Consequently, B/F/B cycles are likely to occur only in cells that lack a DNA damage–inducible cell cycle arrest. Consistent with this conclusion, studies in yeast have shown that the loss of cell cycle regulation due to mutations in MEC1, an ATM-related protein involved in cell cycle regulation, can promote inversion translocations after telomere loss, consistent with fusion of sister chromatids (35). Similarly, the importance of cell cycle regulation in preventing chromosome instability through B/F/B cycles is shown by studies in telomerase-deficient mice. The progressive telomere shortening in telomerase-deficient mice results in only a modest increase in cancer (36) because in addition to promoting chromosome fusion, telomere shortening can also serve as a tumor suppressor mechanism (37). However, mice deficient in both telomerase and p53 show a dramatic increase in the frequency of carcinomas that are similar to those seen in humans (14, 38). Cytogenetic analysis of these tumors showed chromosome rearrangements consistent with B/F/B cycles, suggesting that telomere loss and B/F/B cycles play an important role in human cancer. Similarly, an increase in lymphomas in mice with knockouts in both p53 and various proteins involved in nonhomologous end joining was also found to contain chromosome rearrangements consistent with B/F/B cycles (34).

The mechanism by which lost telomeres are restored can also be a major factor in determining the extent of instability resulting from telomere loss. One of the most common mechanisms for acquiring a telomere in subclone G71 is through translocation, which can be either nonreciprocal, in which case the DNA was lost from the donor chromosome, or through duplication, in which case the DNA is retained on the donor chromosome. Combined, these two types of translocations accounted for 48% of the telomeres that were acquired on the marker der16 chromosome in subclone G71 (Table 1). The fact that these translocations originated from a variety of different chromosomes shows that translocation is a frequent mechanism for telomere acquisition during B/F/B cycles in this cell line. Translocations have also been observed on chromosomes involved in B/F/B cycles in mouse embryonic stem cells (13) and in tumors in mice deficient in telomerase (14) or nonhomologous end joining (39). Although both NRTs and translocations involving duplications can stabilize a chromosome that lost a telomere, they differ in their influence on the
stability of the genome as a whole. Although NRTs stabilize the recipient chromosome due to the loss of its telomere, this instability within donor chromosomes is evident by the fact that they commonly have translocations from other chromosomes (Figs. 2C and 4A) and are more frequently lost from the cell than other chromosomes. The absence of a telomere and frequent translocation on chromosomes donating NRTs also shows that like the marker chromosome, they may undergo B/F/B cycles and extensive DNA amplification and deletions. These cascades of instability can then be passed on to other chromosomes donating NRTs until a telomere is eventually acquired by a mechanism other than NRT. In fact, one metaphase spread in our study contained five successive NRTs and a duplication (data not shown). Thus, not only can the loss of a single telomere generate a variety of NRTs in different cells but it can also result in multiple NRTs within individual cells. Telomere loss is therefore a highly efficient mechanism for generating NRTs, which is an important chromosome rearrangement commonly associated with human cancer (40, 41). These translocations could occur through misrepair involving the end of the chromosome without a telomere and terminal fragments of other broken chromosomes. However, they may also occur by a break-induced replication-related process when the end of the chromosome without a telomere invades and initiates replication at sites on other chromosomes. Evidence for this mechanism is found in the presence of duplications at the sites of NRTs associated with B/F/B cycles in mouse tumors (42).

In contrast to NRTs, translocations involving duplications do not pass the instability on to the donor chromosome because it does not lose its telomere in the process (Figs. 3 and 4A). However, telomere acquisition involving these large duplications is not without consequences because it leads to allelic imbalance, which is another important type of genome rearrangement associated with cancer (40, 41). In addition to the duplication of arms of other chromosomes, we have also observed the duplication of the entire other arm of the same chromosome, resulting in the formation of an isochromosome (Fig. 4B). The consequences of both mechanisms are similar: the duplication of large portions of chromosomes. However, the mechanism involved in the formation of isochromosomes seems to be distinct from the mechanism responsible for the large duplications of the arms of other chromosomes. The isochromosomes observed in our studies were only observed after the complete loss of a chromosome arm, consistent with studies on isochromosomes involved in human genetic disease (43). Fusion of the sister chromatids after the complete loss of one arm would result in centromeres that are in close proximity. This close proximity of the centromeres has been proposed to result in the stability of dicentric chromosomes, either because it promotes inactivation of one of the centromeres, or because it reduces the likelihood that the two centromeres will be pulled in opposite directions during anaphase (43, 44). Telomere loss resulting in isochromosome formation also occurs in vivo in lymphocytes of patients with ataxia telangiectasia (45), consistent with the high rate of spontaneous telomere loss in ataxia telangiectasia cells (46).

Specific rearrangements previously described as telomere-centromere translocations followed by a double duplication (45) can now be explained as having involved a B/F/B cycle, NRT, and isochromosome formation, similar to that observed in our study.

Another mechanism by which chromosomes without telomeres can generate chromosome instability is through fusion with other chromosomes. This type of chromosome rearrangement is likely to be cell line dependent because it will be influenced by the frequency of broken chromosomes or chromosomes that have lost their telomeres. At the time of analysis of subclone G71, the marker der16 chromosome was without a telomere in 17% of the cells in the population (Table 1). Because the frequency of anaphase bridges involving the marker der16 chromosome was previously found to

**FIGURE 6.** Model demonstrating how the loss of a single telomere can lead to extensive chromosome instability involving multiple chromosomes. B/F/B cycles resulting from telomere loss on one chromosome allows for the clonal expansion of cells containing a broken chromosome without a telomere. Telomere acquisition through NRTs and end-to-end fusions with other chromosomes results in the transfer of instability between chromosomes and chromosome loss, whereas telomere acquisition through translocations involving duplications stabilizes the genome but generates allelic imbalances in the process.
correspond to the frequency of marker der16 chromosomes without telomeres, most of the chromosomes without telomeres seem to undergo sister chromatid fusion. Because we detected only eight dicentric chromosomes involving the marker der16 chromosome in 297 metaphase spreads (3%) at the time of analysis (Table 1), the frequency of fusion between sister chromatids is ~10 times more common than fusion between chromosomes in this cell line. However, despite their rarity, these dicentric chromosomes can significantly affect the stability of the genome. Although dicentric chromosomes are often rapidly lost from the population (47), their breakage during anaphase can occasionally result in complex rearrangements involving multiple chromosomes (18), as has been observed in subclone G71 (12).

Some forms of telomere acquisition can stabilize chromosomes involved in B/F/B cycles without resulting in further rearrangement of the genome. Hybridization with subtelomeric probes showed that in 8% of the cells, the telomere on the q arm of the marker der16 chromosome was acquired by small duplications of the q arm (Fig. 5). Although less frequent than translocations, these small duplications are not rare events because they were observed at similar frequencies in several subclones of G71. These small duplications seem to occur through a mechanism that is distinct from the large duplications involving other chromosomes or the other arm of the same chromosome. This conclusion is based on the fact that despite an extensive analysis, we did not observe small duplications of the ends of other chromosomes, and the large duplications of the other arm of the same chromosome only occurred on chromosomes that had lost an entire arm through NRT. One possible mechanism for these small duplications is break-induced replication. Similar duplications of a telomere from the other end of the same chromosome have been found to involve break-induced replication in yeast (48), which occurs at sites with homology to the end of the chromosome lacking a telomere (48, 49). As in yeast, the small duplications of telomeric regions in G71 may therefore result when break-induced replication occurs close enough to the other end of the chromosome to allow replication through to the telomere. Our inability to detect similar duplications originating from other chromosomes therefore suggests that the duplication of the other end of the same chromosome is somehow favored, possibly due to their close proximity in the nucleus. However, whether these duplications originated from the other end of the marker der16 chromosome or its homologue was not determined.

In addition to small duplications, direct addition of telomeres to the ends of broken chromosomes by de novo synthesis by telomerase could also prevent or terminate B/F/B cycles without resulting in further rearrangement of the genome. Direct addition of telomeres involving de novo synthesis by telomerase may have occurred in some cells in our study, as indicated by the fact that the marker der16 chromosome in 12% of the metaphase spreads showed no detectable translocation or duplication and telomeres were present on all of the other chromosomes (Table 1). However, it is also possible that these chromosomes contain small translocations or duplications that are too small to be detected cytogenetically using painting or subtelomeric probes. In fact, in 16% of the metaphase spreads in which the marker der16 chromosome had a telomere without a detectable NRT or duplication, a telomere was missing on one of the other chromosomes. Because NRTs and translocations involving duplications occur at nearly the same frequency, it is likely that most of the metaphases in which all of the other chromosomes contain telomeres resulted from duplications of the ends of other chromosomes. Thus, direct addition of telomeres by de novo addition by telomerase seems to have little influence on preventing or terminating B/F/B cycles in this human cancer cell line. In contrast, direct addition of telomeres to the end of the marker chromosome was previously observed near the site of the initial telomere loss in some HSV-tk–deficient subclones in both EJ-30 (12) and mouse embryonic stem cells (50). In fact, in mouse embryonic stem cells the direct addition of telomeric repeat sequences was the most common event observed after the initial telomere loss. Despite this observation, telomere acquisition in mouse embryonic stem cells during B/F/B cycles also resulted primarily from translocations (13). Direct addition of telomeres therefore seems more likely to occur after the initial loss of a telomere in mouse embryonic stem cells, whereas translocations are a more likely mechanism for telomere acquisition during B/F/B cycles. This could reflect the close proximity of the initial break to a telomere, which may favor de novo addition by telomerase. Studies in yeast have shown that de novo telomere addition occurs preferentially near regions containing telomeric repeat sequences that can serve as nucleation sites for telomere-binding proteins and telomerase (51). In addition, de novo telomere addition at double-strand breaks occurring at interstitial sites may be actively prevented by proteins similar to the yeast PIF1 helicase, which has been shown to prevent de novo initiation of telomeres by telomerase (52).

The combined results of these studies show that in addition to the types of rearrangements commonly associated with B/F/B cycles, amplification and deletion of DNA on the ends of chromosomes, telomere loss can also cause many other types of rearrangements, such as NRTs, large duplications, and chromosome loss. In addition, we have shown that these rearrangements are not confined to the chromosome that lost a telomere, but can occur on other chromosomes as well. Because many of these rearrangements are commonly associated with human cancer (40, 41, 53), establishing approaches to limit this process is likely to prove useful in cancer prevention. Chromosome instability involving B/F/B cycles can be limited by cell cycle arrest or apoptosis, both of which can prevent continued replication of cells that have lost a telomere. As we have shown here, the mechanism used for addition of a telomere to the end of a broken chromosome can also be used to limit the extent of chromosome instability resulting from B/F/B cycles, not only for the chromosome that lost its telomere but also for the genome as a whole. Limiting the instability resulting from B/F/B cycles could be achieved by promoting de novo addition of telomeres, as has been observed in PIF1 mutants in yeast (52). Understanding the pathways that influence telomere loss and chromosome instability involving B/F/B cycles should therefore provide additional approaches to prevent or limit chromosome instability in human cancer.
Materials and Methods

Cell Culture

The EJ-30 cell line (obtained from Dr. William Dewey, University of California, San Francisco) was subcloned from the EJ bladder cell carcinoma cell line. Clone B3, isolated from EJ-30 after transfection with the pNCT-tel plasmid, contains a single copy of the plasmid integrated at the end of a chromosome (12). Both EJ-30 and clone B3 are telomerase positive, as shown by TRAPEze XL assay system (Chemicon, Temecula, CA) and real-time PCR. The HSI-Ik–deficient subclone G71 was selected using medium containing both ganciclovir (50 μmol/L) and G418 (400 μg/mL) to ensure that some portion of the plasmid was present to allow for analysis of the recombination events involved. G71 has been fully characterized with respect to the site of fusion, DNA amplification, and anaphase bridges involving chromosome 16 (12, 13, 17).

Chromosome Analysis

For analysis of NRTs and large duplications, cytogenetic analysis was done in two sequential steps, involving hybridization with a protein nucleic acid telomeric probe followed by M-FISH for chromosome painting of all chromosomes. Preparation of metaphase chromosomes was done as previously described (54, 55). Chromosomes were fixed overnight in methanol/acetic acid (3:1 v/v). Telomere analysis was done as previously described (56) using protein nucleic acid probes labeled with Cy3 (Perceptive Biosystems, Ramsey, MN). M-FISH was done using multi-FISH probes (MetaSystems GmbH, Altuslussheim, Germany) according to the manufacturers’ recommendations. Images of hybridized metaphases were captured with a charge-coupled device camera (Zeiss, Thornwood, NY) coupled to a Zeiss Axiosplan microscope and were processed with ISIS software (MetaSystems). Probes for each subtelomeric region were obtained from CytoCell (Cambridge, UK), and hybridizations were done according to the manufacturer’s recommendations.

For analysis of duplications occurring on the q arm subtelomeric region of chromosome 16, cytogenetic analysis was done in five sequential steps, consisting of (1) the protein nucleic acid telomeric probe, (2), the subtelomeric probes, (3) the cosmid probes located adjacent to the integration site, (4) the chromosome 16–specific painting probe, and (5) the painting probe specific for the q arm of chromosome 16. Preparation of metaphase chromosomes and hybridizations with the cosmid and chromosome painting probes were done as previously described (18, 19). Chromosomes were fixed overnight in methanol/acetic acid (3:1 v/v). The first hybridization to be done was with protein nucleic acid probes for telomere analysis to minimize loss of signal. Telomere analysis was done as previously described (56) using protein nucleic acid probes labeled with Cy3 (Perceptive Biosystems). The RT99 (GenBank accession no. AC004653) and 317H7 (GenBank accession no. AC005569) cosmid clones that were used as probes have been mapped to the end of chromosome 16p (57). These cosmid were isolated from partially digested DNA libraries made from flow-sorted human chromosome 16 (58). The subtelomeric BAC probes (CytoCell) were hybridized according to the manufacturer’s protocol. The subtelomeric BAC clones for chromosome 16 consisted of GS-121-I4, which is located a maximum of 160 kb from the telomere on the short arm, and GS-240-G10, which is located a maximum of 200 kb from the telomere on 16q (59). The chromosome 16–specific painting probe was obtained from Adgenix (Voisins le Bretonneux, France).

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The Loss of a Single Telomere Can Result in Instability of Multiple Chromosomes in a Human Tumor Cell Line

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