Telomere Maintenance in Telomerase-Deficient Mouse Embryonic Stem Cells: Characterization of an Amplified Telomeric DNA

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Telomere dynamics, chromosomal instability, and cellular viability were studied in serial passages of mouse embryonic stem (ES) cells in which the telomerase RNA (*mTER*) gene was deleted. These cells lack detectable telomerase activity, and their growth rate was reduced after more than 300 divisions and almost zero after 450 cell divisions. After this growth crisis, survivor cells with a rapid growth rate did emerge. Such survivors were found to maintain functional telomeres in a telomerase-independent fashion. Although telomerase-independent telomere maintenance has been reported for some immortalized mammalian cells, its molecular mechanism has not been elucidated. Characterization of the telomeric structures in one of the survivor *mTER*^{−/−} cell lines showed amplification of the same tandem arrays of telomeric and nontelomeric sequences at most of the chromosome ends. This evidence implicates *cis/trans* amplification as one mechanism for the telomerase-independent maintenance of telomeres in mammalian cells.

Telomeres are special structures at the ends of eukaryotic chromosomes. Since the original work of Muller and McClintock, the telomere has been thought to protect the chromosome end from degradation and fusion to other chromosomes (38, 39, 43). In most eukaryotes, telomeric DNA consists of tandem repeats of G-rich sequences, for example, TTA GGG in mammals and other vertebrates (reviewed in reference 15). In humans, all chromosome ends contain about 5 kb of telomeric DNA (36), and telomeres shorten with each cell division in most somatic cells (19, 22). However, cells that divide indefinitely, such as germline cells and tumor cells, maintain the length of their telomeres, suggesting that a telomere maintenance pathway is activated in immortalized cells, as telomere maintenance is essential for immortal cell growth (reviewed in references 11 and 20).

Telomerase is a ribonucleoprotein enzyme which elongates telomeres by synthesizing telomeric DNA sequence onto the 3' ends of chromosomes. Telomerase can compensate for the loss of telomeric DNA resulting from incomplete replication of the 3' end of telomeres by normal cellular DNA polymerases (reviewed in reference 2). In humans, germline cells and more than 80% of primary tumor cells have been shown to express telomerase activity, but most normal somatic cells express low or undetectable levels of telomerase (reviewed in reference 49). These results suggest that telomerase is the dominant pathway to maintain telomere length in human cells.

Cloning and expression of the mammalian telomerase RNA

gene encoding the template for telomeric DNA synthesis and the gene encoding the catalytic reverse transcriptase subunit of the telomerase complex have shown that these two gene products are essential and sufficient to reconstitute telomerase activity (3, 13, 21, 28, 41, 44, 57). Results from two different recent studies have further strengthened the notion that telomerase is the dominant mechanism for telomere maintenance and that telomere maintenance is crucial for cellular viability in mammals. It was shown that the expression of telomerase in normal human somatic cells resulted in the extension of their life span (5, 30, 55, 56). Studies with the telomerase RNA component *TER*-deficient (*mTER*^{−/−}) mice have revealed progressive telomere shortening with each successive generation of the mutant mice (4) and severe chromosomal instability (18). Further studies of the late-generation *mTER*^{−/−} mice demonstrated that telomere dysfunction and associated chromosomal instability resulted in proliferative defects exemplified by germ cell depletion in the testis, reduced growth capacity in highly proliferative organs, and a shortened life span (33, 48). Defective proliferation and a similar chromosomal instability were also observed in *mTER*^{−/−} ES cells (46).

While these previous studies have clearly established the importance of telomerase in the maintenance of functional telomeres, the telomerase pathway does not appear to be the only way in which telomeres can be maintained. For example, some human tumor cells and tumor-derived cell lines do not express detectable telomerase activity and appear to maintain their telomeres by a telomerase-independent, alternative lengthening of telomeres (ALT) pathway (7, 8). Recent analysis of the cells derived from the *mTER*^{−/−} mice has also clearly demonstrated that telomerase-independent mechanisms are capable of maintaining and elongating telomere length under some circumstances (18). While it has been shown that telomeres in ALT cells are very long and hetero-

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geneous in size, the details of the molecular mechanism of the ALT pathway(s) need to be elucidated.

Telomerase-independent telomere maintenance has been described for cells from other species. *Drosophila* spp. maintain their telomere length by transposition of a set of retrotransposons (reviewed in reference 37). The yeasts *Saccharomyces cerevisiae*, *Kluyveromyces lactis*, and *Schizosaccharomyces pombe* utilize recombination as a backup mechanism for telomere maintenance (32, 35, 40, 45). Indeed, telomere or subtelomeric sequence-mediated recombination has been described (12, 24, 47). In this report, we describe further studies with *mTER*^{-/-} ES cells. We observed telomerase-independent telomere maintenance in *mTER*^{-/-} cells that survived and proliferated after prolonged culture. Analysis of the telomeric structure in such survivors provided new insights into the nature of telomerase-independent telomere maintenance.

MATERIALS AND METHODS

Cells. *mTER*^{-/-} ES cells were generated and cultured as described previously (46).

Pulsed-field gel electrophoresis and fragment analysis. Genomic DNA from ES cells was prepared as described before (42). DNA (15 µg) was digested with restriction endonucleases and separated on a 1% agarose gel in 0.5× Tris-borate-EDTA at 14°C with a CHEF DR-II pulsed-field apparatus (Bio-Rad). Pulsed-field electrophoresis was performed at 6 V/cm for 12 to 18 h at a ramped pulse of from 1 to 10 s. The gel was dried at 60°C for 1.5 h, denatured in 1.5 M NaCl-0.5 M NaOH solution for 30 min, neutralized in 1.5 M NaCl-0.5 M Tris-HCl (pH 8.0) buffer for 30 min, and hybridized to 5'-[γ-³²P]T₂AG₃ telomeric DNA oligonucleotides in 5× SSC-5× Denhardt's solution-0.1× P wash (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate; P wash is 0.5 mM pyrophosphate plus 10 mM Na₂HPO₄) at 37°C for 12 h. Following high-stringency washes in 0.1× SSC at 20°C for 1 h, the gel was autoradiographed. The 555-bp nontelomeric DNA probe was amplified by PCR with oligonucleotides F01 (5'-TCT CTA TTA TGG GGA TTA AAG G-3') and F02 (5'-TCC ATT TAT TCT CCA CAA CCG C-3'). Quantitative slot blot analysis for telomeric DNA contents was performed as described before (46). The mouse major satellite DNA26 oligonucleotide probes were used as a reference for signal intensity.

Telomere FISH analysis. Metaphase chromosomes from ES cells at different population doublings (PDL) were prepared, and fluorescence in situ hybridization (FISH) with Cy-3-labeled C₃TA₂ peptide-nucleic acid (PNA) probe and subsequent quantitative analysis of digital images were performed as described before (18, 59). FISH analysis of centromere (mouse major satellite DNA) and telomere was carried out by standard methods as described before (53). Centromere and telomere probes were produced by using primers MM18a (5'-TACA CACTTTAGGACGTG-3') and MM18b (5'-CACGTCCTAAAGTGTGTA-3') and telo-1 [5'-(T₂AG₃)₅-3'] and telo-2 [5'-(C₃TA₂)₅-3'] according to the protocols reported previously (25, 26).

Cloning of telomeric DNA. Genomic DNA from DKO741 at 860 PDL was digested with *Hinf*I, and the DNA fragments were separated on a 1% low-melting-point agarose gel. DNA fragments of about 1.6 kb were isolated from the gel, subcloned into the pZErO-2 vector (Invitrogen), and transformed into a Max Efficiency STBL2 competent cell (Gibco-BRL). Each single colony was formed on a Luria-Bertani-agarose plate containing 25 µg of kanamycin per ml at 30°C for 16 h. Clones containing the TTAGGG repeat sequences were screened by colony hybridization with the 5'-[γ-³²P]T₂AG₃ telomeric DNA oligonucleotide probe. Positive clones were isolated and sequenced by a dye terminator method with an ABI Prism 377 sequencer (Applied Biosystems). As described in the legend to Fig. 6, sequencing was initiated from both ends of the insert, and the sequence at the junction of the nontelomeric and telomeric regions was determined.

FISH with cloned nontelomeric DNA. Approximately 3 to 5 µg of nontelomeric DNA in the vector was labeled with Biotin-16-dUTP by nick translation and then precipitated with ethanol, using salmon sperm DNA as a carrier. The probe was dissolved in hybridization buffer containing 50% deionized formamide, 2× SSC, 10% dextran sulfate, and 50 mM phosphate buffer (pH 7.0) to a concentration of 20 ng/µl. Metaphase spreads at selected PDL from wild-type (WT) and *mTER*^{-/-} ES cells were hybridized as described earlier (17, 18). Slides were incubated with pepsin (0.005%) in 10 mM HCl for 10 min at 37°C, washed with phosphate-buffered saline (PBS) containing 50 mM MgCl₂, and then treated with 1% formaldehyde in PBS-MgCl₂ for 10 min at room temperature. After one more wash in PBS, slides were dehydrated in a 70, 90, and 100% ethanol series. The labeled probe was diluted with hybridization buffer to a final concentration of 4 to 8 ng/µl, and 20 µl was added to each slide. The probe and the target DNA were denatured simultaneously at 80°C for 3 to 4 min. Hybridization was carried out overnight at 37°C in a moist chamber. After hybridization, the slides were washed three times for 5 min each in 50% formamide-2× SSC buffer (pH 7.0)

FIG. 1. Growth of *mTER*^{-/-} ES cells in long-term culture. The growth characteristics of *mTER*^{+/+} (WT), *mTER*^{+/-} (KO6), and *mTER*^{-/-} (DKO301 and DKO741) ES cells were monitored during long-term culture.

at 37°C, three times in 0.1× SSC at 60°C, and twice for 5 min each in 2× SSC at room temperature. For immunofluorescence detection, the slides were incubated with avidin-fluorescein isothiocyanate (Vector Labs) for 30 min at 37°C in a humid chamber. The signal was amplified using biotinylated goat antiavidin antibody (Vector Labs). After dehydration in an ethanol series, the slides were embedded with Vectashield mounting medium (Vector Labs) containing 1 µg of propidium iodide (Sigma) per ml. Slides were observed under a Zeiss Axioplan2 microscope (Carl Zeiss) equipped with suitable filters and with a charge-coupled device camera (SensiCam PCO).

Telomerase activity measurement. Telomerase activity of the *mTER*^{-/-} ES cells was measured with a Telomerase detection kit (Toyobo) as described before (52).

BAL-31 nuclease digestion. Genomic DNA (15 µg) was digested with 2.5 U of BAL-31 nuclease (Takara) in 80 µl of 1× BAL-31 buffer at 30°C for 10 and 20 min. The reaction was stopped by adding 20 µl of 0.5 M EDTA and phenol-chloroform extraction. Digested DNA was precipitated with 100 µl of isopropanol, washed with 70 and 100% ethanol, and then resuspended in Tris-EDTA. One third of the digested DNA (5 µg) was further digested with *Bam*HI, *Hap*II, or *Hinf*I and used for terminal restriction fragment (TRF) analysis as described above.

Nucleotide sequence accession numbers. The GenBank accession numbers of the nontelomeric sequences are AB040048 and AB040049.

RESULTS

Isolation of survivors from late-passage cultures of *mTER*-deficient ES cells. Two independent *mTER*^{-/-} ES cell lines (DKO301 and DKO741) were established as previously described (46). These mutant ES cells had no telomerase activity and showed progressive telomere shortening under long-term culture conditions. Once telomere length reached a critical size, chromosomal instability was induced, and growth of the mutant cells was strongly suppressed. The growth-retarded *mTER*^{-/-} ES cells were mostly large cells that apparently stopped proliferation at about 450 PDL. However, cultures were continued, and from 2 × 10⁴ to 10 × 10⁴ cells were replated every 3 to 4 days. Rare subpopulations of both *mTER*^{-/-} ES cell lines survived the growth arrest and resumed proliferation, as shown in Fig. 1. The doubling time of the DKO301 and DKO741 survivors was about 24 h, and they continued to grow up to 850 PDL, the endpoint of the experiment. The size of survivor *mTER*^{-/-} ES cells was similar to that of WT cells, and they were still telomerase negative (Fig. 2). The growth of control WT and *mTER*^{+/+} (KO6) ES cells

FIG. 2. Telomerase activity of *mTER*^{-/-} survivor ES cells. Total-cell lysates were prepared from WT, DKO301 at 663 PDL, and DKO741 at 638 PDL. The assay was performed multiple times, and both DKO301 and DKO741 survivors still exhibited undetectable levels of telomerase activity. IC, internal control.

was constant over 2 years, with a rate of 12 to 16 h per cell division. Due to the limitations of the culture conditions employed in the present study, it was not possible to determine the exact frequency of survivor cells in the *mTER*^{-/-} ES cells. Also, it is uncertain if the survivors were selected by mutation with genetic changes or induced by a physiologically programmed pathway.

Telomere dynamics and chromosomal instability in survivor *mTER*-deficient ES cells. To analyze telomere dynamics in the survivor *mTER*^{-/-} ES cells, we performed Southern blot analysis of the TRFs containing telomeric DNA sequences. As described previously (46) and shown in Fig. 3A, Southern blot analysis of DNA digested with restriction enzyme *HinfI* demonstrated that both the size and the relative signal intensity of the TRFs hybridized with the T₂AG₃ oligonucleotide probe were progressively reduced in both *mTER*^{-/-} ES cell lines up to the growth crisis stage (444 PDL for the DKO301 cells and 428 PDL for the DKO741 cells). After growth crisis, the reduction in TRFs continued in DKO741 survivor cells (from 655 to 834 PDL) but was reversed in DKO301 survivor cells (from 569 to 824 PDL). Based on the reduction in signal intensity during the first 200 PDL (46), no T₂AG₃-hybridizing signals were expected for either *mTER*^{-/-} ES cell line after 600 PDL. However, analysis of short restriction fragments revealed a fragment of about 1.6 kb which hybridized strongly to the T₂AG₃ probe in the DKO741 survivor cells at 614 PDL, which was not observed in WT, KO6, DKO301, or precrisis DKO741 (187 PDL) cells (Fig. 3B). Using other 4- or 5-base restriction enzymes (which were also not able to digest the TTAGGG sequence), we further analyzed the DNA that hybridized with the telomere probe in the DKO741 survivor cells. As shown in Fig. 3C and D, two types of fragments were generated with such restriction enzymes. Like *HinfI* digestion, *AluI*, *HaeIII*, *MboI*, and *RsaI* digestion generated a dominant single fragment of about 1 kb. *HhaI* and *HapII*, however, produced more than one discrete size (about 6, 8, and >12 kb) and larger smears (>12 kb) of restriction fragments. *HapII*-digested DNA from the *mTER*^{-/-} DKO741 survivor cells at different PDL showed ladders of fragments of different sizes (Fig. 3E). Such short distinct fragments and ladders were not observed with DNA from DKO301 survivor cells digested with *HapII* and other restriction enzymes (Fig. 3E and data not shown). Quantitative slot blot analysis also confirmed recovery of

T₂AG₃-hybridizing signals in both *mTER*^{-/-} survivor ES cells between 448 and 764 PDL in DKO301 and 432 and 764 PDL in DKO741 cells (data not shown). Therefore, we conclude that telomeric DNA in the *mTER*^{-/-} survivor ES cells was maintained or accumulated in a telomerase-independent manner.

To further analyze telomere dynamics and chromosomal stability in the *mTER*^{-/-} ES survivor cells, we performed cytogenetic analysis of metaphase spread chromosomes by quantitative FISH (Q-FISH) with the (C₃TA₂)₃ PNA probe (31, 59). Representative FISH images of metaphase spreads from the WT and *mTER*^{-/-} ES cells at different passages are shown in Fig. 4. As summarized in Fig. 5, the mean telomere fluorescence units (TFU) of p-arm and q-arm telomeres was 3.3 ± 0.2 and 9.3 ± 0.3 TFU, respectively, in DKO301 cells and 2.9 ± 0.2 and 10.0 ± 0.3 TFU, respectively, in DKO741 cells at the growth crisis stage (about 450 PDL) (values are means $\pm$ SE). These values were between 10 and 21% of those for WT ES cells at 10 PDL. Different results were obtained with DKO301 cells at 692 PDL and the DKO741 cells at 683 PDL. The values in these survivor cells were 4.7 ± 0.3 and 24.7 ± 0.7 TFU, respectively, and 0.3 ± 0.1 and 23.5 ± 0.5 TFU, respectively, or 16, 58, 1, and 55% respectively, of those in WT cells at 10 PDL. These results are compatible with recovery of telomeric DNA sequences on the q-arm but not the p-arm telomeres.

Cytogenetic analysis revealed that the progressive telomere shortening in the *mTER*^{-/-} ES cells with prolonged culture resulted in increasing chromosomal instability. The number and frequency of fused chromosomes in the WT and *mTER*^{-/-} ES cells at different passages are summarized in Table 1. The chromosomes of the WT ES cells were quite stable, and only three fusion events in 18 metaphases were observed at 1,134 PDL (0.17 chromosome per metaphase). The number of fused chromosomes was significantly increased in both *mTER*^{-/-} ES cell lines. Even before the growth crisis stage, the DKO301 cells at 142 PDL and the DKO741 cells at 154 PDL contained 1.5 and 1.2 fusions per metaphase, respectively. Furthermore, the number of fused chromosomes was increased 10 times at the growth crisis stage in both mutant cells (11.1 to 16.3 and 13.6 to 14.9 fusions per metaphase in DKO301 and DKO741 cells, respectively). These fusions were predominantly of a Robertsonian type (p- to p-arm), but other types of fusions, including a dicentric, p- to q-arm, trisomic, ring, and more complicated types, were also observed.

Once survivors emerged from the cultures, the percentage of fused chromosomes per metaphase was further increased; the frequency of fused chromosomes observed was increased, with 19.1 and 26.5 fusions per metaphase at 692 PDL in DKO301 cells and at 683 PDL in DKO741 cells, respectively; therefore, 19.1 of 27.9 (68%) and 26.5 of 25.5 (>100%) chromosomes were fused, respectively. Interestingly, essentially all chromosomes in metaphase spreads from these survivors were end-to-end Robertsonian type fusions, as shown in Fig. 4. Hybridization with a mouse major satellite probe specific for a majority of centromeres in WT cells (Fig. 4I) showed variable staining of centromeres in the *mTER*^{-/-} survivor ES cells (Fig. 4J), confirming the metacentric nature of the chromosomes in these cells. We conclude that the vast majority of the original p-arm telomeres in the *mTER*^{-/-} survivor ES cells are no longer present at the ends of the chromosomes, which may explain why recovery of TFU on the p-arm telomeres was not induced in these cells (Fig. 5).

Cloning and sequencing of elongated telomere DNA. To further investigate the nature of the telomerase-independent acquisition of telomeric DNA in the *mTER*^{-/-} survivor ES cells, we cloned and sequenced the 1.6-kb *HinfI* TRF from the

DKO741 survivor cells (Fig. 3B). Five independent clones that hybridized with the T₂AG₃ oligonucleotide probe were obtained. Sequencing 200 to 300 bp of both ends on each insert demonstrated that they were all identical. As shown in Fig. 6, further sequencing of the 1.6-kb insert demonstrated that it consisted of the TTAGGG repeats (about 700 bp) flanked with nontelomeric sequences. No sequence with significant homology to the nontelomeric sequences was identified in the GenBank database. Mapping with the 4- and 5-base restriction enzymes (Fig. 6B) showed that the sizes of each fragment

FIG. 3. Telomere dynamics in *mTER*^{-/-} ES cells. Genomic DNAs from *mTER*^{+/+} (WT) and *mTER*^{-/-} (DKO301 and DKO741) ES cells at selected PDL were digested with *HinfI* and separated on a pulsed-field gel (A) or a 0.8% agarose gel (B) or digested with *HapII* and separated on a pulsed-field gel (E). Genomic DNAs from WT cells at 4 PDL, DKO301 cells at 824 PDL, and DKO741 cells at 764 PDL were digested with each 4- or 5-base restriction endonuclease and separated on a pulsed-field gel (C) or a 0.8% agarose gel (D and F). The 5'-[³²P](T₂AG₃)₃ telomeric DNA oligonucleotides (A to E) and 555-bp nontelomeric DNA fragment (F) were used as probes.

containing the telomeric DNA region were consistent with those seen in the TRF Southern blot analysis of the DKO741 survivor cells. Further hybridization analysis demonstrated that the 1.6-kb *HinfI* fragment hybridized to the nontelomeric DNA probe (555-bp PCR fragment amplified from the nontelomeric region 5' to the telomeric DNA region, as shown in Fig. 6B) was specifically and highly amplified in DKO741 survivors (Fig. 3F). Some additional bands (<500 bp) were detected in DKO741 survivor DNA digested with *AluI* and *MboI*, and their sizes were also consistent with the mapping of the 1.6-kb fragment (Fig. 6B). In WT and DKO301 cell DNA, very faint bands were detected (~0.5 kb for *HinfI*, ~0.3 kb for *AluI*, and ~1.0 kb for *RsaI*). Slot blot analysis demonstrated that the nontelomeric DNA signal was amplified by >100-fold in the DKO741 survivor cells (not shown). Furthermore, rehybridization confirmed that the fragments hybridizing to the T₂AG₃ probe shown in Fig. 3D also hybridized to the nontelomeric DNA probe (not shown).

To confirm the localization of the cloned 1.6-kb sequence at the chromosome ends, we performed FISH analysis on WT and both *mTER*^{-/-} ES cell lines with the nontelomeric sequence probe which was used for the previous TRF Southern blot analysis. As shown in Fig. 7C, most of the chromosome ends (85 ± 6%) from DKO741 survivors at 683 PDL specifically hybridized to the nontelomeric DNA probe (green signals). However, no specific signals on the metaphase spread

FIG. 4. Metaphase spreads from WT and *mTER*^{-/-} ES cells. FISH with a PNA-telomere probe for WT (A and B), DKO301 (C to E), and DKO741 (F to H) at selected PDL. Two-color FISH with the telomere (green) and mouse major satellite (pink) probes for WT (I) at 1,134 PDL and DKO741 (J) at 683 PDL. Chromosomal stability and telomeric DNA content have been highly sustained in the WT over 1,000 PDL. On the other hand, telomeric DNA content in the *mTER*^{-/-} ES cells has decreased progressively until the growth crisis stage (C and D; F and G) but regained after the crisis by telomerase-independent mechanisms (E and H). Furthermore, many fusions (mostly p-arm-to-p-arm fusions) were induced during the growth crisis stage (D and G).

chromosomes in WT, DKO301, and precrisis DKO741 (at 154 PDL) cells were observed (Fig. 7A and B and data not shown). The endogenous locus most likely escaped detection by FISH due to the small size of the oligonucleotide probe. This FISH

analysis clearly showed accumulation of the cloned nontelomeric/telomeric DNA sequence at the ends of chromosomes specifically in DKO741 survivor cells.

To further investigate the terminal nature of the 1.6-kb DNA unit, DNA from DKO741 survivor cells at 638 PDL and control cells (WT and DKO301 survivors at 663 PDL) was digested with BAL-31 nuclease for increasing lengths of time. Because this enzyme progressively shortens DNA from the ends, sequences at chromosome ends such as telomeric sequences are sensitive to BAL-31 digestion (29). As shown in Fig. 8, TRFs of WT DNA generated by *HapII* digestion were progressively shortened by BAL-31 predigestion. The average TRF length of DKO301 survivor DNA was also decreased in a time-dependent fashion. However, DKO741 survivor DNA behaved somewhat differently. The amounts of higher-molecular-

FIG. 5. Q-FISH analysis. Mean telomere fluorescence of *mTER*^{+/+} (WT) and *mTER*^{-/-} (DKO301 and DKO741) cells at selected PDL. Fluorescence is expressed in TFU, where 1 TFU corresponds to 1 kb of T₂AG₃ repeat in plasmid DNA (36). For the survivors (DKO301 at 692 PDL and DKO741 at 683 PDL), the q-arm TFU show a more appropriate telomere length at the ends of chromosomes. Error bars indicate the SE.

weight *HapII* fragments (>23 kb) were diminished after 10 min of BAL-31 predigestion, but no further TRF shortening was observed after further incubation with BAL-31 (20 min [Fig. 8] and up to 60 min [not shown]). This result indicates two possibilities. (i) The tandem arrays of the telomeric/nontelomeric DNA which were observed at the end of most of the chromosomes in the DKO741 survivor cells by FISH analysis are not

the very end of the chromosomes, and (ii) the telomeric/nontelomeric DNA is at the very end of the chromosomes but resistant to BAL-31 digestion due to the unusual structure of these arrays or the poor degradation rate of the nontelomeric DNA. In the absence of any conclusive evidence, we prefer the second possibility. If the first possibility is true, it is difficult to envisage the role of such ends as "functional telomeres" (te-

TABLE 1. End-to-end fusions observed in the *mTER*^{+/+} and *mTER*^{-/-} ES cells

Sample and no. of PDL	No. of:								
	Metaphases analyzed	Chromosomes per metaphase	Fused chromosomes ^a	Fusions ^a	Fused chromosomes ^b				Fragments ^a
					RLC	Dic/Tric	Rings	Others	
E14 <i>mTER</i> ^{+/+} cells									
10	17	40.59 ± 0.2	0	0	0	0	0	0	0
1,134	18	40.39 ± 0.4	3 (0.17)	3	3 (100)	0	0	0	0
<i>mTER</i> ^{-/-} cells									
DKO301									
142	20	40.95 ± 0.4	29 (1.5)	29 (1.5)	25 (86.2)	0 (0)	3 (10.3)	1 (3.5)	0 (0)
457 ^c	16	30.31 ± 2.6	260 (16.3)	283 (17.7)	207 (79.6)	17 (6.5)	16 (6.2)	20 (7.7)	8 (0.5)
465 ^c	17	26.94 ± 0.8	188 (11.1)	286 (16.8)	141 (75.0)	37 (19.7)	4 (2.1)	6 (3.2)	0 (0)
692	19	22.92 ± 0.5	362 (19.1)	379 (20.0)	315 (87.0)	33 (9.1)	4 (1.1)	10 (2.8)	2 (0.1)
DKO741									
154	17	41.41 ± 0.8	21 (1.2)	22 (1.3)	12 (57.1)	0 (0)	8 (38.1)	1 (4.8)	5 (0.3)
423	19	26.16 ± 1.4	259 (13.6)	342 (18.0)	213 (82.2)	34 (13.1)	8 (3.1)	4 (1.54)	8 (0.4)
435 ^c	15	24.20 ± 2.3	224 (14.9)	274 (18.3)	190 (84.8)	18 (8.0)	13 (5.8)	3 (1.3)	2 (0.1)
683	18	25.50 ± 1.9	477 (26.5)	598 (33.2)	439 (92.0)	31 (6.5)	5 (1.1)	2 (0.4)	1 (0.1)

^a Numbers in parentheses indicate frequency per metaphase.

^b Numbers in parentheses indicate percentage of total fusions observed. RLC, Robertsonian fusion like configurations; Dic/tric, dicentrics/tricentrics. Other fusions include q-p fusions and complex fusions.

^c Growth crisis PDL.

FIG. 6. Sequencing and structure of the 1.6-kb *HinfI* TRF from the DKO741 survivor ES cells. Genomic DNA from DKO741 at PDL 860 was digested with *HinfI*. Fragments of about 1.6 kb were isolated and subcloned. (A) Nucleotide sequence of the cloned gene. (B) Structure of the cloned 1.6-kb *HinfI* fragment. Open and shaded boxes indicate telomeric and nontelomeric DNA regions, respectively. The recognition sites of the 4- and 5-base restriction endonucleases used in Fig. 2 are indicated: A, *AhaI*; HIII, *HaeIII*; M, *MboI*; Hi, *HinfI*; R, *RsaI*. The sizes of the restriction fragments are shown. The numbering of restriction sites, 3' to the telomeric region is based on the 3'-most *HinfI* site as base 1600. With the T₂AG₃ probe, we expected fragment sizes of 868, 1,315, >1,070, 1,600, and >1,432 bp with *AhaI*, *HaeIII*, *MboI*, *HinfI*, and *RsaI*, respectively. With the 555-bp nontelomeric DNA probe, we expected fragment sizes of 320 and >232, 1,315, >302 and 231, 1,600, and >1,432, respectively, for the same enzymes.

FIG. 7. FISH analysis of nontelomeric DNA sequence. FISH with the 555-bp nontelomeric DNA probe used for Fig. 3F (lower panel). (A) WT cells at 10 PDL; (B) DKO741 at 154 PDL; (C) DKO741 at 683 PDL.

FIG. 8. BAL-31 nuclease treatment assay. Genomic DNA (5 μ g) from WT at 1,008 PDL, DKO301 at 663 PDL, or DKO741 at 638 PDL was digested with BAL-31 nuclease for 0, 10, or 20 min. Then, DNA was further digested with *HapII* and used for TRF analysis. (T₂AG₃)₃ was used as the probe.

lomic sequence arrays existed only in the characterized $\sim$ 1-kb telomeric/nontelomeric TRF fragments, such as shown in Fig. 3D).

DISCUSSION

In the present study, we demonstrate that telomeres in the *mTER*^{-/-} survivor ES cells were maintained independently of telomerase activity. Furthermore, cloning and sequencing analysis of the TRF from the DKO741 survivor cells provides the first evidence at the nucleotide level that telomere length maintenance and extension in telomerase-negative mammalian cells may involve amplification of (sub)telomeric repeat sequences that is independent of the "telomerase" system.

Growth and telomere dynamics in *mTER*-deficient ES cells.

As described previously (46), growth retardation and chromosomal instability were coincidentally induced in *mTER*^{-/-} ES cells under long-term culture conditions. Complementation of *mTER* gene expression in the *mTER*^{-/-} ES cells before the growth crisis stage prevented growth retardation. However, as telomere length continued to shorten, chromosomal stability was significantly maintained (H. Niida and Y. Shinkai, unpublished data). Since *mTER*^{-/-} ES cells complemented with the *mTER* gene contained less than 5% of the telomerase activity measured in WT ES cells, we speculate that the low level of telomerase activity may prevent end-end fusions but not telomere shortening. Similar results were recently reported by Zhu et al. (58), who showed that ectopic expression of telomerase in normal human fibroblast cells may result in the maintenance of chromosome stability and an extended life span despite an initial overall shortening of telomere length. Most likely, the growth defect of the *mTER*^{-/-} ES cells was similarly associated with chromosomal instability of specific chromosomes with a shorter-than-average telomere length.

Growth or proliferative defects were previously observed in *mTER*-deficient mice with shortened telomeres (33, 48). Typically, in the sixth generation (G6) of *mTER*^{-/-} mice, the development of male germ cells was impaired and the proliferative capacity of the skin and hematopoietic cells was decreased. Furthermore, aneuploidy and chromosomal fusions were significantly increased in splenocytes from G6 mice. Mouse embryonic fibroblasts (MEFs) derived from the late-generation animals showed biphasic cell cycle arrest at an early passage (9). However, the MEFs have never shown growth defects even after many chromosomal fusions were induced due to severe telomere shortening (4, 18). On the other hand, in the *INK4a*^{-/-} background, loss of telomere function was associated with a decreased rate of Myc/RAS focus formation (growth defect) in late-generation *mTER*^{-/-} MEFs (14). Although we do not have a clear explanation for this discrepancy, the response to telomere shortening and the capacity to grow in the presence of chromosomal instability may vary among cell types or genetic backgrounds. A possible mechanism is differential checkpoint thresholds. A good example is the well-known difference between cells upon activation of p53; some cells will die by apoptosis, and others will respond with cell cycle arrest. Indeed, the growth defect of the *INK4a*^{-/-} *mTER*^{-/-} MEFs and apoptosis of specific cell types in G6 mice were p53 dependent (9, 14). This evidence may suggest that the growth defect of our *mTER*^{-/-} ES cells was also p53 mediated. In addition, the much more severe phenotype of *mTER*^{-/-} C57BL/6 mice (23) compared to *mTER*^{-/-} mice on a mixed C57BL/6/129 background (33, 48) supports the idea of the genetic background difference.

Once survivor cells predominated in the cultures of *mTER*^{-/-} ES cells, essentially all chromosomes were fused and the total number of chromosomes per metaphase spread was reduced to about half (Table 1). Most of the fusions can be characterized as (pseudo-)Robertsonian fusions. In early passages of WT ES cells, the mean TFU value on q-arm telomeres was about 1.5 times greater than that on p-arm telomeres (Fig. 3). The appearance of Robertsonian-type fusion as the dominant type in the *mTER*^{-/-} ES cells would be expected if telomere size were most critical for induction of end-to-end fusion. Other types of fusions were also observed in both lines of *mTER*^{-/-} ES cells at the growth crisis stage. However, fewer such abnormalities were observed in later passages of the survivor cells, suggesting that cells with other types of fusions than (pseudo-)Robertsonian fusions had a growth disadvantage. Nevertheless, 8 to 13% of chromosomes in the *mTER*^{-/-} sur-

vivor ES cells at >680 PDL were still contained in dicentrics, tracentrics, and other chromosomal abnormalities. One possibility is that only one centromere was active not only in the (pseudo-)Robertsonian-type fusions, but also in the dicentric or tracentric chromosomes, as described elsewhere (51). In this case, breakage-fusion-bridge cycles could not be triggered. Therefore, once further end-to-end fusions were suppressed by telomerase-independent telomere lengthening, the growth defect resulting from the chromosomal instability in the surviving *mTER*^{-/-} ES cells may have been attenuated.

In the DKO741 survivor cells at 683 PDL, 92% of the chromosomes were (pseudo-)Robertsonian-type fusions (Fig. 3) with a mean p-arm telomere value of 0.3 ± 0.1 TFU. The critical size of telomeric DNA sequences required for chromosomal stability in mammals is most likely higher than this estimate, as many of the observed fusions are likely to have evolved from more than one breakage-fusion-bridge cycle. In general, our results support the idea that a minimum telomere length is required at chromosome ends to maintain chromosomal stability and prevent chromosome fusions.

Telomerase-independent telomere maintenance. Although telomerase has been shown to be the dominant pathway to maintain telomere length in mammalian cells, it was reported that some human tumor cells and tumor-derived cell lines do not express detectable levels of telomerase activity and appear to maintain their telomeres by the ALT pathways (7, 8). Recent studies of cells derived from *mTER*^{-/-} mice also clearly demonstrated that the telomerase-independent ALT mechanisms can maintain and elongate telomeres in murine cells under some circumstances (18). While it has been shown that telomeres in ALT cells are typically very long and heterogeneous in size, the details of the molecular mechanism(s) involved in ALT remain to be elucidated.

As described for the *mTER*^{-/-} ES cells, telomerase-defective cells from certain yeast strains have shown a very similar phenotype of telomere dynamics and growth abilities (34, 40, 45, 50). Accompanying the progressive telomere shortening, a severe growth defect was induced once the telomere length reached a critical size. Then, a minor subpopulation of mutant cells without an apparent growth defect emerged. Genetic studies further demonstrated that telomerase-independent telomere maintenance in the surviving mutant yeast strains was dependent on the *RAD52* gene, suggesting the involvement of recombination (32, 35, 40). In *S. cerevisiae*, an unusual telomeric DNA structure consisting of tandem arrays of telomeric and subtelomeric DNA sequences was observed in the telomerase-defective survivor cells (35). Such arrays can be amplified by homologous recombination between two distant telomeric or subtelomeric DNA sequences. However, the surviving cells from *K. lactis* and *S. pombe* seemed to elongate and maintain the telomeric DNA sequence only (40, 45). Therefore, the specific telomeric structure observed in the *S. cerevisiae* survivor strains was considered nonessential for recombination-mediated telomere maintenance and simply the by-product of recombination reaction.

One of the *mTER*^{-/-} survivor ES cell lines, DKO741, showed a telomeric structure similar to that of telomerase-negative *S. cerevisiae* survivors in that most of the (fused) chromosome ends contained repeats of a DNA sequence unit containing both telomeric and nontelomeric (subtelomeric?) sequences. Based on this similarity, it could be hypothesized that the recombination-based reaction is also involved in telomerase-independent telomere maintenance in the DKO741 survivor cells. That is, the DNA sequence unit consisting of the telomeric/nontelomeric/telomeric (or vice versa) sequences may exist on a border between the subtelomeric and telomeric

regions. Alternatively, the nontelomeric DNA may contain a fragile site, and this site may have been preferentially broken following the breakage-fusion-bridge cycles during the growth crisis stage. Upon fusion with a very short telomere on another chromosome, the telomeric/nontelomeric sequences at the end of a chromosome could be generated relatively easily. Then, once a critical but unidentified change(s) is induced, all chromosome ends in the DKO741 cells may eventually have acquired and amplified this specific DNA unit as a consequence of serial recombination-based reactions. The recombination-based reaction can be initiated by interchromosomal pairing, intrachromosomal pairing, or recently described telomere t-looping (16). On the other hand, DKO301 survivor cells and other telomerase-negative human cell lines (7) have not shown such a specific telomeric structure and appear to have only elongated telomeric DNA sequences on each chromosome end. As described for other telomerase-defective yeast strains (40, 45), they may also have utilized a recombination-based reaction with only telomere repeat sequences as the telomere maintenance pathway. Of course, this is still one of several possible mechanisms. These cells or all of the survivor cells may have used a non-recombination-based mechanism. Further investigation will be needed to elucidate the entire spectrum of the telomerase-independent telomere maintenance mechanisms.

Because telomere maintenance in normal cells appears to be highly dependent on the telomerase pathway, the ALT pathway in normal cells may be negatively regulated at different levels. Therefore, accessibility of the telomere region to the ALT pathway may be different before and after the growth crisis stage. One possible level of regulation could be telomere binding proteins (1, 6, 10). It was reported that inactivation of one such protein, human TRF2, induced severe end-end fusions even though these chromosomes contained long stretches of telomere repeats (54). Another telomere binding protein, Tazlp in *S. pombe*, was also shown to be involved in the regulation of telomeric recombination (45). Therefore, TRF2 may regulate the accessibility of telomeres to the telomerase-independent pathway in mammals. Inhibition of TRF2 was found to result in immediate deprotection of chromosome ends, and TRF2 may be required to form a large duplex loop that protects telomeres from DNA repair machinery, including recombination events (16). The unusual telomeric DNA structure observed in the DKO741 survivor cells may potentially disrupt the regulation mediated by telomere binding proteins such as TRF2 and facilitate access to the ALT pathway(s). Alternatively, the unusual telomeric DNA structure may facilitate t-loop formation, and this telomeric t-loop could indeed self-prime for telomeric DNA extension by an ALT pathway(s). Furthermore, inactivation of TRF2 also induced cell death in an ATM/p53-dependent manner, suggesting that telomeres lacking TRF2 were recognized as broken DNA ends and that cells containing such deprotected telomeres are eliminated by the ATM/p53-mediated apoptotic pathway (27). Recent studies with late-generation *mTER*^{-/-} mice in the *p53*^{-/-} background demonstrating that growth arrest and apoptosis in G6 *mTER*^{-/-} mice is mostly mediated through the p53 pathway are in agreement with this notion (9). Short telomeres themselves (e.g., at a length that is unable to form a t-loop) and abnormal TRF2 may both trigger the DNA damage signal. In cells without telomerase, the only way to revert this block is to extend telomeres by ALT pathways or to bypass the signals involved in the ATM/p53 pathway. Once we solve the problems addressed above, we will have a clearer idea of the essential original proposed function of telomeres, that telomeres protect chromosomes from random degradation and fusion.

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