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Reprogramming of Telomerase by Expression of Mutant Telomerase RNA Template in Human Cells Leads to Altered Telomeres That Correlate with Reduced Cell Viability

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Telomerase synthesizes telomeric DNA by copying the template sequence of its own RNA component. In *Tetrahymena thermophila* and yeast (G. Yu, J. D. Bradley, L. D. Attardi, and E. H. Blackburn, Nature 344:126–131, 1990; M. McEachern and E. H. Blackburn, Nature 376:403–409, 1995), mutations in the template domain of this RNA result in synthesis of mutant telomeres and in impaired cell growth and survival. We have investigated whether mutant telomerase affects the proliferative potential and viability of immortal human cells. Plasmids encoding mutant or wild-type template RNAs (hTRs) of human telomerase and the neomycin resistance gene were transfected into human cells to generate stable transformants. Expression of mutant hTR resulted in the appearance of mutant telomerase activity and in the synthesis of mutant telomeres. Transformed cells were not visibly affected in their growth and viability when grown as mass populations. However, a reduction in plating efficiency and growth rate and an increase in the number of senescent cells were detected in populations with mutant telomeres by colony-forming assays. These results suggest that the presence of mutant telomerase, even if coexpressed with the wild-type enzyme, can be deleterious to cells, likely as a result of the impaired function of hybrid telomeres.

Eukaryotic chromosomes terminate in specialized structures, telomeres, that are necessary for their stability and function (for reviews, see references 7 and 61). Telomeres consist of a complex of G-rich repeated DNA and of sequence-specific DNA binding proteins (for a review, see reference 20). Formation of the complex and telomere function, therefore, have stringent DNA sequence requirements (TTAGGG in vertebrates [27, 44]). Synthesis of telomeric DNA is catalyzed by telomerase, a ribonucleoprotein that utilizes a domain of its own RNA component as the template for de novo addition of nucleotides to the G-rich strand (25, 26, 43). This process compensates for the loss of terminal sequences occurring during semiconservative replication of linear DNA molecules (47, 57) and is instrumental in keeping telomere length at an equilibrium (for a review, see reference 24).

Over the last few years, experimental evidence has supported the presumed role of telomere maintenance in cell life span regulation, making telomerase an essential function for long-term or unlimited cell proliferation. Deletion of the template RNA gene, which is required for enzyme activity (26), results in progressive telomere shortening and in loss of viability of otherwise immortal yeast cells (41, 52). Human somatic cells, which generally lack detectable levels of telomerase (for a review, see reference 31), undergo telomere erosion with cell division in vitro (3, 14, 28; for a review, see reference 5) and have a limited life span (29). Immortal germ line cells (33, 58) and the majority of somatic cells immortalized in vitro or in vivo, on the other hand, express telomerase and maintain their telomeres (14–16, 33, 35, 51; for reviews, see references 6 and 17). Experimental lengthening of telomeres (59) or inhibition of telomerase (21, 46, 53) in immortal human cells has been shown to prolong or reduce, respectively, cell proliferative capacity. Alterations in telomere structure that are independent of telomerase inhibition can also have an impact on cell growth and survival. Proteins that bind telomeric DNA are known to participate in telomere length regulation (20), and in yeast, mutation or deletion of these proteins destabilizes telomeres and impairs cell survival (36, 37). In addition, in both *Tetrahymena thermophila* and yeast, mutations of the template domain of the telomerase RNA and the consequent expression of a mutant enzyme cause loss of telomere length regulation and of cell viability (34, 41, 49, 60).

Experimental manipulation of telomeres in lower eukaryotes has also revealed alternative mechanisms for telomere maintenance that may utilize recombination with internal telomeric repeats or gene conversion at the chromosome termini to restore telomeric sequences (38, 42, 56). Survivors that maintain telomeres by these processes have been consistently rescued from populations of yeast lacking a telomeric protein or telomerase, or expressing a mutant enzyme, suggesting that recombination or gene conversion may operate as a salvage pathway when the normal mode of telomere maintenance is tampered with. The existence of immortal human cell lines that lack telomerase activity is compatible with the possibility that telomeres can be preserved in a similar manner even in human cells (8, 45).

In previous work, we have established a correlation between the immortal phenotype of human cells in vitro and in vivo and telomere maintenance by telomerase (14–16) and showed that in these cells inhibition of the enzyme by antisense template RNA leads to shortening of the telomeres to a critical length and to cell death (21). In the present work, we investigated whether reprogramming of human telomerase by expression of mutant template RNA (hTR) would impair the growth and reduce the viability of immortal human cells, as is the case in lower eukaryotes (34, 41, 49, 60). The rationale for exploring this approach for telomere destabilization as an alternative to the antisense strategy was based on the possibility of a shorter phenotypic lag prior to any effects on cell survival, given that

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telomere structure rather than length would be directly altered. We report here that expression of mutant hTR leads to formation of a mutant holoenzyme and to synthesis of mutant telomeric sequences in human cells and that both of these phenomena can be associated with impaired growth rate and colony-forming ability. These phenotypes were detected in cells whose telomeres were stable, at least as a mass population, unlike those of cells expressing antisense hTR (21). Our results are compatible with loss of cell survival due to formation of aberrant DNA-protein telomere complexes.

MATERIALS AND METHODS

Cells and transfection. The human cell line HT-1080 (50) was grown in α-minimal essential medium with 7.5% fetal calf serum and antibiotics. The adenovirus-transformed 293 human cell line (23) was grown in minimal essential medium F11 with 10% fetal calf serum and antibiotics. Media and supplements were purchased from Gibco/BRL (Mississauga, Ontario, Canada). Transfection of cells to generate stable transformants was performed by the DNA-calcium phosphate coprecipitation protocol (22), and selection of transformants was done in medium with 400 μg of G418 (Geneticin; Boehringer Mannheim, Laval, Quebec, Canada) per ml.

Plasmids. pBSU2-33neo and pBSU2-34neo are derivatives of the pGRN33 and pGRN34 plasmids that encode the wild-type (wt) hTR specifying TTAGGG telomeric sequences (pGRN33) or the MuA mutant hTRs specifying TTTGGG telomeric sequences (pGRN34) (21). In the pBS plasmids, expression of hTR is under control of the promoter of the human U2 small nuclear RNA (snRNA) gene (39) and the hTR transcript is followed by 100 nucleotides (nt) of nontranscribed sequences at the 3′ end. In addition, the plasmids contain the neomycin resistance marker. To generate plasmids pBSdT33 and pBSdT34, products comparable in size were isolated from telomerase ladders obtained by extension of the TS oligonucleotide (see below) with wt or mutant telomerase. Sequence analysis of the products cloned into the pBS vectors revealed that pBSdT33 and pBSdT34 contain 8.5 TTAGGG repeats and 9 TTTGGG repeats, respectively. Enzymes required for the construction of recombinant plasmids were purchased from various sources and used in accordance with the manufacturer's specifications. All plasmids used for transfection of human cells were purified by centrifugation in cesium chloride-ethidium bromide density gradients.

DNA and RNA analysis. Clonal populations of stably transfected cells were screened for the presence of hTR-containing plasmids by Southern blotting with digoxigenin-labeled hTR DNA or full-length antisense RNA as the probe and chemiluminescence reagents (Boehringer Mannheim) for detection. The length and sequence composition of terminal restriction fragments (TRFs) in these cells were determined as previously described (14), by hybridization of end-labeled oligonucleotide probes [$(C_3TA_2)_3$ for wt sequences and $(C_3A_3)_4$ for mutant sequences] to dried 0.5% agarose gels containing genomic DNA digested with *Hin*fI and *Rsa*I. With each probe, a 100-fold excess of the other unlabeled oligonucleotide and of oligonucleotide $(C_4A_2)_3$ was added to the hybridization mixture to reduce background hybridization to subtelomeric sequences (2). For *Bal* 31 digestion, genomic DNA was incubated at 37°C with 0.2 U of enzyme per μg of DNA for the times indicated in Results, precipitated, and digested with *Hin*fI and *Rsa*I prior to electrophoresis and hybridization as described above. TRF blots were exposed to PhosphorImager screens. Expression of exogenous hTRs was detected by primer extension analysis. Total RNA was extracted with TRIZOL (Gibco/BRL). The hTR-specific oligonucleotide 5′-AGGCGAACGGGCCAGCAGCTGACAT T-3′ was gel purified and end labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase. First-strand hTR cDNA was synthesized from 10 μg of total RNA by using Superscript II reverse transcriptase (Gibco/BRL), precipitated in ethanol in the presence of 2 μg of tRNA, separated on 8% denaturing polyacrylamide-urea gels, and exposed to PhosphorImager screens. Sizes of extension products were determined by comparison with a ladder generated by digestion of pBR322 with *Hpa*II and with hTR sequencing ladders generated with the hTR-specific extension primer.

Telomerase assay. Cell extracts were prepared by detergent lysis (33) or by three cycles of freezing and thawing in the absence of detergent (63). Enzymatic activity was detected by the PCR-based telomere repeat amplification protocol (TRAP; 33) with the following modifications: the TS oligonucleotide (5′-AATC CGTCGAGCAGAGTT-3′) was end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase, and the reverse primers and *Taq* polymerase were added following telomerase elongation and warming of the reactions to 92°C (hot start). The reverse primers were ACT for detection of wt telomerase (32) and MuA (5′-ACCCAAACCCAAACCCAAACCCAA-3′) for detection of mutant telomerase (21). PCR amplification conditions were 27 cycles of 94°C for 30 s and 60°C for 30 s for wt activity and 94°C for 10 s and 60°C for 30 s for mutant 34 (MuA) activity. Control experiments ensured that amplification of the wt and mutant 34 products was comparable. Each extract was assayed at different protein concentrations to establish the linearity of the assays (30), as well as assayed after digestion with RNase as a specificity control. Enzymatic activity was quantified from all assays within the linear range by normalizing the total amount of reaction products in each lane to the signal obtained from the internal telomerase assay standard (ITAS) present in the same lane, as described in the TRAP-eze kit protocol (Oncor, Gaithersburg, Md.) and in reference 30). Assays in which amplification of the ITAS was absent or reduced were not included in the quantitation of activity. Average amounts of telomerase activity were obtained from a minimum of three assays of two independently prepared extracts from each population. Telomerase activity in single colonies was not quantified, and the presence or absence of the enzyme was determined by assaying equal volumes of extracts.

Cell growth and viability assays. Cells were counted and subcultured at a split ratio of 1:5 to determine the growth rate of the population, or individual colonies were seeded in 60-mm-diameter plates and the time required for them to attain confluence was determined. For determination of colony-forming ability, cells were trypsinized and counted and 100 cells were plated in each of three 100-mm-diameter plates. Colonies were stained with crystal violet after 8 days. Alternatively, cell suspensions were appropriately diluted to yield 1 cell per well, on average, and distributed into 96-well microtiter plates. After 8 days, colonies were lysed in situ and assayed for telomerase activity or stained with crystal violet and counted. In some cases, colonies were stained for expression of the senescence-associated β-galactosidase enzyme as described by Dimri et al. (19). The plating efficiency of each clone was determined based on the total number of colonies relative to the number of seeded cells. The percentage of small colonies was calculated based on the number of colonies that had extremely reduced size and a sparse morphology and were detectable under magnification. Small colonies with sparse morphology that were still detectable by eye but were found to contain senescent-looking cells by microscopic analysis were also included in this group.

Cytogenetic analysis. Spreads of metaphase chromosomes were prepared by standard procedures, stained with 5% Giemsa stain, and analyzed by light microscopy.

RESULTS

Generation of plasmid vectors encoding hTRs under control of the U2 promoter. HT-1080 cells are telomerase positive and constitutively express wt hTR. In previous experiments, low levels of mutant telomerase activity were detected in HT-1080 cells transfected with mutant hTR under control of genomic upstream regulatory sequences (21). In an attempt to achieve more efficient reprogramming of telomerase by outcompeting the endogenous wt hTR, the mutant 34 (MuA) hTR was placed under control of the promoter of the human U2 snRNA gene, which is transcribed by RNA polymerase II (11). A U2-driven wt hTR was also engineered as a control. The choice of the U2 promoter was based on its strength and on the facts that hTR is a nonpolyadenylated transcript and contains runs of T residues within the transcribed region and its transcription is sensitive to α-amanitin (4, 21). These features are compatible with the possibility that hTR is a polymerase II transcript regulated as an snRNA. Assays of cells infected with a recombinant adenovirus encoding the U2-driven mutant hTR indicated that this transcript was indeed functional as determined by detection of mutant telomerase activity (40). Constructs with the U2 promoter were rescued into plasmids containing the neomycin resistance selectable marker, and the resulting plasmids, pBSU2-34neo (mutant hTR) and pBSU2-33neo (wt hTR), were used for stable transformation of human HT-1080 cells. Transfected cells were grown in medium with G418, and resistant colonies were isolated and screened by Southern hybridization for the presence of the hTR gene (data not shown).

Expression of hTR in transformed cells. Primer extension analysis was used to detect and quantify expression of mutant hTR in stably transfected cells (Fig. 1). In the parental HT-1080 cell line, the endogenous hTR produced two major extension products differing in length by 14 nt. The shorter product extends to the described start site (site 1 in Fig. 1B) of the presumed mature form of hTR (1, 21). The second product may correspond to an immature form of the transcript. Transfection of U2-driven hTRs (wt or mutant) resulted in the appearance of a new extension product 32 to 36 nt longer than that corresponding to the endogenous mature hTR and extending to the expected initiation site (site 3 in Fig. 1B) of the transcript regulated by the U2 promoter in the wt and mutant hTR constructs. (The product generated by the mutant hTR U2-34, contains an extra 4 nt between the promoter and the coding region compared to the hTR U2-33 product, due to

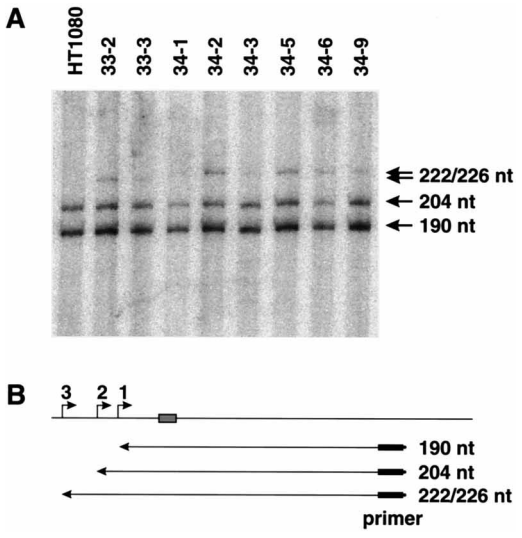


FIG. 1. Primer extension analysis of hTR expressed in transformed cells. (A) Extension products, generated from total RNA by using a labeled hTR primer, are indicated by arrows. Product sizes were determined by comparison with molecular weight markers and sequencing ladders. HT-1080, parental cells; 33-2 and 33-3, cells transfected with wt hTR; 34-1 to 34-9, cells transfected with mutant hTR. Exogenous hTRs are under control of the U2 promoter. (B) Schematic indicating the positions of the primer and extension products relative to the hTR gene. The start sites of the products are indicated by arrows. Site 1 corresponds to the start site of the mature hTR, and site 3 corresponds to the expected start site downstream from the U2 promoter. The box denotes the template region of hTR.

different cloning strategies.) The U2-driven product was clearly detectable in two of two clones transfected with wt hTR and five of seven clones transfected with mutant hTR (Fig. 1A), but its amount was consistently lower than those of products generated by the endogenous transcript, regardless of whether the exogenous hTR was mutant or wt. Together with our previous results obtained with hTR driven by its natural promoter (21), these observations suggest regulation of the total amount of hTR that can accumulate in HT-1080 cells. Although lack of crucial regulatory sequences could also be responsible for the low level of expression of the U2-driven hTR, this seems unlikely since overexpression of these transcripts was achieved in transient assays (40).

Telomerase activity in transformed cells. Clonal populations of transformed cells were assayed for telomerase activity by TRAP, using specific reverse primers for PCR detection of the wt or mutant enzyme as described in Materials and Methods. Mutant activity was detected in the five clones transfected with and expressing mutant hTR, whereas wt activity was ubiquitously present (see Fig. 2 for an example). No mutant activity was present in the parental cell line (Fig. 2) or in cells transformed with wt hTR (not shown). These results confirm that the MuA primer amplifies specifically mutant telomerase products (21). The amount of mutant activity relative to the endogenous wt activity was quantified at early times after clonal isolation (2 to 4 population doublings [PD], corresponding to approximately 30 PD after transfection). The amount of mutant activity varied among positive clones (see Table 1), but expression of the mutant enzyme persisted in all populations until at least 90 to 100 PD after transfection.

Incorporation of mutant sequences into telomeres. We next assessed the in vivo functionality of the mutant enzyme by examining whether its sustained expression during growth of transformants resulted in alteration of telomeric sequences. The composition of TRFs generated by digestion of genomic DNA with frequently cutting restriction enzymes was analyzed by hybridization with oligonucleotide probes specific for mutant sequences at several PD following isolation of the transformed clones. The probe was then removed, and the DNA was rehybridized with a probe specific for wt sequences. Control hybridizations to the pBSdT34 and pBSdT33 plasmids, which contain tandem repeats of mutant or wt sequences, were performed in parallel. As shown in Fig. 3, TRFs containing TTTGGG sequences specified by the mutant hTR U2-34 were detected in the three clones (34-2, 34-4, and 34-5) that expressed the highest levels of mutant telomerase (Table 1). The presence of mutant sequences in the telomeres of these cells demonstrates that the mutant enzyme is functional in vivo. On the other hand, the lack of detectable TTTGGG sequences in populations that did not express a mutant enzyme (e.g., 34-1 and 34-10 and control 33-2) confirms the specificity of the hybridization conditions.

The location of TTTGGG sequences within the terminal restriction fragments was assessed by digestion of genomic DNA with exonuclease *Bal* 31 prior to TRF analysis (Fig. 4A). Gels were hybridized with the mutant probe, and then with the wt probe, and TRF size and the intensities of the hybridization signals were quantified (Fig. 4B). In the case of TTTGGG sequences, the hybridization signal was found to decrease at a faster rate than TRF size as a function of *Bal* 31 digestion, whereas both parameters declined at comparable rates for TTAGGG sequences. This is what would be expected if TTT

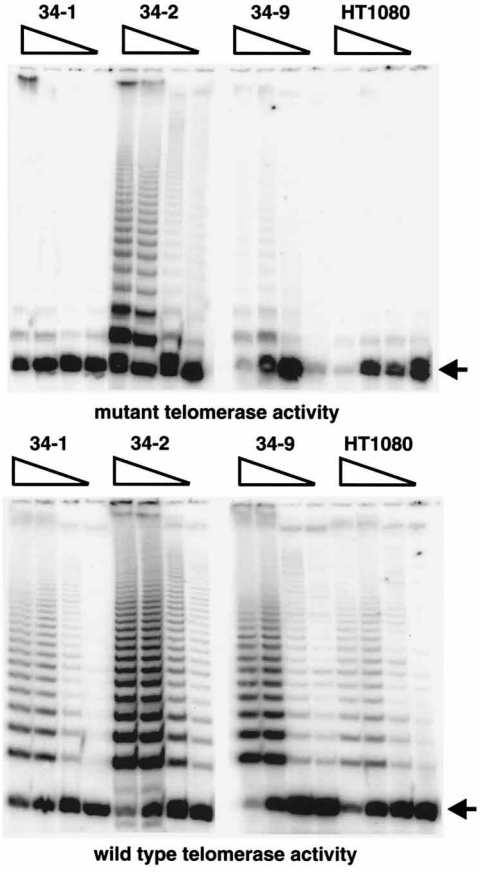


FIG. 2. Telomerase activity in stably transformed cells. Extracts from clonal populations of HT-1080 cells transformed with a plasmid encoding hTR mutant 34 under control of the U2 promoter were assayed for mutant (top) and wt (bottom) telomerase activities. Amounts of protein were 0.01, 0.1, 1.0, and 5 μg per assay. Extracts from the parental HT-1080 cells were assayed as a control. Arrows indicate the ITAS.

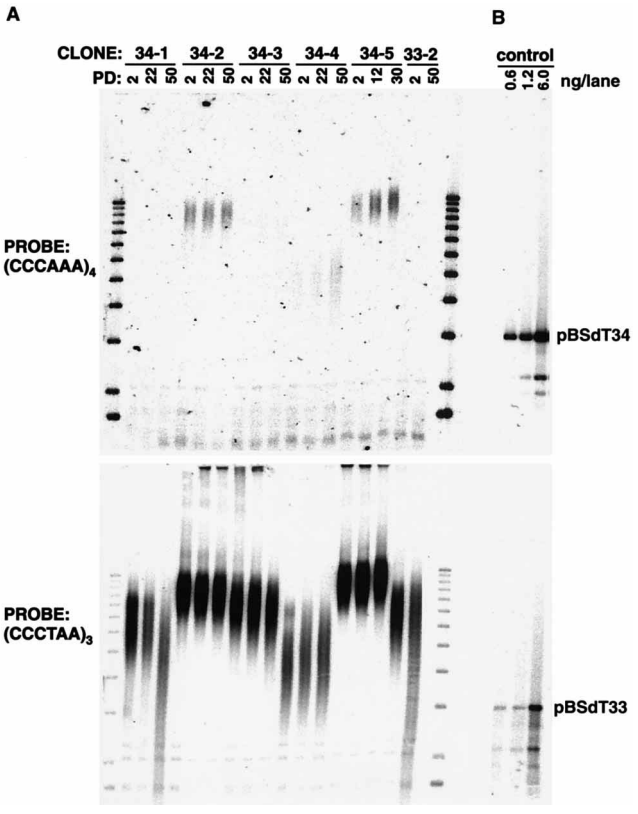


FIG. 3. Analysis of the TRFs of transformed cells. (A) Genomic DNA was extracted from transformed clones at the indicated PD following clone isolation and digested with *Hin*fI and *Rsa*I prior to electrophoresis on a 0.5% agarose gel. The dried gel was hybridized with the $(CCCAAA)_4$ probe for detection of mutant TTTGGG sequences (top). Following exposure to PhosphorImager screens, the hybridization signal was removed and the gel was rehybridized to the $(CCCTAA)_3$ probe for detection of wt TTAGGG sequences (bottom). Clones 34-1 to 34-5 were transformed with mutant hTR; clone 33-2 is a control population transformed with wt hTR. Molecular weight markers shown in the left- and rightmost lanes of both panels A are a radiolabeled 1-kb DNA ladder (Gibco/BRL). (B) Hybridizations to control plasmids pBSdT34 and pBSdT33. The indicated amounts of plasmids were hybridized with the labeled probes used in A and exposed for the same length of time as the corresponding hybridizations in A.

GGG sequences had been recently added to pre-existing and longer TTAGGG tracts which constitute the bulk of the TRFs, indicating that the mutant sequences are indeed at the termini of telomeres. The rate of decline of the TTTGGG hybridization signal was slower in 34-5 cells than in 34-2 cells. This is compatible with the possibility that 34-5 telomeres contain (i) mutant tracts that are also subterminal to wt sequences or (ii) longer terminal mutant tracts.

The average sizes of mutant and wt TRFs in hTR U2-34 transformants were determined from their electrophoretic mobility and the intensity of the hybridization signal in at least two hybridizations such as those in Fig. 3. As shown in Table 1, the average TRF length varied among clones but appeared to be stable within each clone over a large number of PD. (The reduced size of TRFs from clones 34-1 and 33-2 at PD 50 shown in Fig. 3 is likely a result of DNA degradation, since it was not observed in other DNA samples from these populations.) Analysis of telomeres en masse, on the other hand, cannot exclude the existence of a subset of unstable telomeres in the population. The relative amounts of mutant and wt sequences were calculated from the intensities of the signals after correction for the efficiency of hybridization of the $(CCCAAA)_4$ and $(CCCTAA)_3$ probes to the pBSdT34 and pBSdT33 control plasmids. The average amounts of TTTGGG sequences varied from 0.5 to 0.7% of wt sequences in the most positive clones to levels that were too low for quantitation in others. It should be noted that these values do not reflect the relative amount of newly synthesized mutant sequences added to the termini of chromosomes, given the excess of the pre-existing TTAGGG sequences. In addition, telomeric TTT GGG tracts shorter than the nine repeats present in pBSdT34 may hybridize to the probe with lower efficiency, yielding an underestimate of mutant sequences. In the case of TTAGGG sequences, reduced hybridization efficiency to short terminal stretches that may be added onto mutant sequences would have no significant effect on quantitation due to the abundance of the wt sequences in long and uninterrupted internal arrays.

Growth and viability of transformed cells. Mutant telomeres have been shown to affect cell growth and viability in *T. thermophila* and yeast (34, 41, 49, 60). In initial experiments aimed at determining the growth phenotype of hTR U2-34 transformants, the cells were subcultured (1:5 split ratio, when just confluent) for approximately 60 PD. No consistent or significant growth impairment was detected under these conditions (data not shown). Since even in yeast, a significant reduction of growth and viability is difficult to detect in mass liquid cultures but becomes apparent upon streaking of colonies (42), the growth phenotype of transformed human cells was reassessed by colony-forming assays. Following the scheme in Fig. 5, transformed cells were plated (100 cells in each of three plates) at approximately 30 PD after transfection and colonies were allowed to form for 8 days. Two colonies comparable in size were isolated from each transformant, and the plates were stained for determination of plating efficiency. The subclones were expanded for a total of 30 additional PD and then plated as described above. This protocol was repeated a third time with three colonies per transformant from the second plating being replated after a further 40 PD. The cumulative numbers of PD of the parental transfected cells at which plating efficiencies were determined were thus about 30, 60, and 100.

As summarized in Table 2, the average plating efficiency, based on all detectable colonies, varied among clones but was

TABLE 1. Size and structure of telomeres in transformed cells

Clone	Telomerase mutant activity[a]	Telomere[b]			
		Size (kbp) at PD:			Mutant sequence (% of wt)
		2	22	50	
33-2	0	6.7	ND[d]	6.9	0
33-4	0	7.7	ND	ND	ND
34-1	0	6.3	6.9	5.4	0
34-2	0.66	9.9	9.8	9.9	0.5
34-3	0.10	7.8	9.2	7.6	±
34-4	0.71	5.3	5.2	5.2	0.7
34-5	0.86	9.9	10.7	11.2	0.5
34-9	0.17	7.7	ND	8.0	±
34-10	0	ND	ND	8.5	0

[a] Telomerase activity was measured in extracts from clones grown for 2 to 4 PD after isolation (approximately 30 PD after transfection). All extracts were positive for wt telomerase. Average amounts of mutant activity, from assays of independent extracts, are expressed as the fraction of wt activity (see Materials and Methods).

[b] The sizes of telomeres were determined from a minimum of two hybridizations of DNA extracted at the indicated PD following isolation of transformed clones. This number of PD corresponds approximately to 30, 50, and 80 doublings of the original transfected cell.

[c] The mutant sequence percentage is the average of two or more determinations at each PD. ± denotes samples in which a trace of mutant sequences was detected in a single blot at PD 2 but not in subsequent blots at this or other PD.

[d] ND, not determined.

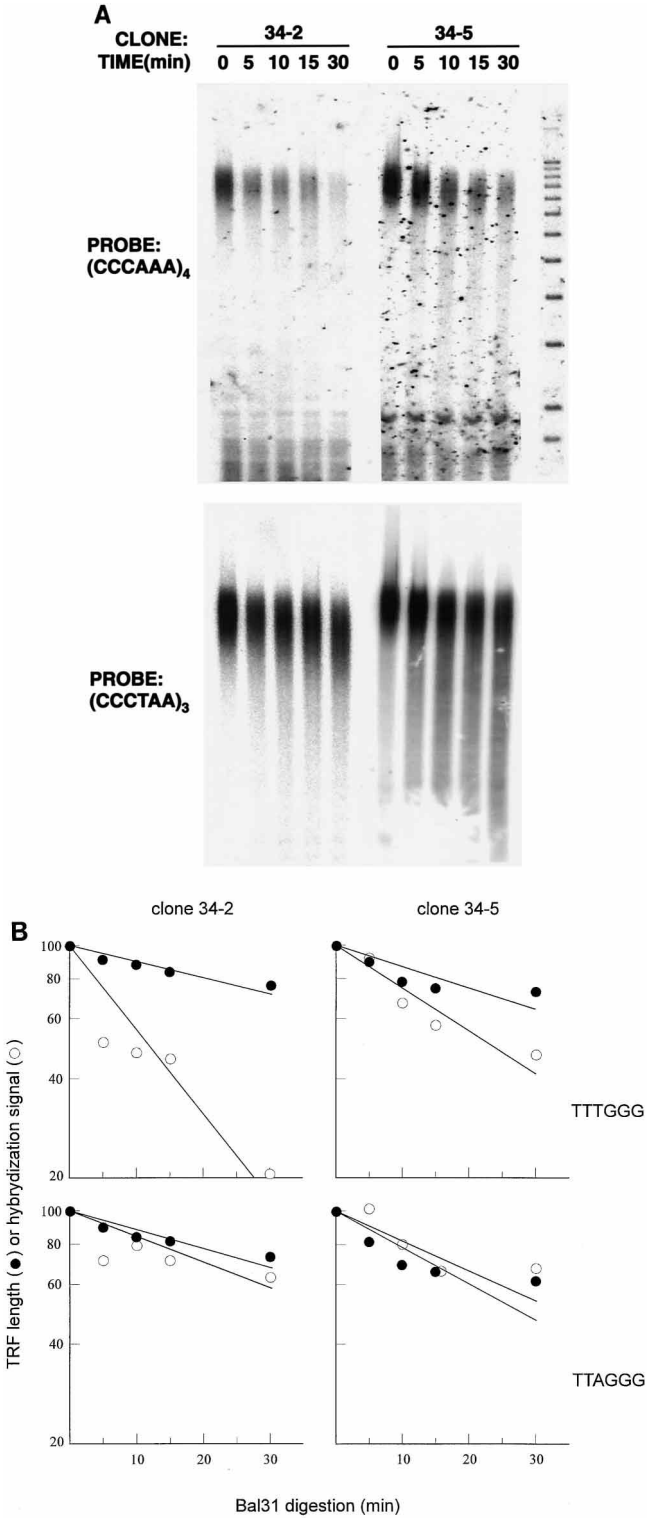


FIG. 4. Sensitivity of mutant TRFs to *Bal* 31 exonuclease. (A) Genomic DNA was extracted from clones 34-2 and 34-5 at about 10 PD after isolation, digested at 37°C for the indicated times with 0.2 U of *Bal* 31 per μg, precipitated, and digested with *Hin*fI and *Rsa*I prior to electrophoresis and hybridizations as described in the legend to Fig. 3. A radiolabeled 1-kb DNA ladder (Gibco/BRL) was used as molecular weight markers (upper panel, rightmost lane). (B) Quantitation of TRF size and hybridization signal of samples in A. All values are percentages of the value for undigested DNA (0 min).

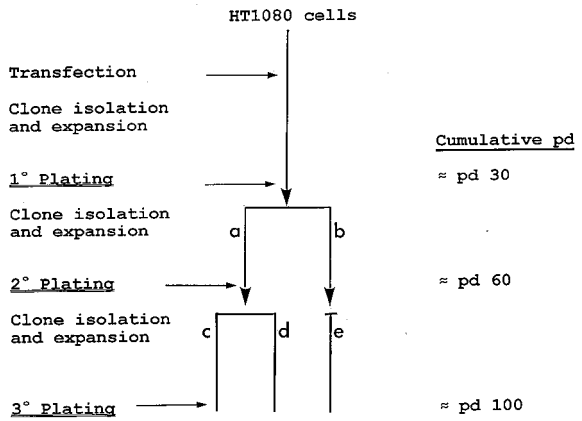


FIG. 5. Scheme for determination of the plating efficiency of transformed-cell populations. For each round of plating, 100 cells of a transformed clone were plated into each of three 100-mm plates. Colony isolation and staining were done after 8 days. Colonies (a to e) were isolated from different plates.

not consistently reduced in populations with mutant telomeres. Striking differences, however, were noticeable in the dimensions and morphologies of the colonies produced by different transformants (PD 30) and their subclones (PD 60 and 100). As shown in Fig. 6A for colonies obtained from plating of subclones at PD 60, cells transfected with wt hTR (33-2 and 33-3) generated mainly large and compact colonies. This was also the case for cells that had been transfected with mutant hTR but were negative for (34-1 and 34-10) or had barely detectable (34-3 and 34-9) mutant telomeric sequences. In contrast, both subclones of 34-2, which had mutant telomeres, produced essentially only small, sparse colonies difficult to detect by eye upon staining. Most of these colonies contained a substantial number of apparently senescent postmitotic cells (Fig. 6B). Cells such as these were found to express the senescence-associated form of β-galactosidase (data not shown). The phenotype of the other two transformants that contained mutant telomeres, 34-4 and 34-5, was more subtle and variable, since in both cases, one of the subclones generated a majority

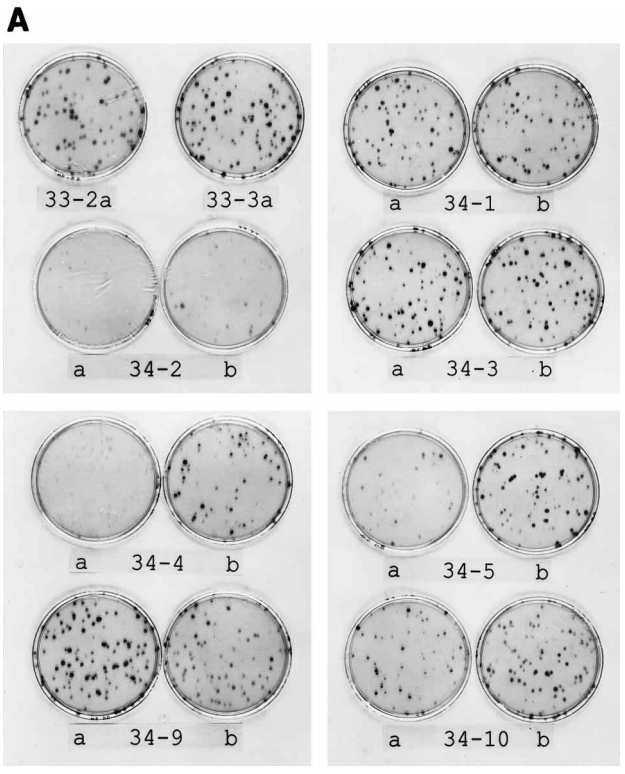
TABLE 2. Plating efficiencies and colony sizes of transfected-cell populations[a]

Clone	Mutant telomeres[b]	% PE, % Sm at:					
		PD 30	PD 60		PD 100		
			Subclone a	Subclone b	Subclone c	Subclone d	Subclone e
33-2	−	86, 4	121, 1	79, 3	57, 10	97, 10	113, 11
33-3	−	68, 8	84, 8	83, 6	55, 14	76, 4	84, 21
34-1	−	105, 13	95, 18	58, 13	103, 21	90, 10	77, 18
34-2	+	68, 74	72, 85	57, 80	94, 79	ND[c]	97, 88
34-3	±	94, 14	71, 14	101, 9	76, 19	67, 16	91, 9
34-4	+	86, 22	96, 97	69, 30	89, 41	83, 34	ND
34-5	+	78, 11	62, 55	91, 29	54, 56	67, 46	68, 35
34-9	±	117, 2	86, 4	108, 5	85, 3	120, 9	90, 14
34-10	−	68, 19	107, 5	69, 9	ND	73, 6	98, 7

[a] Clonal populations of transfected cells and subclones derived from them (a to e) were assayed for colony-forming ability at 30, 60, and 100 PD after transfection as described in the legend to Fig. 5. % PE indicates the average plating efficiency based on the total number of colonies, and % Sm is the average percentage of small colonies with sparse morphology and senescent cells, identified as described in Materials and Methods.

[b] Presence or absence of mutant telomeres is indicated by + and −, respectively. ± identifies clones in which mutant telomeres were not reproducibly detected and were present in too small an amount for quantitation.

[c] ND, not determined.



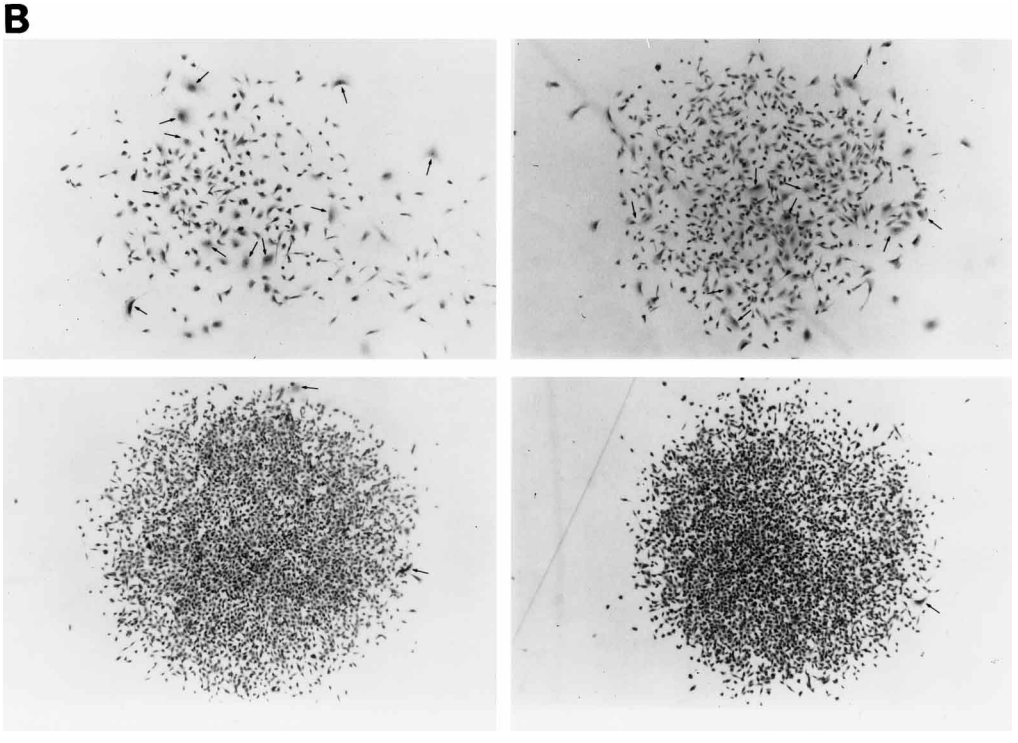


FIG. 6. Colony-forming assay of transformed cells. (A) Results shown are from colony assays performed after 60 PD from transfection, i.e., at the second round of plating (see Fig. 5 and text for details). One plate of three is shown for each subclone. 33-2 and 33-3 are clones transfected with a plasmid encoding wt hTR; all others contain a plasmid with hTR mutant 34. (B) Morphology of colonies generated by different transformants. Top panels, 34-2; bottom left, 33-2; bottom right, 34-10. Examples of apparently senescent cells are indicated by arrows. All of the photographs were taken at the same magnification.

of small colonies and the other generated a majority of nearly normal colonies (Table 2 and Fig. 6A). Similar results were obtained upon plating of additional subclones from each transformant at PD 100 (Table 2). Telomerase assays of a subset of colonies indicated that, regardless of size, all of the colonies tested retained wt enzymatic activity (data not shown), eliminating loss of this mode of telomere maintenance as the cause of stunted growth. Variability in the severity of the growth phenotype was observed between experiments; however, the association between the most severe phenotype and the presence of mutant telomeres was consistent in several experiments.

The growth phenotype of subclones obtained at PD 60 was further examined by determining the time required for them to attain confluence upon reseeding in 60-mm plates. On average,

colonies isolated from populations with mutant telomeres were found to grow significantly slower than those from control populations (data not shown), suggesting that although growth-competent cells were invariably present, affected colonies continued to generate postmitotic cells during their growth. Consistent with this hypothesis, mutant telomerase activity was detected in single colonies from these populations, regardless of their size (data not shown).

Cytogenetic analysis of transformed cells. A subset of transformants with mutant telomeres was examined for the presence of dicentric chromosomes since these structures may originate from fusion of aberrant termini. In the 34-2, 34-4, and 34-5 populations, dicentrics were present at a frequency of five to six per 50 metaphases (data not shown). In contrast, only one dicentric was detected in a total of 100 metaphases from two control subclones.

DISCUSSION

The dependence of eukaryotic chromosomes on functional telomeres for their maintenance and accurate segregation (7, 61), combined with the incomplete replication of termini by DNA polymerase (47, 57), has suggested a potential role for telomeres in cell life span control (48). A variety of experimental data have provided strong support for this hypothesis. In human cells, in particular, elongation of telomeres by introduction of oligonucleotides (59) or inhibition of telomerase by chromosome transfer (46), antisense strategies (21), or chemicals (53) has resulted in a prolonged life span or loss of cell viability, respectively. We have shown here that, as in *T. thermophila* and yeast (34, 41, 49, 60), mutation of telomeric DNA sequences by reprogramming of telomerase with a mutant RNA template can also affect the viability of human cells. This is the first report that alteration of telomere structure, besides telomere length, has an impact on the proliferation of these cells.

The outcome of our experiments is similar to that of studies of lower eukaryotes. There are, however, substantial differences in the systems that affect the quantitative aspect of the results. In yeast, deletion of the telomerase template RNA gene allows very efficient reprogramming of the enzyme by exogenous RNA, which, in turn, leads to clear-cut effects on cell viability (41). Immortal human cells with the hTR gene deleted are not available, and immortal cells that lack expression of hTR and maintain telomeres by other mechanisms do not re-express telomerase upon introduction of exogenous hTR (9). Therefore, we sought overexpression of hTR via the use of an active promoter as a means to overcome the endogenous transcript. This strategy has been successful in *T. thermophila*, in which mutant RNA expressed by efficiently replicating plasmids was essentially the only telomerase template species detected (60).

In the present study, we were able to detect mutant telomeres in cells stably transformed with mutant hTRs and were able to correlate the presence of these structures with a reduction in cell viability. In our experimental system, however, overexpression of exogenous hTR relative to the endogenous wt hTR was not achieved and transformed cells invariably retained the endogenous wt telomerase in larger amounts than the mutant enzyme. This would predict that mutant and wt sequences should be interspersed, resulting in the formation of hybrid telomeres that may retain function due to constant "repair." Indeed, unlike the case in yeast, where telomeric addition of exclusively mutant sequences results in loss of telomere length control (41), telomere length apparently remained stable during growth of the cell populations. Nevertheless, some of the human cell transformants were significantly affected in viability, as shown by colony size and morphology and by the accumulation of apparently senescent cells. A suggestive increase in the number of dicentric chromosomes, compatible with alteration of telomere function, was also detected in these populations. These phenotypes were not related to loss of telomerase activity (10) and, although variable in severity, were prevalent in populations with detectable mutant telomeres. Since the total amounts of mutant sequences did not significantly differ among affected populations, the length and/or position of mutant tracts relative to wt tracts may be responsible for the phenotypes of different clones.

Telomere function depends upon the proper formation of a specific DNA-protein complex, and in yeast, alterations of this complex by deletions of telomeric protein can reduce cell viability (13, 36, 37, 55). We propose that an altered telomere structure, resulting from inadequate binding of telomeric proteins to mutant telomeres, may be similarly responsible for the loss of viability of the human cells we have characterized. The findin that human telomeric protein hTRF1, which binds specifically to double-stranded TTAGGG tracts (12), does not interact with the TTTGGG sequences specified by hTR34 (18) is compatible with this hypothesis. Efficient DNA binding by hTRF1 appears to require more than six contiguous TTAGGG repeats (62). Perhaps, in affected cells, intercalation of mutant and wt sequences results in TTAGGG tracts that are too short for optimal interaction of hTRF1 with chromosome(s) termini. On the other hand, expression of a dominant-negative mutant form of hTRF1 has been shown to affect telomere length but not cell viability (54). Assuming that expression of a mutant pro tein is fully comparable to mutation of target sequences, these observations suggest that hTRF1 is not involved in, or is not solely responsible for, the phenotype of cells with mutant telomeres. Indeed, proteins that bind to single-stranded telomeric DNA (20, 55) may also fail to associate with TTTGGG tails. Formation of abnormal telomere structures in a stochastic fashion in different cells and cell cycles may explain the variability in the severity of the growth phenotypes of different clones.

In lower eukaryotes, the proliferative crisis of populations with destabilized telomeres is invariably followed by the outgrowth of survivors that maintain telomeres by telomerase-independent mechanisms (37,41). Although telomerase-negative immortal human cells exist (8, 45), the viable cells in the transformed populations we have characterized do not belong to this class. Telomerase activity, both wt and mutant, is still present in populations grown for 90 to 100 PD, and telomeres are not elongated as in the case of telomerase-negative cells. It seems most plausible, therefore, that cell viability is associated with the presence of the wt enzyme and, hence, repair of telomeres.

In summary, we have shown that expression of mutant hTR can reprogram telomerase in human cells even in the presence of wt hTR. The mutant holoenzyme is functional in vivo and directs the synthesis of altered telomeres which, in turn, may affect cell viability. The application of this approach to cells containing plasmid-tagged telomeres (27) amenable to cloning may further our understanding of telomere structure and function in human cells.

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