Effect of telomere length on telomeric gene expression

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ABSTRACT

Telomeres gradually shorten as human somatic cells divide and a correlation has been observed between the average telomere length and cell senescence. It has been proposed that the genes responsible for cell senescence are located near the telomere and are activated when telomere length reaches a critical point. This is consistent with evidence from Saccharomyces cerevisiae, in which genes are regulated differently depending on their distance from the telomere. We investigated the possibility that differential gene expression is conferred by telomere length in human cells. A plasmid containing the neomycin phosphotransferase (neo) gene was transfected into the SV40-transformed human fibroblast cell line LM217. In one transfectant the plasmid was integrated at the telomere of chromosome 13. Subclones of this cell line that had various lengths of telomeric repeat sequences on the end of this chromosome were isolated. No effect on neo gene expression was found when the length of the telomere varied between 25 and 0.5 kb, as demonstrated by colony forming ability, growth rates and RNA blot analysis. These results therefore suggest that putative chromatin structural differences conferred by telomere length do not affect the expression of genes located near telomeres.

INTRODUCTION

Telomeres are unique structures made up of short DNA repeat sequences and associated proteins that have multiple functions, including protection of the ends of chromosomes, chromosome organization and segregation and regulation of nearby genes (1). The importance of telomeres is inferred by the similarities in the structures (5–10).

Although the length of telomeres in germline cells is maintained, the length of telomeres in most human somatic cells decreases with age (11,12). Consistent with this observation, telomerase activity is not found in most somatic cells (13,14). However, more recent studies have reported telomerase activity in some somatic cells (15,16) and therefore other factors besides the presence of telomerase activity also appear to be involved in the regulation of telomere length. Regardless of the mechanism for regulation of telomere addition, the correlation between aging and telomere shortening has led to the hypothesis that short telomeres may be the signal for senescence (17).

Several different mechanisms have been proposed for how telomere shortening may lead to senescence. One possible mechanism involves the regulation of a gene or set of genes located near a telomere in which expression is influenced by telomere length (18). Numerous studies have shown that the expression of genes can be influenced by proximity to heterochromatin or telomeric repeat sequences. Early evidence that genes can be regulated by unique chromatin structures was shown in Drosophila, in which a gene involved in eye pigmentation was placed adjacent to pericentric heterochromatin, resulting in variegated eye color (19,20). Furthermore, in Schizosaccharomyces pombe, a gene placed within the central domain of the three centromeres showed variegated expression characteristic of position effect variegation (21). Formation of a new telomere next to genes in Saccharomyces cerevisiae resulted in reversible repression (22). Likewise, genes located near a telomere in S.cerevisiae were found to be transcriptionally repressed and this silencing, called the telomere position effect (TPE), was relieved in a distance-dependent manner (22,23). Recently, telomeric sequences positioned internally in a chromosome have been found to act as silencers in S.cerevisiae (24).

Proteins are important components involved in the silencing function of the telomeres. In yeast, mutations in specific genes, such as SIR2, SIR3, SIR4, NAT1, ARD1 and HHF2, resulted in the relief of silencing of a gene adjacent to a telomere (25). TPE silencing can be enhanced by overexpression of SIR3 (23), whereas the presence of other transcription factors can decrease the silencing caused by TPE in a dose-dependent manner (26). Many proteins, such as Rap1p, Est1p, Rif1p, Pif1p, cdc17p, Tel1p, Tel2p, Sir3p and Sir4p, have also been found to have profound effects on telomere length (27–33).

Although it is clear from the above studies in yeast that gene expression can be influenced by proximity to telomeres or other

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heterochromatic regions, it is unclear whether a similar phenomenon occurs in mammalian cells. The telomeres of mammalian cells have been shown to have a structure that is distinct from that of bulk chromatin (8–10). In addition, the herpes simplex virus thymidine kinase (HSV-tk) promoter was shown to be inhibited by its proximity to centromeres (34,35). In contrast, the simian virus 40 (SV40) early promoter was found not to be inhibited by its proximity to either telomeres or α satellite DNA in hamster cells (36). Whether these differences in suppression are due to cell type, integration site or the nature of the transcriptional promoters used is unclear.

To further investigate the role of telomeres in suppression of gene expression, we have studied the effect of telomere length on expression of the HSV-tk promoter in human cells. For these studies we used an immortal SV40-transformed human fibroblast cell line (KB319) that had a plasmid containing a selectable neo gene integrated at the end of chromosome 13 (37). The presence of the plasmid sequences at the telomere was previously used to investigate the dynamics of changes in length of this telomere (38). In the current study, subclones of this cell line with various lengths of telomeric repeat sequences at this telomere were analyzed to determine whether the length of the telomeric repeat sequences influenced expression of the adjacent telomeric neo gene.

MATERIALS AND METHODS

Cell lines and cell culture

The KB319 cell line contains a plasmid (pMSTK-neo) integrated at a telomere (37). KB319 was derived from LM217, an immortal SV40-transformed human fibroblast cell line. The cell lines E9-6D, E8, E1-6, E9-7 and E9-2 are all second and third generation subclones of KB319. Cell line 2.0-23 has the pMSTK-neo plasmid integrated at an interstitial site (39).

Cell growth experiments involved culturing cells for 8 days in triplicate in medium containing 0, 200 or 600 µg/ml G418. The cells were then harvested and counted and the number of cells was compared with the number of cells grown in non-selective medium. LM217, which does not contain the neo gene, was used as a control. In another growth rate experiment, cells were grown in 400 µg/ml G418 and harvested at 0, 24, 48 or 170 h after addition of the selective medium. For the survival experiments, the cells were plated in duplicate in medium with or without 400 µg/ml G418. After 3 weeks the flasks were stained with methylene blue and the colonies counted.

Southern, RNA and fluorescence in situ hybridization analysis

Southern blot analysis was performed after digestion of cellular DNA with XhoI, which cuts the integrated plasmid sequences ~3 kb from the telomeric repeat sequences at the end of the chromosome (37). The probe used for analysis of the length of the marker telomere consisted of human sequences contained within the plasmid (Fig. 1). These human sequences are also found at their original location in these cells and produce a single band that serves as an internal control for a stable fragment. Photographs from Southern blots run under identical conditions (38) were scanned and placed adjacent to each other by using computer manipulation so that relative telomeric band migration could be easily observed.

mRNA was isolated from the KB319 subclones E9-6D, E8, E1-6, E9-7 and E9-2 and the cell line 2.0-23 using a Fasttrack mRNA isolation kit (Invitrogen). The RNA was quantified and RNA blot analysis was performed with 2 µg mRNA (40). The blot was hybridized with a probe specific for the neo gene and subsequently washed and reprobed with an actin probe. The amount of mRNA hybridized was determined by using a PhosphorImager (Molecular Dynamics) and densitometric analysis.

Fluorescence in situ hybridization was performed as previously described (38), with the entire pMSTK-neo plasmid used as probe.

RESULTS

The integrated pMSTK-neo plasmid at the end of chromosome 13 in an immortal SV40-transformed cell clone KB319 (37) was used to study the dynamics of changes in length of a single telomere (38). Sequences within the plasmid were used as a probe for Southern blot analysis to determine the length of the terminal restriction fragment containing the telomeric repeat sequences. The integrated pMSTK-neo plasmid in this cell line contains a selectable marker gene (neo) with a HSV-tk transcriptional promoter (Fig. 1) that is located within 4 kb of the telomeric repeat sequences. Thus, this cell line provided an opportunity to test the effect of telomere length on gene expression. Because of the extensive telomere polymorphism in KB319, subclones selected at random demonstrated a wide distribution in the length of the telomere on the marker chromosome (38). Representative KB319 subclones E9-6D, E8, E1-6, E9-7 and E9-2, which were isolated and grown without G418 selection, had telomeres of ~25, 6, 3, 1 and 0.5 kb respectively (Fig. 2). Fluorescence in situ hybridization demonstrated that the subclones E8, E1-6, E9-7 and E9-2 have only a single copy of chromosome 13 with the integrated plasmid at its end, although in each subclone a small percentage of cells in the population have two copies (data not shown). One subclone, E9-6D, contains two copies of chromosome 13 with the telomeric plasmid (data not shown). Subclone E9-7 also has a small percentage of cells in the population (<=5%) with interstitial sites (data not shown). The terminal fragment containing the telomeric repeat sequences is easily identifiable on Southern blots by its extensive polymorphism, which is observed as a diffuse band. For comparison, a homogeneous band from a stable interstitial
Figure 2. Southern blot analysis of the relative telomere lengths in the KB319 subclones. (a) Photographs from Southern blots for the parental cell line without the telomeric plasmid sequences (LM217) and KB319 subclones E9-6D, E8, E1-6, E9-7 and E9-2 (40) were scanned and placed adjacent to each other by means of computer manipulation so that relative telomeric band migration could be compared. The telomere band is polymorphic in length as compared with the control band from an interstitial sequence (asterisk). The approximate size of size standards is shown at the left (in kb). (b) The length of the telomeric repeat sequences in the KB319 subclones as determined from the Southern blots in (a) after subtracting the length of the subtelomeric plasmid sequence (3 kb). The sequence served as a convenient internal control. The length of the telomeric sequences (Fig. 2b) was determined from the average size of the diffuse bands after subtracting the length of the subtelomeric plasmid sequence (3 kb). The relative equivalence of the plasmid copy number in the various clones is also evident by comparison of the internal control bands and the telomeric bands seen by Southern blot analysis (Fig. 2a).

Transcription levels of the neo gene in the KB319 subclones with telomeres of various lengths were analyzed by RNA blot analysis (Fig. 3a). mRNA was isolated from the KB319 subclones and from the cell line 2.0-23, which has the neo plasmid integrated at an interstitial site. All of the KB319 subclones and the 2.0-23 cell line had been cultured without selection for the neo gene for extended periods of time (>30 cell doublings) before mRNA isolation. The RNA blot was first hybridized with a neo probe and subsequently washed and reprobed with an actin probe (Fig. 3a). Transcription from the neo gene in the KB319 subclones and 2.0-23 cell line was quantified by densitometric scanning (Fig. 3b). Variability in loading amounts was normalized by standardization with the actin band densities. The amount of neo mRNA in each of the five KB319 subclones reproducibly showed only modest variation. However, the 2.0-23 cell line, which contains the interstitial neo gene, showed a 3- to 4-fold higher level of expression than cell lines containing the neo gene integrated at a telomere.

RNA blot analysis can determine only the average amount of mRNA in all of the cells in the population. To learn more about the influence of telomere length on neo expression in individual cells, we tested the KB319 subclones for colony forming ability and growth rate in G418. The colony forming ability of the KB319 subclones was tested by plating cells in duplicate with or without 400 \( \mu \)g/ml G418. The number of colonies from the flasks with non-selective medium was standardized to 100% for each cell line. No significant differences were observed in any of the KB319 subclones (Fig. 4). For the study of growth rates, the KB319 subclones containing the telomeric neo gene were grown for 8 days in triplicate in medium containing 0, 200 or 600 \( \mu \)g/ml G418. All the cell lines had equivalent growth rates in the presence of increasing amounts of G418, with a slight decrease in cell survival at higher concentrations (Fig. 5). The control cell line LM217, which does not contain the neo gene, showed complete inhibition of growth at all G418 concentrations tested. The KB319 subclones were also grown for various lengths of time with or without 400 \( \mu \)g/ml G418 and cell numbers were determined. Again, no major differences in growth rates were observed in any of the KB319 subclones containing the telomeric neo gene or in the 2.0-23 cell line (data not shown).

DISCUSSION

As shown by RNA blot analysis (Fig. 3), the level of expression of the telomeric neo gene was essentially invariant for all of the KB319 subclones having telomere lengths ranging from 0.5 to 25 kb (Fig. 2). Similarly, growth rate and colony forming ability did not differ among the KB319 subclones. These results suggest that the control of gene regulation is not influenced by telomere length. The results therefore do not support the hypothesis that specific genes near telomeres are involved in the regulation of cell senescence.

Several factors must be taken into consideration when interpreting these results. First, did telomere length shorten sufficiently to influence telomere expression? The average length of all telomeres in senescent fibroblasts is \( \sim \)6 kb (11). However, the length of some telomeres is much shorter and therefore it is impossible to determine if a specific telomere length is associated with senescence. Although the length of the single telomere analyzed in our study was reduced to 0.5 kb, the size distribution of the band demonstrated that this telomere was much shorter in...
Figure 3. RNA blot analysis of neo gene expression in subclones of the KB319 cell line. (a) RNA blot analysis of mRNA from the KB319 subclones E9-6D, E8, E1-6, E9-7 and E9-2, each containing the telomeric plasmid, and from cell line 2.0-23, which has the neo plasmid integrated at an interstitial site. mRNA was hybridized with a neo probe and subsequently washed and hybridized with an actin probe. (b) Quantification of mRNA in the cell lines shown in (a) by densitometric scanning; values were normalized by standardization with the actin band densities.

Figure 4. Influence of telomere length on colony forming ability of the KB319 subclones in the presence of G418. Cells from the parental cell line without a neo gene (LM217) and the KB319 subclones E9-6D, E8, E1-6, E9-7 and E9-2, containing the telomeric plasmid, were plated in duplicate in medium with or without 400 µg/ml G418. The number of surviving colonies in G418 was standardized by comparison with the number of colonies grown without G418.

a substantial proportion of the cells in the population. Second, was the neo gene close enough to the telomere to allow suppression to occur? Studies in yeast demonstrated that the suppression due to chromatin conformation was distance dependent, with suppression seen at 3–12 kb, but not at 20 kb (22,23,41). The neo gene in our study was within 4 kb of the telomeric repeat sequences and therefore should have been susceptible to suppression. mRNA analysis showed that the neo gene in the telomeric plasmid in KB319 was expressed at one third the level of the same plasmid integrated at an interstitial site. However, many factors could influence the level of expression at different integration sites and therefore it cannot be concluded that this difference in expression is due to suppression by telomeric repeat sequences. Regardless, the lack of effect of telomere length on neo expression demonstrates that telomere shortening does not influence gene regulation in our system. Finally, is the transcriptional promoter used in this study resistant to the TPE? Studies in yeast demonstrated that a strong transcriptional promoter can overcome the TPE (26). However, the HSV-tk promoter used here has previously been shown to be relatively weak and can be suppressed easily by epigenetic mechanisms (42) and by proximity to centromeres (34,35). The HSV-tk promoter is therefore clearly susceptible to suppression and should have been sensitive to TPE had it been present. However, we cannot rule out the possibility that specific transcriptional promoters are required for expression of the TPE. A definitive answer on the role of telomeres in gene regulation may therefore have to await the discovery of endogenous genes located adjacent to telomeric repeat sequences.

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Figure 5. Influence of telomere length on the growth rate of the KB319 subclones in the presence of G418. The number of cells after 8 days of culture in 200 or 600 µg/ml G418 were compared with control cultures grown without G418. The rate of growth was determined for the subclones E9-6 (o), E8 (n), E9-7 (q) and E9-2 (+) containing the telomeric plasmid, the 2.0-23 cell clone (Δ) containing the interstitial plasmid and the parental LM217 cell line (solid line) without the integrated plasmid.