Structural stability and chromosome-specific telomere length is governed by cis-acting determinants in humans

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Single telomere length analysis (STELA) of the XpYp telomere has revealed extensive allelic variation and ultra-short telomeres in senescent cells. Superimposed on end-replication losses are additional mutational events that result in large-scale changes in telomere length. In order to establish if the dynamics of the XpYp telomere are typical of human telomeres, here we describe an analysis using STELA of the telomeres of 2p, 11q, 12q, 17p and XpYp. The dynamics of telomere loss (erosion rates and stochastic length changes) was conserved among 2p, 11q, 12q and XpYp within the same cell strains and was dependent on the replicative kinetics of the cells in culture. However, of the telomeres analysed, the telomere of 17p was more stable with a striking paucity of large-scale length changes, and exhibited the shortest recorded allelic distribution (300 bp) in senescent cells and displayed a general, but not absolute, trend towards being the shortest telomere. Ectopic over-expression of hTERT homogenized both allelic and chromosome-specific telomeric distributions. However, telomerase-expressing cancer cells displayed both allelic variation and chromosome-specific telomere length, with 17p displaying the shortest allelic telomere length. Although other telomeres in the genome may share the properties of 17p, these data suggest that physiological levels of telomerase allow differential telomere length regulation and indicate the presence of cis-acting factors that govern both telomeric stability and chromosome-specific telomere length in the presence of telomerase.

INTRODUCTION

Telomeres are essential structures that cap the ends of eukaryotic chromosomes. In humans they are composed of the DNA sequence TTAGGG reiterated into arrays of up to 20 kb. As a consequence of end-replication losses, telomeres shorten with every cell division (1). Ultimately this results in the loss of telomeric function and the triggering of a p53-dependent DNA-damage checkpoint termed replicative senescence (2). The limit on replicative lifespan that telomere erosion provides is considered to have evolved as a tumour suppressive mechanism in long-lived species. However, in order to maintain tissue homeostasis ongoing cell division occurs during life, and results in a loss of telomeric sequences as a function of age (3). This raises the possibility of a telomere-dependent accumulation of senescent cells in human tissues, which may underlie age-related tissue deterioration, disease and cancer predisposition (4–6). Thus, it is important to understand at the molecular level, the mechanisms that underlie telomere erosion and the nature of the telomeric signal that triggers replicative senescence. We have previously described an analysis of telomere dynamics using single telomere length analysis (STELA) at the XpYp telomere. In clonal cell populations, this analysis revealed that with ongoing cell division, the mean of the XpYp telomere length distributions decreased and the variance increased (7). These observations were consistent with the telomere dynamics predicted as a consequence of end-replication losses (1,8) coupled with a
putative C-strand resecting activity (9). However, end-replication losses were superimposed by large-scale telomere deletion events (7). Telomere deletion events have been observed in yeast, and referred to as telomere rapid deletion (TRD), resulting in the rapid resetting of an artificially lengthened telomere to the genome average telomere length (10,11). TRD is an intra-chromatidal process and is dependent on the MRX complex (11,12). The telomere deletion events we observe in humans (hTRD) result in deletion from genome average to a potentially critical length and therefore may result from distinct mechanisms to those described in yeast. However, in humans intra-allelic telomeric mutation is observed in the germline (13–15) and the introduction of specific alleles of TRF2 result in telomeric mutation events that are consistent with intra-allelic process such as the processing of putative Holliday junctions formed at the base of T-loops (12,16). The hTRDs that we observed in the cells grown in culture did not appear to accumulate with ongoing cell division; this implied that these events were either repaired back to the mean XpYp length, that telomeres had fused, or that the cells that had suffered an hTRD event had exited the cell cycle. At the XpYp telomere, hTRDs occurred at a frequency of ~3%; if this frequency is conserved at all telomeres in the genome, then hTRD could represent a significant contributor to genome-wide telomere loss with potential implications for the maintenance of genomic stability and the kinetics of cell division.

In addition, STELA analysis of multiple fibroblast strains revealed extensive allelic variation in telomere length that was set in the zygote as a consequence of differing maternal and paternal telomere length contributions. A new minimal mean telomeric allele length was observed in senescent cells of 1.2 kb, with some telomeres almost completely denuded of telomere repeats. There was considerable variation (upto 6 kb) in the XpYp telomere lengths of senescent cells, with some cell strains, for example IMR90 and IMR91, displaying longer telomeres (4.9 kb and 7.4 kb, respectively) at senescence. From these data, we concluded that the behaviour of the XpYp telomere was inconsistent with it acting as an universal ‘clock’ whose erosion to critical levels was sufficient to trigger senescence in all strains analysed. Indeed, if other telomeres displayed similar levels of variation and dynamics as the XpYp telomere then it was difficult to envisage any such telomere acting as an ‘universal clock’. Instead, we hypothesized that in each zygote a unique spectrum of telomeres displays similar levels of variation and dynamics as the XpYp telomere then it was difficult to envision any such telomere acting as an universal clock’. Instead, we hypothesized that in each zygote a unique spectrum of telomeres displays similar levels of variation and dynamics as the XpYp telomere then it was difficult to envision any such telomere acting as an universal clock’. Instead, we hypothesized that in each zygote a unique spectrum of telomeres displays similar levels of variation and dynamics as the XpYp telomere then it was difficult to envision any such telomere acting as an universal clock’. Instead, we hypothesized that in each zygote a unique spectrum of telomeres displays similar levels of variation and dynamics as the XpYp telomere then it was difficult to envision any such telomere acting as an universal clock’. Instead, we hypothesized that in each zygote a unique spectrum of telomeres displays similar levels of variation and dynamics as the XpYp telomere then it was difficult to envision any such telomere acting as an universal clock’. Instead, we hypothesized that in each zygote a unique spectrum of telomeres displays similar levels of variation and dynamics as the XpYp telomere then it was difficult to envision any such telomere acting as an universal clock’. Instead, we hypothesized that in each zygote a unique spectrum of telomeres displays similar levels of variation and dynamics as the XpYp telomere then it was difficult to envision any such telomere acting as an universal clock’. Instead, we hypothesized that in each zygote a unique spectrum of telomeres displays similar levels of variation and dynamics as the XpYp telomere then it was difficult to envision any such telomere acting as an universal clock’. Instead, we hypothesized that in each zygote a unique spectrum of telomeres displays similar levels of variation and dynamics as the XpYp telomere then it was difficult to envision any such telomere acting as an universal clock’. Instead, we hypothesized that in each zygote a unique spectrum of telomeres displays similar levels of variation and dynamics as the XpYp telomere then it was difficult to envision any such telomere acting as an universal clock’. Instead, we hypothesized that in each zygote a unique spectrum of telomeres displays similar levels of variation and dynamics as the XpYp telomere then it was difficult to envision any such telomere acting as an universal clock’. Instead, we hypothesized that in each zygote a unique spectrum of telomeres displays similar levels of variation and dynamics as the XpYp telomere then it was difficult to envision any such telomere acting as an universal clock’. Instead, we hypothesized that in each zygote a unique spectrum of telomeres displays similar levels of variation and dynamics as the XpYp telomere then it was difficult to envision any such telomere acting as an universal clock’.

The XpYp telomere is located at the terminus of the p-arm pseudoautosomal region of the sex chromosomes and the telomere and adjacent DNA display unusually high levels of sequence polymorphism and linkage disequilibrium, suggesting that this telomere evolves quickly and has very low levels of recombination (13,14,18). Furthermore, Q-FISH analysis suggested that the inactive X-chromosome exhibits elevated rates of telomere erosion and has unusual dynamics during replicative senescence (19,20). Given these properties, we wished to understand if the dynamics described for the XpYp telomere were representative of other human telomeres, and test whether the information and models based on XpYp were applicable to autosomal telomeres. Thus, here we describe the extension of STELA to four autosomal chromosome ends, 2p, 11q, 12q and 17p; these data provide information about telomere dynamics during the onset of replicative senescence and in the presence of telomerase.

RESULTS

The development of STELA at 2p, 11q, 12q and 17p

With the exception of XpYp, the sub-telomeric regions of the human genome are highly variable, with duplications and repetitive sequence families that are shared among multiple chromosome ends (21). This variation coupled with the paucity of sequence data limits the analysis of single chromosome ends in isolation. However, by utilizing single-base sequence differences between different ends it was possible to use PCR to analyse a subset of telomeres. We have previously used PCR to specifically analyse sequence polymorphism in the 12q telomere and adjacent DNA (14). Using the information gained from this analysis it was possible to design 12q specific PCR primers that were suitable for use with STELA. The telomere-adjacent DNA of 11q contains 183 bp of unique sequence between the start of the telomere repeat array and a GC rich minisatellite (22). The minisatellite is refractory to PCR and thus primer design is limited to the 183 bp of telomere-adjacent DNA; PCR primers for STELA were therefore designed in this region. The available telomere-adjacent sequence of 17p does not extend to the start of the telomere repeat array (22), and the distance between the end of the published sequence and the start of the telomere was estimated to be 5 kb (Harold Rietman, personal communication). The distal 4.8 kb of the published 17p sequence contained greater than 98% sequence homology to interstitial telomeres within chromosomes 1, 7, 9, 11 and 16. Nevertheless, given the apparent importance of the 17p telomere (17,23–25), PCR primers for STELA were designed against single bases that distinguished the 17p sequence from that of the homologous sequences. The telomere-adjacent DNA of 2p contains several sub-telomeric sequence families (22) and primers for STELA were again designed based on single base differences from these sequence families.

To accurately determine telomere length with STELA, the exact distance between the telomere-adjacent primer and the start of the telomere repeat array is required. This was known for the telomeres of 2p, 11q, 12q and XpYp but not for 17p. The known 17p telomere-adjacent sequence displayed strong homology with five interstitial telomeres and we therefore speculated that this homology might extend to the telomere repeat array of 17p. We used the sequence information from these interstitial telomeres to design primers that were capable of PCR amplifying from a 17p telomere-specific primer (17p6) to the start of the putative 17p telomere. These products were sequenced (Genbank accession no. DQ355024) and indeed it was clear that the structure of 17p telomere was homologous (>98%) to these interstitial telomeres and thus...
we were able to define the distance of 17p telomere-adjacent primers from the start of the telomere repeat array.

The specificity of the primers was tested by PCR amplification of the telomere-adjacent DNA and the products examined by sequence analysis. Once appropriate primers and conditions were obtained they were tested for suitability with STELA. Fibroblast strains in culture generally have a heterogeneous telomere length profile (7); when developing STELA at additional chromosome ends, this heterogeneity confounds the interpretation of the specificity of the profiles for a particular telomere of interest. In contrast clonal derivatives, being recently derived from a telomere of a single length, have relatively homogeneous telomere profiles (7,26). Thus, to validate the specificity of STELA at 2p, 11q, 12q and 17p, the assays were developed using a clonal derivative of MRC5 (7).

STELA at 17p revealed homogeneous telomere length distributions that were consistent with specific amplification from a single telomere (Fig. 1). The specificity of the 17p specific primer in generating single telomere length profiles indicates that, despite the 98% homology to the interstitial telomeres, the 17p sequence is unique in its telomere-adjacent location. This was confirmed by undertaking STELA with additional primers closer to the telomere. Although these primers would also be capable of amplifying the family of 17p-like interstitial telomeres, all seven produced STELA profiles consistent with amplification of the same 17p telomere (data not shown). This provided further validation that the 17p sequence that we had generated was indeed adjacent to the 17p telomere.

As with 17p, STELA at 2p and 12q appeared to be robust with the detection of single molecule amplification of telomeres of over 12 kb (Figs 1 and 3). As there were sequence constraints in the primer positioning at 11q, STELA at this telomere had a lower amplification efficiency when compared with the other ends. To ensure the specificity of the analysis, STELA products for all ends were detected following separate Southern hybridizations with a TTAGGG repeat containing probe- and chromosome-specific telomere-adjacent probes.

**Telomere erosion rates**

We examined if the dynamics we described previously at the XpYp telomere were typical of human telomeres. To do this, we analysed clonal populations that, without the confounding influence of the replicative kinetics of bulk populations, allow precise estimates of telomere erosion rates and dynamics. To determine telomere erosion based upon end-replication losses (8), as opposed to hTRD events, we calculated erosion rates by determining the differences as a function of population doublings (PD) between the modes of the telomere length distributions plotted as histograms. We undertook an analysis of the 2p, 11q, 12q, 17p and XpYp telomeres in clonal derivatives of MRC5 and IMR90. Telomere erosion rates were conserved among the different ends within the individual clones. However, there was considerable variation between the different clonal derivatives of IMR90; clones 1, 2, 4, 5, 6 and 8 all exhibited similar rates of erosion (mean 163 bp/PD), whereas clones 3 and 7 exhibited erosion rates of 1235 and 608 bp/PD, respectively (Fig. 2A). These erosion rates displayed a strong negative correlation with proliferative lifespan ($R = 0.78$). This correlation was primarily due to clones 3 and 7, which did not proliferate well in culture, obtaining up to 23 and 25 PD, respectively from a single cell clone. As a consequence, telomere length and telomere erosion rates for these clones were estimated in the final two to three PD of culture. The other IMR90 clones
and those of MRC5 were analysed during the exponential period of growth. In order to investigate this further, a detailed analysis of telomere erosion was carried in two of the long-lived IMR90 clones (clones 1 and 6). It was clear from this that telomere erosion accelerated in the later stages of growth. Clone 6 displayed an erosion rate of 109 bp/PD for the first 51 PD of growth, but in the final three PDs of growth this increased to 314 bp/PD. The mean growth rate of clone 6 in the first 51 PDs (0.35 PD/day) slowed for the remaining three PDs to 0.08 PD/day. Clone 1 displayed an erosion rate of 115 bp/PD increasing to 509 bp/PD in the final four PD of growth with a commensurate decrease in the growth rate from 0.39 to 0.11 PD/day (data not shown).

Even after accounting for the elevated rates of telomere erosion in the short-lived IMR90 clones, there was a significant difference in rates of telomere erosion between the clonal derivatives of MRC5 and IMR90 ($P < 0.001$, Z-test); the mean of the MRC5 clones 112 bp/PD ± 56 (±SD, Fig. 2B), whereas IMR90 clones exhibited erosion rates of 163 bp/PD ± 48.

We also determined rates of telomere erosion at the 2p, 11q, 12q, 17p and XpYp telomeres in bulk cultures of five different fibroblast strains. As with the clonal analysis, telomere erosion was largely conserved among the different ends with the same strain, but was variable between the strains, with HCA2 (MJ) recording the slowest rate of erosion (65 bp/PD) and WI-38 the fastest (132 bp/PD, Fig. 2C).

**Telomeric polymorphism**

As had been previously noted, at XpYp, bimodal telomere length distributions were observed at all the ends analysed with STELA (Fig. 1, and Supplementary Material); this was particularly apparent in the analysis of clonal populations (Fig. 1). The lack of informative telomere-adjacent sequence polymorphism precluded us from formally establishing that the bimodal distributions were allelic by allele-specific telomeric amplification. However, the observation that these distributions displayed approximately equal numbers of amplifiable molecules was reminiscent of the demonstrably allelic distributions observed at the XpYp telomere (7).

In addition, we observed that some of the ends could not be amplified with the available primers. None of the eight MRC clones analysed, displayed a bimodal distribution at 17p, although bimodal distributions were observed at the other ends; this led us to conclude that MRC5 contains just one amplifiable 17p telomere. Furthermore, IMR90 could not be amplified with the 11q telomere (Fig. 1).

**Telomere lengths in senescent cell populations**

17p was the shortest telomere in six out of eight senescent IMR90 clones (Fig. 2D), but was the shortest in only two out eight senescent MRC5 clones (Fig. 2E). Interestingly, the shortest telomeric distribution yet recorded with STELA in senescent cells was 0.3 kb in an IMR90 clone (clone 4, Figure 2. Histograms representing telomere data from IMR90 clones, MRC5 clone and bulk fibroblast strains. (A–C) Telomere erosion rates (bp/PD). (D–F) Telomere length of the shortest distributions in senescent cells.
These distributions were consistent with the analysis of the senescent parental cultures, where it was clear that in IMR90, 17p was the shortest telomere, whereas in MRC5, the telomeres of XpYp and 11q were shortest (Fig. 2F). There was no clear trend for any one telomere being shortest in the eight different senescent fibroblast strains analysed, indeed, within these strains only the telomere of 12q did not register as the shortest telomere at senescence. However, the mean length of the shortest distributions in the eight senescent fibroblast strains was as follows: 17p, 2.6 kb; XpYp, 3.6 kb; 12q, 4.8 kb; 2p, 3.2 kb; and 11q, 3.1 kb (Fig. 2F); indicating that on average, the 17p telomere displayed the shortest alleles in the senescent fibroblast strains. Thus, there appears to be a general trend for 17p being the shortest telomere, but this is not absolute and within the five ends analysed here, the identity of the shortest telomere in senescent cells appears to be random but strain-specific (Fig. 2F).

**Telomere dynamics in the presence of telomerase**

Given the general trend for shorter telomeres at 17p, we considered that the length of this telomere may have a propensity to be set shorter in the presence of telomerase. Thus, we examined the telomere length of MRC5 and HCA2 strains ectopically expressing the retrovirally transduced catalytic subunit of hTERT. In HCA2 cells (PD 82), all the five telomeres displayed heterogeneous length distributions; there were no significant differences in length between the different ends (9.7 ± 0.6 kb, mean ± SD) and there was no evidence of allelic length differences. The telomere length distribution were again heterogeneous in MRC5 cells expressing hTERT (PD 200+), the telomere of 11q but not 17p was significantly shorter than those of 2p, 12q and XpYp (P < 0.05). We reasoned that the forced expression of telomerase used here was likely to result in a gross over-expression of telomerase. Therefore, we examined telomere length in two telomerase-expressing immortal keratinocyte holoclones (27) derived from head and neck squamous cell carcinoma, which would be predicted to express telomerase at more physiological levels (28,29). Analysis of these cells (PD > 40) revealed clear chromosome-specific telomere length differences and bimodal distributions reminiscent of allelic telomere length differences (Fig. 3). In both strains the shortest distributions were observed at 17p (Fig. 3).

**Frequency of hTRD events**

Similar dynamics were observed in terms of end-replication losses between the different ends analysed within the same strains/clones. However, analysis of the clonal derivatives of the telomerase-negative strains IMR90 and MRC5 revealed that the frequency of hTRD events was strikingly different between the different ends (Fig. 4). Assuming normality at telomeres that displayed unimodal distributions we defined hTRD events as outliers from the telomere length profiles at a level of significance P < 0.01 (critical z-value >2.32). In both MRC5 and IMR90 clones, a pair-wise comparison at telomeres displaying unimodal distributions revealed a significant difference (P < 0.001) in the frequency of hTRDs between the telomeres of XpYp (4.8%) and 17p (1.5%, Fig. 4) and between 12q...
DISCUSSION

Here, we have shown that the length of specific autosomal human telomeres can be analysed with STELA. Despite over 98% sequence homology with a family of interstitial telomeres, we were able to obtain telomeric profiles from 17p that were consistent with the amplification of a single telomere. We observed that MRC5 contained just one telomeric allele with the 17p sequence used for STELA and that IMR90 contained no detectable 11q telomere. These data were consistent with previous descriptions of extensive polymorphism in the positioning of human telomeres (30–32). These regions are among the most dynamic and variable regions of the genome with telomeric sequence families being shared among numerous chromosome ends and frequent large-scale polymorphism in the distribution of these sequence families (31–33). Furthermore, as observed at XpYp with STELA (7) and other ends with Q-FISH (34) we observed bimodal telomere length distributions at all the ends analysed, consistent with allelic variation resulting from differing maternal and paternal telomeric contributions.

We utilized the unique ability of STELA to analyse telomere lengths of specific chromosome ends in senescent cell populations. Our analysis of bulk populations of eight fibroblast strains revealed that that nature of the shortest telomere at the five ends analysed appeared to be random, with all except 12q being the shortest in at least one fibroblasts strain. 17p, however, was the shortest telomere in three of the eight strains analysed and overall showed the lowest mean telomere length in senescent cell populations. Furthermore, in clonal derivatives, 17p exhibited the shortest yet recorded telomere lengths in senescent cell populations with one allele displaying a modal telomere length of 300 bp. These observations are consistent with previous reports (17) that there is an apparent tendency for 17p to be a short telomere. Telomere length polymorphism in each individual is established in the zygote (7,35) and the ultimate source of this variation is likely to be the maternal and paternal germline lines. In order to maintain telomere length for subsequent generations, spermatogonial stem cells express telomerase, it is conceivable that cis-acting elements, for example, localized chromatin structure could govern chromosome-specific telomere length. Indeed, we observed that both chromosomal and allelic telomere length differentials can be maintained in the presence of telomerase. In the germline, the intrinsic dynamics of telomeres that generate heterogeneity coupled with the propensity for alleles of the 17p telomere to be set shorter, means that it may be reasonable to conclude that this telomere, or other telomeres that display similar properties, have the potential to act as a semi-universal ‘clock’ telomere whose erosion to critical levels is sufficient to trigger replicative senescence in a majority of individuals. Consistent with this concept, our own data show that when compared with the XpYp telomere, the 17p telomere is considerably shorter in the male germline (15).

The telomeres of senescent cells display markers of the DNA-damage response such as phosphorylated H2AX (γH2AX). It has been previously shown by others that the hybridization to whole genome microarrays of material from senescent MRC5 cells immunoprecipitated with γH2AX revealed a subset of 21 γH2AX positive ends which included 2p, 11q and 17p but not XpYp and 12q (2). Interestingly, the XpYp telomere in our batch of MRC5 cells was with 11q the shortest telomere (0.6 kb). Thus, it is unclear as to why the XpYp telomere did not appear to elicit a DNA damage response. However, it is perhaps relevant to note that at 11q both alleles were superimposed at 0.6 kb, whereas at XpYp the shortest allele was 0.6 kb and longest being 5.4 kb. Therefore, this discrepancy may be a consequence of a lack of resolution of hybridization of CHIP material on microarrays such that single γH2AX positive telomeric alleles were not detected. Furthermore, the heterogeneity in the telomere...
lengths of the ends that elicit a DNA damage response in senescent MRC5 cells (0.7–1.6 kb), suggests that there may be chromosome-specific differences in the telomere length threshold below which replicative senescence is triggered.

We have previously observed that there appears to be at least three separate mechanisms operating to generate telomere erosion (7). At the single cell level, end-replication losses (coupled with a putative C-strand resecting activity) result in a gradual reduction telomere length as a function of PD in the bulk of the STELA profiles with a commensurate increase in the variance (7,8). In addition to end-replication losses, telomeres suffer abrupt changes in telomere length, resulting in stochastically shortened telomeres via a mutational mechanism that is inconsistent with end-replication losses. A third factor that impinges upon the calculated rates of telomere erosion is the kinetics of cell growth in culture. If all the cells in the culture exhibit the same rate of growth, the telomere erosion as a function of PD will reflect telomere erosion at the single cell level. However, as is likely to be the case with most human cell cultures, the rates of cell growth will be heterogeneous (36). In this situation, the fastest dividing cells will contribute disproportionally to the increase in cell number and therefore PD. Thus, telomere erosion as a function of PD will be an over-estimate of the rate at the single cell level; a situation greatly exaggerated in Werner’s syndrome (26). In order to delineate the effects of end-replication losses, hTRD and cell growth kinetics, we analysed clonal populations which when compared with bulk populations exhibit simplified growth kinetics. We then separated out end-replication losses from hTRD events. Using this approach we observed that, in terms of end-replication losses, all the chromosome ends within any one strain/clone were subjected to similar rates of erosion. Importantly, this implies that the telomere length distribution of the cell that initiates the culture (a cloned single-cell or zygote) will represent the telomere length distribution observed at the end of the cultures replicative capacity. Therefore, the shortest ends in the initiating cell will be the first to erode to the critical length that elicits a DNA damage response and triggers replicative senescence. The length at which this would occur has not been precisely defined and may vary between ends, but here we were able to define a new minimum telomere length in senescent cells of 300 bp at 17p.

Although the rate of telomere erosion was conserved among the different ends within the same cell strain, they were clearly different between the different strains. This was most notable in the clonal analysis where specific clones exhibited greatly elevated rates of erosion that correlated with their replicative capacity. Furthermore, telomere erosion rates were not consistent throughout the life of the culture; instead, they increased in the later stages as the rate of growth of the culture slowed. The onset of replicative senescence is a heterogeneous process with an increasing proportion of cells exiting the cell cycle as the culture on the whole approaches senescence (36–38). Thus, with a decreasing proportion of cells whose division is responsible for the increase in cell number and thus PD, the ratio of PD to cell division will change as the culture approaches senescence. This predicts that telomere erosion at the single cell level will remain constant, whereas telomere erosion as a function of PD will increase. Furthermore, because it is clear that cell strains exhibit differing sensitivities to cell culture in 20% oxygen, and that those with increased sensitivities, for example IMR90 (39), will display a reduced ratio of PD to cell growth. We conclude that telomere dynamics at the population level (but not the single cell level) are influenced by the kinetics of cells in culture, which in turn is influenced by both the cultures replicative history and culture conditions.

The telomeres of 12q and XpYp displayed hTRDs ≈4% and visual inspection of the bimodal distributions of the 11q and 2p telomeres suggested a similar frequency of hTRDs. The occurrence of these molecules is not consistent with end-replication losses (8); instead, it is more likely that these molecules may be generated as a consequence of intra-allelic mutational events such as oxidative damage and recombination (13,16,40). What was striking about these data in fibroblast strains was that when compared with other ends analysed here, the 17p telomere had very low levels of hTRD events. This is not specific to fibroblasts cultured in vitro as we have observed that when compared with XpYp, the telomere length distributions of 17p are considerably less heterogeneous in DNA derived from peripheral blood leukocytes (J. Rowson and D. Baird, unpublished data). The biology underlying the apparent stability of 17p is unclear, it is conceivable that it may be a consequence of the localized DNA and chromatin structure acting in cis to create a less mutable telomere; this has the potential to interfere with the lengthening of 17p by telomerase, perhaps by restricting access to the terminus and accounting for reduced length in telomerase-expressing cells. However, hTRD events at 17p were observed (but not quantified) in telomerase-expressing cells and our observations in the male germline show that 17p displays similar levels of hTRD to XpYp (15); thus it appears that the relative stability of 17p may be dependent upon the absence of telomerase, which would be capable of stabilizing a critically eroded telomere. The loss of telomeric function can lead to gross chromosomal rearrangements and loss of heterozygosity (LOH) via cycles of anaphase bridging-breakage and fusion (41,42). Furthermore, as pointed out by others (17), it may be pertinent to note that the p53 tumour suppressor gene is located on 17p, and that LOH at this chromosome arm is observed in early-stage neoplasia (43) and is a predictor of progression in Barrett’s oesophagus (23). Therefore we speculate, that a potential explanation for the apparent stability of the 17p telomere or indeed other telomeres that display similar properties in the absence of telomerase could be that; in order to protect against LOH this telomere has evolved to elicit a more stringent response to a critically eroded telomere from the DNA damage machinery, such that cells that suffer hTRD at 17p exit the cell cycle quicker than other chromosome ends.

**MATERIALS AND METHODS**

**Cell culture**

The fibroblasts and MRC5 clonal derivatives used in this study were as previously described (7). We obtained fibroblast strains IMR-90, IMR-91, WI-38, AG05229, AG08049, AG11241 and AG07119A from the Coriell Cell Repository.
and MRC5 human diploid fibroblasts from the European Collection of Cell Cultures. HCA2 fibroblasts, HCA2-hTERT (44), MRC5-hTERT (45) have been described previously. Fibroblast cells were cultured in Eagle’s minimum essential medium supplemented with Earle’s salts containing, 2× non-essential amino acids, 15% (v/v) fetal calf serum, 1 × 10⁵ IU/l penicillin, 100 mg/l streptomycin and 2 mM glutamine. The onset of replicative senescence was defined as at least two weeks of no cell growth and BrdU-labelling indices of <1% (46). To obtain populations of immortal keratinocytes, cell lines derived from head and neck squamous cell carcinoma were plated at low densities to generate clonal colonies. Holoclone colonies, identified by morphological criteria, were isolated with cloning rings.

Telomere length analysis

DNA extractions and STELA reactions were carried out as described previously (7), with the following adaptations: the Taq/Pwo ratio was adjusted from 25:1 to 10:1 and we cycled the reactions with an MJ PTC-225 thermocycler (MJ Research) under the following conditions: 22 cycles (24 for 11q13B) of 94°C for 15 s, 65°C (XpYPE2 and 2p2) or 59°C (17p6, 17pseq1B, 17pseqrev1) or 66°C (12q-550C, 2q-197A and 11q13B) for 30 s and 68°C for 10 min. To ensure specific hybridization of telomeric bands only those that hybridized to both the telomere-adjacent and telomere repeat containing probe were analysed. We calculated the molecular weights of the DNA fragments using the Phoretix 1D quantifier (Nonlinear Dynamics) and undertook statistical analysis of the fragment sizes using Microsoft Excel.

Seven primers were designed at the 17p telomere that was capable of generating identical STELA profiles from the same DNA. The majority of the analysis was undertaken with primer 17p6, the HCA2 and MRC5 parental strains were analysed with 17pseq1B as these DNAs had been previously digested with restriction enzymes (Hinf I and Rsul) that cut the DNA between 17p6 and the telomere. The keratinocyte holoclones were analysed with 17pseqrev1. Two primers were designed at the 12q telomere, the majority of the analysis was undertaken with 12q-550C, the HCA2 and MRC5 parental strains were analysed with 12q-197A.

Telomere-adjacent probes

Telomere-adjacent probes were generated by PCR using the following oligonucleotides: 17p–17p6 and 17p2 primers, 17pseq1A and 17pseqrev1; 12q–12qA and 12qB primers; 2p–2p1 and 2p2; 11q–11q13B and 11q12 primers. Southern hybridization was undertaken in 0.5 M sodium phosphate, pH 7.0, 7% SDS, 1% bovine serum albumin, 1 mM EDTA at 60°C for the (TTAGGG)n, 17p and 12q probes, and 45°C for the 2p probe; and for the 11q probe 50°C in Denhardt’s hybridization buffer (47).

Oligonucleotides

Several primers were designed based on previously published telomere-adjacent sequences (14,22). For those that are used for STELA, the distance from the 5’-end of oligonucleotide to the start of the telomere repeat array are stated in parenthesis.

17p2: 5’ GAGTCAGATTCCATTTGACC 3’
17p6: 5’ GGCTGAACTATAAGCCTTCTG 3’ (3078 bp)
17pseq1B: 5’ CCAAGCGGTTTGAGGTCCTAGG 3’ (50 bp)
12q B: 5’ CCCTCCTGAAATGAGCCTAT 3’
12qA: 5’ GGGACACGATTCTCTT 3’
12q-197A: 5’ GGGAGATCCACCACTGACGTCA 3’ (217 bp)
12q-550C: 5’ ACAGCCTTTGAGGTCCTAGG 3’ (570 bp)
11q12: 5’ CCGATATATCCAGGCGAAAGG 3’
11q13B: 5’ CAGACCTTTGAGGACGCTCTCG 3’ (105 bp)
2p1: 5’ CTAAGCAGCCTAATGTTG 3’
2p2: 5’ GAGCTGCTTTTGCTTAGAC 3’ (183 bp)

17p sequencing

Primers were designed based on sequences showing strong homology to the 17p sub-telomeric sequence. Putative 17p telomere adjacent sequences were amplified using 17pseq1 and 17pseq2, together with 17p6 primer (specific for 17p telomere) and then a 17p telomere-adjacent sequence contig was constructed by sequencing the PCR products with 17p6, 17p2 and the following primers:

17pseq1A: 5’ CCTACGCTCTTCAACCTCGT 3’
17pseq2: 5’ GGTTGCTGGGAAAACATTC 3’
17pseq3: 5’ AGAATCCTGCTCTCAAACTGT 3’
17pseq4: 5’ GTCTACAGTGGCAATCCATGTG 3’
17pseq5: 5’ TTGCAGATATAGGATGTCTGGT 3’
17pseq6: 5’ ACACAGGCAGATTGTTACGT 3’
17pseqrev1: 5’ GAATCAGGTTGTTTGGTTGTC 3’ (311 bp)
17pseqrev2: 5’ CCTTAGCTGTTGGGTGATG 3’ (1113 bp)
17pseqrev3: 5’ TTATAAGCTTTACTGTCTCTCTC 3’ (1548 bp)
17pseqrev4: 5’ GATCATGGGAGGATCATATCTGCG 3’ (1919 bp)
17pseqrev5: 5’ TTGGTGAAATGTGTATTTAAGT 3’ (2367 bp)
17p1: 5’ GCAAAGCAACCACTTAAAAATTGAA 3’

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement

The authors have no conflict of interest.

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