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Repression of an alternative mechanism for lengthening of telomeres in somatic cell hybrids

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Some immortalized cell lines maintain their telomeres in the absence of detectable telomerase activity by an alternative (ALT) mechanism. To study how telomere maintenance is controlled in ALT cells, we have fused an ALT cell line GM847 (SV40 immortalized human skin fibroblasts) with normal fibroblasts or with telomerase positive immortal human cell lines and have examined their proliferative potential and telomere dynamics. The telomeres in ALT cells are characteristically very heterogeneous in length, ranging from very short to very long. The ALT × normal hybrids underwent a rapid reduction in telomeric DNA and entered a senescence-like state. Immortal segregants rapidly reverted to the ALT telomere phenotype. Fusion of ALT cells to telomerase-positive immortal cells in the same immortalization complementation group resulted in hybrids that appeared immortal and also exhibited repression of the ALT telomere phenotype. In these hybrids, which were all telomerase-positive, we observed an initial rapid loss of most long telomeres, followed either by gradual loss of the remaining long telomeres at a rate similar to the rate of telomere shortening in normal telomerase-negative cells, or by maintenance of shortened telomeres. These data indicate the existence of a mechanism of rapid telomere deletion in human cells. They also demonstrate that normal cells and at least some telomerase-positive immortal cells contain repressors of the ALT telomere phenotype.

Keywords: alternative lengthening of telomeres; immortalization; senescence; somatic cell hybridization; telomerase

Introduction

Immortalization of human cells is regarded as a key feature of the malignant phenotype in human cancers (for a review see Bryan and Reddel (1994)). It is dependent not only on a series of key genetic changes but also on maintenance of telomeres which are the specialized repeat sequences found at the ends of linear chromosomes (reviewed in Blackburn (1991)). It has been shown that the telomeres of a normal somatic cell shorten with progressive cell divisions (Olovnikov, 1971; Harvey *et al*., 1990; Hastie *et al*., 1990; Lindsey *et al*., 1991; Allsopp *et al*., 1992; Allsopp and Harley, 1995). This appears to be due, at least in part, to passive loss caused by the 'end replication problem' (Watson, 1972; Levy *et al*., 1992), and recent evidence suggests that there may also be active shortening of the C-rich telomeric strand (Makarov *et al*., 1997; McElligott and Wellinger, 1997). According to the telomere hypothesis of senescence the limited replicative potential of normal cells is due to the telomeres reaching critically short lengths after a number of cell divisions and consequently triggering senescence (Olovnikov, 1971), for review see Reddel (1998).

Unlike normal somatic cells, the majority of immortalized human cell lines express telomerase (Kim *et al*., 1994; Bacchetti and Counter, 1995), a ribonucleoprotein enzyme that adds telomere repeats to chromosome ends thus compensating for telomere shortening (Greider and Blackburn, 1985). Some immortalized cell lines, however, do not have any detectable telomerase activity but have a highly characteristic pattern of telomere lengths that range from very short to abnormally long (Murnane *et al*., 1994; Bryan *et al*., 1995, 1997). This pattern of heterogeneous telomere lengths is maintained over many hundreds of population doublings (Rogan *et al*., 1995), indicating that telomere maintenance can occur in the absence of detectable telomerase activity. This is referred to as Alternative Lengthening of Telomeres (ALT) (Bryan and Reddel, 1997). To date all immortalized cell lines examined have been found to have either telomerase activity or evidence for ALT. Telomere maintenance thus appears to be an important aspect of the unlimited replicative potential of immortalized cells.

The nature of the ALT mechanism is currently unknown. In order to identify this mechanism and the genes involved it will be important to determine whether ALT occurs due to a dominant activating mutation or due to the loss of repressors which are present in normal cells.

About two decades ago experiments involving the fusion of normal and immortal human cells showed that the immortalized phenotype is recessive (Bunn and Tarrant, 1980; Muggleton-Harris and DeSimone, 1980; Pereira-Smith and Smith, 1983). Such somatic cell hybrids have a finite replicative lifespan, presumably due to factors present in the normal cells that are able to reimpose normal proliferation control. It has also been shown that fusion of different immortal cells with each other may result in a finite replicative lifespan,

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that is, different immortal cells may complement each other for the mortal phenotype (Pereira-Smith and Smith, 1983). At least four complementation groups for immortality have been defined (Pereira-Smith and Smith, 1988) and this may be interpreted to mean that immortalization may occur due to different genetic lesions.

To determine how the ALT mechanism is controlled we have fused immortal ALT cells to normal cells, and also to immortal telomerase-positive cells in the same complementation group as the ALT cells. We present evidence that the ALT mechanism is repressible by factors present in normal somatic cells, leading to significant loss of telomeric DNA. We also show that the fusion of ALT cells with telomerase-positive cells is able to repress the ALT telomere phenotype. The results indicate that repression of ALT leads to loss of large telomere tracts and conversely reactivation of ALT results in rapid relengthening of telomeres.

Results

Characterization and growth analysis of GM847 × HFF5 hybrids

Following fusion of the SV40-immortalized cell line GM847 that has the ALT phenotype (Bryan *et al*., 1995) with HFF5 normal diploid fibroblasts, ten colonies of hybrid cells were seeded and cultured individually. Each hybrid clone proliferated for at least 23 population doublings (PD) before entering a period of growth arrest (Figure 1) accompanied by morphological features of senescence (not shown). This growth arrest persisted for a different length of time for each clone with G and J showing the most prolonged period of senescence.

Escape from senescence occurred in every case due to revertant subpopulations which have previously been documented to arise at a rate of 2×10^{-6} in normal × immortal somatic cell hybrids (Bunn and Tarrant, 1980). These were readily visualized as small patches of dividing cells among the arrested cells (not shown). Clones, F, H and I show only marginal retardation of their growth curves which is most likely due to outgrowth of at least one revertant at an early timepoint. This is further evidenced by similar pre- and post-senescent growth rates for these three clones such that revertant clones could begin to take over the mass culture at early PDs (Figure 1). Other clones that have proliferation arrest periods of between 25–75 days have slower growth rates following escape from senescence (Figure 1) and this presumably reflects later reversion by slower growing immortal clones that would not outgrow the cultures at earlier PDs.

To verify that each clonal culture is a GM847 × HFF5 hybrid, PCR fingerprinting analysis was performed at the vWA31, THO1, F13A1 and FES/FPS loci for pre- and post-senescent cultures. The resulting band peaks for pre-senescent clones are listed in Table 1. Each of these contained bands that corresponded to a combination of GM847 and HFF5 alleles at each locus indicating that both genomes are

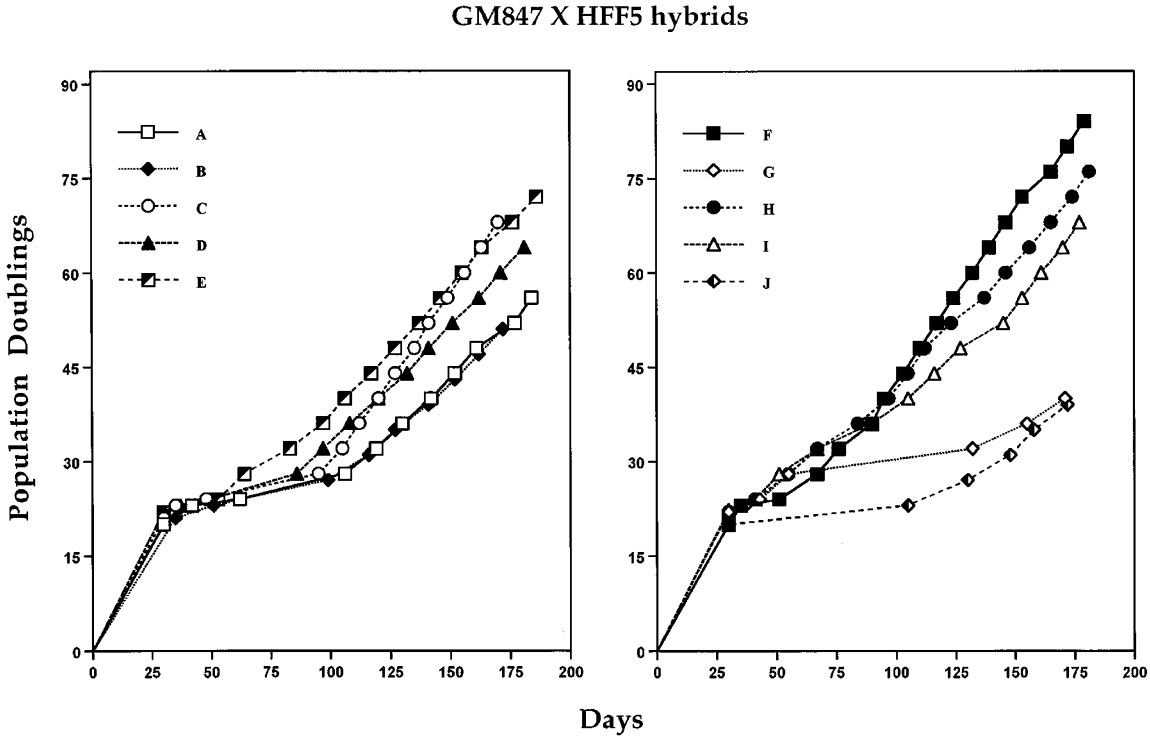

Figure 1 Growth curves of GM847 × HFF5 hybrid clones. GM847 SV40-immortalized fibroblast cells (ALT) were fused with HFF5 normal fibroblasts using polyethylene glycol on day 0 and selected with HAT medium and ouabain. Ten individual colonies (A–J) were isolated and passaged separately. Cumulative population doublings were calculated at each passage

present in these cultures. Analysis of post-senescent cultures (data not shown) indicates that all of the GM847 loci are still present but that there has been major loss of HFF5 loci from clones C, D, E and I. We detected no additional alleles in any clone other than the vWA31 149 bp marker detected in clones B and I which may be due to chromosomal rearrangements or clonally derived from the parent population. 6/10 hybrid cultures retained between 8–14 of the markers tested following escape from senescence.

DNA flow cytometric analyses showed that the pre-senescent cultures did not consist of mixtures of cells with differing DNA contents (data not shown), thus excluding the possibility that the parental cells were co-existing as an unfused mixed culture. Thus, the data clearly indicate that hybrid cultures were obtained. An incidental finding was that clones D and E had a DNA content approximately 30% smaller than the sum of the DNA content of the two parental cells indicating that substantial chromosome losses had occurred from the nuclei of these two hybrid clones. Additionally all of the pre- and post-senescent cultures were positive for SV40 T-antigen expression as determined by immunohistochemistry (data not shown). Repression of ALT

Table 1 DNA fingerprint analyses of HFF5 × GM847 hybrid clones

	Allele size (bp) at locus[a]			
	vWA31	*TH01*	*F13A1*	*FES/FPS*
Parental cells				
HFF5	140, 153	160, 172	188, 196	223, 227
GM847	144	163, 167	184, 192	211, 227
Hybrids				
A	140, 145, 153	164, 168, 171	184, 188, 192	211, 223, 227
B	140, 145, 149, 153	160, 164, 168, 172	184, 188, 192, 196	211, 223, 227
C	140, 144, 153	160, 164, 168, 172	184, 188, 192, 196	211, 227
D	140, 145, 153	160, 164, 168, 172	184, 188, 192, 196	211, 227
E	140, 145, 153	160, 164, 168, 172	184, 188, 192, 196	211, 227
F	140, 145, 153	160, 164, 168, 172	184, 188, 192, 196	211, 223, 227
G	140, 145, 153	160, 164, 168, 172	184, 188, 192, 196	211, 223, 227
H	140, 145, 153	160, 164, 168, 172	184, 188, 192, 196	211, 223, 227
I	140, 145, 149, 153	160, 164, 168, 172	184, 188, 192, 196	211, 224, 228
J	140, 145, 153	160, 164, 168, 172	184, 188, 192, 196	211, 224, 228

[a]Allele sizes at the indicated loci were determined by PCR amplification as described in Materials and methods

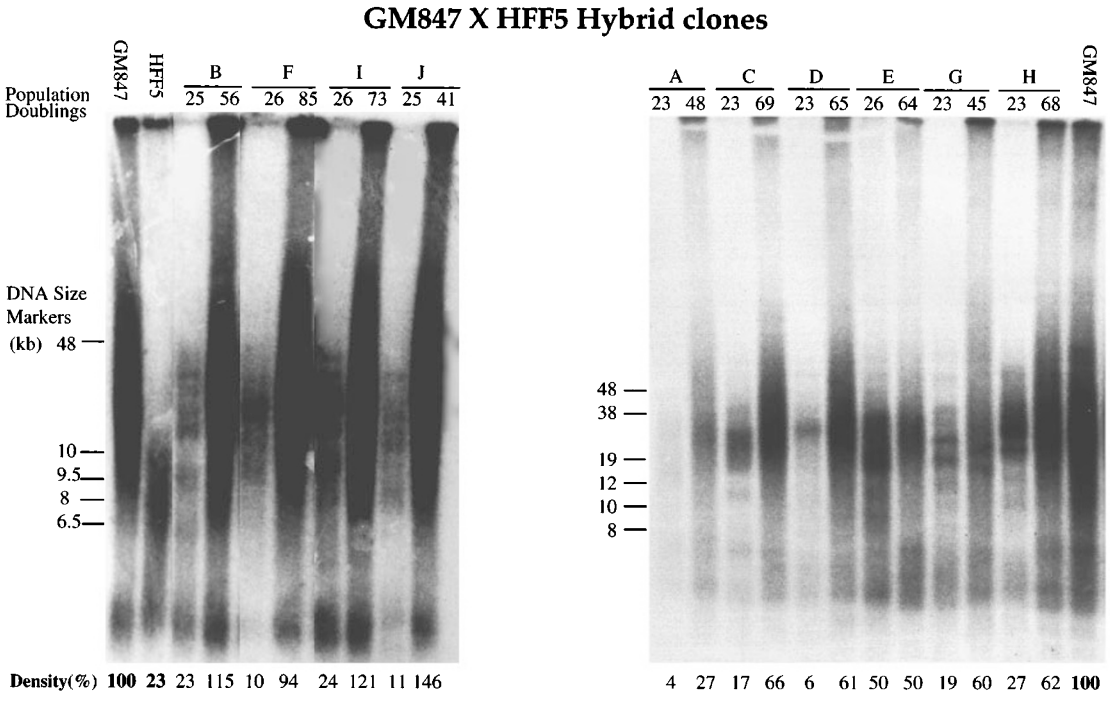

Figure 2 Terminal restriction fragment analyses of GM847 × HFF5 hybrids and parental cells. Hybrid clones were analysed at or before senescence (at population doublings 23–26) and after revertant cells had escaped from senescence. Equal amounts of genomic DNA digested with Rsa1 and Hinf1 restriction enzymes were loaded into each lane and subjected to pulsed-field gel electrophoresis. Dried gels were hybridized to a radiolabeled $(TTAGGG)_3$ probe. Each lane was scanned by a densitometer and the total density was expressed as a percentage of that for GM847

was therefore not due to repression of T-antigen expression.

Telomere dynamics in pre- and post-senescent GM847 × HFF5 hybrids

As is characteristic of telomerase-negative immortalized human cells, GM847 cells have terminal restriction fragment (TRF) lengths that range from very short to abnormally long (Bryan *et al*., 1995). To determine whether normal cells contain factors that repress the telomere maintenance mechanism in these ALT cells, the TRF lengths of pre- and post-senescent hybrid cultures were analysed, using pulsed field gel electrophoresis to separate the TRFs as described (Bryan *et al*., 1995). The results illustrate the difference in TRF patterns between the GM847 and HFF5 cells (Figure 2). The TTAGGG smear pattern present in the GM847 track is characteristic of ALT cells and the low molecular weight 'bands' in many of the lanes in Figure 2 are the result of the pulsed field electrophoresis conditions. These bands do not appear in standard gels. *Bal 31* nuclease digestion of TRF samples of ALT cell types described previously has revealed that the majority of the hybridization signal is telomeric (Bryan *et al*., 1995). Densitometric analyses showed that the HFF5 and GM847 cultures had mean TRF lengths of approximately 5 kb and 20 kb respectively, and the total (TTAGGG)n hybridization signal of HFF5 was 23% that of GM847 (Figure 2). Fusion of GM847 and HFF5 nuclei would be expected to result in some reduction of hybridization intensity due to 'dilution' of the long GM847 telomeres with HFF5 telomeres. DNA flow cytometry showed that the DNA content of GM847 cells is 1.3-fold greater than that of HFF5 cells. Assuming that this difference is reflected in telomere numbers, cell fusion would therefore reduce the hybridization intensity to $([1 \times 23\%] + [1.3 \times 100\%])/2.3$, or 67% of the GM847 intensity.

In 9/10 hybrid clones prior to senescence there was much more striking reduction in telomeric DNA. Densitometry of clone E showed equal tract densities at both PDs tested and this is presumably due to successful outgrowth by immortal revertants by PD 26 and, consistent with this, the growth curve for clone E indicates a shorter period of growth retardation (Figure 1). The total (TTAGGG)n hybridization signal of all other clones ranged from 27% of that in GM847 cells (in pre-senescent hybrid clone H) down to 4% (in pre-senescent hybrid clone A). When these cultures escaped from senescence, however, the TRFs reverted to the ALT pattern. These data are consistent with repression of the ALT telomere maintenance mechanism in the hybrids, followed by its reactivation in the immortal revertants that escape from growth arrest.

Interestingly, the observed telomere loss in the pre-senescent hybrids appear to be greater than would be expected to occur at the rate seen in normal telomerase negative cells. Loss at the rate of 50–200 bp per PD as in normal somatic cells (Cooke and Smith, 1986; Harley *et al*., 1990; Hastie *et al*., 1990) would result in 1.3–5 kb loss over the 23–26 PD prior to growth arrest, which would correspond to a reduction in hybridization intensity to 40–58% of GM847, but we show a greater reduction than this, with some clones

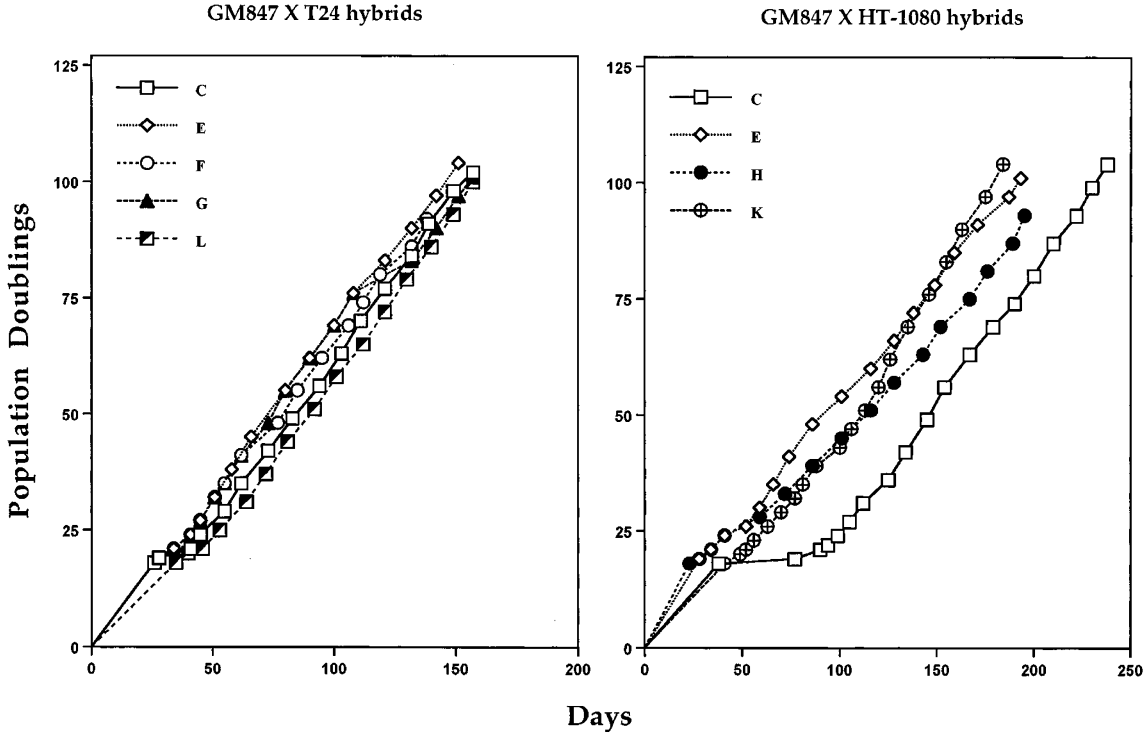

Figure 3 Growth curves of GM847 × T24 and GM847 × HT-1080 hybrid clones. At day 0 GM847 (ALT) cells were fused with T24 or HT-1080 (telomerase-positive) cell lines and hybrids were selected and passaged as described for Figure 1. Cumulative population doublings were calculated at each passage

having very much greater reductions in TTAGGG hybridization intensity (Figure 2). This suggests that there is an active mechanism at work that causes a more rapid attrition of telomeric DNA.

Although the total (TTAGGG)n hybridization intensity was greatly reduced in these clones this was not always reflected by a corresponding decrease in mean telomere length. Figure 2 shows that the mean telomere lengths in the pre-senescent clones were only marginally reduced, and this was confirmed by densitometry (not shown). The simplest explanation is that a very substantial proportion of the cell population, that was very close to entering a senescence-like state at the time the TRF analyses were performed, had lost the great majority of telomeric DNA. In this circumstance, a small subpopulation of immortal revertant cells that had already lengthened their telomeres would have a greatly disproportionate effect on the apparent mean telomere length.

The rate of telomere lengthening in the immortal revertant cultures is difficult to assess because of the small number of cells available immediately after reversion: standard TRF analyses require microgram quantities of DNA and therefore a considerable expansion of the cell population before the first possible timepoint. Densitometric analyses (not shown) indicated that the mean TRF length of clone J increased 17 kb between PD 25 and 41, an average lengthening of >1 kb/PD. However, this calculation ignores the considerable (but unknown) number of cell divisions required for the immortal revertant cells to overgrow the culture initially. Also in the absence of TRF analyses at early time points the data cannot preclude the possibility that very rapid lengthening occurred in the first few PD after reversion to immortality.

The results indicate that normal cells are able to repress the ALT telomere phenotype and that immortal reversion is associated with reactivation of telomere maintenance via ALT. We have also fused GM847 cells with two other strains of normal human diploid fibroblasts (MRC-5 and WI38) and in both cases the results confirmed those obtained with the GM847 $\times$ HFF5 fusions.

Telomere dynamics in hybrids of ALT and telomerase-positive cells

To study the effect of fusing telomerase-positive cells with GM847 ALT cells upon both telomerase activity and the ALT telomeres, we analysed clones from two sets of fusions, GM847 $\times$ HT-1080 (G/HT) and GM847 $\times$ T24 (G/T). The telomerase-positive HT-1080 and T24 cell lines were chosen because they have been assigned to the same immortalization complementation group as GM847 cells (group A) (Pereira-Smith and Smith, 1988; Whitaker *et al*., 1995). These hybrids would therefore be expected to have an immortal phenotype. All but two of the hybrid clones proliferated without any period of growth arrest (Figure 3). A telomere repeat amplification protocol (TRAP) assay showed that each of the four G/HT and five G/T hybrids had telomerase activity (Figure 4) and single locus DNA fingerprinting of each clone at the same timepoint at which the TRAP assay was performed confirmed the presence of both parental alleles in each clone and that they were genuine hybrids (data not shown).

To assess the impact of the fusions upon the ALT telomeres in these immortal $\times$ immortal hybrids we analysed the TRF lengths of the hybrid clones at various PD levels (Figure 5). In each case there had been a marked reduction in telomere length by the first time point at which DNA was available for analysis. This was not due to dilution of the long telomeres by the telomeres from the telomerase-positive parent because a mixture of the parental DNAs showed the characteristic ALT TRF pattern (Figure 5). Thus the presence of telomerase activity does not prevent shortening of the ALT telomeres. Although there was clear evidence of shortening in each of the hybrids, the amount of shortening seen at the first available PD level varied among the clones with the least amount of shortening being seen in G/HT clone E and the most in G/HT clone K.

Analysis at later PD levels showed continued shortening of TRFs in all clones except G/HT clone K (Figure 5). DNA was only available at a single PD time point for analysis of G/HT clone H. The rate of shortening can be quantitated most accurately for the discrete bands, and measures between 38–81 bp/PD which is consistent with the rate of normal somatic cell telomere shortening (Cooke and Smith, 1986; Harley *et al*., 1990; Hastie *et al*., 1990). G/HT clone K already had short telomeres by PD 32, the earliest PD level for which DNA was available, and this length was maintained at PD 71 and 97. A faint high molecular weight band seen in most lanes of Figure 5 was not

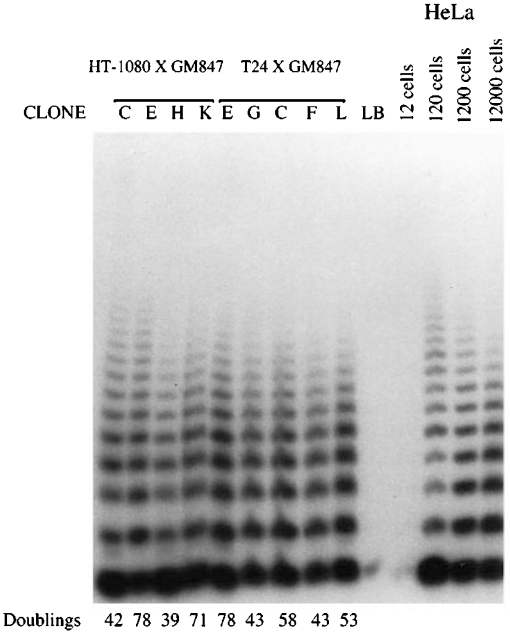

Figure 4 Telomerase activity of hybrids between GM847 (ALT) and either HT-1080 or T24 (telomerase-positive) cells. The TRAP assay was performed on a CHAPS detergent cell lysate equivalent to 12 000 cells for each hybrid clone at the population doubling level indicated. Lysis buffer (LB) was used as a negative control and HeLa cell lysate at the indicated number of cell equivalents (12, 120, 1200, 12 000) was used as a positive control

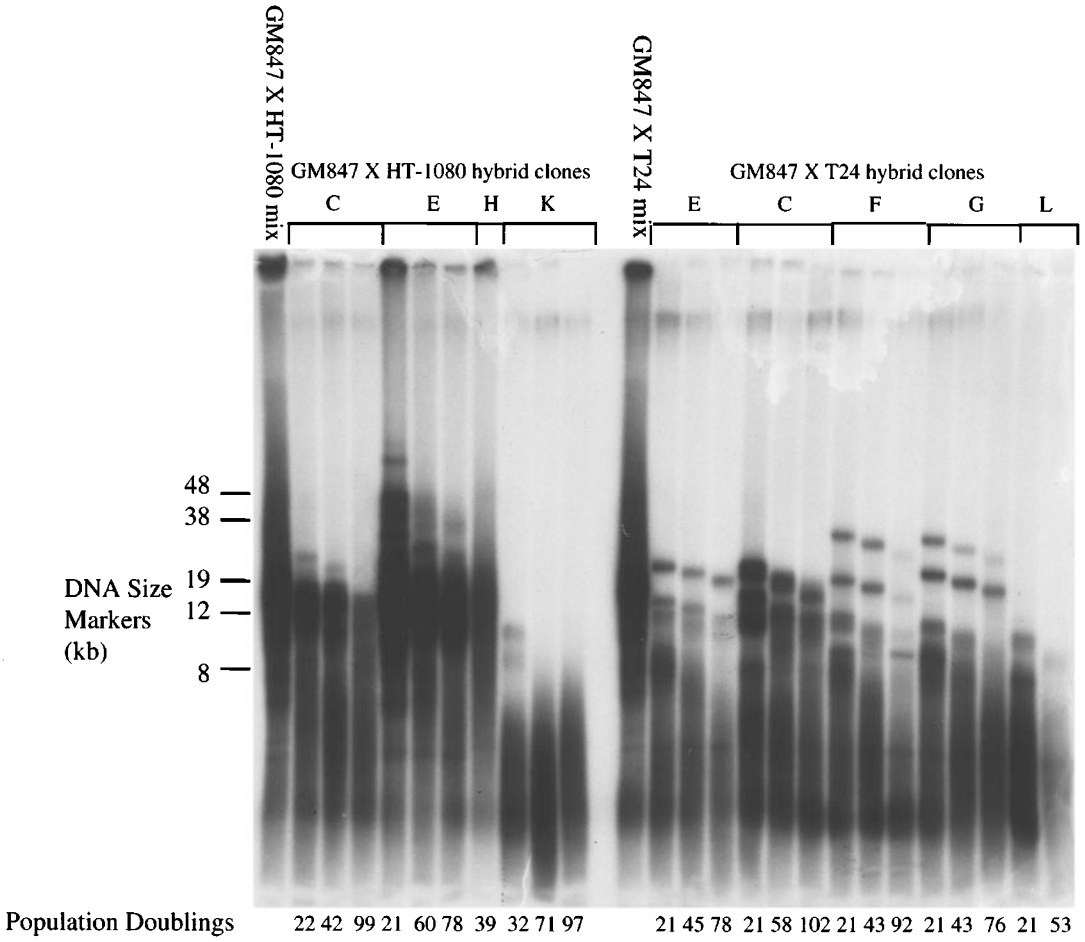

Figure 5 Terminal restriction fragment analyses of hybrids between GM847 and either HT-1080 or T24 at the population doubling levels indicated were performed as described for Figure 2. Controls were 1 : 1 mixtures of digested genomic parental DNA corresponding to each set of fusions. Both T24 and HT-1080 parental TRFs (not shown) show band smears up to the 8 kb size marker

seen in previous gels, even at long exposures, where these same DNA samples were analysed. We consider this band to be a non-specific binding artifact.

Discussion

These data demonstrate that the ALT telomere phenotype is repressed in hybrids between ALT cells and either normal cells or telomerase-positive cells. This is the first report of repression of ALT by fusion of normal and immortal cells, and the first report of the repression of ALT in non-complementary fusions of immortal cell lines. Taken together these data indicate that one or more repressors of ALT exist in both normal cells and immortal telomerase-positive cells. The repression of ALT in immortal × immortal hybrids that retain telomerase activity indicates that these telomere maintenance mechanisms may be regulated independently. Repression of ALT has previously been seen in the immortal revertant cells from one hybrid clone formed by fusion of GM847 (immortalization complementation group A) and BET-1A (group D) cell lines (Bryan *et al*., 1995). The results with the immortal cell lines are in contrast to those in a recent study of non-complementary fusions between ALT and telomerase-positive cell lines in Group D, in which no evidence for repression of ALT was found (Katoh *et al*., 1998).

The existence of at least two repressors of ALT was initially suggested by the observation that ALT cell lines are found in more than one immortalization complementation group (A and D) (Whitaker *et al*., 1995). One repressor appears to be located on chromosome 7 because reduction of telomere length has been shown in an ALT cell line, SUSM-1, following transfer of a normal copy of this chromosome (Nakabayashi *et al*., 1997). Chromosome 7 only causes senescence in cells assigned to group D (Ogata *et al*., 1993, 1995), so it is unlikely that it would repress ALT in GM847 cells which are in group A (Pereira-Smith and Smith, 1988). Like the nature of the ALT mechanism itself, the nature of these putative repressors is unknown. One possibility would be that they are transcriptional repressors of genes encoding

products that are involved in the ALT process. Another possibility would be that they are molecules that inhibit ALT. There is evidence that telomeric recombination can provide a backup mechanism for telomere maintenance in yeast (Lundblad and Blackburn, 1993; McEachern and Blackburn, 1996). If ALT involves recombination, it is possible that there are telomere binding proteins that can act as repressors of ALT by making telomeric DNA inaccessible to the recombination machinery.

The telomere loss in our hybrids that initially occurred at a rate greater than that seen in telomerase-negative normal cells raises the possibility that there may be an active telomere shortening mechanism. In support of this suggestion, there is evidence for a rapid telomere deletion mechanism in yeast that may be controlled by telomere binding proteins and involve recombination (Li and Lustig, 1996). Cleavage of long telomeres to wild type size may occur within a single cell division (Li and Lustig, 1996). Further, tagged telomeres in telomerase-negative immortal human cells were occasionally found to undergo rapid shortening and subsequent rapid relengthening, indicating that telomerase activity is not essential for rapid telomere shortening (Murnane *et al*., 1994). The telomere loss evidenced in our GM847 × HFF5 hybrids suggests that ALT is also not required for rapid shortening as presumably it has already been repressed. This suggests the possibility that telomere maintenance via ALT primarily involves rapid relengthening to balance out continuing shortening, either gradual or rapid.

Telomerase activity present in the immortal × immortal hybrids does not maintain the telomeres that are still long but these shortening telomeres have not reached lengths that will trigger cell cycle exit. In the single clone (G/HT clone K) where rapid shortening resulted in a telomere length within the range typically seen in telomerase-positive cell lines by PD 32, the telomere length was maintained over the subsequent 65 PDs (Figure 5). This is consistent with a feedback mechanism of telomere length control which has been proposed in yeast (Shore, 1997). Also in support of such a proposal are studies that were conducted in our laboratory with telomerase positive 293 cells which revealed shortening high molecular weight TRF bands (Bryan *et al*., 1998). Another study of a human thyroid cancer cell line also showed occasional telomere shortening with no variation in levels of telomerase activity over continuing passages in culture (Jones *et al*., 1998). Although all of our immortal × immortal hybrids have been cultured past 100 PDs their continuing survival, and consequently immortalization status, depends upon telomeres being maintained as seen for G/HT clone K.

The question arises why expression of telomerase is not repressed in hybrids between the telomerase-negative GM847 cells and the telomerase-positive HT-1080 or T24 cells. It is likely that whereas GM847 cells cannot activate telomerase they also cannot repress its activity and thus separate factors are involved. An alternative explanation is that GM847 cells do in fact contain a telomerase repressor but that this is usually lost from the hybrids shortly after fusion.

The mechanism(s) responsible for telomere maintenance in telomerase-negative immortalized cells are currently unknown. The results described here indicate that the activation of ALT involves the loss of repressors present in normal cells and in some telomerase-positive immortalized cells. We also present evidence for an initial rapid reduction of telomeric sequence in hybrid cells in which the ALT phenotype has been repressed, and subsequent more gradual telomere attrition in telomerase-positive hybrids suggesting a telomere length feedback control mechanism. These data have obvious implications for designing strategies to identify genes involved in ALT and other aspects of telomere length control.

Materials and methods

Cell lines

SV40 immortalized human skin fibroblast GM847DM ('double mutant', ouabain and 6-thioguanine resistant) universal hybridizer cells were obtained from Dr O Pereira-Smith, Baylor College of Medicine, Houston, Texas, USA, and HFF5 cells (normal human foreskin fibroblasts) were obtained from Dr R Böhmer, Ludwig Institute for Cancer Research, Melbourne, Australia. HT-1080 (fibrosarcoma) and T24 (bladder carcinoma) cell lines were obtained from the American Type Culture Collection. All parental and fused cells were cultured in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 50 μg/ml gentamicin.

Cell-cell hybridization

GM847DM cells were placed into 75 cm^2 flasks (2×10^6 cells/flask) 4 h prior to addition of the cells to which they were to be fused (HFF5, HT-1080 or T24; also 2×10^6 cells/flask). After an additional 4 h the culture medium was removed and the cells were fused by treatment for 1 min with 48% polyethylene glycol (Sigma). The cells were then washed three times with DMEM medium and incubated overnight in DMEM containing 10% FBS prior to addition of selection reagents. GM847DM universal hybridizer cells are resistant to 10^{-6} M ouabain and sensitive to HAT, medium (DMEM/10% FBS supplemented with 10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, 1.6×10^{-5} M thymidine (Sigma)) whereas HFF5, HT-1080 and T24 cells are sensitive to ouabain and resistant to HAT medium. Selection for fused cells was carried out by incubation with HAT medium supplemented with 10^{-6} M ouabain (Sigma). Controls were established by self fusion of parental cells for each cell type and also by mixing unfused parental cells; none of the controls survived the selection. All hybrid clones were expanded and passaged continuously to determine their proliferative potential.

DNA fingerprinting

GM847 × HFF5 fusion hybrid clones were analysed by DNA fingerprinting using the ABI PRISM STR primer set (Perkin Elmer) for the following loci: vWA31 (von Willebrand's factor), THO1 (Tyrosine hydroxylase gene), F13A1 (Coagulation factor XIII subunit), and FES/FPS (fes/fps proto-oncogene). PCR was carried out upon genomic DNA from each of the parental cell types and from all of the hybrid clones according to the manufacturer's instructions. Resulting band peaks were analysed by an Applied Biosystems Genescan analyser. Hybrid clones were identified by the appearance of band peaks representing alleles from both parental cell types. GM847 × HT-1080 and GM847 × T24

hybrids were fingerprinted using a single locus MS43A probe as previously described (Moy *et al*., 1997).

Terminal restriction fragment (TRF) analysis

Genomic DNA was prepared from the parental and hybrid cultures and 40 μg was digested with restriction enzymes Hinf1 and Rsa1 which cut throughout the genome but not within telomeres. The digested DNA was quantitated by fluorometry and 0.6 μg was loaded onto a 1% agarose gel in 0.5 × Tris-borate-EDTA (TBE) buffer and separated by pulsed-field electrophoresis using a CHEF-DRII apparatus (BioRad) in recirculating 0.5 × TBE buffer at 14°C with a ramped pulse speed of 1–6 s at 200 V for 14 h. The gels were dried, denatured and hybridized to a γ-^{32}P-dATP 5′ end labeled telomeric oligonucleotide probe $(TTAGGG)_3$, and exposed to Kodak XAR film at room temperature for 18 h as described (Bryan *et al*., 1995).

Telomere repeat amplification protocol (TRAP) assay

The PCR based TRAP assay for telomerase activity was used as described (Kim *et al*., 1994). Cell lysates were prepared using the CHAPS detergent lysis method and 12 000[3] cells were used in each assay. The protein concentration of the lysates was measured using the BioRad Protein Assay Kit. For lysates containing telomerase activity the PCR amplification results in a 6 bp ladder seen when the products are separated on a 10% non-denaturing polyacrylamide gel.

Computing densitometry

TRF gel lanes were scanned by a computing densitometer (Molecular Dynamics, Sunnyvale CA, USA) and total optical density (OD) of TTAGGG repeats was calculated for each lane. Mean TRF length was calculated as $\Sigma\ (OD_i)/\Sigma(OD_i/L_i)$ where OD_i is the densitometer output and L_i is the DNA length at position *i* in the lane.

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