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Telomere dynamics in an immortal human cell line

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The integration of transfected plasmid DNA at the telomere of chromosome 13 in an immortalized simian virus 40-transformed human cell line provided the first opportunity to study polymorphism in the number of telomeric repeat sequences on the end of a single chromosome. Three subclones of this cell line were selected for analysis: one with a long telomere on chromosome 13, one with a short telomere, and one with such extreme polymorphism that no distinct band was discernible. Further subcloning demonstrated that telomere polymorphism resulted from both gradual changes and rapid changes that sometimes involved many kilobases. The gradual changes were due to the shortening of telomeres at a rate similar to that reported for telomeres of somatic cells without telomerase, eventually resulting in the loss of nearly all of the telomere. However, telomeres were not generally lost completely, as shown by the absence of polymorphism in the subtelomeric plasmid sequences. Instead, telomeres that were less than a few hundred base pairs in length showed a rapid, highly heterogeneous increase in size. Rapid changes in telomere length also occurred on longer telomeres. The frequency of this type of change in telomere length varied among the subclones and correlated with chromosome fusion. Therefore, the rapid changes in telomere length appeared occasionally to result in the complete loss of telomeric repeat sequences. Rapid changes in telomere length have been associated with telomere loss and chromosome instability in yeast and could be responsible for the high rate of chromosome fusion observed in many human tumor cell lines.

Key words: chromosome fusion/immortality/telomere instability

Introduction

The ends of eukaryotic chromosomes, called telomeres, are composed of short repeat sequences (Blackburn, 1990, 1991). In mammals this repeat sequence is TTAGGG (Moyzis *et al.*, 1988). Telomeric repeat sequences are added on by the enzyme telomerase (Greider and Blackburn, 1985) and are believed to form specialized structures that protect the ends of chromosomes and prevent chromosome fusion (McClintock, 1941; Hastie and Allshire, 1989). Telomerase activity has not been demonstrated in primary human cells; telomeres become shorter with age, both in culture (Harley *et al.*, 1990) and *in vivo* (Harley *et al.*, 1990; Hastie *et al.*, 1990). Repeat sequences may be lost both by degradation by exonucleases and by the inability of DNA polymerase to complete synthesis on the lagging strand of linear DNA molecules (Watson, 1972). The loss of telomeric repeat sequences has been proposed as a possible mechanism for cell senescence (Harley, 1991).

Immortal mammalian cells commonly express telomerase (Morin, 1989; Counter *et al.*, 1992, 1994), which has been proposed to be necessary for the continuous protection of chromosome ends. Telomerase can heal broken chromosomes (Wilkie *et al.*, 1990) but could theoretically also promote terminal deletion and chromosome rearrangement by competing with repair enzymes for DNA strand breaks (Murnane and Yu, 1993). As a result, plasmid DNA containing telomeric repeat sequences is commonly found at the ends of truncated chromosomes after transfection (Farr *et al.*, 1991; Barnett *et al.*, 1993). Plasmid DNA that had telomeric repeat sequences added to both ends intracellularly was also found at the site of a break on chromosome 13 in the immortal simian virus 40 (SV40)-transformed human cell line KB319 (Murnane and Yu, 1993). The creation of the interstitial telomeric repeat sequences that were also seen at this integration site could be another mechanism for chromosome rearrangement, because these sequences have been associated with chromosome breakage (Hastie and Allshire, 1989; Farr *et al.*, 1991; Bouffler *et al.*, 1993).

The loss of telomeric repeat sequences from the ends of chromosomes is another possible mechanism for chromosome instability. Loss of telomeres has been proposed to explain the increased rate of chromosome fusion seen in many tumor cells (Hastie and Allshire, 1989), which could initiate the breakage–fusion–bridge cycles associated with gene amplification (Smith *et al.*, 1992; Toledo *et al.*, 1992; Ma *et al.*, 1993). Despite the presence of telomerase in tumor cells, telomeres are generally shorter in tumor cells than in cells from normal tissues (DeLange *et al.*, 1990; Hastie *et al.*, 1990), although no correlation has been seen between average telomere length and the rate of chromosome fusion (Saltman *et al.*, 1993). However, because the studies cited looked at the average length of telomeres on multiple chromosomes, variation in length on individual chromosomes would not have been detected. Considerable variation in individual telomere length could therefore occur in tumor cells without changes in the average telomere length.

A previous study that demonstrated the presence of a plasmid integrated at the telomere of chromosome 13

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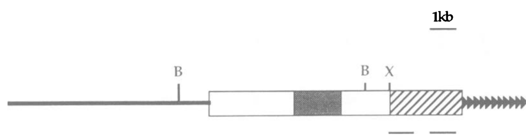

Fig. 1. The structure of the integrated plasmid sequences at the end of the truncated chromosome 13 in cell line KB319. The integrated plasmid (boxed area) contains a selectable *neo* gene (dark box) as well as nonrepetitive human sequences (hatched box). The chromosomal DNA (solid line) and repeat sequences (triangles) on the new telomere are shown. The restriction sites for *Xba*I (X) and *Bam*HI (B) and the location of the human sequences in the plasmid that were used as a probe for Southern blot analysis (underlined) are indicated.

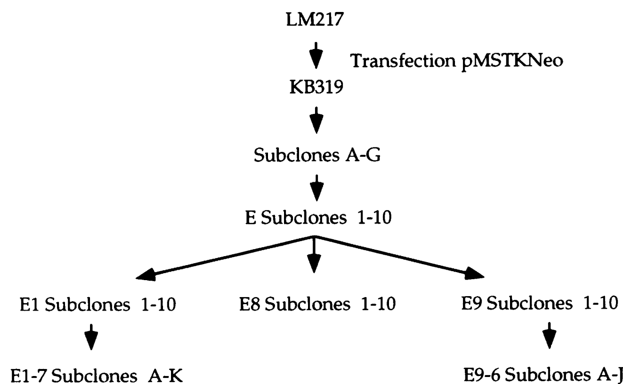

Fig. 2. The lineage of various subclones of cell line KB319. Cells were grown for ~25 cell generations between each subcloning.

(Murnane and Yu, 1993) provided the opportunity to study telomere dynamics on a single chromosome. The use of an integrated sequence as a probe avoided the problem of cross-hybridization of subtelomeric sequences, and therefore the minimal telomere length could be established. Initial studies demonstrated that considerable polymorphism occurred at this telomere (Murnane, 1990). In the present study we further investigated the variability in the length of the telomere on this chromosome to study the mechanisms of addition and loss of telomeric repeat sequences and their role in chromosome instability in mammalian cells.

Results

Analysis of telomere repeat polymorphism on a single chromosome

An immortal SV40-transformed cell line, KB319, contains an integrated plasmid that acquired telomeric repeat sequences after transfection (Figure 1) and is located on the end of a truncated long arm of chromosome 13 (Murnane and Yu, 1993). Sequences within the plasmid were used as a probe for Southern blot analysis, which revealed telomere length polymorphism on the end of this chromosome (Murnane, 1990; Murnane and Yu, 1993). As described previously in yeast (Shampay and Blackburn, 1988), specific DNA fragments containing telomeric repeat sequences produce diffuse bands because of loss or gain of nucleotides from the end of the chromosome. The size of these bands depends on the length of the telomeric repeat sequences on the chromosome in the founder cell from which each subclone was derived. The first-generation (A–G) and second-generation (E1–E10) subclones of KB319 (Figure 2) showed extensive polymorphism in telomere length on chromosome 13 (Murnane, 1990). The extent of polymorphism was remarkable in view of the facts that all of the cells in the second-generation subclones (E1–E10) were descended from a single founder cell from which the first-generation subclone (E) was derived, and that only ~25 cell doublings had occurred before subcloning and Southern blot analysis. The polymorphism appeared to be confined to the telomeric repeat sequences, because no polymorphism was observed in the subtelomeric plasmid sequences (Murnane, 1990; Murnane and Yu, 1993).

In the present study, three of the second-generation subclones (E1, E8 and E9) were selected for additional subcloning (Figure 2) and analysis of telomere polymorphism. *In situ* hybridization confirmed that each of these subclones contained only a single marker chromosome 13 with the plasmid sequences at the telomere, although cells in the population that had doubled their chromosome number had two copies of this chromosome (unpublished observation). As in a previous study (Murnane, 1990), Southern blot analysis was performed by digesting DNA with *Xba*I, which cuts 3.0 kb from the telomeric repeat sequences on the end of the chromosome (Murnane and Yu, 1993), and nonrepetitive human DNA contained within the integrated plasmid was used as a probe. Because the human DNA used as a probe was also found at its original location in the genome, the stable band generated by this fragment served as an excellent internal control. One of the subclones selected for further analysis (E8) showed a diffuse band of ~9 kb, which, after subtracting the length of the subtelomeric plasmid DNA (3.0 kb), indicated telomeric repeat sequences with an average length of 6 kb (Figures 3 and 4); a second subclone (E9) showed a diffuse band of ~4 kb, indicating telomeric repeat sequences with an average length of 1 kb (Figure 5); a third subclone (E1) showed only a smear with no apparent band, indicating extreme length polymorphism within the telomeric repeat sequences (Figure 6).

The subclones of E8 (Figure 3), which originally had 6 kb of telomeric repeat sequences on the end of chromosome 13, commonly showed both a main telomere band and additional lighter bands. The main bands were similar in size to those seen in the parental E8 cell line, although in five of 10 subclones the bands in the subclones were somewhat smaller. Most changes in telomere length therefore appear to be gradual. The lighter bands that were also present were much more heterogeneous in size, varying from ~4.5 kb (Figure 3, lanes 5, 9 and 11) to >23 kb (Figure 3, lanes 7 and 10). In addition to the bands, there was a low level of background hybridization that extended over many kilobases. This low level of background hybridization is more clearly seen with the use of a restriction enzyme that produces larger telomere fragments (see Figure 8 in Murnane, 1990). It was not due to nonspecific hybridization, because it was not detected in the parental cell line, which did not contain the integrated plasmid at its telomere (Figure 3, lane 1). The presence of heterogeneous lighter bands and diffuse background within the subclones was apparently due to variations in telomere length that occurred after subcloning, because all of the cells in the E8 subclones were descended from the single founder cell for E8. The combined results with the E8 subclones show that most

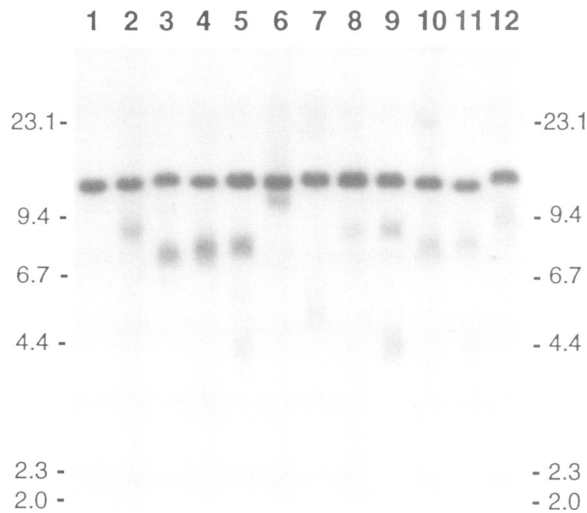

Fig. 3. Southern blot analysis demonstrating polymorphism within the telomeric repeat sequences on the end of the truncated chromosome 13 in the second-generation KB319 subclone E8. DNA was digested with *Xba*I and hybridized with a fragment of the integrated plasmid (see Figure 1). DNA was analyzed from the original SV40-transformed immortal cell line LM217 (lane 1), from the second-generation E8 subclone (lane 2), and from 10 subclones of E8 (lanes 3–12). In this and subsequent figures, subclones were cultured for ~25 cell generations before Southern blot analysis. Molecular weight markers ($\times 10^{-3}$) consisted of lambda bacteriophage DNA digested with *Hin*dIII.

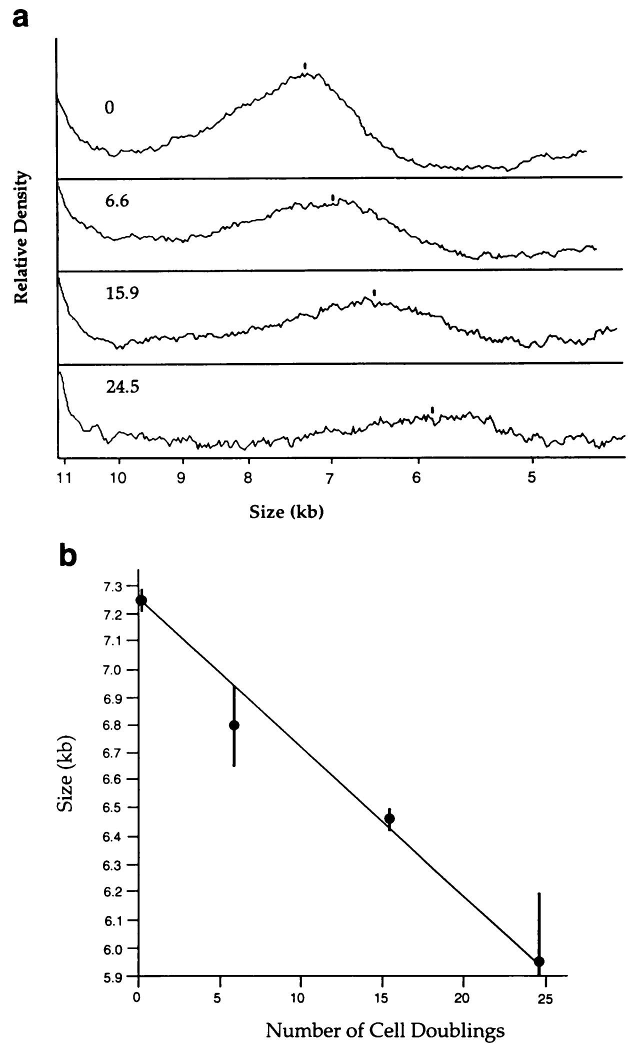

Fig. 4. Size distribution of the telomere fragment in an E8 subclone (see Figure 3, lane 4) with increasing times in culture. (**a**) Densitometric tracings of the bands produced on Southern blots by the telomere fragment after the number of cell doublings indicated. The stable internal control fragment (shoulder of peak shown on the left) was used as a standard. Hatch marks denote the average size of the fragments in the bands. (**b**) The average size of the telomere fragments in relation to the number of cell doublings. Results are expressed as the average and standard deviation from two different Southern blots.

changes in size are small, although occasionally there are more dramatic changes in telomere length.

Because the main bands in several E8 subclones were smaller than those in the parental E8 line, the small changes seemed to result from a gradual loss of telomeric repeat sequences during subcloning. To characterize further the loss of telomeric repeat sequences, we cultured the E8-2 subclone (Figure 3, lane 4) for various times and determined the size of the telomeric repeat sequences on chromosome 13. The band representing the telomere fragment rapidly became more diffuse and decreased in size (Figure 4a). In ~25 cell generations the average length of the telomeric repeat sequences decreased by 1300 bp (Figure 4b), a rate of ~52 bp per cell division. The fact that the band rapidly became so diffuse indicates a high degree of variability in either the rate of loss of repeat sequences in each cell division or the rate of growth of various cells in the population.

Subclones of E9, which originally had ~1 kb of telomeric repeat sequences on the end of chromosome 13, also had main bands that were usually smaller (in eight of 10 subclones) than those in the parental cell line, as well as lighter bands that showed more dramatic changes in size (Figure 5a). Because ~25 cell generations had elapsed during cloning, the reduced size of the telomeres in most E9 subclones (0.5–1 kb) suggested that telomeric repeat sequences were lost at a rate of ~20–40 bp per cell division. A lower limit of ~3 kb was clearly evident in the size of the diffuse bands in these subclones, which was also noted in the first-generation subclones when a different restriction enzyme was used (see Figure 8 in Murnane, 1990). This lower limit corresponds to the size of the restriction fragment without telomeric repeat sequences (Murnane and Yu, 1993); therefore, the gradual loss of terminus DNA did not extend into the subtelomeric sequences. The absence of continued shortening beyond the telomeric repeat sequences appeared to be due to the increase in length of telomeres after the telomeric repeat sequences became less than a few hundred base pairs in length. When the telomere-specific bands in the E9 subclones approached ~3 kb, they became much lighter and were replaced by extensive polymorphism that ranged from 3 kb to >23 kb (Figure 5a, lanes 5, 7, 8, 10 and 11). Additional subcloning of the E9-6 subclone (lane 8) was consistent with the interpretation that rapid telomere elongation had occurred. The E9-6 subclones generally had bands that were less diffuse than in E9-6, yet were larger than those seen in E9 (Figure 5b). The wide variation in the size of the bands (>23 kb in Figure 5b, lane 5) demonstrates that the length of the sequences added to the telomere is highly heterogeneous in the various

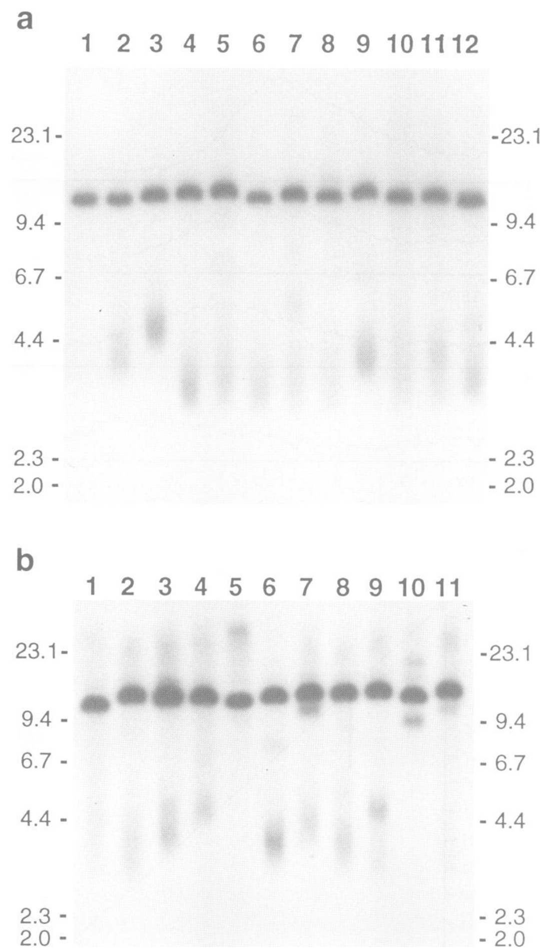

Fig. 5. Southern blot analysis demonstrating polymorphism within the telomeric repeat sequences on the end of the truncated chromosome 13 in the second-generation subclone E9. DNA was digested with *Xba*I and hybridized with a fragment of the integrated plasmid (see Figure 1). (**a**) DNA from the original LM217 cell line (lane 1), the second-generation E9 subclone (lane 2), and 10 subclones of E9 (lanes 3–12). (**b**) DNA from the third-generation subclone E9-6 (lane 1) and 10 subclones of E9-6 (lanes 2–11). Molecular weight markers are described in the legend to Figure 3.

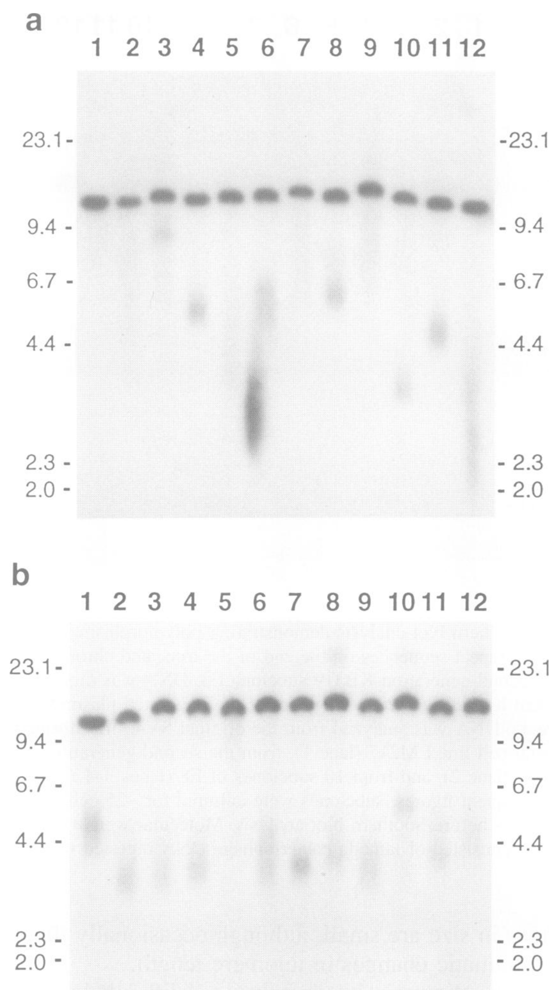

Fig. 6. Southern blot analysis demonstrating polymorphism within the telomeric repeat sequences on the end of the truncated chromosome 13 in the second-generation subclone E1. DNA was digested with *Xba*I and hybridized with a fragment of the integrated plasmid (see Figure 1). (**a**) DNA from the original LM217 cell line (lane 1), the E1 subclone (lane 2), and 10 subclones of E1 (lanes 3–12). (**b**) DNA from the third-generation subclone E1-6 (lane 1) and 11 subclones of E1-6 (lanes 2–12). Molecular weight markers are described in the legend to Figure 3.

subclones. The rapid increase in length of the telomere was not due to selection against cells that have lost the marker chromosome, because no decrease in plating efficiency or cell doubling time was seen in subclones with a short telomere on this chromosome, and a similar increase in telomere length was seen in subclones that contain three other copies of chromosome 13, and therefore no selection against loss of the marker chromosome would occur (unpublished data).

Like the parental E1 cells, several E1 subclones (Figure 6a) showed highly diffuse bands (lanes 5, 7 and 9; the smeared regions in the lower portion of lanes 6 and 12 are nonspecific background). This extensive polymorphism occurred on telomeres of various sizes and was therefore distinct from the polymorphism seen in E9 and its subclones (Figure 5a and b), which occurred only on telomeres that had lost nearly all of their telomeric repeat sequences. Other E1 subclones appeared to have reverted to a more stable telomere length, although a high degree of heterogeneity was evident in the size of the bands, which varied from 3.5 kb (Figure 6a, lane 10) to 9 kb (lane 3). Further subcloning of one of the more stable subclones (E1-6, lane 8) showed relatively stable telomeres that were shorter than those in the parental line (Figure 6b), although not short enough to promote the addition of new telomeric sequences. One of the E1-6 subclones, however, again showed a telomere fragment with extensive polymorphism (Figure 6b, lane 5).

Analysis of telomere polymorphism on other chromosomes

To determine whether polymorphism was also evident on the telomeres of other chromosomes, we performed Southern blot analysis on the DNA of E1 and its subclones, using the TelBam8 fragment as a probe (Figure 7a). The TelBam8 fragment has been shown to be specific for the telomere of chromosome 7 (Brown *et al.*, 1990). DNA from the various subclones was digested with *Bam*HI, which produces a terminal fragment of 8 kb, not including

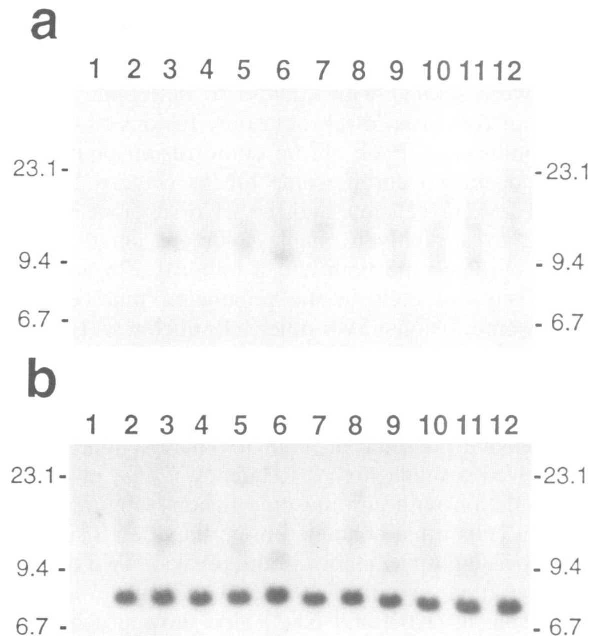

Fig. 7. Southern blot analysis demonstrating polymorphism within the telomeric repeat sequences on the end of chromosome 7. *Bam*HI-digested DNA from the original LM217 cell line (lane 1), the E1 subclone (lane 2), and 10 subclones of E1 (lanes 3–12) was hybridized with either the TelBam8 probe alone (**a**) or the TelBam8 probe in combination with plasmid DNA (**b**) as a control to show stable bands. Molecular weight markers are described in the legend to Figure 3.

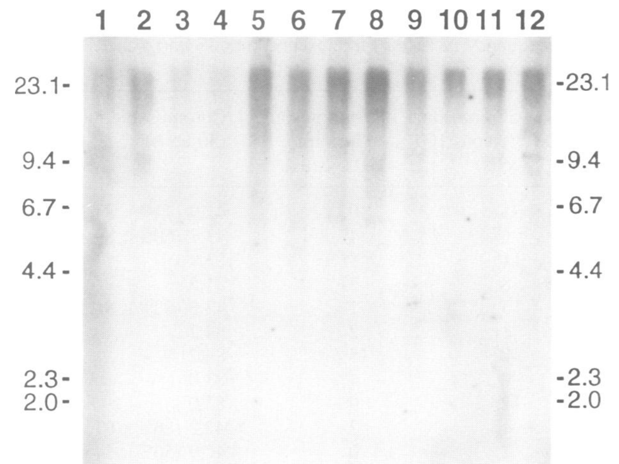

Fig. 8. Southern blot analysis of the average length of telomere fragments on all the chromosomes in various immortal SV40-transformed cell lines. DNA from E1 (lane 1), E1-5 (lane 2), E1-6 (lane 3), E1-9 (lane 4), E8 (lane 5), E8-1 (lane 6), E8-2 (lane 7), E8-4 (lane 8), E9 (lane 9), E9-2 (lane 10), E9-4 (lane 11), and E9-6 (lane 12) was digested with *Hin*fI and *Rsa*I and hybridized with a TTAGGG-specific probe. Most genomic DNA was found as low molecular weight fragments at the bottom of the gel and did not hybridize to the telomeric probe. Molecular weight markers are described in the legend to Figure 3.

telomeric repeat sequences. For comparison with a stable DNA fragment, an identical Southern blot was probed with TelBam8 in combination with a fragment of pMSTK-Neo (Figure 7b). This plasmid DNA hybridized to an internal fragment of the integrated plasmid at the new telomere of chromosome 13 (Figure 1), which did not show polymorphism. Many heterogeneous bands were seen, consistent with the presence of multiple copies of chromosome 7 in these transformed cells. The size of these bands varied from ~9.5 kb (Figure 7a, lanes 6 and 9–11) to 20 kb (lane 8), indicating telomeres from 1.5 to 12 kb, and in some subclones a diffuse background appeared to extend to >23 kb. Although the presence of multiple copies of chromosome 7 makes the data much more difficult to interpret, the results clearly show that a high degree of polymorphism was also present on the telomere of chromosome 7 in this cell line.

The wide distribution in telomere length seen in the KB319 cell line and its subclones appeared much greater than that reported for another immortal SV40-transformed human cell line (Counter *et al.*, 1992). To confirm that this extensive polymorphism was due to telomeric repeat sequences we performed Southern blot analysis, using the telomeric repeat sequence (TTAGGG) as a probe. By Southern blot analysis, the E1, E8 and E9 subclones and their next-generation subclones reproducibly showed a broad distribution in hybridization (Figure 8). Because hybridization with a TTAGGG-specific probe is proportional to the length of the telomere, longer telomeres are overrepresented; however, a high degree of polymorphism in the length of telomeric repeat sequences is clearly characteristic of these subclones, and the telomeres on some chromosomes appear to be very long. This high degree of variability in the average size of telomeres as seen by Southern blot analysis is common to many, but not all, immortal SV40-transformed human cell lines (unpublished observations).

Chromosome fusion

The loss of telomeres has been suggested to play a role in chromosome fusion (McClintock, 1941; Hastie and Allshire, 1989). Using chromosome 13-specific probes, we studied the frequency of fusions in chromosome 13 in the various subclones to determine whether a correlation with telomere length could be observed (Table I). The KB319 cell line and its subclones are aneuploid. KB319 and subclones E, E9, E9-2 and E9-6 all had two populations, one near diploid and another near tetraploid; E1 and E8 and their subclones were almost all near tetraploid (Table I). Most of the near-diploid cells had two homologs of chromosomes 13 and 4, whereas most of the near-tetraploid cells had three or four homologs. The original cell from which KB319 and subclones E, E9, E9-2 and E9-6 were generated therefore appears to have been near diploid, although there is a strong tendency for these cells to become tetraploid. Despite their differences in chromosome number, the E1, E1-7, E8 and E9 subclones were shown by fluorescence *in situ* hybridization to contain only one copy of the marker chromosome 13 with the plasmid sequences at the telomere (unpublished observation). Although some of the cells within these subclones had doubled their chromosome number, and therefore contained two marker chromosomes, this could not account for the telomere length polymorphism seen in some subclones. Cells that had doubled their chromosome number were extremely rare in the subclones with extensive telomere length polymorphism (E1 and E1-7), while the subclones that frequently doubled their chromosome number (E, E9, E9-2 and E9-6; Table I) had relatively stable telomere lengths.

Table I. Frequency of fusions in chromosomes 13 and 4 in KB319 and its subclones

Cell line	Chromosome 13 (fusions/100 cells)	Chromosome 4 (fusions/ 100 cells)	Chromosome number (50 cells)[a]
KB319	1	0	42.9 (74%); 84.2 (26%)
E	6[b]	4[b]	43.1 (60%); 83.3 (40%)
E1	14[c]	8[c]	83.9
E8	8[c]	2	81.5
E9	3	1	43.3 (82%); 81.3 (18%)
E1-6	1	2	84.2
E1-7	18[c]	15[c]	83.2
E8-2	9[c]	4[b]	83.0
E8-4	12[c]	9[c]	83.0
E9-2	1	2	43.2 (86%); 87.7 (14%)
E9-6	2	0	42.9 (56%); 84.0 (44%)

[a]Some cell lines are composed of both near-diploid and near-tetraploid populations; the percentage of cells in each population is indicated.
[b]Significantly different from the parental KB319 cell line ($P < 0.05$; tailed Student's *t* test).
[c]Significantly different from the parental KB319 cell line ($P < 0.01$; tailed Student's *t* test).

Chromosome fusions were identified by the association of the end of an apparently full-length chromosome 13 (as determined by comparison with the other chromosome 13 homologs in the cell) with any other chromosome, without the production of the reciprocal translocation fragments (Figure 9). The frequency of chromosome fusion was relatively low in the parental KB319 cell line and in subclones that had a very short telomere on the marker chromosome 13 (E9, E9-2 and E9-6). The highest frequency of fusion involving chromosome 13 was seen in subclones that demonstrated the most extensive polymorphism in telomere length (E1 and E1-7). The rapid changes in telomere length in these subclones occurred continually on telomeres of any length, unlike other subclones where rapid changes occurred primarily on very short telomeres. Consistent with this correlation, a revertant subclone of E1 with more stable telomeres (E1-6) showed a much lower frequency of chromosome 13 fusions (Table I). E8 and its subclones showed levels of fusion that were intermediate between those of E1 and E9. The absence of multiple cells containing identical fusions demonstrates that the frequency of fusions represents the rate at which they occur in the various subclones. Despite the high frequency of chromosome fusions in some subclones, no clones were observed that contained translocations involving fragments of chromosome 13, demonstrating that breakage of the fused chromosomes was a rare event.

The fusions of chromosome 13 occurred at either end and involved other apparently random chromosomes, although fusions between two homologs of chromosome 13 were occasionally observed (data not shown). The factors responsible for the increased fusion in some subclones therefore appeared to be acting *in trans*. To determine whether other chromosomes showed a similar increase in frequency of fusion in these subclones, we used a chromosome 4-specific probe. The relative frequency of fusions in chromosome 4 was proportional to that in chromosome 13 in the various subclones, although the absolute number of fusions in chromosome 4 was generally lower (Table I).

Because of the high frequency of fusions involving chromosomes 4 and 13 in some subclones, the chromosomes were stained with Giemsa to determine the frequency of formation of chromosome fusions or rings on all chromosomes. Each chromosome fusion or ring was measured as two chromosome breaks (Figure 10). The original LM217 cell line and the E1-6 subclone that had reverted to a relatively stable telomere length on the marker chromosome both had a minority (28 and 33%, respectively) of cells in the population that contained chromosome fusions. Two other E1 subclones (E1-2 and E1-9) that had also reverted to a more stable telomere length showed similar results (unpublished observation). In contrast, the E1-7 subclone, which continued to show the extensive polymorphism in telomere length seen in E1, showed a much higher frequency (74%) of cells in the population with chromosome fusions (Figure 10). In addition, cells often contained more than one fusion, and as many as eight (16 chromosome breaks). Two other E1 subclones that continued to show extensive telomere polymorphism (E1-3 and E1-5) also showed a high frequency of chromosome fusion (unpublished observation). The chromosome fusions were not confined to specific chromosomes, and therefore the instability within these subclones is a general mechanism that affects all of the chromosomes.

Discussion

Our analysis of the kinetics of telomere length variation on a single chromosome clearly showed the presence of extensive polymorphism within the telomeres of subclones of the immortal KB319 cell line. This polymorphism was not confined to the telomere on the marker chromosome 13, because a probe specific for chromosome 7 gave similar results. A previous study suggested that telomeres become more stable after immortalization (Counter *et al.*, 1992); however, because those results were based on the average telomere length of all chromosomes, the heterogeneity in telomere length on individual chromosomes may not have been detectable. The changes in telomere length on chromosomes of immortal mammalian cells may therefore be much more extensive than previously thought. This observation is not entirely unexpected, because the maintenance of telomeres in immortalized somatic cells is not a normal process.

Mechanism of gradual changes in telomere length

Our analysis of the E8 and E9 subclones showed that most changes in telomere length are gradual, similar to those seen in yeast telomeres (Shampay and Blackburn, 1988). The rate of loss of telomeric repeat sequences seen in the E8 subclone (52 bp per cell division) was nearly identical to the estimated rate of loss (48 bp per cell division) of telomeres from somatic human cells that do not contain telomerase (Hastie *et al.*, 1990). However, telomeric repeat sequences did not appear to be completely lost, as demonstrated by the lack of polymorphism in the subtelomeric DNA and the rapid increase in size of the telomeric fragments as they approached the 3 kb limit of the subtelomeric fragment without telomeric repeat sequences. Telomeric repeat sequences, therefore, appear to be added when the size of telomeres is reduced below

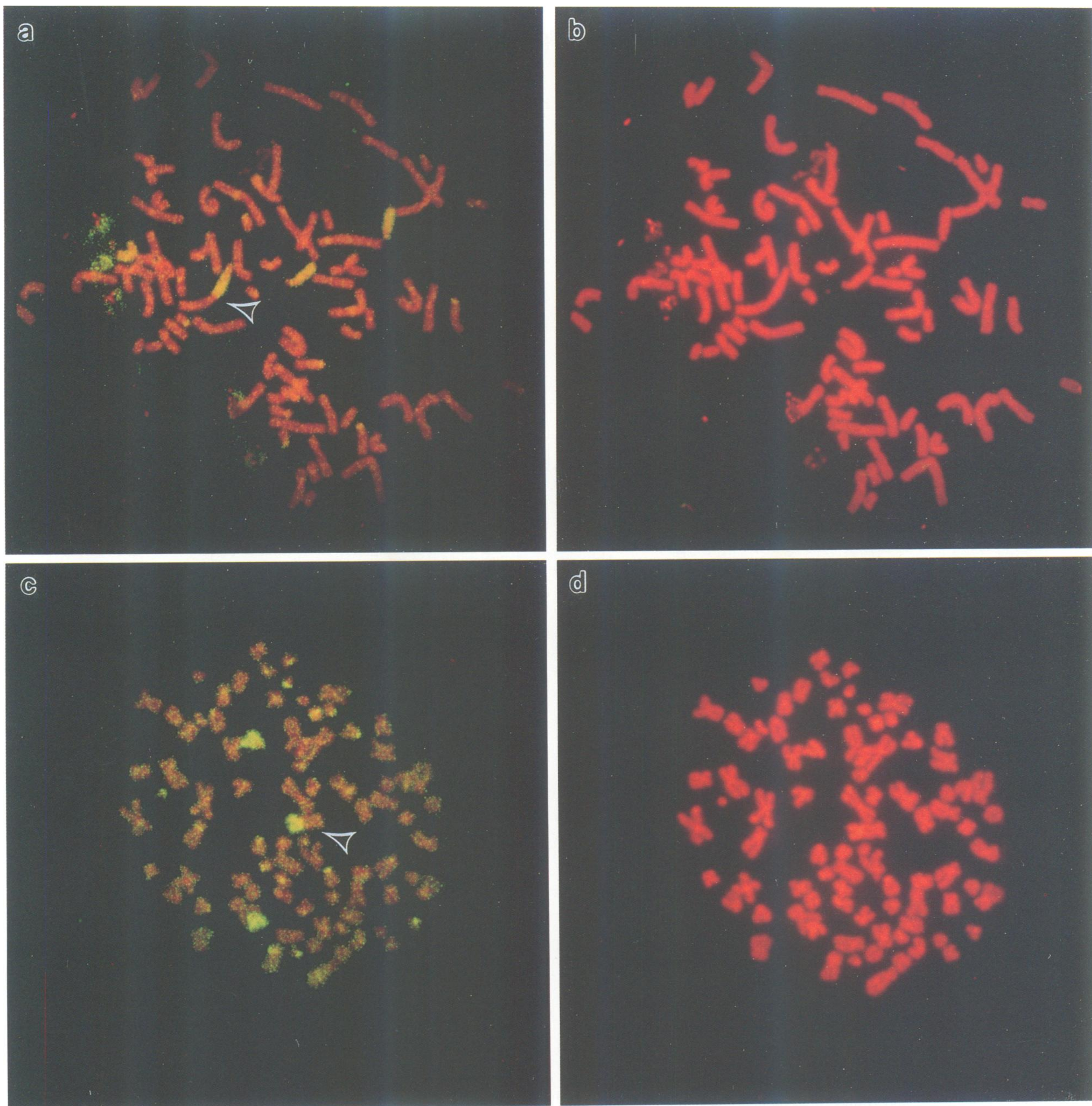

Fig. 9. Analysis of telomeric fusions in chromosome 13. Metaphase chromosomes are shown for subclone E1-6 (**a** and **b**) and subclone E1-7 (**c** and **d**). Fusions of chromosome 13 (arrows) which was labeled with fluorescein (green) by means of a chromosome 13-specific probe (**a** and **c**), were confirmed by comparing the same chromosomes stained with propidium iodide (**b** and **d**). In this way, chromosomes with telomere fusions could be distinguished from chromosomes that were only in close proximity.

a critical limit, although the heterogeneity in the increase in length indicates that this is not a highly regulated process. The mechanism of this rapid addition of sequences to short telomeres is unclear. No telomerase activity has been detected in the original immortal LM217 cell line, the parental KB319 cell line containing the integrated plasmid at its telomere, or in four generations of KB319 subclones (Dr Calvin Harley, personal communication); however, this could be due to inhibitory factors or to intermittent telomerase activity that is below the level of detection. The inability to detect telomerase could suggest that nonreciprocal recombination may be the primary mechanism involved in maintenance of telomeres in this cell line and its subclones. If so, this cell line may provide important information about the influence of recombination on telomere maintenance and stability. Recombination has previously been shown to promote the addition of both telomeric repeat (Wang and Zakian, 1990) and subtelomeric sequences (Lundblad and Blackburn, 1993). Why telomere elongation should occur preferentially on short telomeres is unknown. One possible scenario would be that recombination does not occur on longer telomeres owing to the presence of telomere-specific binding proteins, and that the rate of recombination increases when the telomeres become too short to bind these proteins (Figure 11a).

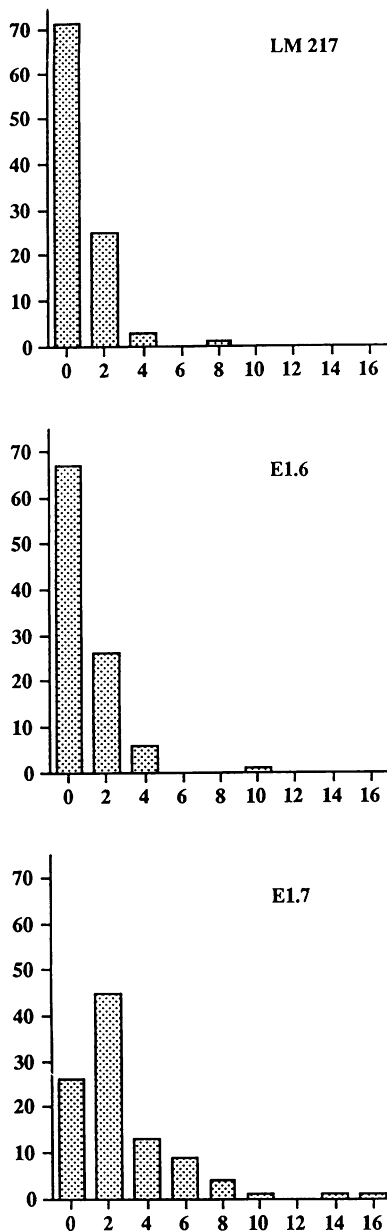

Fig. 10. The distribution of mitoses (percentages) with chromosome rearrangements (dicentrics and rings) in the LM217 cell line and the E1-6 and E1-7 subclones. Dicentrics and rings are scored as two chromosome breakpoints.

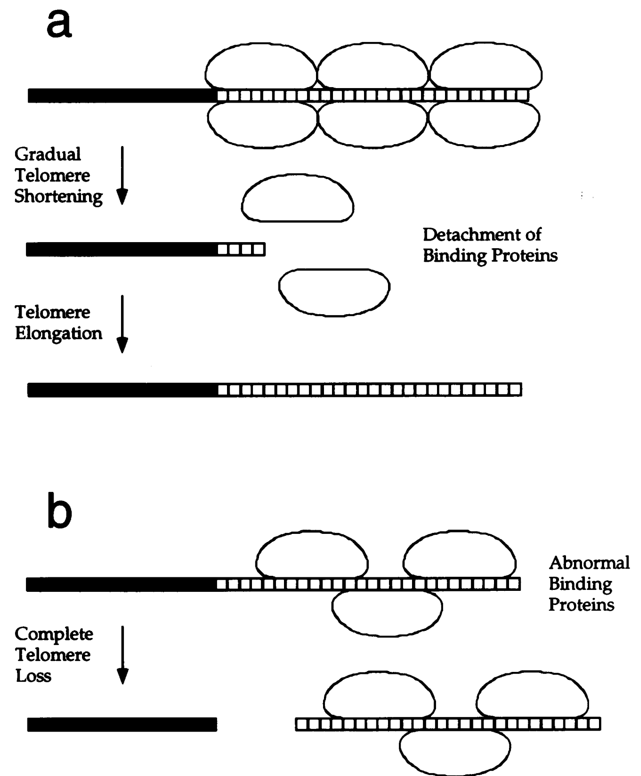

Fig. 11. Possible mechanisms for the two types of telomere polymorphism. (**a**) Gradual loss of telomere repeat sequences (open squares) continues until too few repeat sequences remain for attachment to telomere-binding proteins (ovals). The remaining unprotected repeat sequences then become substrates for telomerase or recombination enzymes, resulting in telomere elongation. (**b**) Cells with altered expression or mutations in telomere-binding proteins fail to protect telomere repeat sequences, leading to a high rate of telomere rearrangement that occasionally results in complete loss of telomere repeat sequences.

Mechanism of rapid changes in telomere length

In addition to the gradual reduction in the length of telomeres and the rapid addition of sequences to short telomeres, rapid changes were also occasionally observed on long telomeres. These rapid changes were independent of telomere length, resulted in increases or decreases in size by many kilobases and varied greatly in frequency among the subclones. Some subclones (E9) showed very few rapid changes on long telomeres and some (E8) showed intermediate numbers of changes resulting in numerous lighter bands; in some subclones (E1), the rapid changes were so frequent that only extremely diffuse bands were observed regardless of telomere length. The frequency of the rapid changes on long telomeres was a heritable characteristic that could be passed on to subsequent cell generations; however, some subclones had frequencies much higher or lower than those in the parental cell line (Figure 6a and b). It is unclear whether these rapid changes on long telomeres are due to the same process that promotes the elongation of short telomeres. An increase in rapid changes could result from abnormalities in various telomere-binding proteins that influence the rate of recombination within telomere repeat sequences (Figure 11b). Mutations or overexpression of specific proteins are known to influence telomere polymorphism greatly, as seen with *RAP1* mutants in yeast (Conrad *et al.*, 1990; Kyrion *et al.*, 1992), in which polymorphism appears to result from a combination of increased telomere length and rapid deletion events (Kyrion *et al.*, 1992).

Telomere polymorphism and chromosome fusion

Regardless of the mechanism involved, the rapid changes in telomere length may have important implications for the cell. An apparent correlation can be seen between the prevalence of the rapid changes in telomere length and the frequency of chromosome fusion (Table I, Figure 10). The highest levels of fusion were seen in the E1 cell line and in its subclones that had the most extensive telomere length polymorphism, whereas revertant E1 subclones with more stable telomeres had a low frequency of fusions. These rapid changes in telomere length may therefore sometimes result in the complete loss of the telomeric repeat sequences required to protect the end of the chromo-

some. The loss of telomeres has previously been shown to correlate with the fusion of sister chromatids in corn (McClintock, 1941) or chromosome loss in yeast (Lundblad and Szostak, 1989; Yu *et al.*, 1990; Sandell and Zakian, 1993), and has been proposed to account for the increased number of dicentric chromosomes in senescent mammalian cells (Benn, 1976; Bender *et al.*, 1989; Counter *et al.*, 1992) and human tumor cells (Hastie and Allshire, 1989). Consistent with this interpretation, mutations in yeast genes that cause extensive telomere polymorphism also appear to cause complete telomere loss and chromosome instability (Conrad *et al.*, 1990; Kyrion *et al.*, 1992).

Implications of telomere instability in immortal cells

The telomere instability found in the KB319 cell line may be an important mechanism for the genomic instability associated with cancer. The fusion of sister chromatids in Chinese hamster cells, which may occur after either chromatid breaks or telomere instability, has been found to initiate the breakage–fusion–bridge cycles responsible for gene amplification (Smith *et al.*, 1992; Toledo *et al.*, 1992; Ma *et al.*, 1993). Chromosome fusion, which results in the formation of dicentric chromosomes, has been found to result from intermediates involved in fusion of sister chromatids (Toledo *et al.*, 1993). Alternatively, fusion between different chromosomes may occur directly after the loss of their telomeres. The increased numbers of dicentric chromosomes seen in a variety of tumor cells have been proposed to result from the loss of telomeric repeat sequences (Hastie and Allshire, 1989). Although a previous study showed that average telomere length alone was not the determining factor in chromosome fusion (Saltman *et al.*, 1993), the rapid changes in telomere length, as seen in the KB319 cell line, could occasionally result in complete loss of telomeric repeat sequences even in cells that do have telomerase. Increased chromosome fusion might therefore result from altered expression or mutations in genes that influence telomere instability. The absence of detectable translocations of chromosome 13 in subclones with a high frequency of chromosome fusion, however, suggests that, as in human tumor cells (Saltman *et al.*, 1993), chromosome fusion does not necessarily result in chromosome breakage. Despite this fact, fusion could result in rare breakage events and therefore could account for gene amplification, which occurs at a much lower frequency than chromosome fusion in mammalian cells. Further studies are required to determine whether there is a correlation between chromosome fusion and genomic instability in these cells.

Materials and methods

Cells and culture conditions

The LM217 cell line was established by transfection of primary human fibroblasts with the pLR309 plasmid, which contains SV40 sequences with a defective origin of replication (Murnane, 1986). This cell line passed through crisis after 6 months in culture and was subsequently subcloned. KB319 is the name given to a cell clone that was previously shown to have an integration site with extreme restriction fragment length polymorphism (see Figures 7 and 8 in Murnane, 1990), which turned out to be due to its location at the telomere of chromosome 13 (Murnane and Yu, 1993). This cell clone was derived from LM217 after transfection with a pMSTK-Neo plasmid that contains a duplicated 2.0 kb human DNA fragment (pMSTK-Neo 2.0). The pMSTK-Neo plasmid also contains a selectable *neo* gene with a thymidine kinase gene transcriptional promoter and termination sequences, a mouse metallothionein gene and the pBR322 vector (Murnane, 1990).

Second-generation subclones of the original KB319 cell line (Figure 2) were selected for further analysis: E1 (see Figure 7B, lane 1, in Murnane, 1990), E8 (see Figure 7B, lane 8, in Murnane, 1990) and E9 (see Figure 7B, lane 9, in Murnane, 1990). Cell lines were subcloned by one of two methods. The cells were first maintained at subconfluence for two passages and replated at low density the day before subcloning. After trypsinization the cells were then checked under a microscope to confirm a single cell suspension. The cells were then transferred into either 75 cm^2 cell culture flasks (~10–20 colonies per flask) or microwell plates (~1 colony per well). The colonies in the flasks were isolated by ring cloning (first-generation subclones A–G, and second-generation subclones of E). The microwells were scanned with a microscope to identify the location of single cells, and single colonies in the vicinity of the original cell were later trypsinized and replated (subclones of E1, E8, E9, E1-6 and E9-6). Cell cultures were maintained in minimal essential medium (Gibco) containing 10% fetal calf serum (Gibco) and were incubated at 37°C in a humidified incubator with 5% CO_2.

DNA preparation and Southern blot analysis

High molecular weight cellular DNA was prepared as described previously (Murnane *et al.*, 1985). DNA was digested with restriction enzymes according to the manufacturer's instructions (Bethesda Research Laboratories). Southern blot analysis and nucleic acid hybridization were performed as described previously (Murnane, 1986). Hybridization probes for specific telomeres consisted of either a portion of the 2.0 kb human fragment from plasmid pMSTK-Neo 2.0 that does not contain an Alu sequence, or the chromosome 7-specific TelBam8 fragment (Brown *et al.*, 1990) kindly provided by Dr William Brown (Oxford University). Southern blot analysis of the telomeres on all chromosomes was performed in a similar manner, except that only 2 mg of DNA was added per lane and no salmon sperm DNA was used in the hybridization solution. The telomere probe consisted of 40 TTAGGG repeat sequences contained in the *Pst*I fragment in the pHuR 93 plasmid (Moyzis *et al.*, 1988). The average molecular weight of DNA fragments was estimated by densitometric analysis on an ImageQuant densitometer (Molecular Dynamics).

Cytogenetics and fluorescence in situ hybridization

Plasmid vectors containing human chromosome 4- and chromosome 13-specific DNA sequences (pBS4 and pBS13, respectively) were kindly provided by J.W.Gray (University of California, San Francisco). The plasmids were purified by using a Qiagen maxi-prep system and were labeled with biotin using a BioNick kit (Bethesda Research Laboratories). The hybridization and cytochemical detection protocols were adapted from a chromosome *in situ* system (Oncor) as described previously (Marder and Morgan, 1993). Briefly, slides were treated with RNase [100 mg/ml in 2× SSC (1× SSC is 0.15 M sodium chloride and 0.015 M sodium citrate)] for 1 h at 37°C and dehydrated in an iced ethanol series (70, 80 and 95%). After drying, chromosomes were denatured in 70% formamide/2× SSC at 70°C for 2 min and dehydrated in an iced ethanol series (70, 80, 90 and 100%). Fifty nanograms of labeled probe were mixed with 250 mg of human Cot-1 DNA (Bethesda Research Laboratories) and 250 mg of human placenta DNA (Sigma) in a 10 ml total volume containing 50% formamide, 2× SSC and 10% dextran sulfate. The DNA was denatured at 70°C for 5 min, chilled on ice and prehybridized at 37°C for 1 h to remove repetitive sequences. The prehybridized probe was then added to the slide with the denatured chromosomes under a glass coverslip and sealed with rubber cement. After a 5 day hybridization at 37°C in a humidified chamber, slides were washed in 50% formamide and 2× SSC at 37°C for 20 min with agitation and rinsed twice in 2× SSC at 37°C for 4 min each time. Slides were immersed in phosphate-buffered detergent (Oncor). For cytochemical detection, the slides were treated at ambient temperature with a blocking reagent for 5 min and then incubated with fluorescein-conjugated avidin for 20 min at 37°C in a humidified incubator. Slides were washed three times with phosphate-buffered detergent, and the fluorescein signal was amplified by two rounds of incubation with anti-avidin and fluorescein-conjugated avidin. Antifade (Sigma) and propidium iodide were then applied to the slides. Metaphase chromosomes were scored on a Zeiss Axioskop microscope equipped with a fluorescence condenser and a fluorescein isothiocyanate/rhodamine dual-band pass filter set (Chroma Technology).

Metaphase chromosome preparations and Giemsa staining for analysis of fusions involving all chromosomes, and *in situ* hybridization to determine the number of copies of chromosome 13 with the integrated plasmid at its telomere in the various subclones, were performed according to published methods (Dutrillaux and Couturier, 1981; Lemieux *et al.*, 1992). Briefly, the marker chromosome 13 was identified with the pMSTK-Neo plasmid probe that was nick-translated with biotin-14-dUTP according to the manufacturer's protocol (Gibco-BRL). The unincorporated nucleotides were removed by gel filtration. Hybridizations were performed overnight at 37°C, and washing consisted of two washes (3 min each) in 50% formamide, 2× SSC and two washes (3 min each) in 2× SSC at 37°C.

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References

Barnett,M.A., Buckle,J., Evans,E.P., Porter,A.C.G., Rout,D., Smith,A.G. and Brown,W.R.A. (1993) *Nucleic Acids Res.*, **21**, 27–36.
Bender,M.A., Preston,R.J., Leonard,R.C., Pyatt,B.E. and Gooch,P.C. (1989) *Mutat. Res.*, **212**, 149–154.
Benn,P.A. (1976) *Am. J. Hum. Genet.*, **28**, 465–473.
Blackburn,E.H. (1990) *Science*, **249**, 489–490.
Blackburn,E.H. (1991) *Nature*, **350**, 569–573.
Bouffler,S., Silver,A., Papworth,D., Coates,J. and Cox,R. (1993) *Genes Chrom. Cancer*, **6**, 98–106.
Brown,W.R.A., MacKinnon,P.J., Villasante,A., Spurr,N., Buckle,V.J. and Dobson,M.J. (1990) *Cell*, **63**, 119–132.
Conrad,M.N., Wright,J.H., Wolf,A.J. and Zakian,V.A. (1990) *Cell*, **63**, 739–750.
Counter,C.M., Avilion,A.A., LeFeuvre,C.E., Stewart,N.G., Greider,C.W., Harley,C.B. and Bacchetti,S. (1992) *EMBO J.*, **11**, 1921–1929.
Counter,C.M., Hirte,H.W., Bacchetti,S. and Harley,C.B. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 2900–2904.
DeLange,T., Shiue,L., Myers,R.M., Cox,D.R., Naylor,S.L., Killery,A.M. and Varmus,H.E. (1990) *Mol. Cell. Biol.*, **10**, 518–527.
Dutrillaux,B. and Couturier,J. (1981) *La Pratique de l'Analysée Chromosomique.* Masson, Paris.
Farr,C., Fantes,J., Goodfellow,P. and Cooke,H. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 7006–7010.
Greider,C.W. and Blackburn,E.H. (1985) *Cell*, **43**, 405–413.
Harley,C.B. (1991) *Mutat. Res.*, **256**, 271–282.
Harley,C.B., Futcher,A.B. and Greider,C.W. (1990) *Nature*, **345**, 458–460.
Hastie,N.D. and Allshire,R.C. (1989) *Trends Genet.*, **5**, 326–331.
Hastie,N.D., Dempster,M., Dunlop,M.G., Thompson,A.M., Green,D.K. and Allshire,R.C. (1990) *Nature*, **346**, 866–868.
Kyrion,G., Boakye,K.A. and Lustig,A.J. (1992) *Mol. Cell. Biol.*, **12**, 5159–5173.
Lemieux,N., Dutrillaux,B. and Viegas-Pequignot,E. (1992) *Cytogenet. Cell Genet.*, **59**, 311–312.
Lundblad,V. and Blackburn,E.H. (1993) *Cell*, **73**, 347–360.
Lundblad,V. and Szostak,J.W. (1989) *Cell*, **57**, 633–643.
Ma,C., Martin,S., Trask,B. and Hamlin,J.L. (1993) *Genes Dev.*, **7**, 605–620.
Marder,B.A. and Morgan,W.F. (1993) *Mol. Cell. Biol.*, **13**, 6667–6677.
McClintock,B. (1941) *Genetics*, **41**, 234–282.
Morin,G.B. (1989) *Cell*, **59**, 521–529.
Moyzis,R.K., Buckingham,J.M., Cram,L.S., Dani,M., Deaven,L.L., Jones,M.D., Meyne,J., Ratliff,R.L. and Wu,J.-R. (1988) *Proc. Natl Acad. Sci. USA*, **85**, 6622–6626.
Murnane,J.P. (1986) *Mol. Cell. Biol.*, **6**, 549–558.
Murnane,J.P. (1990) *Somat. Cell Mol. Genet.*, **16**, 195–209.
Murnane,J.P. and Yu,L.-C. (1993) *Mol. Cell. Biol.*, **13**, 977–983.
Murnane,J.P., Fuller,L.F. and Painter,R.B. (1985) *Exp. Cell Res.*, **158**, 119–126.
Saltman,D., Morgan,R., Cleary,M.L. and de Lange,T. (1993) *Chromosoma*, **102**, 121–128.
Sandell,L.L. and Zakian,V.A. (1993) *Cell*, **75**, 729–739.
Shampay,J. and Blackburn,E.H. (1988) *Proc. Natl Acad. Sci. USA*, **85**, 534–538.
Smith,K.A., Stark,M.B., Gorman,P.A. and Stark,G.R. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 5427–5431.
Toledo,F., Le Roscouet,D., Buttin,G. and Debatisse,M. (1992) *EMBO J.*, **11**, 2665–2673.
Toledo,F., Buttin,G. and Debatisse,M. (1993) *Curr. Biol.*, **3**, 255–264.
Wang,S.-S. and Zakian,V.A. (1990) *Nature*, **345**, 456–458.
Watson,J.D. (1972) *Nature*, **239**, 197–201.
Wilkie,A.O.M., Lamb,J., Harris,P.C., Finney,R.D. and Higgs,D.R. (1990) *Nature*, **346**, 868–871.
Yu,G.-L., Bradley,J.D., Attardi,L.D. and Blackburn,E.H. (1990) *Nature*, **344**, 126–132.

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