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Telomeric structure in cells with chromosome end associations

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Abstract. End-to-end associations of metaphase chromosomes have been observed in a variety of human tumors, ageing cells, and several chromosome instability syndromes. Since telomeres of tumor cells and ageing tissues are often reduced in length, it has been suggested that chromosome end associations may be due to loss of telomeric repeats. We report the molecular structure of telomeres of two human tumor cell lines with frequent end-to-end associations of metaphase chromosomes. These telomeres were shown to be severely reduced compared with most other human cells with functional telomeres. However, we also describe two cell lines with severely shortened telomeres that are not detectably compromised in their function. We suggest that telomeric length is not the only determinant of the fusigenic behavior of human telomeres in tumor cells.

Introduction

Telomeres are composed of repetitive DNA that protects chromosome ends from degradation, fusion, and incomplete replication (for reviews see Zakian 1989; Blackburn 1991). In plants, chromosome ends without telomeres are fusigenic resulting in dicentric chromosomes, which may enter repeated breakage and fusion cycles wrecking havoc in the genome (McClintock 1941). Apparent end-to-end fusion of metaphase chromosomes has been observed in human cancer, senescent cells, the Thiberge Weissenbach syndrome, viral infection, and ataxia telangiectasia (Yerganian et al. 1962; Benn 1976; Dutrillaux et al. 1978; Taylor et al. 1981; Fitzgerald and Morris 1984; Mandahl et al. 1985, 1988; Morgan et al. 1986; Kovacs et al. 1987; Dewald et al. 1987; Pathak et al. 1988; Saltman et al. 1989; Aledo et al. 1989; Schwartz et al. 1989; Abruzzo et al. 1991). Since it is not known whether chromosome end associations are reversible, it is not clear to what extent this phenomenon might contribute to genome instability in mammals. In addition, it is not known what causes end-to-end associations. It has been suggested that fusigenic chromosome ends lack functional telomeric DNA (Hastie and Allshire 1989; Harley et al. 1990; Hastie et al. 1990). This idea is mainly based on the observation that human telomeres decline from 10–20 kb to less than 4 kb during tumorigenesis and ageing, as well as during normal human development (Cooke and Smith 1986; Allshire et al. 1988; de Lange et al. 1990; Hastie et al. 1990; Harley et al. 1990). However, data on the structure of telomeres in cells with chromosome end associations have been lacking.

We have performed a detailed cytogenetic and molecular characterization of two human tumor cell lines with frequent telomeric associations and several control cell lines with apparently functional telomeres. A melanoma cell line (Melu) showed telomeric associations in 9 out of 100 metaphase spreads; a large cell lymphoma cell line (SU-LL-1) showed telomeric associations in 35% of the metaphases. Our molecular data show that the telomeres in these cells are significantly reduced in length. However, we identified similarly shortened telomeres in two control cell lines, indicating that telomeric length is not the only factor that determines whether chromosome ends become associated at metaphase.

Materials and methods

Cytogenetic analysis. The SU-LL-1 cell line was established from a patient with anaplastic large cell lymphoma. The karyotype of the lymphoma patient's tumor was determined from unstimulated cultures of blood. The Melu cell line was established from a patient with metastatic malignant melanoma. Cytogenetics were not performed on the primary melanoma tumor. The SU-LyB-1 cell line is a normal lymphoblastoid cell line that grew spontaneously from a culture of bone marrow cells. Hela is a cervical carcinoma cell line. RD is a rhabdomyosarcoma cell line. The latter two cell lines were obtained from the ATCC. Metaphase chromosomes spreads of the cell lines were prepared by adding 0.1 μg/ml ethidium bromide (Sigma) 2 h prior to harvesting and 0.1 μg/ml Colcemid (Gibco) 1 h before harvesting. Standard techniques were used for har-

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vesting and preparation of slides. Chromosomes were G-banded using trypsin Wright's stain. In the case of the SU-LL-1 primary tissue, 20 metaphases were examined. There were 100 or more metaphases analyzed from each cell line.

DNA isolation, Bal31 digestion, and genomic blotting. All procedures for DNA isolation, Bal31 digestion, and genomic blotting analysis are described in detail in de Lange et al. (1990). The insert of pTH2Δ (de Lange et al. 1990) was labeled using the random hexamer labeling technique. The TTAGGG repeat probe is a 800 bp fragment composed entirely of TTAGGG repeats (pSP73.Sty11, T.d.L., unpublished), which was labeled using a 5′CCCTAACCCTAACCCTAA 3′ primer and Klenow enzyme. The data were quantitated with a Molecular Dynamics PhosphorImager.

Results

Cytogenetic analysis

The representative karyotypes from the primary lymphoma and the SU-LL-1 cell line are as follows: patient, 48,XY,+1,−2,+der(5)t(2;5)(p23;q35),+7,−21,+der(21q),−22,+3mar (Fig. 1); SU-LL-1 cell line, 49,XY,+1,−2,−5,+der(5)t(2;5)(p23;q35),+7,−21,+der(21q),+3mar. There were no end-to-end chromosome associations or ring chromosomes in the patient's metaphases at presentation or at relapse 4 months later. By contrast, 35% of the metaphases from the SU-LL-1 cell line had end-to-end chromosome associations and rings (Fig. 2). The involvement of individual chromosomes in end-to-end associations was non-random (Tables 1 and 2). Chromosome 3p is most frequently involved in telomeric associations (23% of the association events, see Table 1). Chromosomes 1 and 7, which were duplicated in the primary tumor, showed frequent end-to-end associations (Table 2). Chromosomes 1p and 7p or 7q were joined to each other in 3% of the telomeric associations. Only two chromosomes, 3 and 7, were nonrandomly associated with their homologs (Fig. 2B; Table 2). Nonrandom telomeric associations have been noted before (Fitzgerald and Morris 1984; Mandahl et al. 1985; Kovacs et al. 1987; Dewald et al. 1987; Schwartz et al. 1989; Pathak et al. 1988; Saltman et al. 1989; Aledo et al. 1989; Abruzzo et al. 1991), but a different subset of chromosome ends is involved in each case. Many of the metaphases from the SU-LL-1 cell line contained small G-negative fragments that were either attached to the ends of chromosomes or were extrachromosomal (Fig. 3). These fragments were not observed in the primary tumor cells.

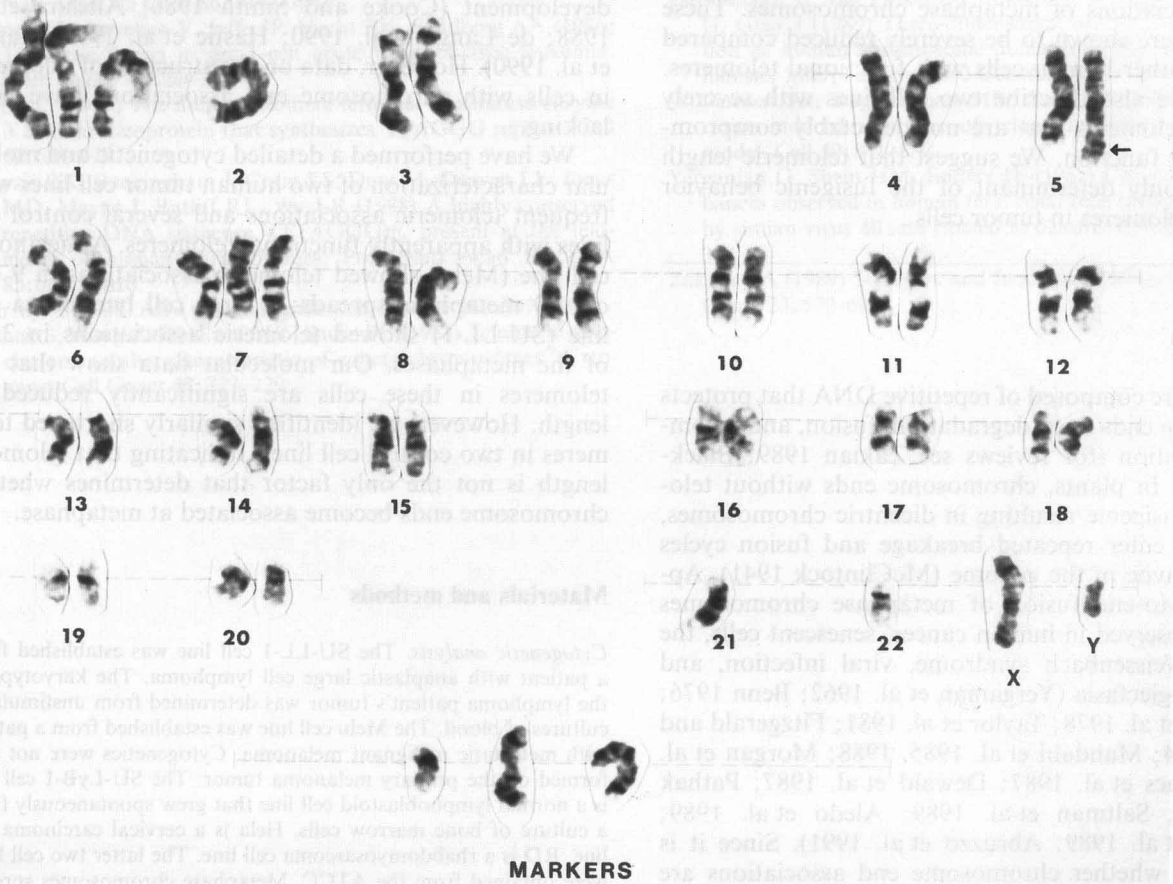

Fig. 1. G-banded karyotype of SU-LL-1. The karyotype shown was derived both in the SU-LL-1 primary tumor and in the major clone of the SU-LL-1 cell line. The *arrow* indicates the der(5) also present in the karyotype of the cell line

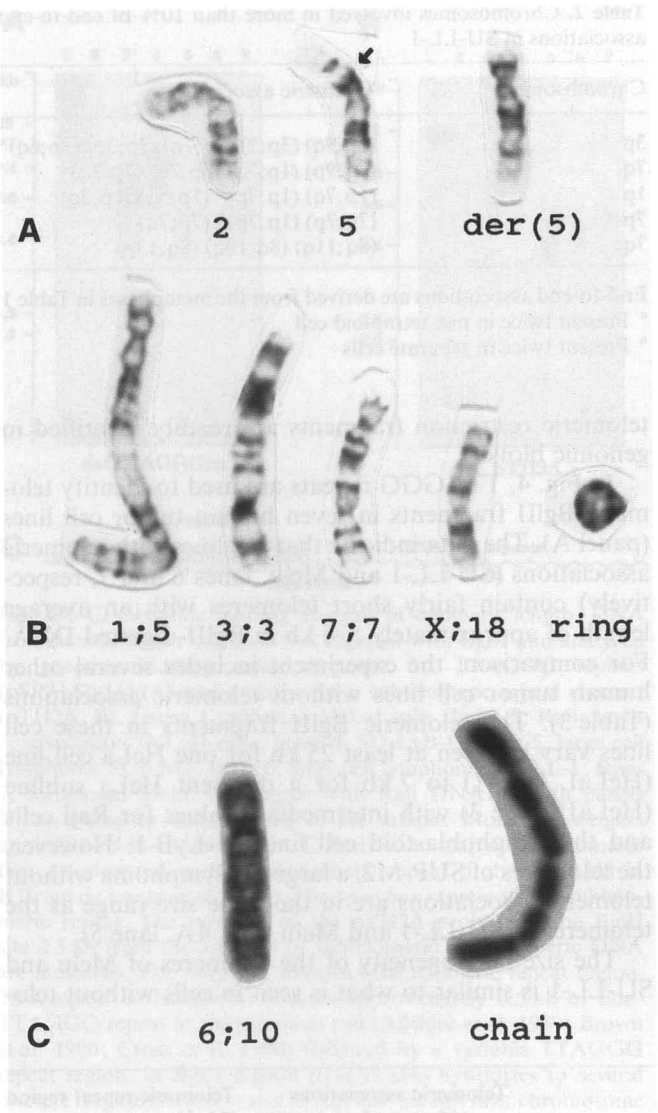

Fig. 2A–C. Examples of end-to-end chromosome associations from the SU-LL-1 and Melu cell lines. **A** Partial karyotype from SU-LL-1 metaphase showing the t(2;5). The *arrow* indicates chromosome 19 associated with 5p. **B** Telomeric associations and an unidentified ring chromosome from the SU-LL-1 cell line. **C** Telomeric association and a multicentric chromosome chain from the Melu cell line

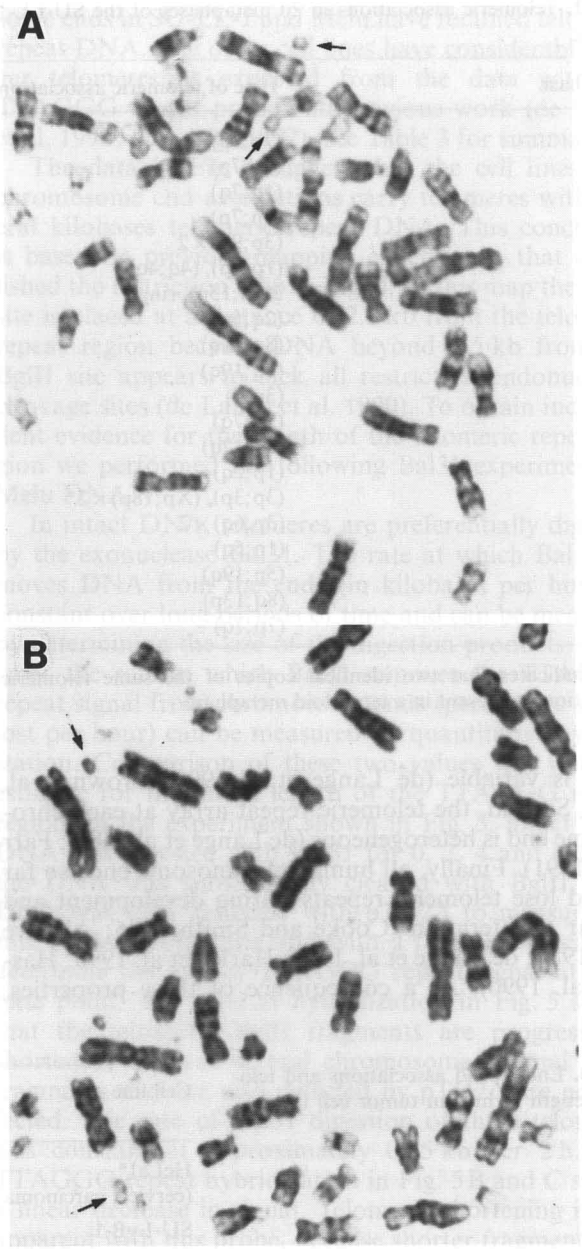

Fig. 3A, B. G-positive fragments in SU-LL-1 metaphases. **A** and **B** show two metaphases of SU-LL-1 with multiple G-positive fragments (*arrows*) either associated with chromosome ends or free

The karyotype of the Melu cell line derived from a metastatic melanoma was as follows: 56,X,−X,+2,+5,+7,+8,12q+,+15,+19,+20,+22,+3mar. Telomeric associations were found in 9 out of the 100 cells examined (Table 3). The frequency with which individual chromosomes were joined end-to-end appeared random in this small data set. Most associations involved only two chromosomes, but in two of the cells there were multicentric chains (Fig. 2C).

The SUP-M2 and SU-LyB-1 cell lines were analyzed repeatedly over 1 year and no telomeric associations or ring chromosomes were observed (Table 3). In addition, no telomeric associations were found in RD and HeLa (Table 3).

Telomeric length in cells with and without telomeric associations

Human telomeres are composed of 2 to 30 kb tandemly arranged telomeric repeats with the sequence (TTAGGG)n in the strand that runs to the 3′ end of the chromosome (Moyzis et al. 1988; Allshire et al. 1988; de Lange et al. 1990; Harley et al. 1990; Hastie et al. 1990). In most human DNA preparations TTAGGG repeat probes hybridize to heterogeneously sized restriction fragments with an average molecular weight that varies in different cell lines and tissues. Variation in the length of telomeric restriction fragments has several causes. First, the physical map of subtelomeric

Table 1. Telomeric associations in 20 metaphases of the SU-LL-1 cell line

Metaphase	Type of telomeric association
1	(7q;9p)
2	(7p;7p)
3	(1p;7q)
4	(1p;7p)
5	(3p;9q) × 2
6	(1p;7p), (4q;4q)
7	(12p;15p), ring
8	(2q;5p)
9	(8q;11q)
10	(8q;10q)
11	(3p;?)
12	(3p;7q)
13	(5q;12q)
14	(1p;2q)
15	(3p;3p), (Xp;18p) × 2[a]
16	(3p;6q) × 2
17	(1p;5q)
18	(5p;19q)
19	(8q;13p)
20	(7p;7q)

[a] × 2 indicates that two identical copies of the same telomeric association are pesent in a tetraploid metaphase

Table 2. Chromosomes involved in more than 10% of end-to-end associations in SU-LL-1

Chromosome	Telomeric associations
3p:	(3p;9q) (3p;?) (3p;7q) (3p;3p) (3p;6q)[a]
7q:	(7q;9p) (1p;7q) (3p;7q) (7p;7q)
1p:	(1p;7q) (1p;7p)[b] (1p;5q) (1p;2q)
7p:	(7p;7p) (1p;7p)[b] (7p;7q)
7q:	(8q;11q) (8q;10q) (8q;13p)

End-to-end associations are derived from the metaphases in Table 1
[a] Present twice in one tetraploid cell
[b] Present twice in separate cells

DNA is variable (de Lange et al. 1990; Brown et al. 1990). Second, the telomeric repeat array at each chromosome end is heterogeneous (de Lange et al. 1990; Farr et al. 1991). Finally, all human chromosome ends so far studied lose telomeric repeats during development and cellular proliferation (Cooke and Smith 1986; Allshire et al. 1988; de Lange et al. 1990; Harley et al. 1990; Hastie et al. 1990). As a consequence of these properties, telomeric restriction fragments are readily identified in genomic blots.

In Fig. 4, TTAGGG repeats are used to identify telomeric BglII fragments in seven human tumor cell lines (panel A). The data indicate that cell lines with telomeric associations (SU-LL-1 and Melu, lanes 6 and 7, respectively) contain fairly short telomeres with an average length of approximately 5–6 kb in BglII-digested DNA. For comparison, the experiment includes several other human tumor cell lines without telomeric associations (Table 3). The telomeric BglII fragments in these cell lines vary between at least 25 kb for one HeLa cell line (HeLaI, lane 1) to 7 kb for a different HeLa subline (HeLaII, lane 3) with intermediate values for Raji cells and the lymphoblastoid cell line SU-LyB-1. However, the telomeres of SUP-M2, a large cell lymphoma without telomeric associations are in the same size range as the telomeres of SU-LL-1 and Melu (Fig. 4A, lane 5).

The size heterogeneity of the telomeres of Melu and SU-LL-1 is similar to what is seen in cells without telo-

Table 3. End-to-end associations and telomeric length in human tumor cell lines

Cell line	Telomeric associations per 100 metaphases	Telomeric repeat region (kb)[b]
HeLaI[a] (cervical carcinoma)	n.d.	25–30
SU-LyB-1 (nontransformed lymphoblastoid)	0	10–15
Raji (Burkitts lymphoma)	n.d.	7–12
HeLaII[a] (cervical carcinoma)	0	3–6.5
RD (rhabdomyosarcoma)	0	2.5–4.5[c]
Melu (melanoma)	9	2.5–4.5
SUP-M2 (large cell lymphoma)	0	1.5–3.5
SU-LL-1 (large cell lymphoma)	35	1.5–3.5

n.d., not determined
[a] HeLaI and II are HeLa cell lines that differ with respect to the length of their telomeres
[b] The telomeric repeat region was measured by genomic blotting and includes 0.5 kb of non-TTAGGG repeats in addition to TTAGGG repeats (see Figs. 4, 5, and text for discussion)
[c] The length of telomeres in RD is taken from de Lange et al. (1990)

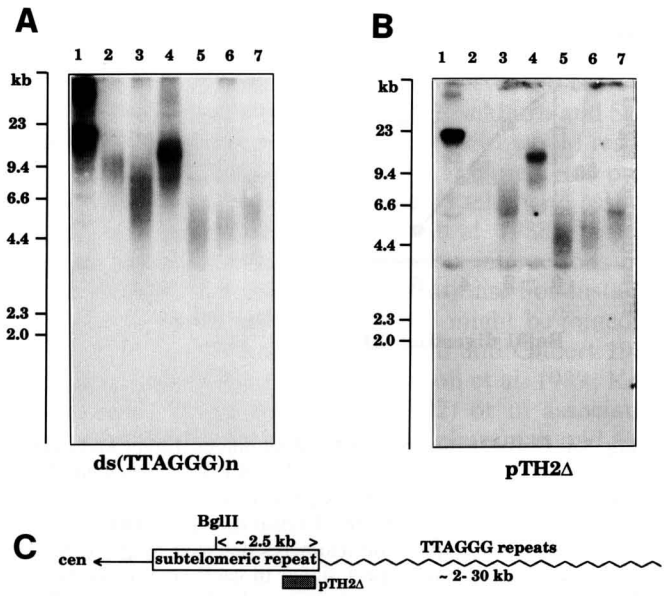

Fig. 4A–C. Genomic blotting analysis of telomeric length. DNA from human tumor cell lines was digested with BglII and analyzed by genomic blotting using a probe for TTAGGG repeats (pSP73.Sty11) (**A**) and a probe for a subtelomeric repeat element (pTH2Δ, **B**). Lanes 1, adherent HeLaI cells; lanes 2, Raji; lanes 3, suspension HeLaII cells; lanes 4, SU-LyB-1; lanes 5, large cell lymphoma SUP-M2; lanes 6, large cell lymphoma SU-LL-1; lanes 7, melanoma Melu. The signal with Raji DNA in both panels is low owing to underloading of the sample. Molecular weights are indicated in kilobase pairs. **C** Restriction map of the telomeres that are detected with a subtelomeric repeat probe (pTH2Δ) in **B** (from de Lange et al. 1990). The *open box* represents the subtelomeric repeat. The position of the pTH2Δ probe and the BglII site 2.5 kb from the junction of subtelomeric and telomeric DNA are indicated. The *wavy line* indicates the telomeric repeat region. This region is thought to contain approximately 0.5 kb of non-TTAGGG repeat at the proximal end (Allshire et al. 1989; Brown et al. 1990; Cross et al. 1990) followed by a variable TTAGGG repeat region. In BglII digests pTH2Δ also hybridizes to several discrete fragments (4, 6.5, and 15 kb) that are not near chromosome ends

meric associations (Fig. 4). However, chromosome ends that have lost most of the telomeric repeat region are not expected to be detected with TTAGGG repeats. Such severely shortened chromosome ends might still contain sequences proximal to the TTAGGG repeats. Previously, we have described the structure of a subtelomeric repeat that is present on several human chromosome ends (de Lange et al. 1990). These chromosome ends have well defined and nearly identical restriction maps (Fig. 4C) and can be detected with a cloned DNA probe for the subtelomeric repeat (pTH2Δ). Using the known restriction map of these telomeres and pTH2Δ as a probe, telomeric length was determined in the same set of cell lines as used in panel A of Fig. 4. In SU-LL-1 DNA, pTH2Δ detects a set of BglII fragments of 4–6 kb that according to the restriction map include 1.5 to 3.5 kb telomeric repeat DNA. A similar telomeric length is found for the SUP-M2 cell line, while Melu cells are deduced to contain 2.5–4.5 kb telomeric repeat DNA. These values are consistent with the findings with the TTAGGG repeat probe and argue that most chromosome ends in SU-LL-1 and Melu have retained telomeric repeat DNA. The other cell lines have considerably longer telomeres as expected from the data with the TTAGGG repeat probe and previous work (de Lange et al. 1990; de Lange 1992) (see Table 3 for summary).

The data in Fig. 4 suggest that the cell lines with chromosome end associations carry telomeres with several kilobases telomeric repeat DNA. This conclusion is based on previous mapping experiments that established the restriction map in Fig. 4. In this map the BglII site is placed at a distance of 2.5 kb from the telomeric repeat region because DNA beyond 2.5 kb from the BglII site appears to lack all restriction endonuclease cleavage sites (de Lange et al. 1990). To obtain independent evidence for the length of the telomeric repeat region we performed the following Bal31 experiment on Melu DNA.

In intact DNA, telomeres are preferentially digested by the exonuclease Bal31. The rate at which Bal31 removes DNA from the ends (in kilobases per hour) is constant over long periods of time and can be measured by determining the size of the digestion products. Similarly, the rate at which Bal31 removes the TTAGGG repeat signal from chromosome ends (percentage signal lost per hour) can be measured by quantitative hybridization. Comparison of these two values will yield an estimate for the initial length of the TTAGGG repeat region. In the experiment shown in Fig. 5, intact Melu DNA was digested with Bal31 for 0, 2, 4 and 6 h and the DNA was subsequently cleaved with BglII. This DNA was then analyzed with pTH2Δ to measure the rate of Bal31 digestion and with TTAGGG repeats to determine the loss of TTAGGG repeat signal at each time point. The pTH2Δ hybridization in Fig. 5 shows that the telomeric BglII fragments are progressively shortened, whereas several chromosome-internal BglII fragments that are also detected by pTH2Δ are not affected. The rate of Bal31 digestion of these telomeres was constant at approximately 0.85 kb per 2 h. The TTAGGG repeat hybridization in Fig. 5B and C shows a linear decrease in signal. Telomeric shortening is less apparent with this probe, because shorter fragments hybridize less to TTAGGG repeats. Quantitative analysis of the TTAGGG repeat signals show that Bal31 removed approximately 29% of the TTAGGG repeats in Melu telomeres per 2 h (panel C). Therefore it appears that the Melu telomeres contain in the order of 3 kb ($100/29 \times 0.85$ kb) of TTAGGG repeat DNA. In addition to TTAGGG repeats, the telomeric repeat region is thought to include approximately 0.5 kb of non-TTAGGG repeats at the proximal end of the telomere (Allshire et al. 1989; Brown et al. 1990; Cross et al. 1990). Control experiments show that these repeats are not detected by the TTAGGG repeat probe under the stringent hybridization conditions used in the experiment in Fig. 5 (data not shown). Thus the total length of the telomeric repeat region (TTAGGG repeats + non-TTAGGG repeats) is estimated to be approximately 3.5 kb based on our Bal31 data. This value is in good agreement with our estimate of 2.5–4.5 kb of telomeric repeat DNA based on the mapping data. Therefore we

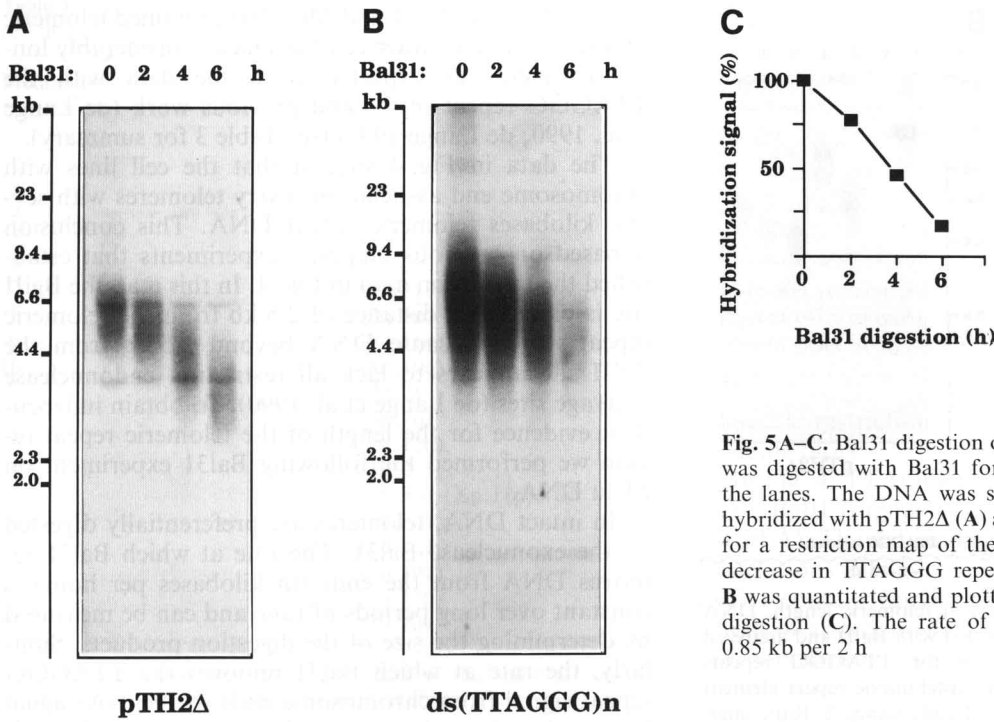

Fig. 5A–C. Bal31 digestion of Melu telomeres. Intact Melu DNA was digested with Bal31 for 0, 2, 4 and 6 h as indicated above the lanes. The DNA was subsequently cleaved with BglII and hybridized with pTH2Δ (**A**) and TTAGGG repeats (**B**). See Fig. 4 for a restriction map of the telomeres detected by pTH2Δ. The decrease in TTAGGG repeat signal in each lane in the blot in **B** was quantitated and plotted against the duration of the Bal31 digestion (**C**). The rate of Bal31 digestion was approximately 0.85 kb per 2 h

conclude that our estimates of the length of the TTAGGG repeat region at human telomeres based on the restriction map in Fig. 4 are correct. Table 3 summarizes the estimated length of the telomeric repeat region (including 0.5 kb of non-TTAGGG repeats) in each of the cell lines analyzed here.

Discussion

We have identified a large cell lymphoma cell line (SU-LL-1) and a melanoma cell line (Melu) with prominent telomeric associations and analysed the telomeric DNA in these cells. Our data indicate that the length of the TTAGGG repeat region of SU-LL-1 and Melu chromosome ends is 1–3 kb and 2–4 kb respectively. These values are derived from mapping experiments and were verified by Bal31 analysis of Melu DNA. The telomeres of SU-LL-1 and Melu are significantly shorter than the telomeres in most human cells. However, similarly short or shorter telomeres are present in two other cell lines without telomeric associations. Thus, telomeric reduction does not necessarily lead to chromosome end associations.

Several explanations may be given. First, the cell lines with chromosome end associations may contain a minority of chromosome ends that lack TTAGGG repeats altogether and are fusigenic for this reason. Using a subtelomeric probe we have not detected chromosome end fragments that could represent such TTAGGG repeat deficient chromosome ends. We have also found no evidence for an increase in the size heterogeneity of telomeres in these cell lines. However, we cannot exclude that chromosome ends without TTAGGG repeats also lack the subtelomeric DNA that was used as a probe in these experiments. In addition, since the telomeric associations involve less than 1% of the telomeres in SU-LL-1 and MeLu, they may be caused by a minor fraction of the chromosome ends that is not detectable by genomic blotting.

A second explanation for the variable phenotype associated with reduced telomeres is that short TTAGGG repeat tracts may behave differently in different cells. Perhaps some cell lines simply lack enzymes required for telomeric joining or express an activity that enhances the protective activity of reduced telomeres. Finally, it is conceivable that short telomeres do not associate unless they have an additional deficiency, e.g. loss of the correct terminal structure or complex. For instance, telomeres of hypotrichous ciliates have a 3′ protrusion of several G-rich telomeric repeats that are encapsulated by a telomeric protein (Klobutcher et al. 1981; Pluta et al. 1982; Henderson and Blackburn 1989; Gottschling and Cech 1984; Gottschling and Zakian 1986; Price 1990). Clearly, alterations in the maintenance and encapsulation of telomeric termini might contribute to loss of telomeric function and induce chromosome end associations. Such a deficiency may also result in shortening of the telomeres, for instance due to loss of the correct primer for telomerase mediated telomeric maintenance (Morin 1989), leading to an apparent correlation between telomeric reduction and chromosome end associations.

It has been proposed that the dicentric chromosomes created by telomeric associations cause genome instability in human cancer (Hastie and Allshire 1989) and senescent cells (Harley et al. 1990). A prerequisite for this model is that telomeric associations are stable through mitosis. The most stable telomeric association is a double stranded DNA ligation. However, as suggested by

Murray (1990), ligated telomeres may be resolved during replication. In *Tetrahymena* telomeres, the C-rich strand contains several nicks, which may serve to dissociate inadvertently ligated telomeres (see Blackburn and Szostak 1984). Replication through such nicks would resolve one of the two daughter molecules. The predicted products, dicentrics with single chromatid telomeric associations, have been observed (Pathak et al. 1988). In addition to ligation, several non-covalent interactions may be responsible for telomeric associations. For instance, 3′ single stranded ends of telomeres might be joined either by DNA-DNA interaction (Sen and Gilbert 1988; Sundquist and Klug 1989; Williamson et al. 1989; Kang et al. 1992; Smith and Feigon 1992) or in association with proteins (Lipps et al. 1982; Raghuraman and Cech 1989). Furthermore, the association of telomeres could be mediated by nuclear matrix or envelope components (Rabl 1885; Cremer et al. 1982; Agard and Sedat 1983; van Dekken et al. 1989; Chung et al. 1990; Rawlins et al. 1991; de Lange 1992). None of these interactions would be expected to persist and telomeric associations of this kind may have little impact on the stability of the genome.

Irrespective of the mechanism of telomeric association we can ask to what extent this phenomenon contributes to genome instability in human cells. Mandahl et al. (1985) observed histiocytoma cells with pairs of identical telomeric associations. The simplest interpretation of these duplications is that a telomeric association resulted in a dicentric, which persisted through mitosis, underwent non-disjunction, and was maintained through the following G1-, S-, and G2-phases. This indicates that telomeric associations are not necessarily resolved in each cell cycle and can lead to non-disjunction. However, a clonal expansion of a telomeric association has never been reported. Thus, dicentrics formed by telomeric association must have a limited life-span. In addition to non-disjunction, dicentrics may be lost by two routes. Their telomeric association could resolve or the dicentric could break. Breakage of dicentrics is suggested to contribute to genome instability by initiating repeated breakage-fusion-bridge cycles with other chromosomes. However, there is little cytogenetic evidence for these kinds of events. For instance, while we have found a high frequency of telomeric associations in the SU-LL-1 cell line, we do not observe non-clonal chromosomal rearrangements in these cells. Similarly, the histiocytoma of Mandahl et al. (1985) has multiple telomeric associations in nearly every metaphase but lacks the secondary rearrangements expected from breakage-fusion-bridge events. Thus, the cytogenetic data currently available indicate that telomeric associations may contribute to chromosome loss and duplication. The resulting loss of heterozygosity could play a role in tumorigenesis and cellular senescence. By contrast, there is as yet no reason to believe that dicentrics formed through telomeric associations undergo breakage-fusion-bridge cycles. Therefore, the idea that telomeric associations could lead to chromosomal rearrangements remains hypothetical.

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