

## Structure and Variability of Human Chromosome Ends

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Received 21 August 1989/Accepted 17 October 1989

Mammalian telomeres are thought to be composed of a tandem array of TTAGGG repeats. To further define the type and arrangement of sequences at the ends of human chromosomes, we developed a direct cloning strategy for telomere-associated DNA. The method involves a telomere enrichment procedure based on the relative lack of restriction endonuclease cutting sites near the ends of human chromosomes. Nineteen (TTAGGG)<sub>n</sub>-bearing plasmids were isolated, two of which contain additional human sequences proximal to the telomeric repeats. These telomere-flanking sequences detect BAL 31-sensitive loci and thus are located close to chromosome ends. One of the flanking regions is part of a subtelomeric repeat that is present at 10 to 25% of the chromosome ends in the human genome. This sequence is not conserved in rodent DNA and therefore should be a helpful tool for physical characterization of human chromosomes in human-rodent hybrid cell lines; some of the chromosomes that may be analyzed in this manner have been identified, i.e., 7, 16, 17, and 21. The minimal size of the subtelomeric repeat is 4 kilobases (kb); it shows a high frequency of restriction fragment length polymorphisms and undergoes extensive de novo methylation in somatic cells. Distal to the subtelomeric repeat, the chromosomes terminate in a long region (up to 14 kb) that may be entirely composed of TTAGGG repeats. This terminal segment is unusually variable. Although sperm telomeres are 10 to 14 kb long, telomeres in somatic cells are several kilobase pairs shorter and very heterogeneous in length. Additional telomere reduction occurs in primary tumors, indicating that somatic telomeres are unstable and may continuously lose sequences from their termini.

Eucaryotic chromosomes end in specialized structures, called telomeres (31), that are thought to fulfill at least three functions. First, telomeres protect natural double-stranded DNA ends from degradation, fusion, and recombination with chromosome-internal DNA (28). Second, cytogenetic observations indicate that telomeres are located at the nuclear periphery, suggesting a role for chromosome ends in the architecture of the nucleus (1, 36). Third, telomeres must provide a solution to the end-replication problem (46): because all known polymerases require a primer and synthesize DNA from 5' to 3', the 3' ends of linear DNA pose a problem to the replication machinery.

The single common structural feature of eucaryotic telomeres is the presence of a tandem array of G-rich repeats which, according to genetic studies in *Saccharomyces cerevisiae*, are necessary and sufficient for telomere function (26, 44). Although all telomeres of one genome are composed of the same repeats, the terminal sequences in different species vary. For instance, *Oxytricha* chromosomes terminate in TT TGGGG repeats (24), *Tetrahymena* utilizes an array of (TTGGGG)<sub>n</sub> (7), plant chromosomes carry the sequence (TTTAGGG)<sub>n</sub> (37), and trypanosomes and mammals have TTAGGG repeats at their chromosome ends (see below) (6, 9, 16, 30, 45). The organization of the telomeric repeats is such that the G-rich strand extends to the 3' end of the chromosome. At this position, telomerase, an RNA-dependent DNA polymerase demonstrated to be present in *Tetrahymena thermophila* and other ciliates, can elongate telomeres, probably by using an internal RNA component as template for the

addition of the appropriate G-rich sequence (20–22, 42, 47). This activity is thought to complement the inability of polymerases to replicate chromosome ends, but other mechanisms of telomere maintenance may operate as well (35).

Proximal to the essential telomeric repeats, some chromosome ends harbor additional common elements called subtelomeric repeats or telomere-associated sequences (12, 15, 17, 45; see also reference 18 and references cited therein). Unlike the telomeric repeats, these sequences are not conserved and their function remains unclear (32).

Chromosome ends of unicellular organisms often show structural instability. Frequent rearrangements of subtelomeric sequences occur in trypanosomes (8, 17), *S. cerevisiae* (10, 23), and plasmodia (15), and changes in the telomeric repeat region can be observed in protozoa (4, 34, 45), ciliates (25), and fungi (11, 26, 27). As much as 3.5 kilobase pairs (kb) was seen to be added to telomeres of *Trypanosoma brucei* in a process that appears gradual and continuous, and was calculated to result from the addition of 6 to 10 base pairs (bp) per end per cell division (4, 34, 45). A similar gradual telomere elongation, compatible with the addition of telomeric repeats by telomerase, occurs in continuously growing *T. thermophila* (25) and a cell cycle mutant (*cdc17*) of *S. cerevisiae* (11). In wild-type *S. cerevisiae* (41), however, and in *T. thermophila* grown in batch cultures (25), the tandem array of telomeric repeats is maintained at constant length. At least four genes (*CDC17*, *EST1*, *TEL1*, and *TEL2* [11, 26, 27]) govern the length and stability of yeast telomeres; their mode of action is not understood.

Much less is known about the structure and behavior of chromosome ends of multicellular organisms. Recently, mammalian telomeres became amenable to molecular dissection with the demonstration that telomeric repeats of

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plants and *T. thermophila* species cross-hybridize to vertebrate chromosome ends (3, 37). Subsequently, it was shown that human DNA contains tandem arrays of TTAGGG repeats, probably at chromosome ends only, providing further evidence for the evolutionary conservation of telomeres and a tool for the isolation of telomeric DNA (30). Two strategies to obtain human chromosome ends have proven successful: an indirect isolation protocol that relies on human telomeres to be functional in *S. cerevisiae* (9, 16) and direct cloning in *Escherichia coli* (see below).

Here we describe the first detailed characterization of the structure and variability of human autosomal chromosome ends. The chromosome ends we have studied share a subtelomeric repeat of at least 4 kb that is not conserved in rodent genomes. The germ line configuration of these chromosome ends is characterized by a long stretch of DNA (of up to 14 kb) that lacks restriction enzyme cutting sites and may be entirely composed of TTAGGG repeats. From this region sequences are lost during development, leading to shortened, heterogeneously sized telomeres in somatic tissues, primary tumors, and most cell lines.

### MATERIALS AND METHODS

**Southern blotting.** Solid tissues were stored at  $-70^{\circ}\text{C}$ . Cell lines, peripheral blood, and semen were processed without storage. Solid tissues were minced in TNE (10 mM Tris hydrochloride [pH 7.5], 100 mM NaCl, 10 mM EDTA) in a Polytron at setting 3. Sodium dodecyl sulfate (0.5%) and proteinase K (100  $\mu\text{g}/\text{ml}$ ) were added, and the DNA was incubated at  $55^{\circ}\text{C}$  for at least 1 h. The DNA was isolated by phenol-chloroform extraction and isopropanol precipitation. The proteinase K incubation and extraction were repeated once, and the DNA was dissolved in TE (10 mM Tris hydrochloride [pH 7.5], 0.1 mM EDTA) at 200 to 500  $\mu\text{g}/\text{ml}$  and stored at  $4^{\circ}\text{C}$ . Peripheral blood DNA from buffy coat cells and DNA from cell lines were prepared similarly. Sperm DNA was isolated as follows. Semen was diluted 10-fold in phosphate-buffered saline and centrifuged at 4,000 rpm in a Sorvall HG-41 rotor to collect spermatozoa. Pellets were suspended in 10 mM Tris hydrochloride (pH 8.0)–10 mM EDTA–100 mM NaCl–40 mM dithiothreitol–20  $\mu\text{g}$  of proteinase K per ml, and sodium dodecyl sulfate was added to 2%. Further treatment was carried out as described above. Southern blots were prepared with 8 to 12  $\mu\text{g}$  of DNA per lane separated on 0.8 to 1.2% agarose gels run at 10 to 30 V for 20 cm. Gels were treated with 0.25 N HCl for 20 min, 0.5 M NaOH–1.5 M NaCl twice for 30 min each, and 0.5 M Tris hydrochloride (pH 7.5)–3 M NaCl twice for 30 min each prior to blotting onto Hybond membrane in  $20\times$  SSC ( $1\times$  SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The DNA was cross-linked to membranes by UV irradiation. Hybridizations were done as described by Church and Gilbert (13). The (TTAGGG)<sub>70</sub> repeat probe (45) was labeled by nick translation (38); all other probes were labeled by using random primers (19). Final washes were at  $65^{\circ}\text{C}$  in 40 mM sodium phosphate buffer (pH 7.2)–1 mM EDTA–1% sodium dodecyl sulfate.

**BAL 31 digestion of genomic DNA.** Human kidney DNA (200  $\mu\text{g}$ ) was treated with 50 U of BAL 31 (mixed activity; International Biotechnologies, Inc.) in 800  $\mu\text{l}$  of BAL 31 mix (International Biotechnologies). Samples were phenol extracted at 0, 1, 2, 3, and 6 h; the DNA was collected by ethanol precipitation, and 30  $\mu\text{g}$  of DNA from each time point was digested with *Ava*II. Three identical sets of DNA (10  $\mu\text{g}$  per lane) were size fractionated on a 0.8% agarose gel and blotted as described above.

**Isolation of telomere-derived clones.** A telomere-enriched library was constructed as follows. Human kidney DNA (100  $\mu\text{g}$ ) was treated with 10 U of BAL 31 (mixed activity) for 1 h at  $30^{\circ}\text{C}$  to remove about 100 bp and create flush ends. The DNA was isolated and cut simultaneously with *Alu*I, *Dra*I, *Eco*RV, *Hinc*II, *Rsa*I, and *Xmn*I, and 20  $\mu\text{g}$  of *Eco*RI linkers (10-mer; New England BioLabs, Inc.) was ligated to the fragments. The mixture was treated with 1,000 U of *Eco*RI for 2 h and size fractionated through 0.5% low-gelling-temperature agarose (Bethesda Research Laboratories, Inc.) at 1 V/cm for 20 h. Ten gel slices in the high-molecular-size range (2 to 25 kb) were analyzed by Southern blotting to identify (TTAGGG)<sub>n</sub>-containing fractions. The three largest fractions (10 to 25 kb) were positive and were processed further. DNA was isolated by agarase hydrolysis (Calbiochem-Behring) and ligated to *Eco*RI-digested, dephosphorylated pSP73 (Pharmacia, Inc.). The ligation mixture (160  $\mu\text{l}$ ) was used to transform 40 aliquots (50  $\mu\text{l}$ ) of competent *E. coli* HB101, and the resulting transformants were plated on Hybond filters. The library was screened with a nick-translated double-stranded (TTAGGG)<sub>70</sub> probe (45). Nineteen positive clones were amplified and sequenced on supercoiled DNA by using Sequenase (Pharmacia) and primers flanking the insert.

**Subcloning.** pTH2 $\Delta$  and pTH14 $\Delta$  were derived as follows. pTH2 and pTH14 were linearized by using a site in the polylinker next to the TTAGGG repeats, treated with BAL 31, and subsequently digested to release the insert. Appropriately shortened fragments were subcloned into pSP73 and sequenced to verify the absence of TTAGGG repeats. The inserts in the subclones are 380 bp for pTH2 $\Delta$  and 400 bp for pTH14 $\Delta$ .

### RESULTS

**Isolation of human telomeres and their flanking sequences.** We used a double-stranded (TTAGGG)<sub>70</sub> probe, derived from a trypanosome telomere (45), to hybridize to human DNA that had been digested with the exonuclease BAL 31 for increasing periods and subsequently cut with different restriction enzymes. In agreement with Moyzis et al. (30), we found that this sequence preferentially recognizes BAL 31-sensitive DNA fragments, i.e., chromosome ends (see, for example, Fig. 3). In addition, we found through the use of more than 60 different restriction enzymes that most endonucleases yield telomeric fragments larger than 10 kb in the DNA we used. This suggested a way to enrich for telomeric sequences: by digesting genomic DNA with several enzymes that cut frequently in chromosome-internal loci but not near telomeres, chromosome ends should be enriched in the high-molecular-size fraction of genomic DNA.

Our strategy for the isolation of human chromosome end sequences was as follows (Fig. 1). Human kidney DNA was briefly treated with BAL 31 nuclease to modify (blunt) the very end of the telomere for subsequent cloning steps. The DNA was digested with *Alu*I, *Dra*I, *Xmn*I, *Hinc*II, *Eco*RV, and *Rsa*I. These enzymes yield blunt-ended telomeric fragments that are larger than 10 kb, even though their combined activity degrades genomic DNA to an average size of about 800 bp. DNA fragments larger than 10 kb were isolated by agarose gel electrophoresis and inserted in a plasmid vector by using *Eco*RI linkers. Based on DNA quantitation, we estimate that telomeric fragments are enriched at least 100-fold by this procedure.

A library of about 10,000 recombinant plasmids contained 19 clones that hybridized to a double-stranded (TTAGGG)<sub>70</sub>

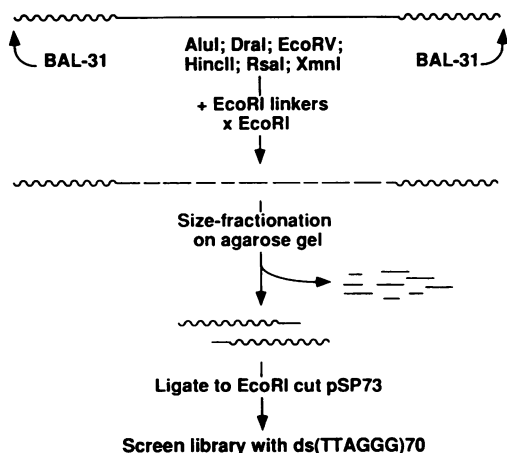


FIG. 1. Schematic representation of the procedure used to clone telomeric DNA from the human genome. See the text for discussion and experimental details.

probe. Sequence analysis showed that all but two of these plasmids have inserts that are composed solely of TTAGGG repeats (Fig. 2). The inserts, varying from 60 to 900 bp, are much shorter than the DNA that was ligated to the vector (which was at least 10 kb), indicating that substantial recom-

bination had occurred during cloning. We also found that two plasmids had lost part of the vector sequence. Similar instability of cloned telomeric sequences in *E. coli* has been reported previously (37, 45).

Two plasmids (pTH2 and pTH14 [Fig. 2]) contained additional human sequences adjacent to TTAGGG repeats. pTH2 contains eight complete TTAGGG repeats preceded by a 390-bp G+C-rich (80%) sequence that contains six copies of a 29-bp direct repeat. pTH14 has a 410-bp sequence of more moderate G+C content (50%), with no homology to pTH2, and terminates with approximately 80 TTAGGG repeats.

For the experiments described below, we removed all TTAGGG repeats from both clones by deletion and subcloning (see Materials and Methods). These probes, called pTH2 $\Delta$  and pTH14 $\Delta$ , were hybridized to genomic DNA that had been treated with BAL 31 nuclease and subsequently digested with *Ava*II. The results show that both probes detect heterogeneous large fragments that are progressively shortened by BAL 31 nuclease (Fig. 3). This indicates that pTH2 and pTH14 are indeed derived from human telomeres. The linkage of TTAGGG repeats to DNA that detects BAL 31-sensitive genomic loci provides further evidence that human telomeres contain TTAGGG repeats, as suggested by the work of Moyzis et al. (30). The flanking sequence in pTH14 detects many additional DNA fragments that are not

pTH1; pTH3-13; pTH15-19:

TTAGGG TTAGGG TTAGGG TTAGGG TTAGGG TTAGGG TTAGGG TTAGGG TTAGGG (TTAGGG)<sub>1-90</sub>

pTH2:

GACCGCGCCA CCCCCGGCGT CCCC GCCCGG CCGCGTCAGA CTGTTCCGGT GTAACAGGCC CCGCCACCAT 70  
 CAGCCAGCG ACCTGGCTCG GTGCCCTGC GCCCGCTCA ATCCCGCTCG CCCAGCGACA CCTCCCCTCT 140  
 GGGAGCGCC CCGCCTGCC CAATGACCTA CACCGCTCT CCCAAAGCG\* CCGCGCCTC TCTGCCCTG 210  
 CGCGGGCGG\* CCGCCCTCT CTCCGCTCG CCGGGCGCG\* CGCGCTCTC TCGCCTCGG CCGGCCCGC 280  
 CGCCTCTCT CCGCCTCGG CCGCGCGCG\* CGCCTCTCG CGCCTCGCG GCGCGCGCG\* GCCTCTCTG 350  
 GCCTCGCGG CCGCCCGCCT TTTGGGAGGA TCGAGTTGG (TTAGGG)<sub>8</sub>

pTH14:

GACCGCGAGG CCGGAGTGC GTTCTGCTCA GCACAGACCT GGGGTCACC GTAAGGTGG AGCAGCATT 70  
 CCCTAAGCAC AGACGTTGG GCCACTGGCT GGCTTTGGGA CAACTCGGG CGCATCAACG GTGAATAAAA 140  
 TCTTCCCGG TTGCAGCCGT GAATAATCAA GTCAGAGAC CAGTTAGAGC GGTTCAGTGC GAAAAACGG 210  
 AAAGCAAAG CCCCTCTGAA TCTGGGCAG CAGATTCTCC CAAGCTAAGG CGAGGGCTG CATTAAAGG 280  
 TCCAGTTGCA GCATCGGAAC GCAAATGCAG CAGTCCTAAT GCACAGATGA TACCCAAAAT ATAACACCG 350  
 CGTTCCTCAT GTCGTTAGG TAGCGTCAGG GTCGGGTCG GGTTCGGGT CAGTTCAGG (TTAGGG)<sub>80</sub>

FIG. 2. Nucleotide sequences of recombinant plasmids from a human telomere-enriched library. The figure shows sequences of the inserts in 19 plasmids that were detected with a nick-translated double-stranded (TTAGGG)<sub>70</sub> probe in a library enriched for telomeres described in the text and Fig. 1. The inserts in 17 clones (shown at the top) are composed solely of variable numbers of TTAGGG repeats. pTH2 and pTH14 contain TTAGGG repeats preceded by telomere-flanking sequences. The asterisks in the pTH2 sequence mark the first base of six direct repeats of 29 bp. The sequences of the inserts in pTH2 and pTH14 may represent a distorted version of their original genomic loci owing to recombination in *E. coli* (see text). The GenBank accession numbers are M29361 for pTH2 and M29360 for pTH14.



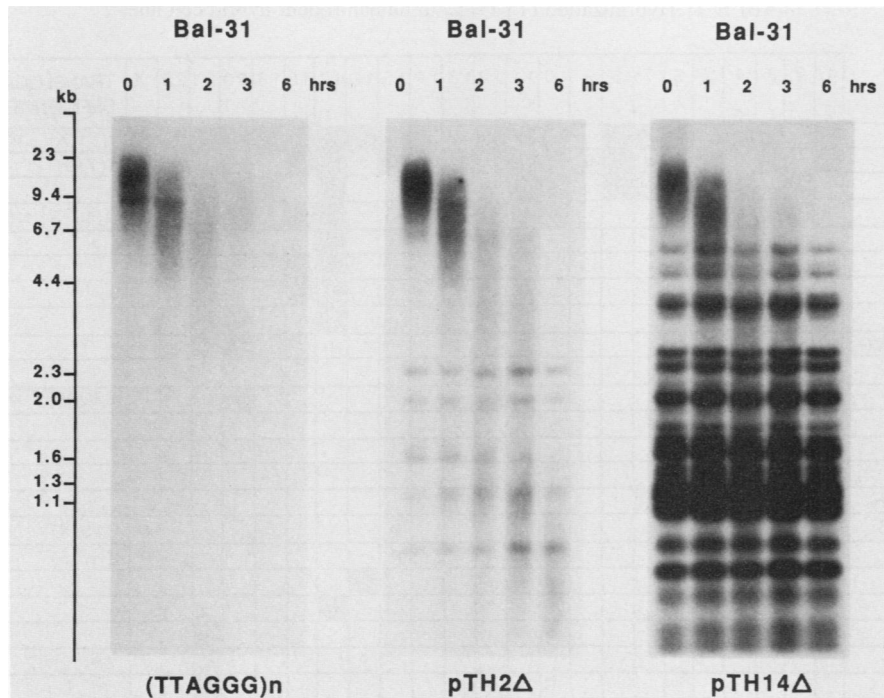


FIG. 3. The proximal sequences in pTH2 and pTH14 detect BAL 31-sensitive loci. Human kidney DNA was treated with BAL 31 for the times indicated and subsequently digested with *Ava*II. Three identical Southern blots were hybridized to double-stranded (TTAGGG)<sub>70</sub>, the proximal sequence in pTH2 (pTH2Δ), and the proximal sequence in pTH14 (pTH14Δ).

affected by BAL 31 digestion. Attempts to analyze the telomere(s) from which pTH14 is derived have been confounded by this hybridization pattern. In contrast, pTH2Δ hybridizes to only a few BAL 31-resistant fragments, which allowed us to characterize this probe in detail.

**pTH2Δ detects a subtelomeric repeat at a subset of the human autosomal chromosome ends.** The copy number of the pTH2Δ sequence was examined by quantitative hybridization analysis of human DNA with pTH2Δ as a standard (data not shown). These experiments suggest that the pTH2Δ is a repetitive element that is present on 10 to 25% of the human chromosome ends. However, because pTH2Δ may detect different chromosome ends with variable efficiency, this copy number assessment could be inaccurate.

pTH2Δ does not cross-hybridize efficiently to rodent DNA. This enabled us to use human-rodent hybrid cell lines to identify chromosomes whose ends are detected by this probe. Table 1 summarizes the results of hybridization of pTH2Δ to 23 human-rodent hybrid cell lines. The most informative data come from inspection of hybrids that contain single human chromosomes. These hybrids show that several chromosomes are detected by pTH2Δ with variable efficiency (e.g., chromosomes 7, 16, 17, and 21), whereas other chromosomes do not hybridize at the stringency used (e.g., chromosomes 3, 4, 15, and X). The hybridization of pTH2Δ to chromosome 21 is probably due to the long arm of this chromosome, because a hybrid that contains only the distal part of 21q, XTR3-BsAgB (Table 1), hybridizes well. This conclusion is corroborated by the use of pTH2Δ to complete a physical map of the last 700 kb of 21q, which includes several previously mapped markers for this chromosome arm (M. Burmeister, D. R. Cox, and R. M. Myers, manuscript in preparation).

Although pTH2Δ detects more than one human chromosome end, we were able to derive a restriction map of its

genomic environment in total human DNA (Fig. 4). Restriction enzyme analysis of isolated human chromosomes (chromosomes 7, 16, and 21) in human-rodent hybrids gives results that are in agreement with this physical map (data not shown). It appears, therefore, that pTH2Δ is part of a subtelomeric repeat that is present near the telomeres of a subset of the human chromosomes, e.g., chromosomes 7, 16, 17, and 21 and possibly others. On the basis of the published physical maps, we surmise that this subtelomeric repeat is different from the two telomere-flanking sequences that were recently isolated by cloning in yeasts (9, 16).

**Polymorphisms in the subtelomeric repeat.** The structure of the subtelomeric repeat shows minor variations. For instance, the distance between the two *Sty*I sites varies from 3.8 to 4.4 kb at different chromosome ends, and some chromosomes lack the most terminal *Sty*I site (Fig. 4). A similar variability in restriction fragment length was observed with other enzymes that yield fragments from within the subtelomeric repeat, e.g., *Ava*II, *Pst*I, and *Sau*96A (data not shown). When DNAs from different sources are examined with these enzymes, a highly polymorphic pattern is observed (Fig. 5) that is reminiscent of the polymorphisms detected by the VNTR (variable number of tandem repeat) probes described by Nakamura et al. (33).

**De novo methylation of subtelomeric *Hpa*II sites.** The sequence of pTH2Δ is very G+C rich and, unlike most human DNA, contains roughly equal numbers of CpG and GpC dinucleotides (68 and 77, respectively [Fig. 2]). In this respect pTH2Δ resembles the CG islands associated with the 5' ends of many genes (5). CG islands are usually not methylated, and it has been suggested that their abundance of CpG dinucleotides is maintained because this DNA is not methylated in the germ line, where deamination of <sup>Me</sup>CpG can result in irreversible loss of CpG dinucleotides. A similar mechanism may operate at telomeres: in germ line DNA,

TABLE 1. Hybridization of pTH2Δ to human-rodent hybrid cell lines<sup>a</sup>

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	TRANSLOCATIONS DEFICIENCIES	pTH2 Δ
XER-11	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	t(11;X), t(X;11)	■
JSR-17G	■	■	■	■	■	■	●	■	●	■	■	■	■	■	■	■	■	■	■	■	■	■	■	t(7;9)	■
WIL-6	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■
TSL-2	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	t(17;3),t(3;17)	■
WIL-2	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■
WIL-13	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■
WIL-5	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■
ICL-15	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■
REW-8-DCSAz-3	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■
JSR-2	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■
XTR-3BsAgB	■	■	●	■	■	■	■	■	■	■	●	■	■	■	■	■	■	■	■	■	■	■	■	t(3;X), 10q <sup>-</sup> , 21q	■
HA(3)BB	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■
HA(30)	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■
9TK	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■
DUA-1-CSAzF	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■
DUA-1CSAzB	■	■	■	■	■	■	■	■	■	■	■	■	■	■	●	■	■	■	■	■	■	■	■	t(15;X)	■
DUA-1A	■	■	■	■	■	■	■	■	■	■	■	■	■	■	●	■	■	■	■	■	■	■	■	t(X;15)	■
HA(16)I	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■
IT22xWER1	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■
MH-21	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■
SSC16-5	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■
CHG3	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■

<sup>a</sup> Somatic cell hybrid DNAs were digested with *SryI*, *EcoRI*, or *MboI* and analyzed by Southern blotting with pTH2Δ as a probe. The column to the right depicts the efficiency with which pTH2Δ detects DNA sequences in the hybrids (■, strong hybridization; ▨, weak hybridization; □, no hybridization). The presence of human chromosomes in the cell lines is indicated. Somatic-cell hybrid clones was considered positive for a given chromosome if more than 10% of the cells contained it (■). If only part of this chromosome (briefly described under translocations and deficiencies) is present, this is indicated by ●. The details of these translocations and deficiencies are as follows: t(11;X) = 11qter→11p11::Xq11→Xqter; t(X;11) = Xpter→Xq11::11p11→11pter; t(7;9) = 7pter→7q22::9p24→9pter; t(17;3) = 17qter→17p13::3p21→3pter; t(3;17) = 3p21→3pter::17p13→17qter; t(3;X) = 3pter→3q21::Xq28→Xqter; t(15;X) = 15qter→15p11::Xp11→Xqter; t(X;15) = 15p11→15qter::Xpter→Xp11; XTR-3BsAgB contains a fragment of chromosome 21 and is positive for a marker for 21q22 (superoxide-dismutase 1); the breakpoint of the 10q<sup>-</sup> chromosome in this cell line is not known.

*HpaII*, which cuts CCGG but not C<sup>Me</sup>CGG, creates very small fragments that hybridize to pTH2Δ, indicating that methylated CpG dinucleotides are scarce (Fig. 6). In contrast, a high level of methylation is observed in peripheral blood DNA, resulting in large *HpaII* fragments that are due to the presence of C<sup>Me</sup>CpG, because digestion with *MspI*, an isoschizomer of *HpaII* that is not sensitive to CpG methyl-

ation, reproduces the germ line pattern (Fig. 6). CpG methylation, as detected with *HpaII*, was observed in all somatic tissues we have analyzed, including adrenal gland, pancreas, salivary gland, kidney, and brain tissues (data not shown). This indicates that the subtelomeric repeat undergoes de novo methylation during the formation of somatic tissues.

**Telomeres contain a long region of TTAGGG repeats.**

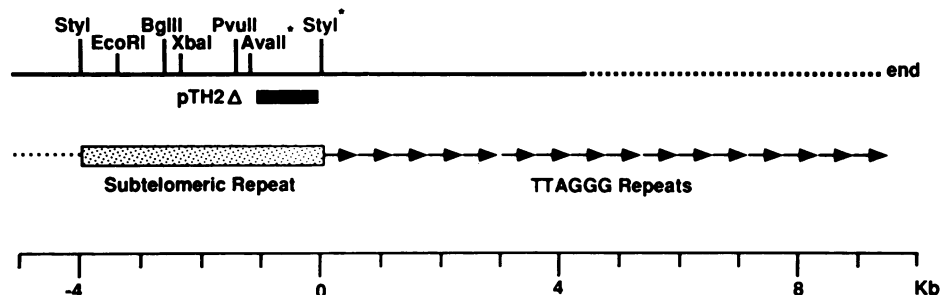


FIG. 4. Structure of chromosome ends that carry the pTH2Δ subtelomeric repeat. The physical map was deduced from single and double digestions of normal kidney DNA with the indicated enzymes. The approximate position of pTH2Δ is indicated below the map. The schematic below represents the inferred structure of these chromosome ends (see the text). The length of the TTAGGG repeat region is highly variable. The *SryI* site marked \* varies slightly in position at different chromosome ends, and at least one copy of the subtelomeric repeat lacks this site. The proterminal position of the *SryI* fragments that represent (part of) the subtelomeric repeat was verified by BAL 31 digestion. The *AvaII* site marked \* in the subtelomeric repeat is also variable: some chromosome ends contain only the *AvaII* site indicated; other subtelomeric repeats contain additional sites (see the text and Fig. 5). The telomeric region (indicated by the TTAGGG repeats) is not cut by the following restriction endonucleases: *AatII*, *AflII*, *AhaII*, *AluI*, *Apal*, *AseI*, *AvaI*, *AvaII*, *BalI*, *BamHI*, *BanII*, *BbvI*, *BclI*, *BglI*, *BglII*, *Clal*, *DraI*, *EagI*, *EcoRI*, *EcoRV*, *FokI*, *FspI*, *HaeII*, *HgiA1*, *HindII*, *HinfI*, *HpaI*, *KpnI*, *MaeI*, *MluI*, *NaeI*, *NarI*, *NciI*, *NcoI*, *NdeI*, *NotI*, *NruI*, *NsiI*, *OxaNI*, *PpuMI*, *PstI*, *PvuI*, *PvuII*, *RsaI*, *SacI*, *SacII*, *Sau3A*, *Scal*, *ScrII*, *SfaNI*, *SfiI*, *SmaI*, *SnaBI*, *SpeI*, *SphI*, *StuI*, *TaqI*, *XbaI*, *XhoI*, and *XmnI*.

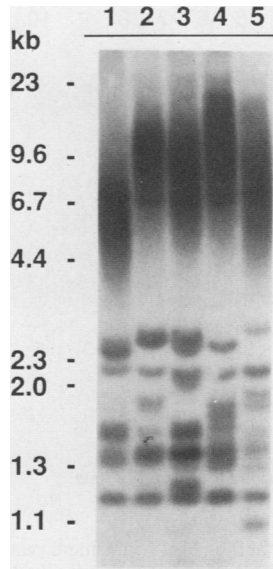


FIG. 5. Restriction (*Ava*II) fragment length polymorphisms. Peripheral blood DNAs from five unrelated individuals were cut with *Ava*II and analyzed by Southern blotting with pTH2Δ as a probe.

Distal to the subtelomeric repeat there is a long terminal region that is not cut by more than 60 restriction enzymes (Fig. 4). This is most easily explained by the presence of a substantial number of TTAGGG repeats at the ends of human chromosomes. The presence of long telomeric repeat regions is in agreement with the observation that (TTAGGG)<sub>n</sub>-containing genomic restriction fragments are usually longer than 10 kb. Although telomeres of most unicellular organisms have a more modest size, similarly long telomeric repeat regions are found in trypanosomes (4, 45). It is not excluded that the "barren" region distal to the *Sty*I site is composed of TTAGGG repeats punctuated with other repetitive sequences that lack restriction sites (see Discussion).

**Terminal heterogeneity.** As mentioned above, restriction fragments that contain the very end of the telomere are often heterogeneous in size and appear as heterodispersed bands that extend over several kilobase pairs (Fig. 4, 5, and 7). The size heterogeneity is prominent in all somatic DNA samples and does not seem to be a consequence of the DNA isolation procedure. Similar heterodispersed patterns are found in hybrid cell lines, including those that contain single human chromosomes, which suggests that terminal heterogeneity exists at individual chromosome ends and arises soon after hybrid formation (data not shown). These data suggest that the presence of large variations in the position of the end of the telomere is an intrinsic feature of somatic human chromosomes. Alternatively, the heterodispersed patterns could be the result of an unusual structure that leads to aberrant migration in agarose gels.

**Loss of telomeric sequences in somatic cells.** Several authors have observed that human chromosome ends are shortened in peripheral blood lymphocytes compared with germline DNA from the same individual (3, 9, 14, 16). The diminution was suggested to result from the loss of sequences from the termini (14), but this issue could not be addressed because the variable region had not been mapped precisely. Here we describe the length variation of the

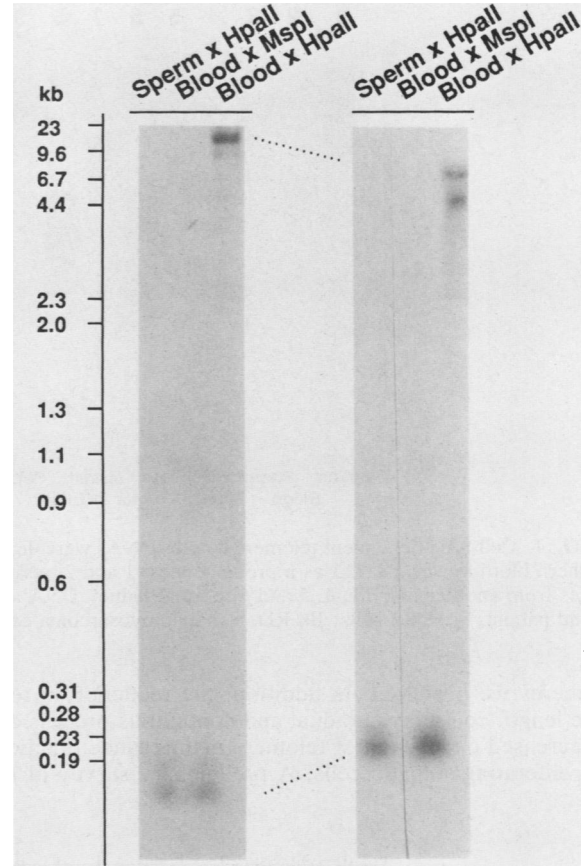


FIG. 6. De novo CpG methylation near telomeres. Sperm and peripheral blood DNA from two individuals was digested with *Msp*I or *Hpa*II as indicated and analyzed by Southern blotting with pTH2Δ as a probe.

chromosome ends detected by pTH2Δ in a variety of human tissues, tumors, and cell lines.

We examined the average length of telomeres in six sets of matched sperm and peripheral blood samples by using pTH2Δ as a probe on DNA digested with *Bgl*II, *Ava*II, or *Pvu*II (Fig. 7, lanes 1 to 4; Fig. 8, lines 1 to 6). In each case the telomeres are several kilobases shorter in blood DNA. For one individual (Fig. 8, line 6), we were able to compare sperm, blood, and salivary gland samples. Again, the germ line DNA contains the longest telomeres. Two additional somatic tissues (kidney and mammary gland [Fig. 8, lines I, II, and III]) for which matching germ line DNA was not available contained telomeres that were shorter (by about 2.0 kb) than the average sperm telomeres (11.5 kb).

To determine where the changes in telomere length occur, digested all DNAs with *Sty*I and hybridized then with pTH2Δ. *Sty*I-digested DNA always yielded fragments of approximately 4 kb (Fig. 7; data not shown), showing that the tissue-specific variability in telomere length reflects changes in the length of the barren region distal to the *Sty*I site at position 0 kb in Fig. 4.

These data suggest that sequences are lost from all telomeres in concert during the generation or expansion of differentiated cell populations such as peripheral blood lymphocytes. Sudden deletions, occurring early in development, outside the germ line, could have generated the shortened



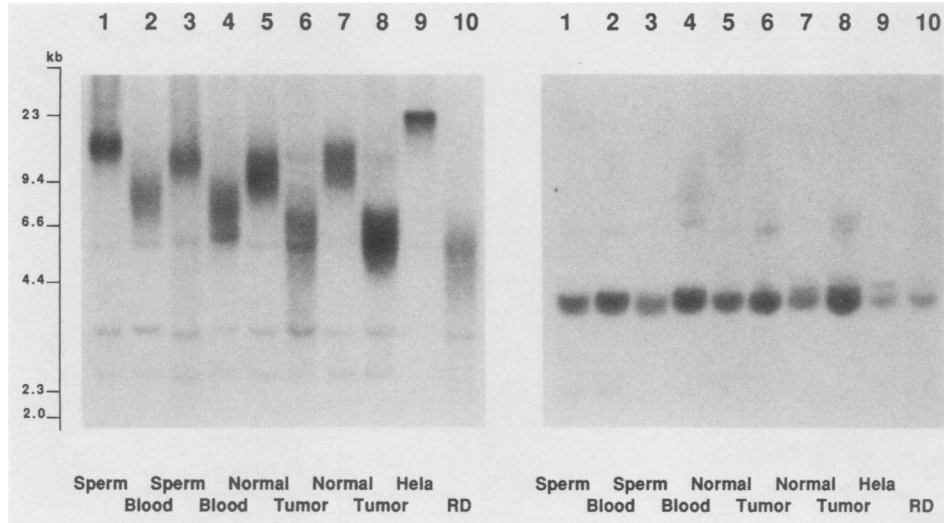


FIG. 7. Cell-type-dependent telomere length. DNAs were digested with *Bgl*II (left-hand panel) or *Sty*I (right-hand panel) and analyzed by Southern blotting with pTH2Δ as a probe. Lanes: 1 and 2, germ line (sperm) and peripheral blood from one individual; 3 and 4, the same tissues from another individual; 5 and 6, normal kidney DNA and Wilms' tumor DNA from one patient; 7 and 8, the same tissues from a second patient; 9, HeLa cells; 10, RD, a rhabdomyosarcoma cell line.

telomeres we observed. In addition, the reduction in telomere length could be a gradual and continuous process due to decreased expression of telomere maintenance functions in proliferating somatic cells. A preliminary survey of hu-

man tumors suggests that proliferating cells continue to lose telomeric repeats. Two DNA samples from individuals with Wilms' tumor (a childhood kidney neoplasia) were compared with neighboring normal kidney DNA and found to contain

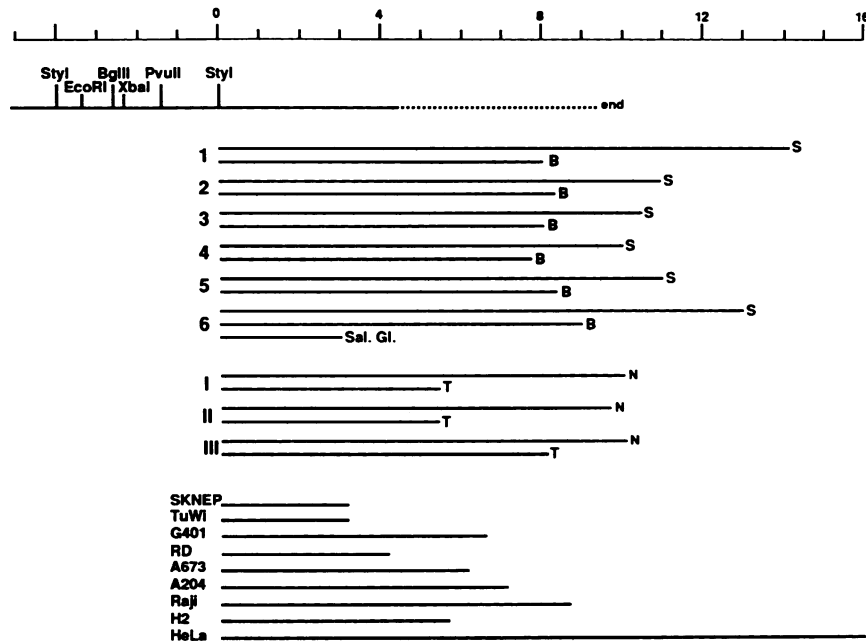


FIG. 8. Schematic representation of the average length of telomeres in human tissues, tumors, and cell lines. The top of the figure shows the restriction map of the chromosome ends that contain the pTH2Δ subtelomeric repeat. The average length of telomeres in human tissues, tumors, and cell lines is depicted below as the distance between the *Sty*I site at position 0 kb and the end of the chromosome. Lines: 1 to 5, germ line (S) and peripheral blood (B) of five individuals; 6, germ line (S), peripheral blood (B), and salivary gland (Sal. Gl.) of one individual; I and II, normal kidney (N) and Wilms' tumor (T) from two patients; III, normal mammary gland (N) and mammary carcinoma (T) from one patient; SKNEP, TuWi, and G401, Wilms' tumor cell lines; RD, A673, and A204, rhabdomyosarcoma cell lines; Raji and H2, EBV-immortalized B-cell lines; HeLa, cervical carcinoma cell line. The average length of the telomeres was determined multiple times for each sample by Southern blotting of *Ava*II-, *Bgl*II-, and *Pvu*II-digested DNA and pTH2Δ as a probe. To reduce the effects of gel-to-gel variability, most samples were analyzed in concert and in all cases different tissues of one individual were analyzed side by side. In addition, all samples were tested with *Sty*I (as in Fig. 7) to verify that the variation in telomere length is confined to the region beyond the last *Sty*I site. HeLa telomeres are larger than 20 kb; their exact length was not determined.

dramatically shortened telomeres (Fig. 7, lanes 5 to 8; Fig. 8; lines I and II). A more moderate reduction in telomere length compared with neighboring normal tissue was observed in a DNA sample from a small breast carcinoma (Fig. 8, line III).

Loss of telomeric sequences could eventually destabilize chromosome ends and thus restrict the life span of somatic cells. The limited number of primary tumors we surveyed do not seem to have reversed this degeneration process, and we were curious to see whether established tumor cell lines have regained the ability to maintain or expand telomeres. Inspection of three Wilms' tumor cell lines, three rhabdomyosarcoma cell lines, and two Epstein-Barr virus immortalized B cell lines showed fairly short telomeres (Fig. 8). In contrast, the cervical carcinoma cell line HeLa contains telomeres that are longer than in any other cell type we have examined, including sperm (Fig. 7, lane 9; Fig. 8).

### DISCUSSION

Studies of human chromosome ends are motivated mainly by two considerations. First, the principles of mammalian chromosomes may be deduced in part from the elements recognized to have *cis*-active function, i.e., centromeres, replication start sites, and telomeres. Second, telomeres define the molecular and genetic limits of chromosomes; thus, they are important landmarks in the evolving physical map of the human genome. Our findings have bearing on both features.

To obtain human chromosome end sequences, we have used a direct cloning strategy in *E. coli* that should be generally applicable to telomeres. The only criteria are that a telomere-specific probe be available and that some enrichment of telomeric DNA be achieved. The first criterion should be easily met, given the strong conservation of telomeric repeats. To enrich for telomeric DNA two procedures are currently available: size fractionation of genomic DNA digested with enzymes that preferentially cut chromosome-internal DNA, as described here, and the use of isopycnic  $\text{Ag}^+/\text{Cs}_2\text{SO}_4$  gradients as described by Brown (9). Unlike the two-step isolation strategy that uses yeast artificial chromosomes as an intermediate cloning vehicle (9, 16), our procedure is simple and rapid and does not rely on heterologous telomeres to be functional in *S. cerevisiae*. However, the protocol we have used yields short telomere-flanking sequences, whereas yeast artificial chromosomes can in principle carry large sections of chromosome ends.

Each of the telomeric clones we have analyzed contains a tandem array of TTAGGG repeats. In two clones, such repeats are preceded at the 5' side by telomere-flanking sequences that detect BAL 31-sensitive fragments in genomic DNA. These findings provide further support for the suggestion that human chromosomes end in the sequence 5'-(TTAGGG)<sub>n</sub>-3', reading from centromere to telomere. Allshire et al. (2) have suggested, on the basis of hybridization studies, that other simple repeats occupy the proximal part of the telomeric repeat region at human chromosome ends. We have found no evidence for such an intermingling of repeats. The approximately 6 kb of telomeric repeats that we have sequenced did not show any deviation from the sequence (TTAGGG)<sub>n</sub>, other than the few imperfect repeats that reside at the boundary of the telomere-flanking sequences. However, certain telomeres or parts of telomeres may have escaped our cloning. Further analysis is required to establish whether (TTAGGG)<sub>n</sub> is the only G-rich repeat at human chromosome ends.

One of the two telomere-flanking sequences described here recognizes a common element that is shared by a subset of human chromosome ends, but is apparently lacking in rodent genomes. Therefore, this repeat can be used as a human-specific marker to determine long-range physical maps of the termini of isolated human chromosomes in human-rodent hybrid cell lines. Such mapping exercises will be facilitated by the terminal position of the marker, because this arrangement allows consecutive restriction sites to be visualized by indirect end labeling of partial digestion products. How many chromosome ends could be analyzed in this manner is not known precisely. We estimate that up to 25% of the 46 human chromosome ends carry this element, and some of these chromosomes (chromosomes 7, 16, 17, and 21) have been identified by using hybrid cell lines. In situ hybridizations may eventually establish the abundance and location of this element; however, the small cloned segment currently available is not a useful tool for this technique.

One of the chromosome ends that contains the subtelomeric repeat (21q) carries a single-copy sequence within 200 kb of the telomere (Burmeister et al., in preparation). It appears, therefore, that the subtelomeric repeat is not larger than 200 kb; restriction analysis showed that its minimal size is 4 kb. Several questions with regard to this element remain. First, the area shows a high frequency of restriction fragment length polymorphism. The nature of this variability and its inheritance is not known. Second, in view of the mobility of telomere-associated sequences in other systems (10, 17, 23), it will be of interest to examine whether human subtelomeric repeats can move to new chromosome ends. Finally, the subtelomeric region shows an unusual pattern of CpG methylation. <sup>Me</sup>CpG is scarce or absent in sperm DNA but abundant in DNA from other sources, indicating that de novo methylation of at least the paternal telomeres occurs during development. Although de novo methylation of maternal sequences has been documented in the early stages of embryogenesis in the mouse, paternal sequences usually start out hypermethylated or remain hypomethylated (29, 39, 40). The only other sequences known to be hypomethylated in the male germ line and to acquire <sup>Me</sup>CpG during development are satellite sequences (40, 43). The importance of these exceptional methylation patterns is unclear.

Our experiments demonstrate that human chromosomes terminate in a long region of TTAGGG repeats from which sequences are lost during the generation of the soma. This process leads to shortened, heterogeneously sized telomeres in somatic tissues. In tumors a further shortening of the telomeres is seen, suggesting that somatic telomeres are unstable and continuously lose sequences, probably from the ends. However, it is not excluded that sudden large deletions are in part responsible for the shortened somatic telomeres. To address this question, the status of chromosome ends in developing (fetal) tissues will have to be examined. In addition, we do not know whether telomere reduction is strictly coupled to cellular proliferation. If the diminution results from incomplete replication of the telomere, such a coupling would be expected; however, other mechanisms, such as exonucleolytic degradation, may operate independent of cell division. In any event, it is clear that the maintenance of telomeres is impaired in somatic cells. An obvious candidate activity that may be reduced or lacking is telomerase. A human telomerase activity that can add TTAGGG repeats to G-rich primers has recently been identified (G. Morin, personal communication). Interestingly, the activity was demonstrated in extracts of HeLa cells, which we found to have exceptionally long telomeres.



Other cell types have not been tested yet, but such experiments could now establish whether telomerase activity is (in part) responsible for the dynamics of human chromosome ends.

Assuming the TTAGGG repeats are essential for telomere function, the loss of these sequences would eventually destabilize chromosome ends. The loss of telomeric repeats in yeasts is thought to result in chromosome loss and ultimately cell death (26). This precedent raises the question of whether chromosome instability in mammalian cells may in some cases be the consequence of telomere loss. With the exception of HeLa cells, all tumor cell lines we have examined contain fairly short telomeres; continued loss of TTAGGG repeats may therefore be one of the factors that leads to chromosome instability in malignant cells. These and other questions regarding telomere function in mammals may best be approached experimentally through the development of linear episomes or artificial chromosomes that carry synthetic telomeres.

#### ACKNOWLEDGMENTS

We gratefully acknowledge the generosity of our Fourth Floor colleagues, who donated materials to this work, and Joel Lavine, who procured blood samples. Margit Burmeister, University of California, San Francisco, and Gregg Morin, Yale University, are thanked for communication of unpublished results. Lex van der Ploeg, Columbia University, contributed a trypanosome telomere plasmid, and T.d.L. gratefully acknowledges his interest and encouragement. T.d.L. also thanks Janis Shampay, Bruce Bowerman, Peter Pryciak, and Paul Bates for technical advice and stimulating discussions and Don Ganem and Vicky Lundblad for critical comments on the manuscript.

T.d.L. is a Lucille P. Markey Scholar, and this work is supported in part by a grant from the Lucille P. Markey Charitable Trust. H.E.V. is an American Cancer Society Professor. This work is supported in part by a grant from the Winegart Foundation to D.R.C., by a grant from The Council for Tobacco Research and the Mather Foundation to S.L.N., and by Public Health Service grants from the National Institutes of Health to D.R.C., R.M.M., A.M.K., S.L.N., and H.E.V.

#### ADDENDUM IN PROOF

Recently we have determined the length of telomeres in two additional HeLa cell lines that, in contrast to the HeLa cells described in this article, grow in a nonadherent manner. Both HeLa cell lines were found to contain telomeres of approximately the same size as those of the Raji telomeres in Fig. 8, i.e., much shorter than the telomeres in the adherent HeLa cells.

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