Letters to the Editor

Am. J. Hum. Genet. 63:648, 1998

Repeat Expansion–Detection Analysis of Telomeric Uninterrupted $(TTAGGG)_n$ Arrays

To the Editor:

In humans, as in all vertebrates, telomeres are nucleoprotein complexes at the ends of chromosomes and primarily consist of tandemly repeated double-stranded $(TTAGGG)_n$ hexamers, to which are bound various specific proteins. The telomeric TTAGGG repeats are replicated by conventional DNA polymerases and by the reverse transcriptase telomerase, which catalyzes the addition of new TTAGGG repeats to the 3′ ends of chromosomes (de Lange 1995; Meyerson et al. 1997). The replenishing of telomeric terminal repeats by telomerase is required in order to compensate for the loss of these sequences in incomplete replication of linear chromosomes during the S phase. As part of the aging process, normal somatic cells undergo a progressive loss of TTAGGG repeats, both in vitro and in vivo, which correlates with lack of expression of telomerase in most somatic cells (de Lange 1995). In transformed cell lines and tumors, this loss is overcome by the up-regulation of the putative human telomerase catalytic-subunit gene (hEST2) and reactivation of telomerase, a process that is stabilized further by the involvement of the human telomeric repeat–binding factor, TRF1 (Meyerson et al. 1997; Nakamura et al. 1997; Van Steensel and de Lange 1997). However, recent data from telomerase knockout mice suggest that the proposed involvement of telomerase with tumorigenesis may be coincidental and of no functional significance (Blasco et al. 1997). In telomerase-negative immortal cell lines and tumors, other, still-unidentified mechanisms must operate to provide for telomere maintenance and elongation (Bryan et al. 1995, 1997). In human cells, functional telomeres have been found to form at previously interstitial sites almost exclusively after transfection with TTAGGG repeats (and, more rarely, after transfection with TTAGGG-related heterologous sequences), thus demonstrating that there are stringent sequence requirements for the formation of human telomeres (Hanish et al. 1994).

Uninterrupted telomeric TTAGGG-repeat arrays in somatic cells currently are believed to be in the range of ~10-kb or more (de Lange et al. 1990; de Lange 1995). The $(TTAGGG)_n$-repeat maximum length has been estimated by physical mapping strategies, on the basis of measurement of genomic DNA restriction-fragment lengths after Southern blotting and hybridization either with a $(TTAGGG)_n$ probe or by chromosome-specific subtelomeric probes (Brown et al. 1990; de Lange 1995; Notaro et al. 1997) and, more recently, by using quantitative FISH (Martens et al. 1998). Accurate measurements by cloning and sequencing have been hampered by the high instability of the telomeric $(TTAGGG)_n$ and have failed to show any clone containing an unadulterated $(TTAGGG)_n$ array >540 bp (de Lange et al. 1990). We have used the repeat expansion–detection (RED) assay (Schalling et al. 1993), originally described for detection of long trinucleotide repeats in the human genome, to measure maximum TTAGGG-repeat lengths and to monitor the relative stability of the repeats in DNA templates from both blood and transformed cell cultures. The RED assay uses genomic DNA as a template for the annealing and ligation of repeat-specific oligonucleotides, does not require flanking sequence determination or single-copy probes, and detects the longest repeat of a given type present in the genome. The RED method is highly reproducible in our hands (Sirugo and Kidd 1995; Sirugo et al. 1997; also see Epicentre Forum Website). We have analyzed DNA templates extracted from whole blood of 21 northern Europeans, for the maximum length of uninterrupted TTAGGG repeats, and have found that the longest uninterrupted $(TTAGGG)_n$ arrays in such templates ranged from ~220 bp to a maximum length of ~480 bp, well below the several-kilobase range described in the literature (de Lange 1995) (fig. 1 and table 1). This range was conserved (and the results were repeatable) in all samples analyzed, suggesting that this is the $(TTAGGG)_n$ maximum-length range that normally is present, in vivo, in human leukocytes.

Unlike the indirect physical-mapping strategies used for measuring the maximum length of the telomeric hexamer, the RED method gives a direct measure of the actual size of the longest uninterrupted TTAGGG repeat in the genome. The observed $(TTAGGG)_n$ maximum length of ~480 bp cannot be explained on the basis of

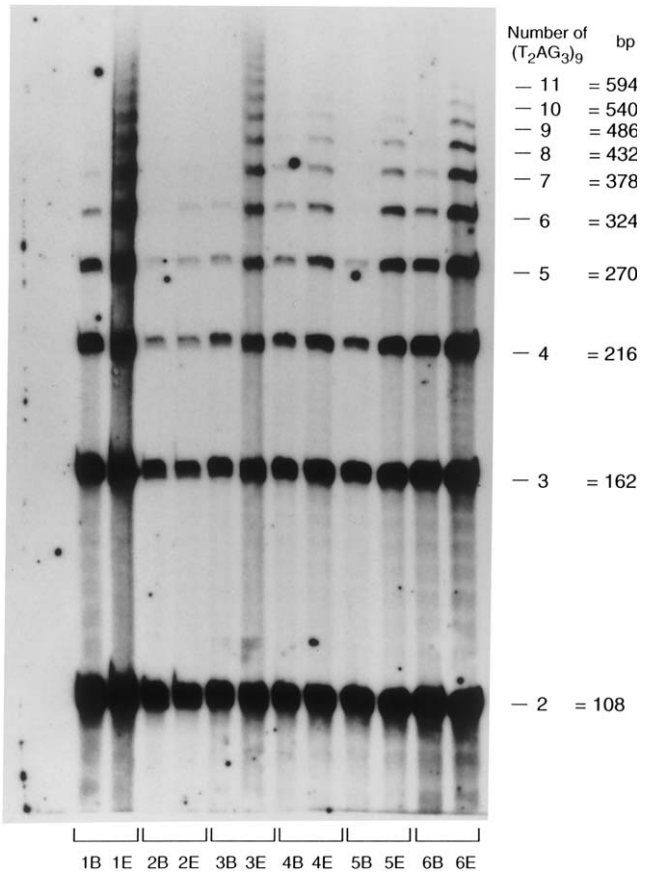

Figure 1 RED of $(TTAGGG)_n$ arrays, performed with a $(TTAGGG)_9$ oligonucleotide and 5 μg of DNA template in the conditions described by Sirugo and Kidd (1995; also see Epicentre Forum Website). Reaction products were fractionated by denaturing PAGE on 6% gels (20:1 acrylamide:bisacrylamide), were transferred onto a nylon membrane, and were detected by hybridization with a radiolabeled $(CCCTAA)_5$ probe. Lanes 1B–6E correspond to RED in DNA from blood/EBV-transformed lymphoblast pairs from six northern Europeans (Sirugo et al. 1997). An additional, seventh lymphoblast pair was analyzed (not shown). "B" and "E" denote, respectively, blood and EBV-cell-line DNA templates for each pair. The number of ligated $(TTAGGG)_9$ for each band is indicated in the figure, along with the corresponding size (in bp). All EBV-cell-line templates have been used for a previously published RED-based study of CTG/CAG-repeat maximum length in populations (Sirugo et al. 1997). Short CTG/CAG arrays were detected by RED in five of the seven lymphoblastoid cell samples (lanes 2E–6E) (Sirugo et al. 1997). We therefore can conclude that the repeat expansions detected in this study are specific for $(TTAGGG)_n$ arrays, after EBV transformation. One of these individuals (lanes 1B and 1E) was shown to carry a large CTG/CAG-trinucleotide–repeat expansion in templates from both blood and the EBV cell line. This rules out the possibility that the short $(TTAGGG)_9$ ligated ladders in blood are determined by poor quality of the DNA template with respect to the EBV-cell-line DNA. Fifteen additional northern European individuals also were analyzed; all carry $(TTAGGG)_n$ maximum lengths of 200–450 bp in their blood-extracted genomic DNAs (not shown) and provide confirmation that this is the normal $(TTAGGG)_n$ size range in this population sample.

aging. According to previously published reports (Vaziri et al. 1993), telomere shortening in leukocytes that is due to senescence would correlate with a size reduction of telomeric restriction fragments, from ~10 to ~5 kb, implying a $(TTAGGG)_n$ maximum length much larger than the ~480 bp observed in our study. It is therefore likely that the ~10-kb, large regions so far believed to contain unadulterated $(TTAGGG)_n$ are, in fact, composed of shorter blocks of uninterrupted arrays intercalated with other motifs every 200–500 bp (Allshire et al. 1989).

From seven of the above-mentioned blood samples, Epstein-Barr virus (EBV)–transformed cell lines were es-

Table 1

$(TTAGGG)_n$ Maximum Lengths in DNA from Blood and EBV Cell Lines

	No. of Ligated $(TTAGGG)_9$ Repeats In DNA from	
Sample	Blood	EBV-Transformed Cell Lines
Paired samples:[a]		
1	7	13[b]
2	6	8
3	7	13 [b]
4	9	11
5	5	10
6	8	12
7	7	13[b]
Unpaired samples:		
8	9	
9	6	
10	8	
11	6	
12	6	
13[b]	7	
14	4	
15	6	
16	5	
17	6	
18	6	
19	6	
20	5	
21	6	
24		13[b]
25		9
26		9
27		13[b]
28		10

[a] $(TTAGGG)_n$ maximum length in templates extracted from blood is always elongated after EBV transformation, with expansions ranging from "moderate" (pairs 2, 4, and 5) to "large" or "very large" (pairs 1, 3, 6, and 7). By a simple one-direction sign test, the probability of all seven transformed cell lines being elongated by chance is significant ($P < .01$).

[b] Arbitrary upper size value for very long but nonmeasurable ligated ladders.

tablished. The RED analysis of DNA templates extracted from these cell lines clearly demonstrated that the $(TTAGGG)_n$ arrays were systematically expanded when compared with the $(TTAGGG)_n$ maximum length detected in DNA extracted from whole blood (fig. 1 and table 1), with maximum lengths in the range of 450–700 bp (or more). Five additional DNAs from EBV-transformed cell lines of other northern European subjects also showed maximum lengths in the same range, of 450–700 bp (or more). RED analysis of control samples from three different mice strains (DNAs extracted from spleen) revealed the presence of $(TTAGGG)_n$ arrays >700 bp (not shown), in agreement with the observation of ultralong telomeres in mouse (Zijlmans et al. 1997). A very long (up to the nonresolving area of the gel) $(TTAGGG)_n$ ladder also was detected when the RED method was tested on a 5–8-kb synthetic $(TTAGGG)_n$ / $(CCCTAA)_n$ polymer generated by PCR (fig. 2). Taken together, these data rule out the possibility that the "short" $(TTAGGG)_n$ maximum lengths detected by RED in templates from whole blood are artifacts due to the inability of the ligation process to proceed beyond secondary structure (e.g., intrastrand hairpins) of $(TTAGGG)_n$ arrays.

Overall, the maximum $(TTAGGG)_n$ length in the 21 samples from blood was found to be significantly shorter (200–500 bp) than the maximum length detected in the 12 EBV cell lines (Mann-Whitney test; $P < .001$). The $(TTAGGG)_n$-array expansions are in accord with telomere lengthening in transformed cell lines (de Lange 1995; Meyerson et al. 1997; Van Steensel and de Lange 1997) and largely consistent with the reported hEST2 up-regulation/telomerase reactivation in transformed cell cultures, although we cannot exclude the possibility that they originate from DNA polymerase slippage or by chromosomal recombination following EBV transformation.

It has been stressed that "in considering telomere dynamics, it is clearly important to establish the length of the $(TTAGGG)_n$ repeat array" (de Lange 1995, p. 266) and that the instability of $(TTAGGG)_n$ might have "some mechanistic similarity to the instability of short tandem repeats that produce variable microsatellite or minisatellite loci, and the instability of trinucleotide repeats that underlie some human genetic diseases" (Kipling 1995, p. 196). It is worthwhile to hypothesize that the interruption of the unadulterated $(TTAGGG)_n$ arrays every 200–500 bp in DNA templates from whole blood may be important for the maintenance of repeat stability, whereas the loss of interruption could be associated with instability and expansion, as part of a dynamic process perhaps not dissimilar to that resulting in expansion mutation of some trinucleotide repeats (Ashley and Warren 1995; Gordenin et al. 1997). The RED method could be used to monitor telomere stability in transformed cell

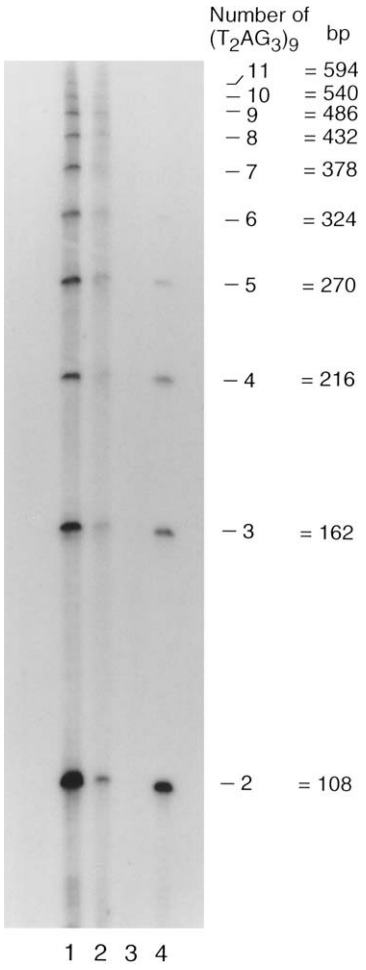

Figure 2 RED of $(TTAGGG)_n$ arrays, using 100 pg (lane 1) and 10 pg (lane 2) of a synthetic polymer generated by PCR with $(TTAGGG)_9/(CCCTAA)_5$ primers in the absence of DNA template. The PCR was performed in a 20-μl final volume, with 200 μM of each dNTP, 2 M Betaine, 5% dimethylsulfoxide, 2 μl of 10 × Klentaq buffer, and 0.2 μl of KlentaqLA-16 enzyme mix (15:1 Klentaq1:*Pfu* DNA polymerase) (Barnes 1994; Baskaran et al. 1996). Samples were taken through 40 cycles of 95°C for 1 min, 37°C for 1 min, and 72°C for 6 min, with a 3-s increase per cycle. The reaction product was run on a 1% agarose gel and was visualized, by ethidium bromide staining, as a smear with a size range of 1–20 kb. PCR products in the 5–8-kb range were gel purified and used as template in a RED reaction with $(TTAGGG)_9$ oligonucleotides. Decreasing amounts of synthetic template yield an equally long ladder but one that is of lower relative intensity (lanes 1 and 2). A control reaction in the absence of DNA template is shown in lane 3, demonstrating that the ladder does not result from self-annealing of $(TTAGGG)_9$ oligonucleotides. Lane 4 shows results for a RED replicate on a DNA template extracted from blood (sample 3B in fig. 1), demonstrating that the shorter length detected in DNA from whole blood is consistently reproduced across reactions.

lines and tumors or to test the effect of genes involved in telomere maintenance or DNA repair after transfection in cell lines with abnormally short telomeres. In conclusion, the results generated by the RED method offer a new perspective on $(TTAGGG)_n$ maximum length and on the relative stability of this telomeric hexamer, both in vitro and in vivo.

Acknowledgments

We thank Drs E. Fritz , S. Meyn, F. Calafell, S. Baserga, G. Isaya, and T. Ashley for helpful discussion and advice, and we thank B. King for her gift of DNA samples from mice. This work was supported in part by National Institutes of Health grants MH44876, MH39239, and MH50390.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Epicentre Forum, http://www.epicentre.com/f2_3/f2_3al.html

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