

Telomeres

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Telomeres are essential for chromosome stability and replication. Maintaining a balance between telomere shortening and lengthening is essential for cell viability. Recent work on telomeres from yeast, *Drosophila* and mammals, and on telomerase has provided insight into the mechanisms of both the shortening and lengthening processes.

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Introduction

Eukaryotic chromosomes consist of one continuous linear DNA molecule. Chromosome ends are 'capped' by specific structures called telomeres which differ from randomly generated breaks in the chromosome. Telomeres appear to have several different functions. Broken chromosomes lacking telomeres are recombinogenic, generating translocations and aneuploidy. Telomeres are also involved in chromosome replication: DNA polymerases require a primer, and synthesize DNA in the 5'–3' direction; strict application of these rules would result in the gradual loss of sequences from the end of a linear DNA molecule. Thus, there must be a special mechanism to replicate the chromosome ends (reviewed in [1]). Finally, telomeres probably play a role in nuclear architecture and chromosome positioning; they are often found associated with the nuclear envelope. Over the past 10 years the cloning and characterization of telomeres from single-cell eukaryotes has elucidated many aspects of telomere structure and function. Much of this work has been reviewed recently [1–4]. Therefore, I will focus mostly on the more recent developments in understanding telomere structure, function and synthesis.

Telomere G-strand structure

Telomeres consist of simple, tandemly repeated G-rich sequences. The particular sequence at the chromosome ends differs from organism to organism (reviewed in [1]). In the ciliates *Tetrahymena* and *Euplotes*, the repeated sequences are d(TTGGGG) and d(TTTTGGGG), respectively, while in yeast there is a more irregular sequence: d(TG_{1–3}) — one dT residue followed by either one, two or three dG residues. Physical studies on single-stranded oligonucleotides containing these G-rich sequences, showed that G strands can associate to form specific Hoogsteen base-paired structures [5–7,8•]. For example, the *Tetrahymena* telomeric sequence d(TTGGGG)₄ will form a four-stranded struc-

ture in which there are four planar G residues all sharing Hoogsteen base-pairs [8•,9•]. This structure was termed the G quartet [8•]. As telomeres from several organisms have a region of single-stranded G residues protruding at the 3' end [10], it was hypothesized that the structure which these sequences form plays a functional role at telomeres. To date the role of G-strand structures remains unclear. Telomerase elongation does not require the G-strand structure: oligonucleotides with inosine substituted for guanosine do not form intramolecular base-paired structures, yet they are elongated by telomerase [9•]. Similarly, binding of telomere proteins (see below) does not require the secondary structure [11•]. It is possible that the G-strand structure may play some other role which can not yet be tested directly, for instance it may function in chromosome stability, telomere associations or protection from degradation.

Telomere-binding proteins

Telomeric sequence tracts can vary in length between 32 and 50 000 bp, depending on the organism [1,2,4]. For some time it has been suggested that there are specific non-histone proteins bound to telomeres *in vivo*. The first such protein to be isolated and characterized was from *Oxytricha*; it consists of two polypeptide subunits, one of 55 kD and one of 43 kD [12,13]. (The smaller polypeptide subunit has recently been cloned [14].) A protein with similar properties has also been identified in *Euplotes* [15].

The binding of *Oxytricha* telomere protein to telomeric DNA has been extensively characterized [12,16]. Reconstitution of purified protein with labeled DNA oligonucleotides showed that two different specific complexes are formed. The complexes were analyzed by methylation interference, methylation protection and non-denaturing gel electrophoresis. One of these two complexes readily polymerizes at high protein:DNA ratios [17,18]. This polymerization may reflect the telomere–telomere interactions which have been seen *in vivo*.

Because purified telomere-binding proteins will bind to synthetic d(TTTTGGGG)₄ oligonucleotides, it is possible to look at the effect of the G-strand structure on protein binding [11•]. The kinetics of both folding and unfolding of the G-strand structure were compared to the kinetics of protein-binding to the oligonucleotide in different buffers. Unfolding was fastest in TE, followed by 50 mM Na⁺ and was very slow in 50 mM K⁺. Protein binding to the oligonucleotide, determined by a filter-binding assay, displayed similar kinetics to G-strand unfolding in each of the buffers, indicating that the folded form must first unfold, in a rate-limiting step, before protein can bind. Thus, the proteins appear to bind only to the unfolded oligonucleotide and not to the folded form.

A number of groups have looked for telomere-binding proteins in yeast. Proteins have been identified which specifically bound to DNA affinity columns containing repeats of the yeast telomeric sequence [19]. Other groups identified telomere-binding proteins through the age-old process of serendipity [20,21]. The protein Rap1 was originally characterized for its ability to bind to the mating-type silencer region in yeast [20–22]. The *RAP1* gene is also known as *TUF*, *GRF1* and *TBA*. Deletion mutants of *RAP1* are not viable, indicating that the gene is essential. When the binding specificity of Rap1 was characterized, the GT richness of the consensus was noted as being telomere-like. Telomere sequences were then tested directly and shown to be very tightly bound by Rap1 [20]. Subsequently, it was shown that Rap1 and the proteins which were purified as telomere-binding proteins on affinity columns were in fact the same protein [23].

Rap1 binding plays a functional role at telomeres *in vivo*. When yeast strains containing temperature-sensitive alleles of *Rap1* are grown at a semipermissive temperature, telomere length shortens gradually over a number of generations [24••]. There are two very intriguing aspects to this shortening. First, the telomeres do not shorten indefinitely, rather they stop at a new shorter equilibrium. Second, when these cells with shortened telomeres are returned to the permissive temperature, telomere length does not just stop decreasing but it actually increases back to the original length. This suggests that there are mechanisms which maintain yeast telomere tracts at a specific genetically determined length and that Rap1 is not only involved in preventing telomere shortening, but also somehow in the re-establishment of normal length.

Overexpression of Rap1 protein causes telomeres to become somewhat longer and more heterogeneous in length, and also generates chromosome instability [25••]. The effects of Rap1 on telomere length are probably mediated, at least in part, through direct binding to the telomere. Antibodies to Rap1 will specifically precipitate telomeric restriction fragments from total yeast chromatin. When a large excess of telomere sequence is introduced into yeast, the telomeres shortened, suggesting that Rap1 and other essential telomere-binding proteins may be titrated out by the excess telomere repeats [26].

Other genes which affect yeast telomere length are *TEL1*, *TEL2*, and *EST1* [27,28]. Mutations in *est1* cause telomere shortening and chromosome instability. Unlike the *rap1* temperature-sensitive alleles, *est1* telomeres appear to shorten continuously and ultimately cause cell death. Homology of the Est1 protein to RNA-dependent polymerases suggests that this gene may be a component of the telomere replication machinery [29]. The study of yeast telomeres has shown that telomere length is determined by a number of different factors which control lengthening, shortening and homeostasis. Because telomere structure is conserved in eukaryotes, understanding the many different factors which regulate yeast telomere length may elucidate these mechanisms in mammalian cells and other systems.

Telomeric silencing

Proteins bound at telomeres may play other roles besides regulation of telomere length. When inserted at a site very near a yeast telomere, some genes are turned off at the transcriptional level [30••]. When *URA3* is inserted immediately adjacent to telomeric TG_{1,3} tracts transcription of the gene is repressed. However, no repression is seen if *URA3* is inserted next to TG_{1,3} tracts at an 'internal' position in the chromosome (20 kb from the telomere). This transcriptional repression is reversible and is inherited in a 'semistable' manner. When the *ADE2* gene is inserted near a telomere, mitotic inheritance of the repressed state can be followed studying the inheritance of the red *ade2*⁻ phenotype (by colony sectoring). Both predominantly red colonies with white sectors and white colonies with red sectors are seen. When white cells within a sector colony are replated, they give rise to both red and white sector colonies. This 'semistable' mitotic inheritance of the repressed state is reminiscent of the epigenetic inheritance of silent mating-type repression in *sir1*⁻ cells [31].

In addition to *RAP1*, several other loci involved in mating-type repression are also involved in transcriptional repression at telomeres (O Aparicio, B Billington and D Gottschling, personal communication). Mutations in the *SIR 2,3,4* genes as well as in *NAT1* and *ARD1* all relieve the transcriptional repression of genes inserted at telomeres. The relief of transcriptional repression is similar to the ability of all of these mutations to relieve repression at the silent mating loci HMR and HML. The mechanism by which SIR, NAT and ARD silence transcription is not known; however, evidence suggests that they affect chromatin structure [32,33]. Because yeast telomeres are rich in repetitive DNA they resemble heterochromatic DNA found at telomeres in higher eukaryotes. Telomeric silencing in yeast may resemble transcriptional position effects seen by nearby heterochromatin. The SIR, NAT and ARD genes may affect silencing at HML, HMR and telomeres by establishing an 'inactive chromatin' state (O Aparicio, B Billington and D Gottschling, personal communication).

Drosophila telomeres

The isolation of *Drosophila* mutants, which apparently lack a telomere on one chromosome has generated some recent controversy in the telomere field: Are telomeres required to prevent chromosome rearrangements? Terminal deficiencies were generated in two different ways: destabilization of a telomeric P element [34•] or X-irradiation in a *Mu-2* background [35,36•]. In each case the chromosome appears to end within the coding region of specific genes, and to have no telomeric sequences on the ends. Consistent with the idea that telomeres are required for complete replication, the 'broken' chromosomes lose approximately 70 bp of DNA from the end with each generation. However, in contrast to broken chromosomes in other organisms, the ends appear not to be susceptible to recombination. 'Healed' versions of the shortening chromosomes have been identified in fly stocks. The healing event places a known repetitive *Drosophila* 'telomere-associated' DNA sequence, HeT, at the unstable end [37•]. Although the healing events apparently stop the progressive chromosome shortening, they may not reflect the structure of real *Drosophila* telomeres because their length is not stable as they are propagated in fly stocks. The acquisition of telomere-associated Y' repeats is also able to rescue *est1* mutants that have lost telomeric sequence from cell death (V Lundblad and E Blackburn, personal communication). Although the mechanism of repeated DNA sequence addition may differ in *Drosophila* and yeast, it is clear in yeast that the Y' elements are not the functional telomeric sequence, but rather their movement may simply provide a salvage pathway for chromosome stabilization. Once the structure of a 'normal' *Drosophila* telomere is defined, the role of HeT DNA at wild-type and mutant telomeres may become clearer.

Telomerase

As conventional DNA replication can not completely replicate the end of linear chromosomes, it has been proposed that a special mechanism for telomere replication must exist. Evidence that accumulated over a number of years suggested that to balance sequence loss resulting from incomplete replication, a telomere terminal transferase enzyme adds sequences onto chromosome ends [38] (reviewed in [1,2,4]). Such an enzyme, telomerase, was originally identified in *Tetrahymena* [39] and has subsequently been found in the ciliates *Oxytricha*, *Euplotes* and in HeLa cells [40–42]. Telomerase is a ribonucleoprotein, containing both essential RNA and protein components [43]. The RNA component from *Tetrahymena* contains the sequence 5' CAACCCCAA 3' which provides the template for the d(TTGGGG) repeats synthesized *in vitro*. Evidence for the RNA template in telomerase initially came from *in vitro* studies using oligonucleotides complementary to the telomerase RNA [44]. The cloning of the *Euplotes* RNA component provided

further evidence that the RNA component is an internal template. The *Euplotes* telomerase synthesizes the sequence d(TTTTGGGG), and the RNA contains the sequence CAAAACCCCAAAC [45]. Definitive evidence for RNA templating came from the recent experiments of Yu *et al* [46••]. The gene for the telomerase RNA component was mutated within the template region and transformed back into *Tetrahymena*. The transformed cells contained telomere repeats with sequences corresponding to the mutation introduced into the RNA. These experiments not only showed that the RNA is used as a template but also that telomerase is responsible for synthesizing telomeric tracts *in vivo*.

The *Tetrahymena* cells with mutant telomere sequences were sick and telomere length was aberrant. In some cases the telomeres became very long and in some cases they became short. In all cases the cells died if the mutant telomerase RNA gene was retained [46••]. This suggests that when the telomere repeat sequence is altered, the mechanism for length regulation is upset. Perhaps the binding of telomere proteins is required and the proteins can not bind the mutant telomere sequences. Thus, the telomere shortening in the telomerase mutants may be similar to the effect of Rap1 temperature-sensitive mutants. These results suggest telomere-protein binding is important for both telomere length maintenance and cell viability.

Mammalian telomeres

Most of the initial work on telomeres was done in single-cell organisms such as *Tetrahymena* and yeast. The conserved structure of simple repeated sequences suggested that telomere structure might be conserved in all organisms. Cooke *et al* [47] first isolated a sequence from the human pseudoautosomal repeat near the telomere on the X chromosome. This probe showed that, like the telomeres in smaller eukaryotes, human telomeres are heterogeneous in length.

The first telomere to be cloned from a multicellular organism was from the plant *Arabidopsis* [48]. The chromosomes in this organism contain tandem repeats of the sequence d(TTTAGGG) similar to those found in single-cell eukaryotes. This *Arabidopsis* probe, as well as a *Tetrahymena* probe [49], hybridized to human DNA, suggesting that human telomeres also consist of simple G-rich sequence repeats [48–50]. This knowledge then led a number of groups to clone human telomeres. Several groups used functional complementation in yeast [51•–54•]. One end of a linear artificial chromosome was removed and fragments of human DNA were ligated onto the end. Upon transformation into yeast only those pieces of DNA which function as telomeres will allow maintenance of the linear chromosome. Because *Tetrahymena* d(TTGGGG) repeats can function as telomeres in yeast, it was expected, and later shown, that the human telomere repeats would also be functional. Human telomeres were also isolated by direct

cloning in *Escherichia coli* [55•]. The human telomere consists of hundreds of tandem d(TTAGGG) repeats, a sequence very similar to that found in both single-cell eukaryotes and plants. Subsequent studies showed that many other mammals have telomeres which hybridize to this d(TTAGGG) sequence, indicating that most if not all of the telomere repeats in mammals are probably d(TTAGGG) [56].

The cloning of human telomeres was important not only for understanding mammalian telomere function, but also for human genome mapping. If the unique DNA adjacent to each telomere is available as a probe, the limits of the human physical maps can be defined. In addition, loci for diseases such as Huntington's disease are known to be located near telomeres [57,58]. Thus, probes for the telomere might allow the isolation of such genes [59].

Like many other organisms, human telomeres have repetitive 'telomere-associated sequences' just internal to the d(TTAGGG) repeat tracts. Telomere-associated sequences are not conserved among eukaryotes and no functional role for them has yet been identified. Sequences which hybridize to either several human chromosomes or to one specific chromosome have been characterized [51•,52•,54•,55•,60•]. Because the human telomere-associated sequences do not hybridize to rodent DNA, hybrid rodent cell lines carrying specific human chromosomes can be used in mapping the telomere-associated sequence, and in constructing long-range physical maps of individual chromosomes. Human telomere-associated sequences are highly polymorphic: length and copy number polymorphisms are seen on a given chromosome between different hybrid cell lines and in different individuals [55•,60•]. Such frequent rearrangement of telomere-associated sequences is also seen in plasmodium, trypanosomes and yeast [61–64].

When human genomic DNA is cut with four base-recognition restriction enzymes, the telomeric fragments are on the order of 10–12 kb in length. This is startlingly long because in yeast and ciliates the terminal fragments are only on the order of 1–2 kb long. The mouse terminal restriction fragments are even longer; they vary between 20–200 kb and must be resolved on pulse-field gels [65•,66•]. In some mouse strains many individual bands are resolved. It is not clear at present what accounts for the difference in length of a 150 kb and a 20 kb telomeric restriction fragment.

Quantitative hybridization suggests that there is more d(TTAGGG) sequence in the mouse than in the human genome. It may be that different telomeres have different amounts of the d(TTAGGG) sequence or simply that the presence or absence of particular restriction sites in telomere-associated sequences generates 'telomeres' of differing length. Some mouse telomere fragments, like trypanosome telomeres, may consist of long 'barren regions' devoid of restriction sites capped by terminal d(TTAGGG) arrays. Further analysis is needed to clarify the structure of mouse telomeres.

Like human telomeres, mouse telomeres are also polymorphic. The length polymorphisms seen with d(TTAGGG) probes on pulsed-field gels may be the result of frequent recombinations of the as yet uncharacterized mouse telomere-associated sequences on these long restriction fragments or of recombination within the d(TTAGGG) tracts themselves. When specific telomere bands are followed in a cross, new bands are found which can not be attributed to either parent. Whether the high frequency of mitotic and meiotic recombination at mammalian telomeres has a function is not known; however, studies of yeast telomere recombination may offer some insight.

Recombination involving the telomeric $G_{1.3}T$ tracts has been seen in yeast and has been suggested to play a role in maintenance of telomere length [67,68]. In addition, the structure, distribution and movement of the telomere-associated Y' sequence have recently been carefully studied in yeast [63,64]. By marking a single telomere-associated Y' sequence in the cell and selecting for either duplication or loss of the marker, the kinds and frequency of recombination events were followed. Tandem arrays of the repetitive element can expand and contract, probably through sister chromatid exchange. In addition, both complete loss of the element from a telomere and gain of the element at a telomere that did not have one are seen. This kind of movement could explain the differences in telomere-associated sequence distribution between individuals observed in humans and mice. In yeast no function has been found for the telomere-associated DNA. Thus, the Y' elements may represent 'selfish DNA' which simply resides and replicates inside the 'host' genome.

Cancer and aging

The cloning and characterization of human telomeres led to a number of interesting observations. First, the earlier observation that sperm telomeres are longer than somatic telomeres was confirmed [55•,69•,70]. Quantitative hybridization shows that this length difference is due to the number of d(TTAGGG) repeats at the terminus [70]. Second, de Lange *et al.* [55•] showed that telomeres are shortened in Wilm's tumors and in many established cell lines. This observation was extended by Hastie *et al.* [69] who looked at a large number of colorectal carcinomas and found telomere length is shorter in the tumor tissue when compared with the adjacent non-tumor mucosa. Third, human telomeres were found to shorten with age. Primary fibroblasts grown *in vitro* have a limited life span [71]. When these primary cells are passaged, the telomeres progressively lose d(TTAGGG) sequences [72•]. Telomere shortening is also seen *in vivo*; blood [69•] and fibroblasts from people of different ages show shorter telomeres with increasing age (R Allsopp and C Harley, personal communication) [72•].

The short telomeres in carcinomas and in older cells suggests that telomeres become shorter with increasing rounds of cell division. Thus, telomere length may be a

direct marker for the number of divisions that a cell has undergone. Does telomere shortening play a causative role in either cancer or senescence? The fact that end-to-end chromosome fusions are seen in both tumor cells (reviewed in [73]) and in senescent fibroblast cultures [74], suggests that the loss of telomeric repeats may play a role in generating the chromosome instability and aneuploidy seen in both of these phenomena.

Clearly both oncogenesis and senescence involve other cellular changes besides telomere shortening. What role might telomere shortening play? The regulation of cell growth may involve a checkpoint [75,76], at which telomere length is monitored. It has been suggested that such checkpoints might play a role in cellular senescence [77]. If telomere length is too short, cell growth will be arrested. Transformed cells bypass this checkpoint and continue cell division even with short telomeres. Similarly, senescent fibroblasts can be forced to undergo additional cell divisions in the presence of the simian virus 40 T-antigen [78]. Thus, short telomeres may be a signal for cell cycle arrest; only when this step is bypassed does chromosome instability arise.

One mechanism for telomere shortening might simply be the absence of telomerase activity in somatic tissue. The presence of long telomeres in sperm and in fetal tissue, and the presence of shorter telomeres in somatic tissues, suggests that telomerase might be active in the germline and inactive somatically. This simple model has become testable since human telomerase activity has been identified [42]. However, the effects of telomere-binding proteins and telomere-associated sequence on yeast and *Tetrahymena* telomere length suggest that telomerase alone is not responsible for maintaining telomere length. Thus, a more complete understanding of all the factors involved in maintaining telomere length is needed before the mechanism of telomere shortening in tumors and aging can be fully understood.

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Mouse telomeres were analyzed on pulse field gels by hybridization to a probe for d(TTAGGG). The segregation of the long terminal restriction fragments in families indicated that new length variants are generated in the germline at high rates. Inbred mice of the same strain kept in different colonies showed different patterns of bands. Digestion with the enzyme MnlI which cuts a variant of the telomeric repeat sequence, released small d(TTAGGG)-hybridizing fragments from the very large telomere arrays. These small fragments also show unusual segregation in crosses.

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