

The Telomere and Telomerase: How Do They Interact?

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Abstract

The ribonucleoprotein (RNP) enzyme telomerase synthesizes telomere DNA and maintains telomere length in eukaryotic cells. This review describes recent findings that provide new understanding of the functions of telomeres and telomerase. Telomerase has an essential RNA moiety in which a short sequence acts as the template for synthesis of telomeric DNA. Recent results show that, besides acting as a template, the telomerase RNA plays essential roles in the enzymatic functions of telomerase that are as critical as those provided by the protein reverse transcriptase subunit of telomerase. Analysis of telomerase RNA mutants in yeast has provided evidence that telomerase is an oligomeric/dimeric enzyme containing at least two telomerase RNA molecules and two enzyme-active sites. Recent data suggest that this telomerase RNP also plays a critical role in capping short telomeres. Thus, the length of a telomere is only one determinant of whether it is sufficiently long to function as a cap, stabilizing the chromosome end. Several lines of evidence converge on the notion that for telomere length regulation and other telomere functions, the very few last repeats at the tip of the telomere are the most crucial. **Key Words:** Telomere, telomerase, human telomerase, yeast telomerase, telomere homeostasis, *Kluyveromyces lactis*, *Saccharomyces cerevisiae*, *Tetrahymena thermophila*.

Introduction

ALL CHROMOSOMES IN A TYPICAL EUKARYOTE bear telomeric DNA at their termini. The amount of telomeric DNA varies from telomere to telomere but is kept within well regulated upper and lower bounds. What are telomeres for? First, they cap the ends of the chromosomes. This capping function includes shielding the telomere from being recognized as damaged DNA, thus causing cell cycle arrest, and shielding telomeres from engaging in inappropriate kinds of recombinations. Capping also protects the telomeric DNA from degradation. In addition, the conventional cellular DNA replication machinery alone appears unable to replicate telomeric DNA completely. This creates a need to top up the telomeres fre-

quently to compensate for this lack of complete replication, and perhaps also to augment DNA lost through nuclease action at telomeres. A major player in the replenishment process is telomerase, as described below. We still don't know all the molecular answers to how capping and replenishment are accomplished. One newly recognized aspect of telomeres that will be emphasized in this review is that both the telomerase ribonucleoprotein (RNP) itself, as well as the very last few telomeric repeats on the chromosome and their associated proteins, are emerging as critical molecular and functional components of the chromosomal cap.

The telomeric DNA sequences of eukaryotic linear chromosomes are repeated sequences, examples of which are shown in Fig. 1. The number of repeats per telomere varies greatly from organism to organism. For example, the ciliated protozoan *Tetrahymena thermophila* has about 50 T₂G₄ repeats whereas humans and many other species have up to thousands of T₂AG₃ repeat units per telomere. Somewhat different-looking repeat sequences are found in *Sac-*

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Tetrahymena	TTGGGG
Vertebrates	TTAGGG
<i>S. cerevisiae</i>	(TG)₁₋₆TG₂₋₃
<i>K. lactis</i>	ACGGATTGATTAGGTATGTGGTGT
<i>C. albicans</i>	ACGGATGTCTAACTTCTTGGTGT

Fig. 1. Telomeric DNA sequences.

Saccharomyces cerevisiae — with its somewhat less regular repeated sequences — and *Kluyveromyces lactis* — another budding yeast related to *S. cerevisiae*, which has a rather long, 25 base-pair repeat unit. Both yeasts have telomeres that are a few hundred base pairs in length, whereas typical human telomeres are thousands of base pairs long. The enzyme telomerase specifies and synthesizes the unit repeat sequence of the DNA strands shown in Fig. 1. Although these sequences differ somewhat from each other, it is becoming very clear that, fundamentally, telomerase and telomeres function very similarly in these different species. The findings from experiments done in the simpler model organisms, therefore, have important implications for humans. We have chosen various systems for the experimental ease by which we can explore certain aspects of telomere function. Some of the experiments described here will be drawn from *Tetrahymena*, others from *S. cerevisiae* or *K. lactis*, and yet others from human cells. Both new and older results emphasize the role of RNA in the capping and DNA synthetic functions of telomerase.

The Roles of Telomerase RNA

Fig. 2 illustrates the basic core machinery of telomerase in the ciliated protozoan, *Tetrahymena*, and its mode of reaction. *Tetrahymena* telomerase was the first telomerase to be discovered, by Carol Greider and me at the University of California, Berkeley, in the mid-1980s (1). Depicted in Fig. 2 is the end of the chromosomal DNA, which has a duplex region, and in at least some fraction of the DNA, an overhanging DNA strand. The 3' end of this overhang, consisting of the T₂G₄ repeat sequence, can be elongated by the addition of nucleotides, using dNTPs as substrates. Elongation occurs by copying a template sequence that is part of the telomerase RNA moiety of this RNP. We showed some years ago that by making mutations in the template sequence we could change the complementary sequence in the telomeric DNA (2, 3).

The core RNP of the telomerase complex contains a reverse transcriptase protein component,

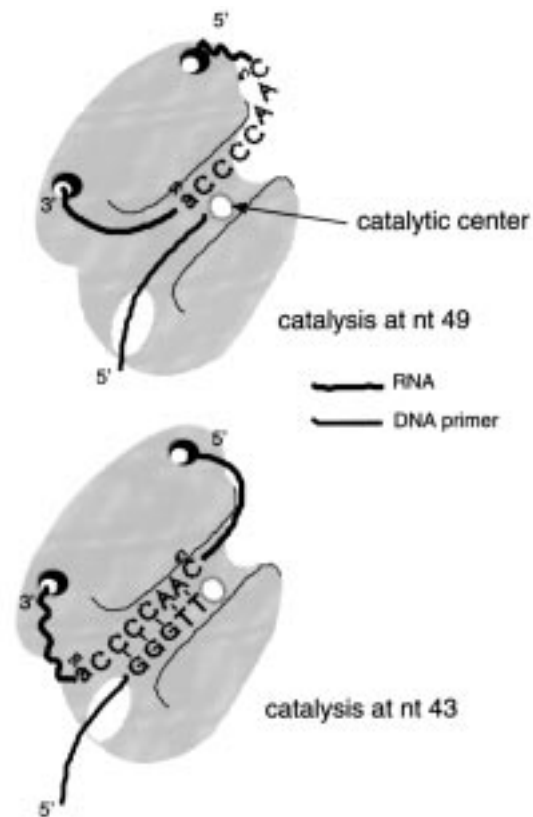


Fig. 2. The basic core machinery of telomerase in the ciliated protozoan, *Tetrahymena thermophila*.

with amino acid motifs characteristic of the reverse transcriptase family (4). These include three aspartate residues that form the catalytic metal-binding sites for the catalysis of polymerization.

Telomerase RNA, in addition to providing its well documented templating function in this and other species' telomerases, also has some very interesting roles in the action of telomerase. These roles have important mechanistic implications for how the active site of this RNP enzyme works, as well as interesting evolutionary implications. Telomerase RNAs vary greatly in size among different species, with the smallest (about 150 nucleotides long) being in the ciliated protozoans, ranging through about 400 nucleotides in mammals, to up to 1.3 kilobases in yeasts such as *K. lactis* and *S. cerevisiae*. There is also an enormous range in primary sequence: within the ciliated protozoa alone, for example, the RNAs diverge by more than 80% in primary sequence. However, ciliates share a remarkably conserved core secondary structure. This, together with the data described below, is consistent with the telomerase RNA playing important roles in the active site functions of telomerase (5, 6).

David Gilley in our group first analyzed the *in vitro* telomerase activities of telomerase containing telomerase RNAs bearing various template mutations (7, 8). Strikingly, he found that making specific changes in the template caused remarkable changes in the way that the enzyme acted. For example, a C to U mutation at position 48 — a tiny change of just an amino group to a keto group on the base at that position — had drastic effects *in vitro* and *in vivo* (2, 7). The wild-type enzyme synthesizes many tandem 6-base T₂G₄ repeats *in vitro*. In contrast, the reaction catalyzed by the 48U base substitution mutant enzyme sputters out very quickly. Through extensive analyses, we concluded that this mutant enzyme breaks some of the cardinal rules for nucleic acid polymerases that perform template-directed synthesis: it has very low fidelity at certain places on the template, misincorporating at rates approaching 50%, even at low triphosphate concentrations *in vitro*. To put this result in perspective, the HIV reverse transcriptase, and other viral reverse transcriptases that are notorious for their low fidelity, have error levels of only 10⁻² to 10⁻³. Not content with that, once the 48U mutant telomerase misincorporates, producing a mispair, unlike any good polymerase that will reject the mispair for further elongation, this mutant telomerase continues to elongate it. Another telomerase RNA template mutant behaved similarly. In addition, certain template mutants make slippage errors, reiterating the copying of some template nucleotides (2, 7). The 48U mutation and some other template mutant enzymes also dissociate the DNA product too quickly from the enzyme, aborting synthesis (2, 8). In summary, template mutations can produce dysfunctional enzymes.

In vivo studies reinforced the conclusion that the 48U mutant enzyme functions poorly. This mutation caused the telomeric DNA to shorten, and after about 20–25 cell divisions the cells stopped dividing (2, 7). Expression of this mutant RNA provided the first demonstration in any system that telomerase is important for cells and that functional telomerase is required for cells to continue dividing (3).

Similar and even more dramatic phenomena were seen by mutating the large yeast telomerase RNAs (9, 10). John Prescott in our lab group made mutations in the template sequence of the telomerase RNA of *S. cerevisiae*. The templating domain sequence of this species' RNA contains some redundancies that may partly account for the irregularity of the sequence made *in vivo*. John made a series of trinucleotide substitutions both in

and next to the template region. When he assayed the *in vitro* enzymatic activity of telomerase containing these substitutions, one of them, called 476GUG, had no telomerase activity. The 476GUG mutant RNA was stable, transcribed and expressed normally, and formed in an RNP particle with the same chromatographic and size properties as normal telomerase. Thus there is nothing obviously wrong with the assembly of this telomerase, leaving us with the conclusion that the problem lies at the level of enzymatic action itself (11, 12).

Similar results were found by Jagoree Roy and Tracy Boswell Fulton in our lab group for the large telomerase RNA of the related yeast *K. lactis*. In this case, changing just a few bases located several hundred nucleotides away from the template produced a completely inactive telomerase enzyme. Again the RNA was stable and assembled into a stable RNP. Emphasizing the specificity of this result, we found that various large deletions of other portions of this RNA caused no problems for enzymatic function (13).

In summary, small changes in the sequence of even very large telomerase RNAs can have a dramatic impact on the telomerase RNP enzyme. I would like to put these results into perspective with respect to our views of enzymatic active sites. A component of a protein enzyme required for the active site of that protein is typically defined by, for example, mutating catalytic aspartates to alanine, as was initially done for the yeast telomerase reverse transcriptase protein component (14). Such a mutation destroys the catalytic activity that produces the phosphodiester bond, as one would expect. However, in addition to catalysis, the active site also has other functions: for example, it has to bind triphosphate substrates, along with the template and primer, and then carry out whatever conformational changes are necessary to get the catalytic aspartates in position to execute the reaction. Notably, as described above, specific small changes in the RNA, as opposed to the protein, of telomerase produce the same result as mutating the aspartates — no enzymatic activity. Therefore, even when the protein is intact, highly specific mutations in the yeast telomerase RNA can prevent the active site from functioning. We normally imagine that an active site is built up through a network of interactions between amino acids in a protein. Here, we propose that the RNA residues also play crucial roles in building up the network of functional interactions. Together, these place the catalytic aspartates in the correct configuration for activity. Therefore, we propose that the active site of telomerase is composed of various func-

tional groups contributed by RNA telomerase as well as protein residues.

The RNP structure of telomerase presents another interesting problem for a nucleic acid polymerase. The template sequence is only a very short, exposed part of the larger RNA that is built into the RNP particle. The template is anchored into the RNP in some fashion. At the beginning of a round of elongation, the DNA primer and built-in template have a particular spatial relationship to the protein's catalytic center. But as polymerization occurs along the template, the primer-template moves past the catalytic center, changing the relationship of the built-in RNA to the protein in the RNP (Fig. 2). Thus, effectively, a new active site is created for each new polymerization step. Perhaps the protein cannot by itself make this new active site at every step, and is helped by the RNA, as described above. The notion of building a new active site at each polymerization step may account for the observation that the properties of the enzymatic reaction are very different at each step of the polymerization — i.e., are template position-specific — during copying along the template (15, 16).

A Telomere Capping Function for the Telomerase RNP

Chromosomal ends are protected from being recognized as damaged DNA, from undergoing inappropriate recombination and from being degraded. Our recent work suggests that the telomerase RNP itself helps provide that protective function. First, the telomerases of two yeasts, *S. cerevisiae* and *K. lactis*, act highly nondissociatively when elongating a telomeric DNA primer *in vitro* (12, 17). In other words, the telomerase stays bound to its DNA product. Hence, these telomerases produce very short products *in vitro*, not because the enzyme is destroyed or degraded after a round of synthesis, but because the product stays associated with the active site and cannot be further elongated. One of the experiments that showed this was simply to allow the reaction to take place, then use gel filtration chromatography to attempt to separate out the telomerase RNP particles (plus any of their bound products) from free DNA products (whose expected fractionation properties were indicated by synthetic oligonucleotide markers). The products of the telomerase reaction remained associated with the RNP. This result suggested the possibility that *in vivo* telomerase may also be associated with its DNA product, the tip of the telomeric DNA (11, 12).

Other findings supportive of a capping function for telomerase were made *in vivo*. Deletion of the telomerase RNA (so there is no telomerase RNP in the cell) causes *RAD52*-dependent recombination to occur at telomeres, even those that are very long (18). This suggests that recombination doesn't normally take place at telomeres, but in the absence of a cap — that is, the absence of any telomerase RNP — recombination can act rather promiscuously on these telomeres.

Another indication of a capping function for telomerase came from experiments with the *S. cerevisiae* template mutations described earlier (11, 12). Unexpectedly we found that the 476GUG mutation, which is unable to carry out polymerization, causes loss of cell viability and senescence to occur sooner than expected if telomere length alone were important. In this mutant, telomeres progressively shorten. Senescence is interpreted to be the response to the DNA damage signal resulting from uncapped (shortened) telomeres. Certain other telomerase RNA mutations, in and outside the template, also cause telomeres to shorten. However, in contrast to the 476GUG mutant, the telomerase in each of these other mutants, although somewhat altered in its action, is enzymatically active, and the cells continue to grow perfectly well with no sign of senescence (11, 13, and yet-unpublished work by J. Roy and E.H. Blackburn). The striking finding was that the telomeres in these actively dividing mutant cells were considerably shorter than those in the (otherwise isogenic) 476GUG mutant at the time of its senescence.

We proposed that an active telomerase may bind to its product, the telomeric DNA, as it does *in vitro*, and thereby help to stabilize short telomeres (12). Thus telomere length is not the sole determinant of when senescence will occur. This is relevant to the situation in human cells. As a very broad-brush generalization, cancer cells often have active telomerase, and their telomeres are maintained to at least some degree, although their telomere lengths can vary. In contrast, many somatic cells lack active telomerase, although often cells that are proliferating or are in regenerating tissues do have it. In cells lacking telomerase, over the human lifetime telomeres generally shorten (19, 20). Thus, we can ask: is it the shortening of telomeres, the presence or absence of telomerase, or a combination of both that is important in signaling to cells when to stop dividing? Very recently, we showed in cells growing in culture, that it is the combination of both (21). We do not know if these findings are directly relevant to normal aging of the human body.

However, it was yeast, a very simple system, that clearly told us that it is not just telomere length per se, but rather a lack of active telomerase combined with some degree of shortening, that together determine when a cell will stop dividing.

Evidence for an Oligomeric/Dimeric Telomerase RNP

In the course of the yeast experiments described above, we obtained other surprising results that forced us to think about telomerase in new ways (11, 12). These results came initially from yeast genetic experiments involving mating the 476GUG mutant cells to wild-type cells and analyzing the resulting cells that now contained both wild-type and 476GUG telomerase RNAs. The 476GUG RNA-containing telomerase, which had been completely inactive when only the 476GUG RNA was present in the cells, now sprang to life! Cloning and sequencing of telomeres synthesized in these heterozygous cells showed that the 476GUG telomerase RNA template (as well as the wild-type template) was now copied into DNA. The classic genetic way of explaining such a result was that the telomerase enzyme works as an oligomer, minimally a dimer, in which the wild-type telomerase RNA is able to rescue the 476GUG RNA function. Through *in vitro* biochemical experiments, we established that this is the case, and that the active telomerase RNP particle minimally is dimeric, with two telomerase RNAs and two enzyme-active sites. Furthermore, in order to be rescued by the wild-type RNA, the 476GUG RNA has to be in the same oligomeric/dimeric telomerase RNP complex as a wild-type telomerase RNA; if the oligomeric/dimeric complex contains only 476GUG RNAs, the 476GUG RNA cannot function (12). We do not yet understand the mechanism of the communication between the two RNAs, nor why 476GUG telomerase RNA cannot function by itself.

Critical Roles for the Telomeric Repeats at the Very Tip of the Telomere

In the last section of this article, I will focus attention on the very tip of the telomere itself. I will first briefly review our current views on the way telomeres regulate their length. In a population of telomeres, one observes a gaussian-like distribution of telomere sizes. This distribution, strikingly, has clear lower and upper limits (Fig. 3). We propose that a homeostatic mechanism normally acts at the level of the telomere to keep

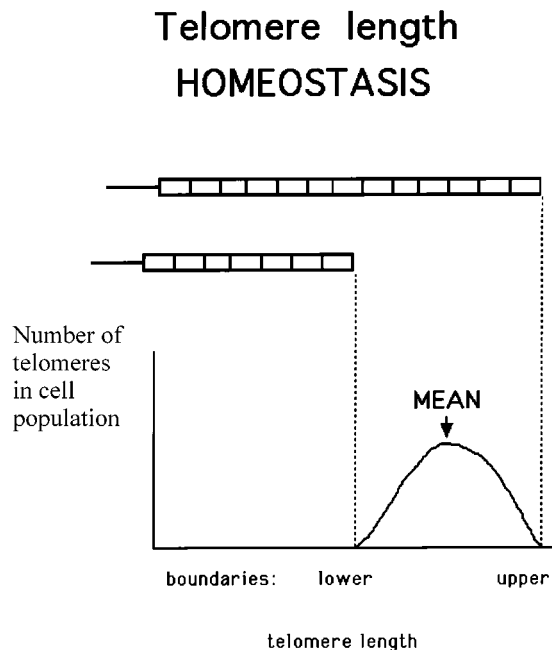


Fig. 3. Telomere length.

telomere lengths within these boundaries (22, 23). Although one can envisage a static model, an alternative dynamic model is currently borne out by much data. In this model, telomerase and the telomere act together as a homeostatic system consisting of both negative and positive regulators of telomere length. We can observe this active, dynamic balance in action in growing cells by making mutations in the telomerase RNA template (24). The appearance of mutant repeats can readily be monitored by, for example, placing a restriction site in the mutant repeat sequence, and following the encroachment of the restriction sites into the telomeres by digesting them with the restriction enzyme. Over time, the original distal repeats of the telomeres become replaced by mutant repeats. This addition is balanced by shortening, perhaps through incomplete replication, and/or nuclease action. The addition and losses occur somewhat stochastically, but normally are balanced overall, i.e., are in equilibrium. Hence, there is a continual dynamic turnover at the telomere tip, but the overall telomere length will stay constant (Fig. 4).

What causes telomeres to be so resistant to further shortening? Much work, done by our lab and by others, provides evidence that the access of telomerase to chromosomal ends is negatively regulated. The negative regulator is the telomere itself (24–27). In yeast, a key player is the Rap1p protein, a sequence-specific, double-stranded telomeric DNA binding protein. Rap1p

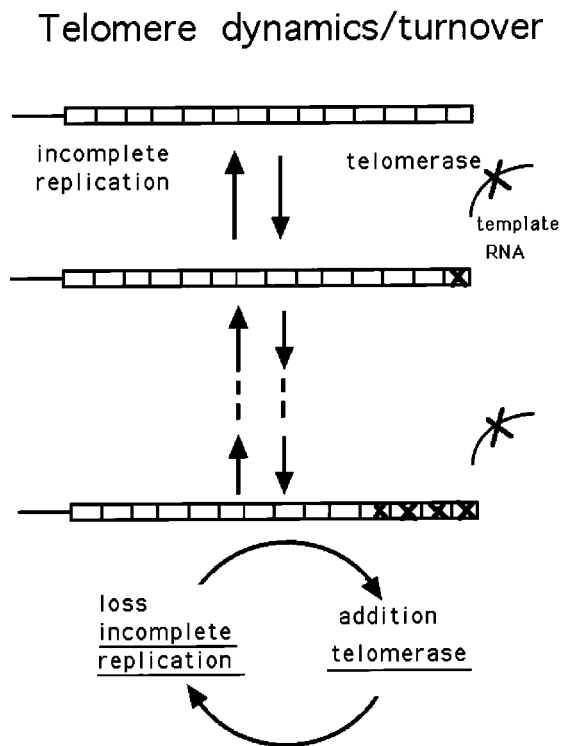


Fig. 4. Dynamic turnover at the telomere tip.

coats the telomeric DNA repeats and then assembles around itself a suite of other proteins, thereby building up a higher-order nuclear protein complex. The C-terminal domain of Rap1p is used to organize the binding of the other proteins to the telomere, but has no effect on DNA binding per se (27). We proposed that this telomeric higher-order complex can switch between a telomerase-accessible and a telomerase-inaccessible state. This two-state model has the requisite features of a robust homeostatic system, based simply on the idea that enough telomerase is present and that the telomere itself can block the access of telomerase to the telomeric end (Fig. 5). Specifically, in this model the longer the telomere, the more likely it is to be in the inaccessible state. Conversely, the shorter it is, the more likely it is to be accessible to telomerase. Thus, the probability of being elongated with telomerase is high for short telomeres and low for long ones. When a telomere is long, telomerase will fail to add more DNA, and the telomere will gradually get shorter over time, increasing its chance of switching into the telomerase-accessible state, at which point the shorter it is, the more likely it is to be elongated.

One interesting consequence of such a homeostasis system is seen if one tries to break out of the normal boundaries, for example by manipulating telomerase activity in cells. We attempted to

Telomere length homeostasis

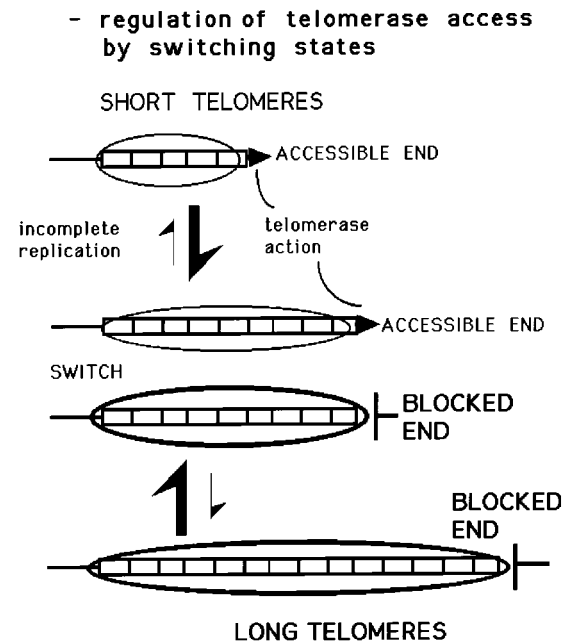


Fig. 5. Telomere length homeostasis.

ablate telomerase activity in human lymphoid cell lines grown in culture. The goal was to shorten telomeres so they were no longer stably maintained, and thereby arrest cell division (28). The chain terminators AZT and dideoxy-guanosine, in their triphosphate form, partially inhibited human telomerase *in vitro*. These inhibitors of telomerase, when added to cells in culture in their nucleoside form, caused progressive telomere shortening over several cell passages. The telomeres then became stably short, but the cells kept dividing. Thus, the telomeres were very resistant to further shortening. By ablating telomerase activity only partially, it appeared that the telomere length equilibrium was reset to a new low level. Hence one implication of a telomere homeostasis system is that any inhibitor of telomerase used in a therapeutic setting will likely have to be very potent.

Mutations made by others and by our group in Rap1p yeasts provide clear evidence for a role for Rap1p in telomere length regulation along the lines described in Fig. 5 (25–27). Here I outline data showing that, in addition, it is the telomeric repeat complex at the very end of the telomere — i.e., the complex assembled onto the very most distal repeats of the telomere — that is particularly crucial for telomere regulation. This is true even in the presence of a full-length, normal complex of internal wild-type repeats. As I will describe, even very small changes at the very

ends of telomeres can cause loss of regulation of telomeres and loss of the capability of telomeres to cap chromosomes. Various experiments all converge on this same conclusion.

First, in the *K. lactis* system, which has the same Rap1 binding site and length regulation system as in *S. cerevisiae*, we made mutations in the telomerase RNA template sequence that to varying degrees lowered the binding affinity of the resulting mutated telomeric DNA to Rap1. The loss of affinity measured *in vitro* correlated with the degree of loss of length control *in vivo* (24, 25). One example of such a mutation was the Acc mutation, a point mutation in the Rap1 binding site in the telomeric repeat that dropped the binding affinity by a couple of orders of magnitude. As soon as one or two Acc repeats were added to the ends of otherwise wild-type telomeres, length control was completely lost, and these telomeres became very long and also very deregulated (uncapped), being degraded as well as lengthened (Fig. 6). The point here is that the internal tract was wild-type (WT) and close to normal length. Thus, at the point when telomere length became deregulated, the bulk of the telomeric complex, with the exception of its very end, was normal.

In a slightly different experiment, we made a different template mutant (called the AA mutant) which by itself had a very small effect on Rap1 binding and telomere length. However, when that mutation was combined with a small mutation in the C-terminal domain of Rap1, only a few mutant repeats had to be added before telomeres rapidly became severely deregulated and degraded (25).

A third type of experiment involved template mutations outside the Rap1 consensus binding site. We checked that the Rap1 binding affinity *in vitro* of such a mutant repeat was normal (25).

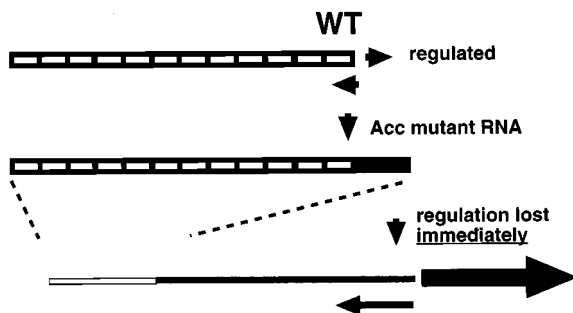


Fig. 6. Putting the wild-type (WT) telomerase RNA gene back into the cells restored telomere length regulation — the Acc mutant.

One example was a mutation that introduced a site for the restriction enzyme KpnI into the telomeric repeat (Bgl mutant). Telomeres in Bgl mutant cells remain well regulated for quite a long time (24). However, when most of the wild-type repeats in a telomere have been replaced by Bgl repeats, through the process of encroachment diagrammed in Fig. 4, quite suddenly there is a complete loss of length regulation. The cells show abnormal enlarged morphologies and a large percentage of them have a much higher than normal DNA content and evidence of missegregated DNA (29).

In all three cases — the Acc mutant, the AA-Rap1-delta C double mutants and the Bgl mutants — putting the wild-type telomerase RNA gene back into the cells restored telomere length regulation (Figs. 7 and 8). The telomeres often remained very long, but were now regulated

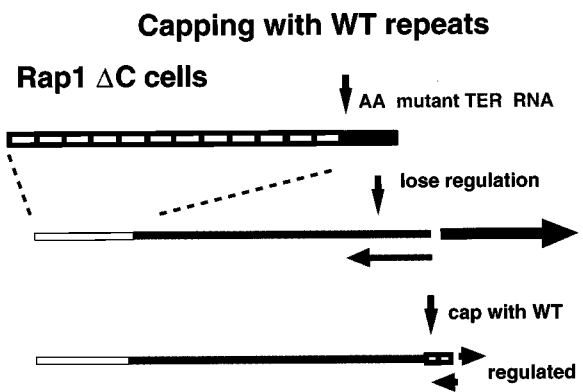


Fig. 7. Putting the wild-type (WT) telomerase RNA gene back into the cells restored telomere length regulation — the AA-Rap1-delta C double mutants.

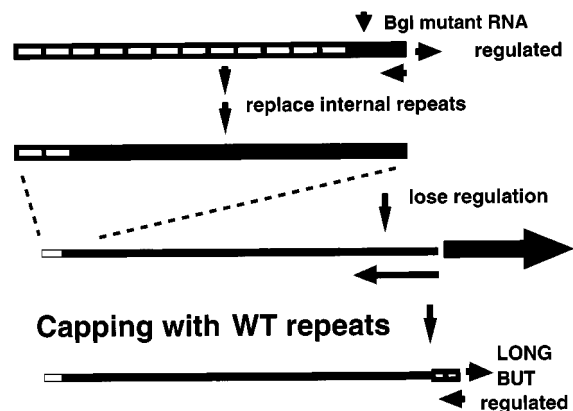


Fig. 8. Putting the wild-type (WT) telomerase RNA gene back into the cells restored telomere length regulation in the Bgl mutant cells.

tightly about their new, elongated lengths (25, 26, 29). Thus, we concluded that these telomeres had become functionally recapped. Interestingly, only a few initial type repeats were needed at the termini in order to restore capping.

The fourth experimental line of evidence came from work initially done in the ciliate *Tetrahymena*. We discovered that in the absence of proper capping function, telomeres fail to separate from each other in anaphase. This was shown when we made mutations in the template of the *Tetrahymena* telomerase RNA. Certain mutants prevented cells from completing anaphase (30). High resolution, 3-dimensional light microscopy indicated that the chromatids became stretched from one pole of the spindle to the other in anaphase, but their ends apparently failed to separate, so that they formed anaphase bridges at very high frequencies. Molecular analyses indicated that the tips of the telomeres in these mutant cells were comprised of mutant repeats, but the bulk of the telomeres were still wild-type (Fig. 9). Thus, this situation appears to resemble that in the Acc mutants in yeast, in which the ends of the telomeres were also mutant, leading to failure of DNA to segregate properly (29). Hence we speculate that, as in *Tetrahymena*, this failure results from the formation of anaphase bridges by fusion of the tips of uncapped telomeres.

In summary, telomeres and telomerase interact in cells to protect the ends of chromosomes and hence the genetic material of a cell. The telomerase RNP complex, which in at least yeast (and probably in other organisms) is oligomeric or dimeric, acts itself as part of the protective cap of telomeres that would otherwise be too short to be stable. In addition, the molecular nature of the very tip of the telomere is a crucially important determinant of whether the telomere can carry out the various functions that, in aggregate, we call capping.

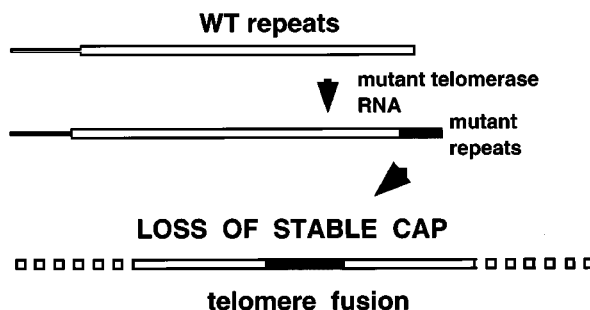


Fig. 9. The tips of the telomeres in the mutant cells were comprised of mutant repeats.

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