

# Tracking Telomerase

# Commentary

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## *The Telomere Problem*

The paper reprinted here, the initial identification of telomerase, resulted from our testing a very specific hypothesis: that an enzyme existed, then undiscovered, that could add telomeric repeats onto chromosome ends. We based this hypothesis on several unexplained facts and creative questions being asked by people who were trying to understand those facts.

Telomeres had been identified, by Barbara McClintock, and defined, by H.J. Muller, in 1938, as the functional chromosome elements that protect chromosome ends, six years before Avery's identification of DNA as the hereditary material (McClintock, 1939; Muller, 1938). In the early 1970s, following the molecular understanding of DNA replication, several researchers recognized that the end of a DNA molecule poses a special problem. Since polymerase uses a primer, how does it replicate the very end of the chromosome? Jim Watson and Alexei Olovnikov stated the problem and proposed possible solutions to it. Watson proposed that phage T7 avoided this end-replication problem by concatamerizing its genome before replication, leaving no ends to replicate; Olovnikov suggested that this replication problem could account for the limited lifespan of human cells (Olovnikov, 1973; Watson, 1972). Thomas Cavalier-Smith imagined hairpin structures at chromosome ends to deal with the problem (Cavalier-Smith, 1974). These early models were imaginative, but without more knowledge of eukaryotic chromosomal telomere structure, they could not be tested.

## *Telomere Sequence Revealed*

The sequence of a natural chromosomal telomere was first identified in the pond-dwelling ciliate *Tetrahymena*. The advantage of *Tetrahymena* lay in the many chromosomes it has in its macronucleus: a single cell contains over 40,000 telomeres. Liz, working in Joe Gall's lab at Yale from 1975 to 1977, wanted to determine the sequence and structure at the ends of a eukaryotic chromosome. She studied the mini-chromosomes that contain the amplified ribosomal RNA gene of *Tetrahymena*. Chromosomal termini from other ciliates were found at that time to have terminal-repeated sequences, but the sequence was not known (Herrick and Wesley, 1978; Wesley, 1975). By 1977, she had found that *Tetrahymena* telomeres consist of tandem repeats of the sequence CCCCAA (Blackburn and Gall, 1978). Furthermore, the heteroge-

neous size of the fragments in gel electrophoresis was the first suggestion of unusual behavior of telomeric DNA. A similar telomere repeat sequence, CCCCAAAA was soon found on natural chromosome ends in other ciliates (Klobutcher et al., 1981). Another very unusual finding regarding these repeated sequences came in 1982: David Prescott found that these repeated sequences are added de novo to ciliate chromosomes during the developmental process of chromosome fragmentation (Boswell et al., 1982). This was the first hint that a special mechanism may exist to add telomere repeats.

The next clue came from work in yeast. In a remarkable example of functional conservation across phylogenetic kingdoms, Liz and Jack Szostak (Szostak and Blackburn, 1982) showed that the *Tetrahymena* telomeric sequences could replace the yeast telomere entirely. A mini-chromosome with these foreign telomeres maintained its linear structure and replicated and segregated properly through mitosis and meiosis. Even more strikingly, the yeast cells added yeast telomeric repeats to the very end of the *Tetrahymena* telomeres (Shampay et al., 1984). This work also established the telomeric sequence of yeast as an irregular GT repeat, extending Walmsley and Petes' evidence for a GT-rich sequence at yeast telomeres (Walmsley et al., 1984).

A final piece of evidence hinting at telomere addition came from trypanosomes: when kept in continuous growth conditions, their telomeres became progressively longer (Bernards et al., 1983). Thus, by 1984 it was apparent that something unusual was occurring at telomeres.

## *Two Models Emerge*

Two classes of models were proposed to account for the addition of telomere repeats onto telomeres. The most popular model was recombination, because it used known mechanisms (Bernards et al., 1983; Walmsley et al., 1984). Several variants of this model suggested that repeated sequences misalign and, through a mechanism similar to gene conversion, one repeat copies from another to achieve a net elongation. Liz, however, preferred an alternative model in which a hypothetical enzyme would add repeats de novo (Shampay et al., 1984). To her, this model better explained the direct addition of yeast telomeric repeats to *Tetrahymena* repeats in vivo, the ciliate data of telomere addition during chromosome fragmentation and the slow progressive growth of trypanosome telomeres. Furthermore, Liz had been struck by McClintock's telling her, in the late 1970s, that she had found a mutant in maize that failed to undergo the healing that normally occurs when a broken chromosome end is introduced into the embryo cells soon after fertilization (McClintock, 1941). This suggested to Liz that healing chromosome breaks might be a normal function of these cells, and de novo telomere addition could explain this healing.

## *Testing the Model*

Liz realized that the best way to determine if de novo addition occurs was to find the enzyme that does the addition. *Tetrahymena*, as a rich source of telomeres, would presumably also be a rich source of any enzymes

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that act on them. In addition, it was known by this time that *Tetrahymena* adds telomere sequences to chromosome ends during the developmental process of chromosome fragmentation (Yao and Yao, 1981).

In early 1984, Liz did some preliminary experiments incubating mixtures of restriction fragments, both with and without telomeric repeats at their ends, and nucleoside triphosphate substrates with *Tetrahymena* extracts. A new signal that hybridized specifically with a CCCCA repeat probe appeared, in a time-dependent manner, as a broad but distinct band on a gel. She presented these results, showing a slide of this gel, in a talk at the April 1984 Keystone conference. Although no one there seemed to take much notice, she was encouraged by these results and convinced that it was worthwhile to look harder for this still-hypothetical activity.

In April 1984, Carol joined Liz's lab as a Ph.D. student and we set out to develop an assay for a telomere-synthesis enzyme. We initially thought that three factors would be critical to get right: the extract, the substrate, and the assay. We guessed right about the extract conditions from the start, but the correct substrate and assay took some time to find. We initially adopted the extract conditions reported by Tom Cech to study processing of ribosomal RNA in *Tetrahymena* extracts (Zaug and Cech, 1980). We purified nuclei in a low-salt buffer containing magnesium and lysed them with detergent. Finding a substrate that would mimic a telomere was more problematic. We began using purified restriction fragments that we designed with telomeric sequence at one end and nontelomeric at the other. The assay was to incubate the restriction fragments in *Tetrahymena* nuclear extract containing  $^{32}\text{P}$ -labeled dG and dC, and unlabeled dA and dT and then purify the fragment back from the extract. We then cut the fragment with a restriction enzyme to distinguish the two ends and resolved the products on an agarose gel. We examined these by autoradiography to determine whether more radioactive label was incorporated onto the telomeric or the nontelomeric end. Because we did not know the structure of the substrate for a potential telomere-synthesis enzyme, we treated the restriction fragments with specific nucleases to generate a 3' overhang, a 5' overhang, or a blunt end. To our chagrin, all the ends became labeled, probably by repair DNA polymerases.

Next, we tried a different plasmid that allowed the generation of a restriction fragment that had telomere repeats immediately at the end. The resulting fragments were much smaller and thus we could analyze the products on a polyacrylamide gel. We saw a hint of increased incorporation of label into a substrate with a 3' overhang. This labeling would not be expected to come from repair synthesis.

The small hint of specific labeling from the acrylamide gel analysis was encouraging, and so, we continued to adjust the assay in small ways. We next used DNA sequencing-type gels to resolve the reaction products and examine their size in detail. We also tried using either  $^{32}\text{P}$ -dC or  $^{32}\text{P}$ -dG as the labeled nucleotide, instead of both together. In September 1984, we did an experiment that showed a telomere substrate with a 3' overhang became longer by about 40 base pairs and labeled specifically with  $^{32}\text{P}$ -dG. It is still not clear, looking at

these gels today, whether this longer product was due to telomerase activity. But it looked promising and encouraged us to change the assay yet again.

We reasoned that if a telomere restriction fragment substrate with a 3' overhang was elongated, then a synthetic DNA oligonucleotide of the telomere G strand TTGGGG should be a substrate too. Furthermore, it would be possible to add this synthetic DNA at significantly higher concentrations than were possible by using restriction fragments, thus potentially greatly increasing the rate of any potential enzyme reaction. Eric Henderson, a postdoctoral fellow in the Blackburn lab at that time, was analyzing the physical properties of TTGGGG oligonucleotides (Henderson et al., 1987). So we simply borrowed some (TTGGGG)<sub>4</sub> to use as a substrate in addition to the restriction fragments we were analyzing. The result was dramatic. When Carol developed the film from the sequencing gel on Christmas Day 1984, the 5 lanes that had (TTGGGG)<sub>4</sub> as a substrate all showed a repeating pattern, with an apparent 6-base periodicity, that extended, ladder-like, up to the top of the gel. This was precisely what we would expect of a telomere-synthesis enzyme. Furthermore, the signal was much stronger for those lanes in which we assayed extracts made not just from vegetatively growing cells, but from cells during the period when telomeric repeats were added to the newly fragmenting chromosomes in mated cells. This biological correlate argued even more compellingly that this was the right activity.

#### *Tempting, but True?*

After that first day of excitement, we began thinking of everything those bands could represent besides de novo telomere synthesis. The most likely explanation seemed that rather than some new activity, the ladder of repeats came from a conventional polymerase copying endogenous CCCCA repeats in the extract or self-associated TTGGGG oligonucleotide (Henderson et al., 1987). Or it might come from contaminating CCCCA oligonucleotide in the TTGGGG preparation that paired with TTGGGG. There were many potential sources of artifacts and we knew we had to rule out even the more far-fetched ones to convince ourselves of the existence of a completely novel enzyme, that added TTGGGG repeats onto TTGGGG oligonucleotides de novo. We then set out to test the validity of these potential alternative explanations for the repeat ladder and we also tried to optimize the assay and improve the signal.

We devised many experiments to determine whether the repeated ladder was in fact de novo addition. We treated the extract with micrococcal nuclease to remove endogenous CCCCA repeats (luckily for us, the telomerase ribonucleoprotein complex is somewhat resistant to micrococcal nuclease treatment, and the endogenous DNA was digested first). We made extracts using a variety of different conditions and from different points in the *Tetrahymena* life cycle. We limited the nucleotides added in the reaction, adding only dT and  $^{32}\text{P}$ -dG or dA and  $^{32}\text{P}$ -dC, or other combinations of radioactive and non radioactive nucleotides. We used dideoxynucleotides that would chain-terminate if incorporated to examine the sequence added. We developed a quantitative incorporation assay. We also tested different oligonucleotide substrates, which it turned out, was the

key to convincing ourselves that we were assaying a new telomere-synthesis enzyme.

#### **Telomere Terminal Transferase**

The persuasive experiment came in June 1985. We were testing different oligonucleotide substrates and decided to try an oligonucleotide that represented the yeast *S. cerevisiae* telomere repeat sequence. We knew that *Tetrahymena* telomeres functioned in yeast and that yeast telomere repeats were added onto the ends in vivo. We decided to test the converse experiment: whether a yeast sequence telomeric oligonucleotide would work as substrate in a *Tetrahymena* extract in vitro. The yeast telomere sequence is an irregular repeat containing a mixture of TG, TGG, and TGGG sequences. In control experiments, an oligonucleotide sequence unrelated to telomeres as well as a (CCCCAA)<sub>4</sub> primer oligonucleotide did not generate a repeated sequence ladder. But when we added TG-rich yeast sequence oligonucleotide to the *Tetrahymena* in vitro reaction, it was indeed elongated and the product was a regular 6 base-pair-repeated sequence indicative of *Tetrahymena* repeats. This was exciting, because it showed that the input oligonucleotide was not simply producing a copy of itself—such copying could not give a regular 6-base repeat pattern. Furthermore, the repeating pattern produced with the yeast oligonucleotide primer was offset by one nucleotide from that produced with a (TTGGGG)<sub>4</sub> primer. The *Tetrahymena* primer ended in four Gs while the yeast primer ended in three Gs; thus, the offset banding pattern suggested that the sequence at the 3' end of the oligonucleotide primer determined the phase of the products that resulted. We now believed that this was a new enzyme activity that added telomere repeats onto telomere oligonucleotide substrates. We went home and celebrated.

In the paper, submitted in August 1985 and published that December, we named this activity telomere terminal transferase, because we thought it added telomere repeats onto telomere substrates in a manner analogous to terminal transferase. That name, *Tetrahymena* telomere terminal transferase however, was a mouthful, and in 1987, on the suggestion of Claire Wyman, a graduate student in Liz's lab, we shortened it to telomerase (Greider and Blackburn, 1987).

#### **Tinkering with Telomerase**

The obvious next question was: where does the information specifying the addition of TTGGGG repeats come from? Liz wondered if it was like the enzyme that adds CCA onto tRNA 3' ends that has sites for two nucleotides and the differential affinity for each depending on the 3' end of the substrate bound (Sano and Feix, 1976; Sternbach et al., 1971). Carol predicted that the enzyme might have a nucleic acid component that could serve as a template for repeat addition. So we set out to test the effect of prior DNase 1 or RNase A digestion of the extract on telomerase activity. In 1986, the molecular biology students at Berkeley invited Tom Cech to give a seminar. Cech—who, four years earlier, had discovered that RNA from *Tetrahymena* would catalytically self-splice (Kruger et al., 1982)—happened to be visiting the Blackburn lab when we were doing the RNase experiment. Throughout the day, he kept checking back in the lab to see if the results were in yet.

RNase indeed inactivated telomerase activity. We

then set out to purify telomerase and clone the genes encoding the RNA. The purification and extensive additional enzyme characterization occurred over the next two years at Berkeley (Greider and Blackburn, 1987). But cloning the gene that encoded the RNA was difficult and was not accomplished until Carol left Berkeley and established her own lab at Cold Spring Harbor. In 1989 when the gene encoding the RNA component was identified, the presence of the CAACCCCAA sequence within the RNA immediately suggested a template mechanism. Biochemical experiments confirmed this mechanism (Greider and Blackburn, 1989).

#### **Tailoring Telomerase**

Gou-Liang Yu in Liz's lab did the final experiment that established the in vivo role of telomerase. He tested mutations made in the template region of the RNA that would specify different telomere sequences by expressing the mutant genes in *Tetrahymena*. The transfected cells had altered telomere repeats specified by the mutant gene (Yu et al., 1990). This confirmed that telomerase is indeed the enzyme that synthesizes telomeres in cells.

Another outcome of these experiments was that one template mutant did not show any detectable addition of the predicted sequence onto telomeres. Rather, the telomeres shortened and the cells grew for a while and then senesced (Yu et al., 1990). This showed that interfering with telomerase in *Tetrahymena* would lead to cell death and effectively limit their lifespan.

#### **Time Will Tell**

Initially, this work had little impact beyond the—then quite small—circle of people interested in telomeres and chromosomes. It was not immediately obvious to some outside this field how far one could generalize *Tetrahymena* or yeast biology to other species. *Tetrahymena* has an unusual life cycle that involves chromosome fragmentation and telomere addition—how did we know it doesn't have a quirky method of telomere synthesis? And yeast was, well, yeast. Skepticism decreased, however, with evidence that human telomeres have properties similar to *Tetrahymena*'s. In 1986, Howard Cooke and coworkers reported heterogeneous terminal restriction fragments from human X and Y chromosomes, similar to those in *Tetrahymena* and yeast. Further, the fragments were shorter in adult blood cells than in germ cells, suggesting that telomere shortening might occur in somatic tissue (Cooke et al., 1985; Cooke and Smith, 1986). In 1988, the human telomere sequence was shown to consist of tandem repeats of TTAGGG (Moyzis et al., 1988). The next year, telomerase activity was documented in human cells (Morin, 1989).

With this human connection, the medical relevance of telomeres and telomerase began to emerge. As described in the accompanying review by Tom Cech, telomerase is linked to cancer and aging. The end-replication problem, where we started, has a role in cellular senescence; forced expression of telomerase can extend the lifespan of cultured cells (Bodnar et al., 1998). Telomerase is activated in most tumors (Kim et al., 1994) and is being actively pursued as a target for cancer therapy. Recently, telomerase was linked to the human genetic disease dyskeratosis congenita, in which limiting telomerase leads to progressive bone marrow failure, and perhaps to failure of other organ systems (Vulliamy et al., 2001).

Time will tell which connections between telomerase and health will endure; further, new, unforeseen connections may yet emerge. We did not set out to find a new approach to cancer therapy or study specific disease mechanisms. We were simply interested in how chromosomes are maintained. It may seem highly improbable: a new medical approach from studying the chromosome fragments of a pond creature? Yet the history of medicine is filled with examples of advances from improbable places. Fundamental mechanisms are conserved across species, although one particular species may accentuate a particular mechanism. Given the diversity of life, no doubt many new fundamental mechanisms remain to be found.

#### Acknowledgments

We thank Nathaniel Comfort for critical reading of the manuscript. Work in the Blackburn and Greider labs is supported by grants from the National Institutes of Health

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