

# TELOMERASE DISCOVERY: THE EXCITEMENT OF PUTTING TOGETHER PIECES OF THE PUZZLE

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by

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The story of telomerase discovery is a story of the thrill of putting pieces of a puzzle together to find something new. This story represents a paradigm for curiosity-driven research and, like many other stories of fundamental discovery, shows that important clinical insights can come from unlikely places. In this paper I describe the process of scientific discovery – at times frustrating, at times misleading and perplexing, but yet also at times wonderfully exciting. The willingness to keep an open mind, to enter uncharted waters and try something new, along with patience and determination, came together to tell us something new about biology. Fundamentally this story shows how curiosity and an interest in solving interesting problems can lead to a lifetime of exciting discoveries.

## IDENTIFYING THE PUZZLE: TELOMERE SEQUENCES DEFINED

Telomeres posed a puzzle for biologists for a many years: it was clear that a chromosome end differed from a chromosome break – but how? In the 1930s, Herman Muller and Barbara McClintock showed that natural chromosome ends have special properties that afforded their protection; they coined the word *telomere* to describe these natural chromosome ends, deriving the word from the Greek *telo* = end and *mere* = part (McClintock, 1939, 1941; Muller, 1938). The puzzle of how these ends functioned remained unsolved for many years, until their molecular structure was characterized.

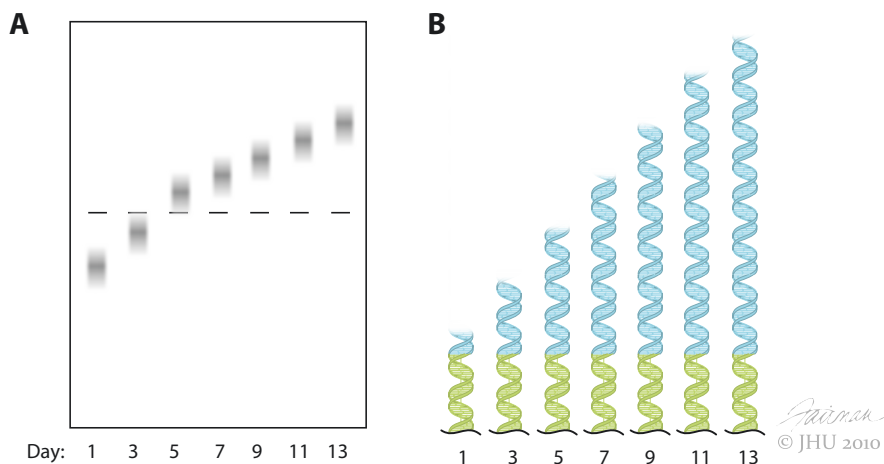
In 1978, Blackburn and Gall identified the first telomere sequence using the ciliate *Tetrahymena*, which contains 40,000 telomeres. They found that the chromosome end was made up of tandem, consecutive repeats of the simple sequence CCCCAA (Blackburn and Gall, 1978). Their discovery of this simple repeated sequence turned out to be the key to understanding telomere function.

The identification of tandem repeats in the telomeres of *Tetrahymena* was followed by the identification of similar repeats in the telomeres of other organisms, including *Oxytricha*, *Physarum*, yeast, and trypanosomes (Bernards *et al.*, 1983; Boswell *et al.*, 1982; Johnson, 1980; Szostak and Blackburn, 1982). This conservation of telomere sequence across a wide variety of organisms

suggested that telomere function might also be similar in these species. This conservation also suggested that telomeres and their function – of maintaining chromosome ends, and hence protecting them from destruction – arose early in evolution, such that all species with linear chromosomes evolved with the same ancestral mechanism of maintenance, albeit with some variation.

#### CURIOUS FACTS ABOUT TELOMERES: SOME PIECES OF THE PUZZLE

Liz Blackburn and others were interested to know how the simple repeats functioned as telomeres to confer protection upon a chromosome end. Soon after the telomere sequence was first identified, several curious facts about telomeres were uncovered. First, telomeres were heterogeneous in length: in a population of cells, different chromosome ends possessed telomeres comprising different numbers of repeats (Blackburn and Gall, 1978). How was this established? When a population of telomeres was digested using restriction enzymes it generated so-called “fuzzy bands” when the digest was separated using agarose gel electrophoresis (Figure 1). Why was this? The variable-length telomeres had generated heterogeneous fragment lengths, which all migrated to slightly different positions on the agarose gel, yielding blurred (or ‘fuzzy’) bands, rather than the sharper, more distinct bands that are typical of restriction fragments which are all of a similar size.



**Figure 1. Telomere elongation in Trypanosomes and Tetrahymena.**

Keeping cells in continuous log phase growth results in progressive elongation of the telomeres. A) Diagram of a gel showing the heterogeneous length telomeres from *Tetrahymena*. Each lane represents increased numbers of cell divisions. B) Diagram of the interpretation of telomere elongation seen in gel in part A.

A second curious fact was that the telomeres in trypanosomes grew longer as the cells were grown in culture (Bernards et al., 1983). The same surprising result was found when similar experiments were performed with *Tetrahymena*: when kept in continuous log phase growth, the telomere fragments grew progressively (Larson et al., 1987) (Figure 1). This elongation was unexpected. In fact, it was the opposite of what had been predicted on the basis of published models for telomere replication. In 1974, James Watson suggested that a linear chromosome should get shorter as cells divided, based on the mechanism by which DNA polymerase replicates DNA during cell division (Watson, 1972). A similar idea was also proposed by Alexi Olovnikov, who suggested that chromosome ends might shorten after rounds of DNA replication (Greider, 1998; Olovnikov, 1973). Why, then, were the telomeres getting longer? And why were they fuzzy?

### COLLECTING MORE PIECES OF THE PUZZLE: TELOMERE SEQUENCE ADDITION

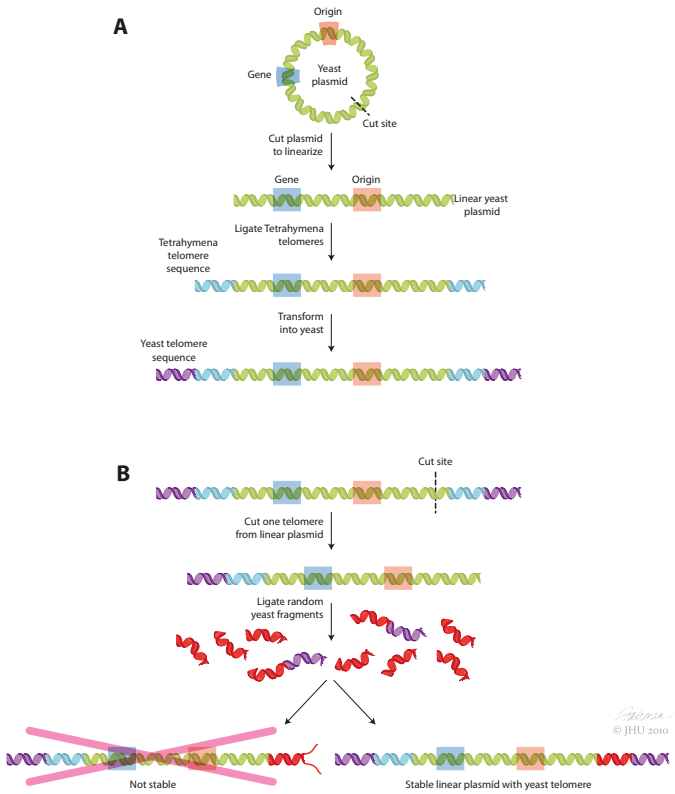
When Liz Blackburn and Jack Szostak met for the first time at a conference, they were both interested in DNA ends. They knew about the curious structure of telomeres and their elongation, and they saw a way to use these puzzle pieces to perform a long-shot experiment: they decided to test whether *Tetrahymena* telomeres could function as telomeres in yeast. The experiment was a long shot because these two species are very distantly related, having diverged from a common ancestor millions of years ago. Despite being a long-shot it was an experiment they could do, so they forged ahead.

Blackburn and Szostak took a circular yeast plasmid and cut it once with a restriction enzyme to make it linear. Jack knew from his earlier experiments in yeast that this linear DNA would be rapidly degraded if put into yeast cells. However, they wondered whether the addition of *Tetrahymena* telomeres to this linear DNA would cause the ends to be protected – as they are in *Tetrahymena* – so preventing the normal rapid degradation of the DNA when transformed into (that is, added to) yeast cells. Liz purified the *Tetrahymena* telomeres and Jack ligated them onto the linearized yeast plasmid (Figure 2). When this construct was transformed into yeast, it was stable: it replicated and was maintained as a linear chromosome fragment.

This maintenance of the linear plasmid was a stunning result: it indicated that the *Tetrahymena* telomeres functioned and protected DNA ends in yeast, a very distantly-related organism (Szostak and Blackburn, 1982). Emboldened by this success, Liz and Jack took one step further forward; they devised a method to identify the naturally occurring yeast telomere sequence. Instead of attaching *Tetrahymena* telomeres to the linear plasmid, they set out to find the parts of the yeast genome that protect and maintain the linear plasmid, and which therefore contain the yeast telomere. To do this, they removed one end of the linear plasmid to which *Tetrahymena* telomeres had been added, and ligated random genomic fragments of yeast

DNA to this free end. Those random fragments that harbored a telomere allowed stable maintenance of the linear plasmid.

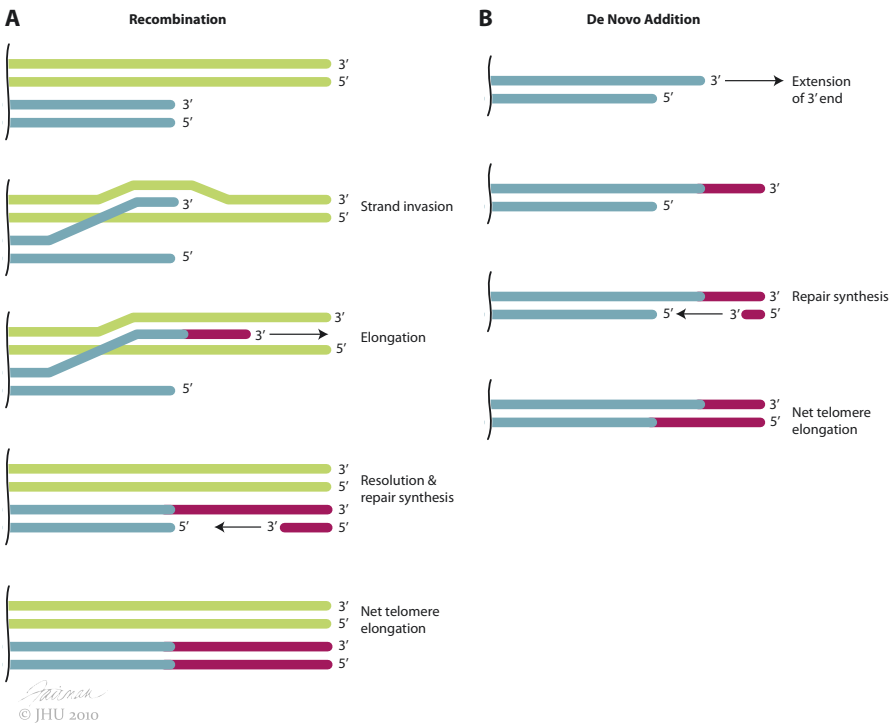
In doing these experiments, Liz and Jack noticed another curious fact: the *Tetrahymena* telomeres on the plasmid maintained in yeast were longer than they had started out. They were curious to know why. So, together with Janice Shampay, they set out to sequence the cloned yeast telomeres and the *Tetrahymena* telomere end. When they sequenced the yeast telomere they found it to have the same kind of tandemly-repeated sequences as had been found in all other organisms so far. More strikingly, determining the sequence of the *Tetrahymena* telomeres from the linear plasmid showed why they were longer: the initial *Tetrahymena* telomeres had been extended by terminal addition of yeast-specific sequences. (Shampay et al., 1984) (Figure 2)



**Figure 2. Evidence for telomere elongation.**

A) *Tetrahymena* telomeres function in yeast and are elongated. A circular yeast plasmid was linearized at a unique cut site and *Tetrahymena* telomeres were added. This linear DNA was transformed into yeast and the linear plasmid was stable. During propagation in yeast, the terminal DNA was elongated by addition of yeast telomeres sequences. B) Cloning of a yeast telomere. The linear plasmid shown in part A was cut to remove one end and random pieces of yeast genomic DNA were ligated onto the end. Those products that had internal yeast DNA added were not stable – they would be degraded by nucleases. However, when a piece of DNA that contained a telomere was ligated, this plasmid was stable and was propagated as a linear plasmid. Yeast telomere fragments were first identified using this strategy.

This last puzzle piece was a very important one. Models of telomere maintenance and elongation had been proposed, which involved telomere-telomere recombination (Figure 3a). Even though Jack’s expertise lay in understanding these recombination models, both he and Liz recognized that the addition of yeast-specific sequences onto the *Tetrahymena* ends could not really be explained by those models. Instead, the data suggested to Liz and Jack the existence of an active elongation mechanism in yeast, whereby DNA was generated *de novo* rather than being the result of a recombination event (Figure 3b). They wrote in their paper, “We propose that terminal transferase-like enzymes are responsible for extending the 3’ G+T rich strand of yeast telomeres.” By continually following the clues (and keeping an open mind) they had come to the conclusion that no known enzyme could do the sequence addition, and so proposed instead that there must be an unknown enzyme that adds telomere sequences.

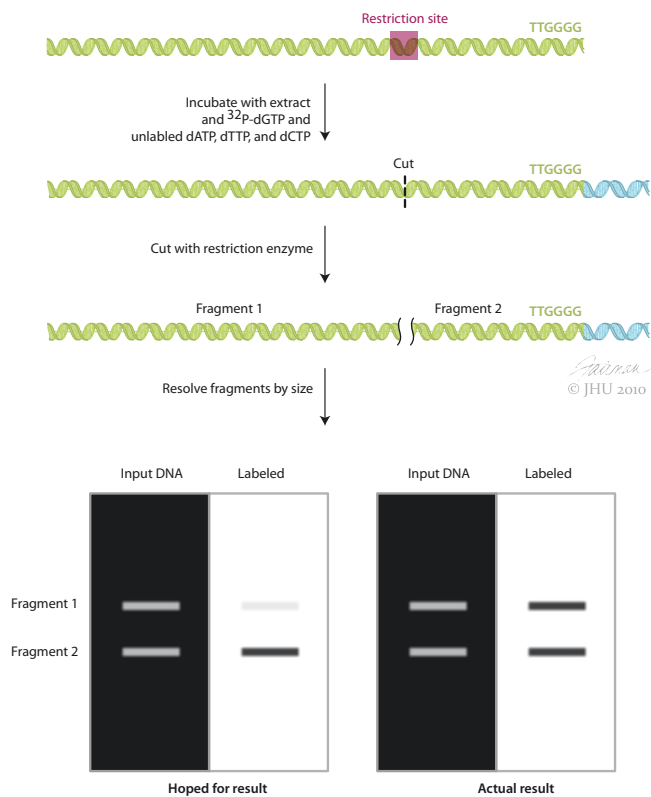


**Figure 3. Two models for telomere elongation.**

A) Recombination can elongate telomeres. Since telomeric repeats are homologous on all chromosome ends a short telomere may copy off of a long telomere in a gene-conversion type of recombination. The 3’ end of the short telomere base pairs with the longer telomere and polymerase extends the end of their strand. The other strand can be copied by conventional polymerase activity. This results in the net elongation of the short telomere, while the long telomere remains long. B) Alternative model for telomere elongation proposes that *de novo* elongation lengthens telomeres. The discovery of telomerase indicated that this mechanism is the predominant mechanisms for telomere elongation in most species.

LOOKING FOR TELOMERE ELONGATION: DEFINING THE EDGES OF THE PUZZLE

When I joined Liz’s lab in May of 1984 I set out to look for this unknown enzyme. We decided to use biochemistry to see if we could identify an enzyme that might elongate telomeres. There was no established protocol for finding an unknown enzyme, so we had fun and made one up. In fact, more precisely, we continually made up new protocols. It was like biochemical improvisation: we started with one concept of an assay that might allow detection of addition onto telomeres, but kept modifying the assay after each set of experiments. We changed the reaction conditions, the substrates, and even method of detection. After nine months we found something (!) and so we had another piece of the puzzle. But how did our nine-month search for this puzzle piece unfold?



**Figure 4. Initial assay for telomere-specific end labeling**

A restriction fragment was purified from a plasmid. The fragment had telomeric DNA on the right end and non-telomeric DNA on the left end. There was a restriction enzyme cut site that would allow two different sized pieces (Fragment 1 and Fragment 2) to be generated. After incubation with  $^{32}\text{P}$  dGTP and  $^{32}\text{P}$  dCTP and unlabeled dATP and dTTP the fragment was purified and cut with the restriction enzyme. The products were resolved on an agarose gel and then the gel was exposed to X-ray film. The hoped for result was that the Fragment 2 with the telomeric end would be preferentially labeled (Left bottom panel). The actual results showed that both ends were equally labeled (Right bottom panel), prompting a revision of the assay.

First we needed an assay – a way to detect if telomere elongation was happening. The first assay we tried explored whether a piece of DNA that included a telomere would incorporate DNA precursors more readily than a piece of DNA containing non-telomeric sequences. The idea was that if there *was* an enzyme that actively elongated telomeres, we might be able to detect it through its activity in association with telomere DNA. For this assay, we developed a substrate that was meant to mimic a telomere in the cell: a linear DNA fragment that contained a telomeric sequence at one end but not at the other (Figure 4). I incubated this linear fragment of DNA with the nucleotides dA, dC, dG and dT – the building blocks of DNA – the dC and dG had been labeled with the radioactive isotope  $^{32}\text{P}$ . We reasoned that an elongation enzyme might preferentially elongate the end of the DNA fragment that contained the telomeres; if it did, we expected to see more of the  $^{32}\text{P}$  label incorporated into the telomeric end than the end lacking a telomere.

I incubated the linear DNA substrate in an extract made from *Tetrahymena* nuclei. The extract was prepared in a manner that we hoped would allow all of the enzymes normally present in the nuclei to be active. We also added radiolabeled dCTP and dGTP and unlabeled dATP and dCTP to serve as DNA precursors. After incubation for an hour, I purified the linear fragment from the extract to examine what had happened to the ends. Following its purification, I cut the DNA fragment to generate two unequal sized fragments to distinguish between the telomeric and non-telomeric ends. (The smaller of the two pieces contained the telomere end.) We could then separate and identify the two different-sized fragments on an agarose gel.

After separating the two pieces by size, we exposed the gel to X-ray film, because the fragments that had incorporated the  $^{32}\text{P}$ -labeled precursor would generate a dark band on the film and we could thus identify them. We then looked to see if there was more radioactive label incorporated in the fragment that had the telomere end than the non-telomere end (Figure 4). Unfortunately, no matter how we prepared the extract, both fragments always incorporated similar amounts of label. We knew we needed a different way to approach this problem.

#### A PUZZLE-SOLVING STRATEGY: GETTING THE ASSAY RIGHT

The most productive way to solve a puzzle is to attack it with the right strategy. Since we did not know precisely what we were looking for, we tested a number of different approaches to see which plan might be successful. After each experiment, we thought of new changes to make to the next experiment. Two of the many changes we tried proved important: using sequencing gels to resolve the DNA fragments after incubation, and using synthetic oligonucleotides as telomere substrates instead of large restriction fragments. How did these two changes affect the outcome of the experiments?

After our initial attempts that I've described above, we sat and puzzled about the fact that both the telomere end and the non-telomere end showed

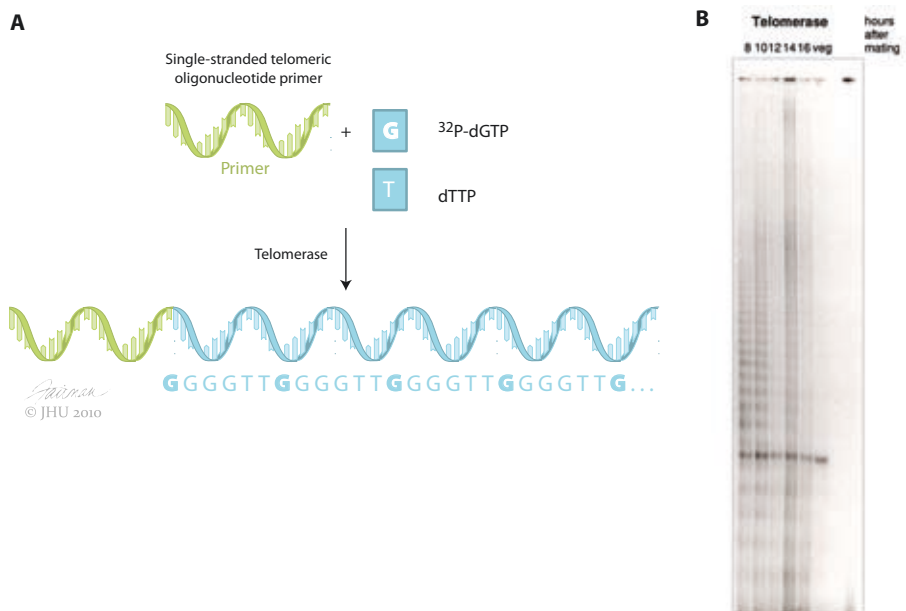
incorporation of the radioactive label. We realized that exonucleases, which were expected to be present in the extract, were likely generating some single-stranded DNA within the linear DNA fragment; repair polymerases would then fill out the single strand region, causing radioactive label to be incorporated into both ends of the fragment.

We thought hard about a way to get around this problem. First, we changed the approach: rather than looking for increased label incorporation, we decided to look for changes in the size of the fragment. If there was an enzyme that extended telomeres not only should the telomeres become labeled with the radioactive precursors, but *also* the size of the fragment should increase as the telomere is extended. (The repair polymerases in the extracts, which could cause both telomere and non-telomere ends to be labeled would not be capable of generating DNA that was *longer* than the fragments added at the start of the assay.)

To see a change in size of a fragment we needed to have a short fragment: a small change in size of a large fragment would be too hard to detect, but a small change in size of a very small fragment would be noticeable. We repeated the experiment as described above but, when cutting the fragment, made the cut very close to the telomere end to generate a telomere fragment just 34 base pairs long. The non-telomere and telomere fragments were then separated on a gel that was usually used for DNA sequence analysis and which could distinguish between fragments that differed in length by just a single base.

I worked from May through December trying different variations of this experiment, staring hard at the sequencing gels but never quite convincing myself there was much of a change in fragment size. So, in December of 1984, we decided to make another change to the assay: we changed the substrate that we were adding to the reaction. Instead of a long linear DNA fragment, I tested a synthetic 18 residue oligonucleotide (TTGGGG)<sub>4</sub> as the substrate. Eric Henderson, a postdoctoral fellow in the lab, was studying the unusual structure of DNA oligonucleotides made of these G-rich telomere sequences, and offered some of his synthetic oligonucleotide, which I decided to use instead of the DNA restriction fragment to see if it might be elongated.





**Figure 5. Telomerase activity assay**

A) Diagram of primer elongation assay. A single stranded DNA oligonucleotide with the sequence (TTGGGG)<sub>4</sub> was added with 32P dGTP and unlabeled dTTP and incubated in *Tetrahymena* cell extracts that we hoped would have telomerase activity. The primer was extended by addition of a six base repeating pattern B) Telomerase elongation of telomeric primer. Extracts from a time course of *Tetrahymena* development were made at different numbers of hours of development (indicated at top by hours). Extracts were also made from vegetatively growing cells (marked Veg). The banding pattern represents the six base repeat of TTGGGG that is added to the primer. This autoradiogram was the first experiment in which telomerase activity was seen on December 25, 1984.

So, I set out to examine the elongation of the telomeric oligonucleotide. I made cell extracts from *Tetrahymena* and incubated them with the oligonucleotide and radiolabeled nucleotide precursors (Figure 5A). It took over a week to set up the experiment, do the reactions, and then run the sequencing gel. To maximize the signal generated by the radioactive label, I exposed the gel to X-ray film for three days. When I went to the lab to develop the X-ray film, I was thrilled to see a repeating pattern of elongation products that extended up the gel. (Figure 5B). The oligonucleotide substrate was being elongated to give products that varied in size by six bases, giving the repeating pattern seen on the gel. This was the first visualization of telomerase activity.

## TESTING OURSELVES: DO THE PIECES REALLY FIT, OR ARE WE FORCING THEM?

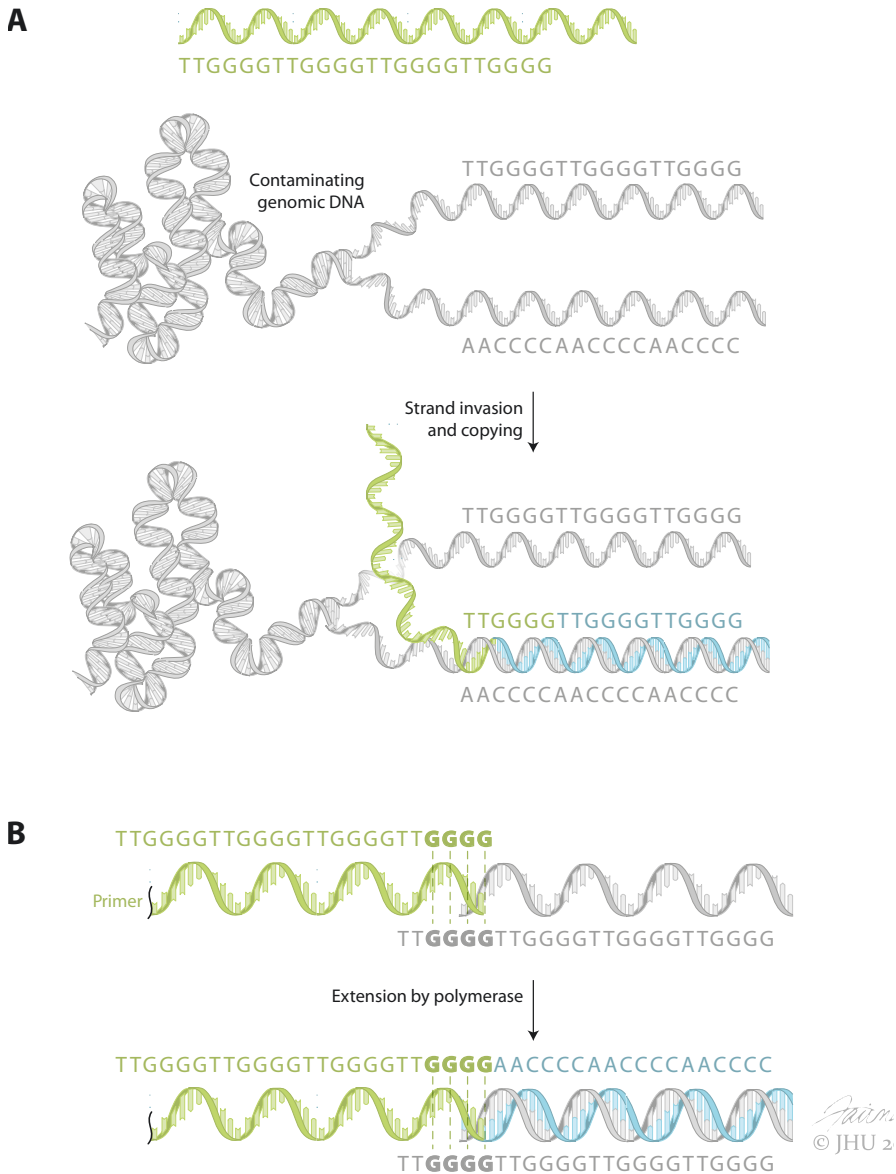
I talked to Liz the next day and showed her the gel. We were both talking at the same time, trying to understand the meaning of the repeating pat-

tern. We knew that this could be the result of the enzymatic activity we were looking for, but we also wanted to be sure the pattern we were seeing was indeed generated by a novel enzyme. Were we truly seeing something new, or was our own wishful thinking coloring our interpretation of the result?

To be sure we were not forcing the interpretation of the elongation pattern, we set out to test various alternative explanations that might generate a repeating pattern like the one we were seeing. For example, we thought the TTGGGG primer might be annealing to double-stranded genomic DNA that might be present as a contaminant, such that a conventional polymerase could generate the TTGGGG repeat addition when replicating the DNA (Figure 6A). Alternatively, the primer might be self-annealing (that is, pairs of the primer might be sticking to one another), generating a double-stranded substrate for a conventional polymerase to copy (Figure 6B).

To address these and other concerns, we devised an ever-evolving set of control experiments to determine if the repeat addition was the result of the activity of a previously identified enzyme. For one control, we treated the samples with aphidicolin, which inhibits conventional DNA polymerases. More importantly, we used a CCCCAA primer, which would be expected to be elongated if simple copying of telomere repeats by DNA polymerase was occurring (rather than nucleotides being added *de novo*). The fact that the CCCCAA primer was not elongated ruled out the trivial explanation that the repeating pattern was coming from the copying of endogenous DNA.

These were exciting times. Once I could repeatedly see the primer elongation activity, it was fun to test various ideas about how it might be generated. I would come in to the lab every day, eager to test the next set of experiments and find something new. The final experiment that convinced both Liz and me that we had something new was when we did the converse of the experiment that Liz and Jack Szostak had done, which had been published in *Cell* in 1982. They had put *Tetrahymena* telomeres into yeast cells and shown that a yeast telomeric sequence was added to the ends. By contrast, we made a synthetic yeast sequence telomere oligonucleotide primer and put it in *Tetrahymena* extracts – and found that the *Tetrahymena* telomere repeats were added to the yeast telomere.



**Figure 6. Diagram of possible telomere elongation artifacts**

A) If the primer telomeric oligonucleotide were to anneal to contaminating DNA in the extract, any conventional DNA polymerase could copy the telomeric sequence and might generate the six base repeating pattern. B) An alternative artifact might come from two telomeric primers self-annealing through G-G non-Watson-Crick base pairing. Extension of these “primer dimers” might also generate a 6 base repeated pattern.

How could we tell that *Tetrahymena* and not yeast telomere repeats were being added? The yeast sequence primer had three Gs at the end, while the *Tetrahymena* telomere sequence had four. The banding pattern of sequences added onto the yeast primer was one base longer than would have been the case if the yeast sequence had been repeated: this extra base was the extra

G required to complete the GGGG found in the *Tetrahymena* sequence. This result was quite stunning. The shift in banding pattern convinced us a new enzyme was in action: we could not imagine how conventional polymerases would elongate a yeast sequence with *Tetrahymena* repeats. We wrote up the paper, which was published in *Cell* in December of 1985 (Greider and Blackburn, 1985).

## THE NEXT PART OF THE PUZZLE: SEQUENCE INFORMATION

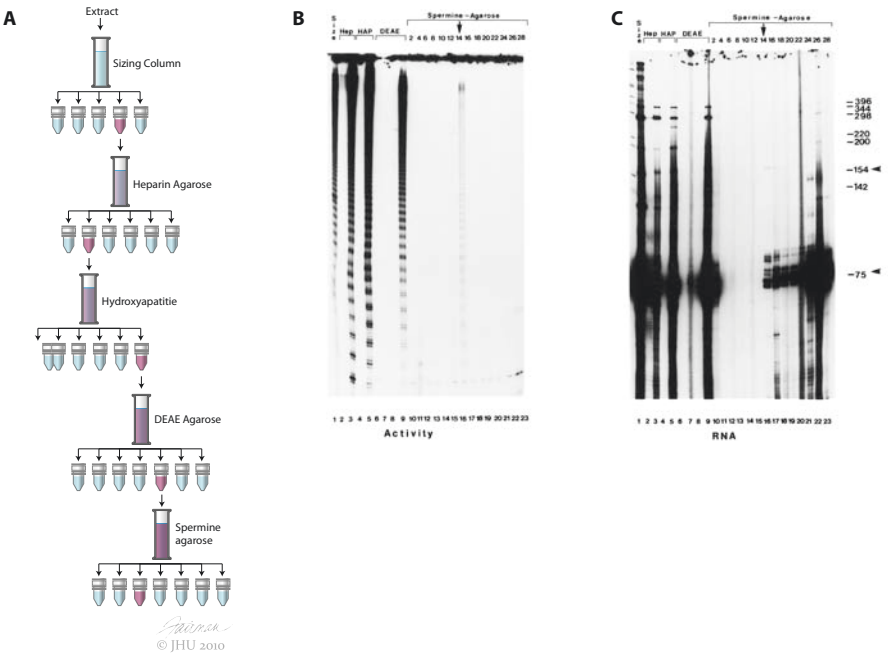
The next big question was where the information for the addition of TTGGGG repeats was coming from. Liz and I talked through several different models. The one I wanted most to test was that there might be an RNA component that specifies the sequences added to the chromosome end. I set out to do an experiment to pre-treat the extract with either DNase (which would digest DNA) or RNase (which would digest RNA) or nothing (as a control) to see if this pre-treatment affected the activity of telomerase. On the day of this experiment, Tom Cech, who had a long-standing interest in both telomeres and RNA, was visiting Berkeley to give a seminar. Liz and I met with Tom in the morning and described my idea of seeing whether the activity would be sensitive to treatment with RNase. He agreed that was an interesting experiment.

Throughout the day, as Tom was being escorted around the department from appointment to appointment, he would stop by the lab and see how the experiment was going. We found that pre-treatment of the *Tetrahymena* extract with RNase did indeed block the elongation activity. Establishing that RNA was needed for elongation was a key clue: it allowed us to think about possible mechanisms by which the enzyme would specify the TTGGGG repeats that were added.

## FOLLOWING THE CLUES: IS THERE A TEMPLATE?

The inactivation of telomerase by RNase treatment suggested a clear hypothesis: the TTGGGG repeats are made by copying from an RNA template, which is a component of the functioning telomerase. The most powerful way to test the validity of this hypothesis was to find the actual RNA template. To isolate a component of telomerase, we needed first to purify the enzyme from all of the other proteins present in the crude extract. I established a multistep purification protocol for telomerase, using conventional column chromatography (Figure 7A). I would separate the extract into fractions and test each fraction for activity (to identify the fraction containing telomerase). I would then take the active fractions and subject them to another, different separation step. I used size exclusion, ion exchange, dye binding, and heparin binding columns to successively purify telomerase (Figure 7B). I then examined the active fraction to look for an RNA that was always present when telomerase was active. I purified the RNAs from active fractions, and labeled them with  $^{32}\text{P}$  (Figure 7C); we then

narrowed down the likely candidates by determining which RNAs reproducibly co-purified with telomerase.



**Figure 7. Purification of telomerase and identification of the co-purifying RNA**

Purification scheme for telomerase. A) Extracts were fractionated first on a sizing column, the fractions were all assayed for activity and those that had activity were again fractionated on a Heparin Agarose column. Further purification continued in the same manner, after each column the active fractions were loaded on a subsequent column including Hydroxyapatite, DEAE Agarose and finally Spermine Agarose B) Gel showing the active fractions for each of the columns diagrammed in part A. C) To identify the co-purifying RNAs, all RNAs from each of the active fractions was end labeled with  $^{32}\text{P}$  and resolved by gel electrophoresis. The faint RNA band in lane 16 migrating near the 154 base marker (indicated on the far right) was later found to be the telomerase RNA. RNA gel Parts B and C are reprinted from the publication *Cell* 51, 887–898; Greider, C.W., and Blackburn, E.H. (1987). The telomere terminal transferase of *Tetrahymena* is a ribonucleoprotein enzyme with two kinds of primer specificity with permission from Elsevier.

We then faced our next challenge: we needed to have the sequence of the RNA component to determine if a template mechanism was, indeed, working. We tried a number of different methods to obtain the RNA sequence, including the very newly developed method termed PCR (polymerase chain reaction), which we had heard about but which had not yet even been published.

After trying to obtain a sequence for a number of months, I decided to take a more direct approach: I used direct RNA sequencing techniques to determine a partial sequence from those RNAs that emerged as good candidates. To do this, the RNAs of interest were cut out of a high-resolution

gel and sequenced. This sequencing revealed that the very small RNAs that co-purified with telomerase activity were, in fact, tRNA contaminants that were present in the active fractions due to the high abundance of tRNA in the cell. For example, I sequenced one RNA that ran near a marker of 175 bases and found it was related to 7SL RNA, an RNA that had recently been associated with the signal recognition particle involved in ribosome function. Finding known RNAs was somewhat reassuring as it told us that the sequencing technique was working. However, it was somewhat of a disappointment as we were hoping to identify a new RNA that might provide the template for telomerase.

One RNA that I had my eye on after staring at many different purification experiments ran near the 154 base marker. No single experiment had pinpointed this RNA as the best candidate; my interest was really a hunch since I had seen this RNA repeatedly in many experiments. I decided to test this hunch, however this “154 base RNA” as I referred to it proved harder to sequence. I was able to obtain only partial sequence from different regions of the RNA, but none of the partial sequences contained a telomere repeat as expected for a template and the full length RNA proved impossible to sequence.

All of the accumulated evidence indicated there must be a template in telomerase, but we did not have the final key piece of the puzzle. Since telomerase was an interesting enzyme and our experiments clearly suggested that an RNA was involved, we decided to write a paper about what we knew about telomerase, despite not having the actual RNA in hand. We wrote the paper on the biochemical characterization of telomerase and the fact that there was likely an RNA component; it was published in *Cell* in 1987 (Greider and Blackburn, 1987). Looking back it is now clear that many of the experiments in this paper that helped to characterize telomerase served as the basis for identifying telomerase in other organisms. After the identification of *Tetrahymena* telomerase, telomerase enzymes from other organisms such as *Euplotes*, *Oxytricha* and humans were characterized by other groups (Lingner and Cech, 1996; Lingner *et al.*, 1994; Morin, 1989; Shippen-Lentz and Blackburn, 1989)

## A CHANGE IN VENUE: SEEING THE PUZZLE FROM A DIFFERENT PERSPECTIVE

I finished my Ph.D. at Berkeley in November 1987 and took a position as an independent fellow at Cold Spring Harbor Laboratory in January 1988. At Cold Spring Harbor I was still focused on identifying the RNA, but my exploration took a different approach. Rather than continuing with more RNA sequencing, I decided to see if I could clone the RNA gene directly from the partial sequence information that I had already obtained.

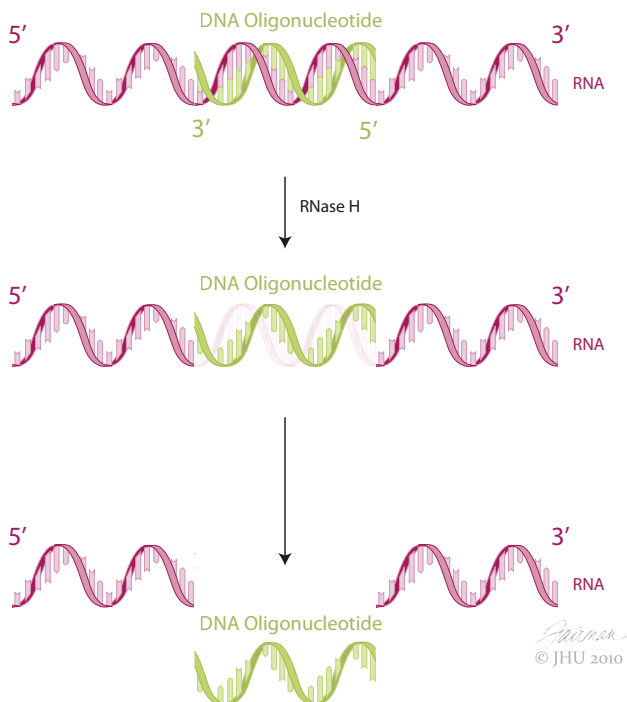
I designed several different oligonucleotide probes that were complementary to the regions of partial RNA sequence I had obtained, and made size- selected genomic libraries that were enriched for sequences that

hybridized to these oligonucleotides. After a number of attempts, I obtained one clone that possessed both the correct partial sequence and the sequence CAACCCCAA. Seeing this sequence on the sequencing gel was exciting, as it mirrored what would be expected if a template mechanism for TTGGGG addition was indeed used by telomerase. This clone was clearly a central puzzle piece. I went on to verify that this RNA was expressed in *Tetrahymena* and was around 160 nucleotides in length. All the signs were that my earlier hunch about “154 base RNA” being a key player in telomerase activity had been right.

### IS THIS THE RIGHT PUZZLE PIECE?

It was exhilarating to have the sequence of an RNA that might encode the RNA component of telomerase in hand. But I needed some evidence that this RNA was indeed the template. Again, just as when we were trying to first identify telomerase, there was no precedent for the experiments I was trying to do. It was fun to be creative and dream up ways to test whether this RNA was the right candidate. I talked to my friends at Cold Spring Harbor, listened to their advice and read about other enzymes and about how functional RNAs were identified.

My next step in characterizing telomerase activity was to test the function of my candidate RNA template. To do this, I decided to use the oligonucleotides I had used in the cloning experiments, which were complementary to telomerase. I thought that if I could inactivate the enzyme by specifically cleaving the candidate RNA, I would have strong evidence for the involvement of this specific RNA in telomerase activity. I think it was Adrian Krainer, a colleague at CSH, who suggested using RNase H in this experiment. RNase H is a specific RNase that will cleave the RNA of a DNA/RNA duplex. The thought was that, if the oligonucleotide hybridized specifically to regions of complementary RNA in telomerase, and subsequent RNase H cleavage inactivated the telomerase, we would have evidence for the involvement of the RNA to which the oligonucleotide had hybridized in telomerase-mediated telomere elongation (Figure 8).



**Figure 8. RNase H cleaves the RNA of a DNA/RNA duplex**

Single stranded regions of RNA will hybridize to single stranded complementary oligonucleotides. When such hybrids are incubated with RNase H, the RNA part of the duplex will be degraded in only the regions that are complementary. In this example the RNA is cleaved in the middle leaving the two RNA halves intact.

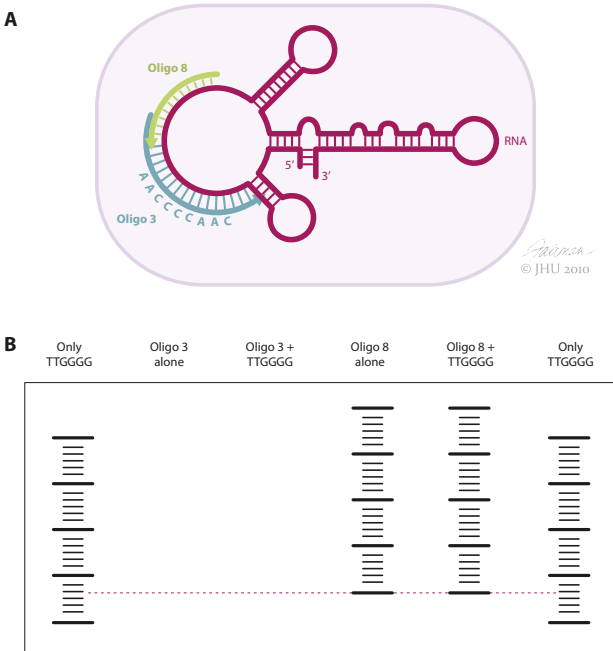
As is often the case in science, unexpected results provided the most important puzzle pieces. The RNase H experiment relied on the oligonucleotide having access to the RNA to be able to hybridize with it, even when the RNA is bound by telomerase proteins. As this wasn't always the case, some of the oligonucleotides had no effect. However, two oligonucleotides did give completely unexpected results. Incubation of telomerase with *Oligo 3* inactivated telomerase even before RNase H was added, while, amazingly, incubation of telomerase with *Oligo 8* resulted in elongation of *Oligo 8* itself (Figure 9). I had to sit and think about what this meant.

At first it was frustrating: if telomerase was already being inactivated by *Oligo 3* and adding RNase H had no further effect, how could I do the experiment? This frustration soon faded when, having talked about this result with my friends and puzzling more, I realized there was a much more interesting explanation for these results. I had fortuitously found additional evidence of a role for the 159 nucleotide RNA in the telomerase reaction. *Oligo 3* was unique in that it hybridized to my 159 nt RNA in a region adjacent to the template region and also across it (Figure 9A). When anchored to the RNA by hybridization, this oligonucleotide would block binding of the TTGGGG oligonucleotide substrate, and thus block telomerase activity (Figure 9B).



Subsequent work showed that RNase H would cleave the 159 nt RNA when incubated with *Oligo 3*, showing that *Oligo 3* does indeed hybridize to the 159 nt RNA as part of telomerase.

The other unusual puzzle piece was *Oligo 8*. This oligonucleotide shared sequence similarity with *Oligo 3* but its 3' end stopped just before the template region. This oligonucleotide also hybridized to the 159 nt RNA and the 3' end was positioned in exactly the right place for it to be elongated by telomerase (Figure 9A). This implied that the CAACCCCA sequence in the putative RNA did indeed serve as a template for TTGGGG repeat addition. Putting all of these pieces of the puzzle together, I felt there was good evidence to support the cloned RNA being the RNA component of telomerase.

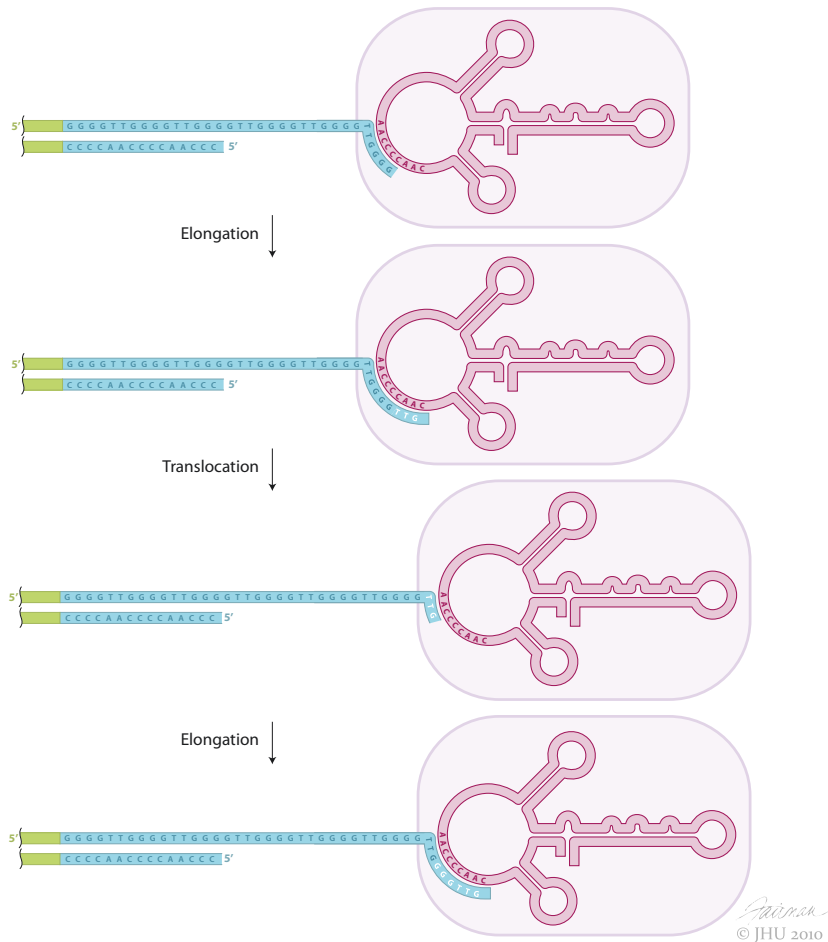


**Figure 9. Elongation of oligonucleotides complementary to the telomerase RNA**

A: the secondary structure of *Tetrahymena* telomerase. *Oligo 8* hybridizes adjacent to the template region with its 3' end positioned one base before the template. *Oligo 3* also hybridized adjacent to the template but also extends across the template region. B: Diagram of gel showing effects of *Oligo 3* and *Oligo 8* on telomerase activity. Lane 1, Telomerase will elongate (TTGGGG)<sub>4</sub> primer when it was added alone. Lane 2, *Oligo 3* alone was not elongated even though the 3' end contains TTGGGG sequence. Lane 3, *Oligo 3* and TTGGGG are added together there is no activity because *Oligo 3* binds to and blocks the active site of telomerase. This inhibition is the basis for the first telomerase inhibitor to be used in clinical trials against cancer (GRN163) (Asai *et al.*, 2003). Lane 4, *Oligo 8* was added alone and it was extended by telomerase generating a distinct banding pattern. This is because *Oligo 8* itself was being extended. Lane 6, When both *Oligo 8* and TTGGGG primer were added, the *Oligo 8* banding pattern was seen because it can out-compete the TTGGGG primer through its ability to hybridize to the telomerase RNA. (Adapted from Greider, C.W., and Blackburn, E.H. (1989), "A telomeric sequence in the RNA of *Tetrahymena* telomerase required for telomere repeat synthesis," *Nature* 337, 331–337.)

# MODELS CAN SHOW THE SOLUTION TO THE PUZZLE

In writing the paper on the identification of the telomerase RNA (Greider and Blackburn, 1989), I was encouraged by Bruce Stillman to include a diagrammatic model for how I thought the enzyme might work.



**Figure 10. Model for telomerase elongation of telomeres**

The sequence AACCCCAAC in the telomerase RNA serves as a template for extension of the telomere TTGGGG strand. The terminal TTGGGG on the telomere can base pair with the AACCCC in the RNA. This leaves three bases that can serve as a template for elongation. The activity of telomerase adds TTG and the end of the template is reached. Translocation can then reposition the 3' end such that base pairing between the terminal TTG and the telomerase RNA is maintained.

I was so caught up in the data that drawing a model was not foremost in my mind. However, I found that drawing out how I interpreted the results made everything even clearer. We knew from our early experiments that there

must be a protein component to telomerase in addition to the RNA. I did not know how many protein components there might be but decided to draw just one for simplicity. The CAACCCCAA sequence in the RNA represented one and a half copies of the complementary strand of the TTGGGG repeat sequence. So the model I drew had the sequence GTTGGG base paired with the CAACCC, which left CAA free in the template sequence to be copied (Figure 10). With this in mind, I proposed that telomerase has an elongation phase during which the CAA is copied, followed by a translocation to reposition the growing sequence for another round of elongation. I wrote up the paper and, together with Liz Blackburn, in whose lab I had begun the sequencing, published a paper in *Nature* describing the RNA (Greider and Blackburn, 1989).

## SOLUTIONS TO PUZZLES SHOW THE WAY TO MORE INTERESTING QUESTIONS

Drawing out the telomere elongation model helped to clarify my thinking about telomerase. Thinking about the model also immediately raised several new questions that I was curious to address. For example, does the proposed translocation step actually occur? That is, does telomerase hold on to the substrate it is elongating for a while, or does one enzyme only add one repeat, with a second repeat added during a second round of binding by a separate enzyme molecule? This question, which I had not thought of before I drew the model, was suddenly a burning one for me. I went on to tackle this next puzzle in a later paper (Greider, 1991). Many other questions arose as we continued our work on telomerase. The many different paths the research took and our later focus on telomerase in cancer and human disease is described in the Nobel Lecture presentation online at the Nobel Foundation website ([www.nobelprize.org](http://www.nobelprize.org)).

Putting together puzzle pieces is challenging, fun, and extremely gratifying, especially when they lead to new understanding in biology. This process of making a hypothesis and following leads is not a linear one: there are many twists and turns in the path. But the key is to keep the excitement and to follow the leads that are the most rewarding. I learned this during the first six years of working on telomerase, and it is the approach that I continue to follow. Many new questions often arise after one part of a puzzle is solved; the rewarding thing about curiosity-driven science is being able to pick from these new questions those that seem the most interesting to me. The pleasure of figuring out the puzzle and finding out things not known before is a great reward. Sharing that experience with friends and colleagues makes the reward even greater.

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Portrait photo of Professor Greider by photographer Ulla Montan.