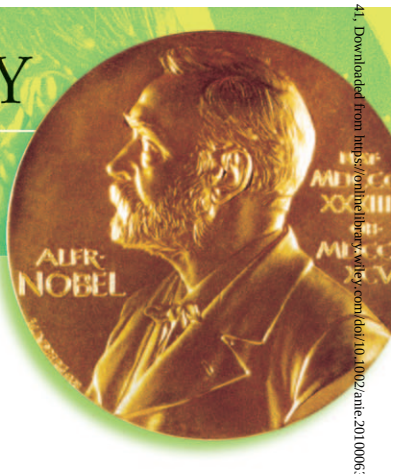
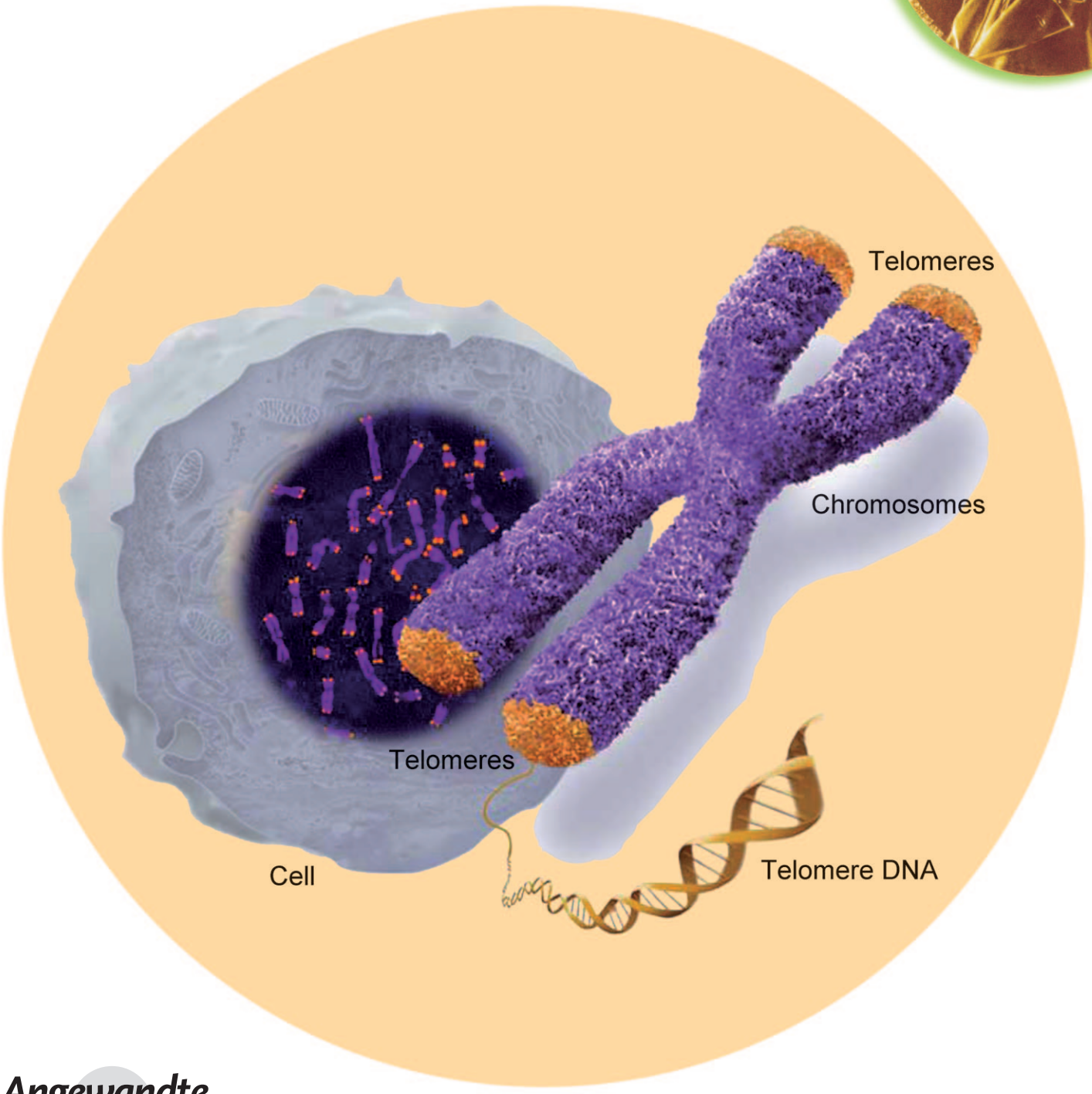
THE NOBEL PRIZE IN PHYSIOLOGY OR MEDICINE 2009





Angewandte International Edition Chemie

Telomeres/Telomerase

DOI: 10.1002/anie.201000635

DNA Ends: Just the Beginning (Nobel Lecture)**

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DNA · Nobel lecture · telomerase · telomeres

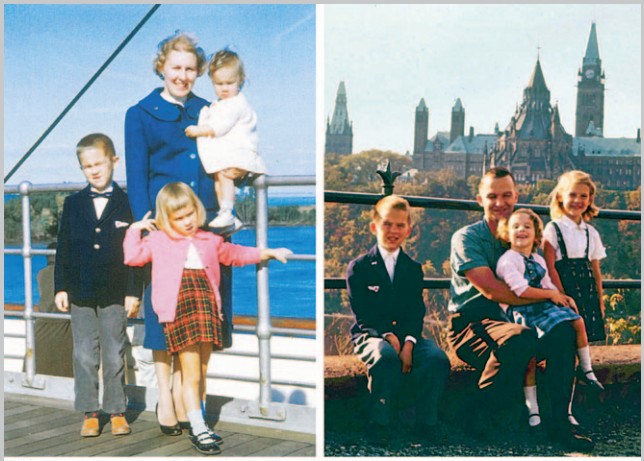
1. Scientific Autobiography

I greatly enjoy reading the biographies of scientists, and when doing so I always hope to learn the secrets of their success. Alas, those secrets generally remain elusive. Now that I find myself in the surprising situation of having to write my own biography, and thus to reflect on my career, I find the same mystery. I do not know why I have always been fascinated by science, or why I have been driven by the intense desire to make some original contribution. And although I have had some degree of success as a scientist, it is hard to say precisely why. Nevertheless, I have attempted to identify some of the incidents and decisions that helped or hindered me at various times, in the hope that these anecdotes might be helpful to those embarking on a scientific career.

I have generally sought to work on questions that I thought were both interesting and approachable, yet not too widely appreciated. To struggle to make discoveries that would be made by others a short time later seems futile to me. This, coupled with a distaste for direct competition, attracts me to areas of science that are less densely populated. On multiple occasions, I have been led into these new areas by talking to people working in fields quite different from mine. The confluence of ideas from distinct fields seems to create a kind of intellectual turbulence that is both exciting and productive.

My knowledge of the details of my family history is rather sketchy. My paternal great-grandfather was born near Cracow, and emigrated to New York City in the late 19th century, but ultimately settled in a small farming town in Saskatchewan, Canada where my father was born. Eager to escape the small town isolation, my father left as soon as he could by joining the RCAF towards the end of World War II. He was trained as a pilot but fortunately the war ended before he could serve in combat, and he was then posted to Ottawa. My mother's family came from England but settled in Ottawa, where my mother was raised and met my father after the war. Shortly after they married my parents moved to England for my father's continued training in aeronautical engineering at Imperial College, London. I was born in London, England during the great fog of 1952, but survived the coal-fueled air pollution with no ill effects and after less than a year in England was carried to Canada by my parents. My father continued to work as an aeronautical engineer for the RCAF for the next twenty years, and our house was always decorated with models of the airplanes he worked on. After he retired my father joined the civil service, and for a time studied issues of Arctic transportation; I remember him telling me about the complex properties of Arctic sea ice. Some of my work has an engineering flavor, in that we build structures and test their properties, and it's possible that may reflect some influence of my early home life. But a more direct influence stems from the fact that my father was often unhappy with his job, chafing at both his superiors and his subordinates. This I am sure made me seek out the academic life for its more egalitarian aspects. I have never felt like I worked for a boss or had employees who worked for me, just colleagues who like me were interested in learning more about the world around us.

My childhood was punctuated by frequent moves, as my father was transferred to different Air Force postings in Germany, Montreal, and Ottawa. At the time many school



With my parents and my sisters in Ottawa.

systems encouraged students to advance as rapidly as possible; as a consequence I was often the youngest in my class. Although socially difficult, this was more than compensated for by making my classes more interesting than they would otherwise have been. Some of my earliest recollections involve grade school math. Learning about fractions was for some reason surprising enough to have stuck with me for the

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rest of my life; similarly, my discovery of quadratic equations in grade 5 was a revelation. Later, at Riverdale High School in suburban Montreal I was fortunate to have some exceptional teachers. Don Hall struggled to answer my strange science questions, and Irene Brun (now Winston) inspired a life-long love of biology. At the same time, my interest in science was encouraged at home. My father built a basement chemistry lab for me, and the experiments I conducted there often made use of remarkably dangerous chemicals that my mother was able to bring home from the company where she worked. My mother also helped me to get my first summer job, in a chemical testing laboratory at the same company. This was a good window into the importance of quantitative analysis, but the repetitive nature of the work was not at all interesting. Some of the experiments carried out in my basement lab were much more dramatic. For example, with my father's assistance, shortly after the tragic Apollo 1 fire, we prepared and collected a jar of pure oxygen. We then carefully lowered a small quantity of methanol into the oxygen reservoir. The transformation of the barely visible pale blue flame in air into an intense jet of fire in oxygen was amazing, but also horrifying in the context of the recent Apollo fire. Less carefully supervised experiments frequently led to explosions, which made chemistry seem much more dramatic than one would guess from the textbooks. My failure to carefully separate the hydrogen evolved during electrolysis from ambient air led to an impressive explosion which resulted in a glass tube being embedded in a wooden ceiling rafter. I also participated in more biologically oriented projects with my high school friend Joachim Sparkuhl. In the basement of his house we constructed a small hydroponics garden, inspired, I believe, by the idea that astronauts living on some future space station would need or want to grow their own fresh food.

In 1968 I began my undergraduate studies at McGill University in Montreal, at the age of 15. My first laboratory work at McGill involved helping a chemistry graduate student to purify cholesterol, the starting material for the synthesis of sterols. We started with large sacks of gallstones, which we would dissolve in hot solvent, and then recover the iridescent crystals of pure cholesterol after the solution cooled. While this was a useful experience, it did not inspire me to remain in chemistry, and the pull of biology increased as new opportunities opened up. To my surprise I was accepted into a summer research program for undergraduates at the Jackson Laboratories, a renowned mouse genetics institute on Mt. Desert Island off the coast of Maine. The environment was idyllic, and the program combined intense scientific education and hands-on experimental work with outdoor activities such as hiking up Cadillac Mountain and observing the beautiful organisms that populated the nearby tidal pools. The Jackson labs are a mouse genetics research facility, and this influenced my future scientific career in an unexpected way. My project, carried out under the guidance of Dr. Chen K. Chai, involved the analysis of thyroid hormones in various mutant strains. This required the careful dissection of the thyroid gland from many mice. Although I was, after much practice, able to remove the thyroid without (at least most of the time) severing any of the many nearby major blood vessels, I strongly disliked the process of killing and dissecting the animals, and by the end of the summer had vowed never again to work on animal models.

Back at McGill the next fall, this time as a resident student (my parents having moved back to Ottawa), I started spending less time in the lectures and more time in the library, and also searching out new labs in which to gain additional experience. I was always surprised when seemingly intimidating Professors welcomed me into their labs and invited me to join in ongoing research projects. During this year and the next I did work in several labs in the Biology and Biochemistry departments, generally on plant biology systems. Field trips with Kurt Meier, a specialist in bryophyte biology, inculcated an enduring affection for the simple mosses and liverworts. I apparently did well enough in a physiology course run by Ron Poole to land a summer job prototyping and testing new lab experiments for the following year's lab course. Although most of my lecture courses were uninspiring, John Southin's course in Molecular Biology was an incredible exception. I'll never forget entering the first class and being handed a thick book of printouts, which I assumed were a set of papers we were supposed to read. In fact the whole book was simply a list of references, which we were expected to read and absorb in the library. These readings from the frontiers of molecular biology were very impressive. We read and discussed the beautiful Meselson–Stahl experiment, which was just over a decade old at the time, and learned how the genetic code had been unraveled only a few years previously. The fact that one could deduce, from measurements of the radioactivity in fractions from a centrifuge tube, the molecular details of DNA replication, transcription and translation was astonishing to me. One of the intellectual highlights of my time at McGill was the open-book, open-discussion final exam in this class, in which the questions were so challenging that the intense collaboration of groups of students was required to reach the answers.

In my senior year, I began a project in Mel Goldstein's lab, together with my friend Joachim Sparkuhl. The subject of our study was the beautiful colonial green flagellate *Eudorina elegans*, a smaller version of the more common *Volvox*. Over the school year and the following summer, we obtained evidence that these algae secreted a peptide hormone that induced spermatogenesis under favorable environmental conditions. This work led to our first scientific publication, which appeared the following year.[1]

In the Fall of 1972 I started my graduate studies at Cornell University in Ithaca, New York. I decided to attend Cornell in part because the A. D. White Fellowship would fully support me, but also because I would be able to pursue my work on *Eudorina* in the Department of Plant Physiology. At the time, I was enamored with a grandiose plan to develop *Eudorina* as a simple model system for studies in developmental genetics. This plan did not work out, for several reasons, not least the fact that this sort of ambitious program cannot be developed in isolation by an inexperienced student. Lacking the necessary genetic expertise, and either unable or unwilling to seek out the necessary help, my project became mired in frustrating technical difficulties.

However, the periods spent waiting for my *Eudorina* cultures to grow allowed for plenty of time for conversations with my fellow graduate student John Stiles. John was approaching graduation, and was thinking about what to do after the completion of his Ph.D., while I was gradually shifting from thinking about *Eudorina* to dreaming up some more productive project. We talked a lot about the emerging methods in molecular biology which were clearly heading towards the ability to explore the structure and activity of individual genes at the molecular level; cloning and sequencing technologies were just beginning to emerge. John and I eventually came up with a specific proposal for a collaborative experiment. Our idea was to chemically synthesize a DNA oligonucleotide of sufficient length that it would hybridize to a single sequence within the yeast genome, and then to use it as an mRNA and gene specific probe. While conceptually simple, our idea was technically challenging. At the time, there was only one short segment of the yeast genome for which the DNA sequence was known, the region coding for the N-terminus of the iso-1 cytochrome c protein, which had been intensively studied by Fred Sherman for many years. The Sherman lab, in a tour de force of genetics and protein chemistry, had isolated double-frameshift mutants in which the N-terminal region of the protein was translated from out-of-frame codons. Protein sequencing of the wild type and frameshifted mutants allowed them to deduce 44 nucleotides of DNA sequence. John and I thought that if we could prepare a synthetic oligonucleotide that was complementary to the coding sequence, we could use it to detect the cytochrome-c mRNA and gene. At the time, essentially all experiments on mRNA were done on total cellular mRNA, rendering efforts to monitor the expression of individual genes almost impossible.

John and I were sufficiently confidant of our ideas to begin contacting labs where we might pursue the work, with me doing the chemistry, and John working on the yeast biology. At Cornell, there was one laboratory that was the obvious place for such an experiment, and that was the lab of Ray Wu in the Department of Biochemistry. Ray was already well known for determining the sequence of the sticky ends of phage lambda, the first ever DNA to be sequenced, and his lab was deeply involved in the study of enzymes that could be used to manipulate and sequence DNA more effectively. John and I approached Prof. Wu, who listened to our proposal and allowed that it was an interesting idea worth exploring. However he was reluctant to appear to be "poaching" a graduate student from another lab and department; another complication was that the work would require a collaboration with Fred Sherman's lab. John applied to Fred's lab in nearby Rochester, New York, for a postdoctoral position, and was accepted. At Cornell, I persisted and eventually Ray allowed me to transfer into his lab and begin the project.

The interlude between wrapping up my work in the Department of Plant Physiology and starting as a transfer student in the Department of Biochemistry provided me with the opportunity for an extended vacation and my first trip to Europe on my own. I began with a visit to Cambridge, England where I was very kindly hosted by Prof. Poole (for whom I had worked at McGill), who was on sabbatical at the University of Cambridge. I explored the town and was incredibly impressed by the Chapel of King's College and the ethereal music therein. Even more impressive was the famous Laboratory of Molecular Biology at the MRC, where I talked with one of the iconic figures of molecular biology, Sydney Brenner. I was asked to wait for Sydney in his office, which I was surprised to notice held two large desks, both piled to the ceiling with papers. When Sydney arrived he told me about his remarkable new project involving the use of the nematode *Caenorhabdites elegans* as a model system for developmental genetics—this was an impressive if somewhat painful lesson on the right way to carry forward such an ambitious project. I also learned why two desks crammed that small office - it turned out that Sydney shared an office with a fellow molecular biologist, one Francis Crick!

After a memorable month of art, architecture and music in Paris, I returned to Ithaca to start afresh in a new lab with a new project. My goal was clear—the chemical synthesis of the oligonucleotide needed for our gene detection scheme. At the time, this was still a challenging endeavor for a student such as myself with minimal synthetic skills. Ray had an ongoing collaboration with Saran Narang, who was developing the solution phase phosphotriester approach to oligonucleotide synthesis. Our plan was to use this approach to prepare large quantities of the five trimers needed to make a 15-mer, then link the trimers together to form 6-mers, a 9-mer and finally a 15-mer. I began the work under the tutelage of Chander Bahl, a postdoc who had some experience with this technology. Unfortunately our lab was better equipped for enzymology than synthesis, and we lacked a critical mass of experienced chemists. After a year of work, I was still far from my goal and becoming increasingly frustrated. Fortunately Ray Wu realized that I needed help, and arranged for me to visit Saran Narang's lab in Ottawa. There I was fortunate to receive training from Keichi Itakura, who later became famous for synthesizing the gene for insulin. After two weeks of intense training, I returned to Ithaca, and attacked my synthesis with fresh energy. A few months later, I was rewarded with several milligrams of our long sought 15-mer. In collaboration with John Stiles and Fred Sherman, who sent us RNA and DNA samples from appropriate yeast strains, we were able to show that we could use the labeled 15-mer as a probe to detect the *cyc1* mRNA, and later, the gene itself. This was quite exciting, and seeing our work published in *Nature*[2] was a great boost to my confidence after years of work with little to show. It was also an important lesson in effective research strategy, imprinting on me the value of seeking help from knowledgeable people when faced with difficulties. One of the delights of the world of science is that it is filled with people of good will who are more than happy to assist a student or colleague by teaching a technique or discussing a problem.

The completion of my Ph.D. in 1977 marked the beginning of a major scientific transition for me. Against all commonsense advice, I decided to remain in Ray's lab for postdoctoral work, but in a very different scientific area. The decision was triggered by the arrival in Ray's lab of a new postdoc, Rodney Rothstein, from Fred Sherman's lab in Rochester. Rod was already a seasoned yeast geneticist, but had little experience with molecular biology; in contrast my graduate work was in

molecular biology but I had no practical experience with genetics. We hit it off and essentially trained each other through our collaborative work on yeast transformation. Our frequent discussions were long and often loud, sometimes triggering mild protests from Ray who would emerge from his office and ask us to turn it down a notch when he needed a quieter atmosphere in which to work. The combination of the molecular biology I learned in Ray's lab and the genetics I learned there from Rod prepared me well for the next decade of my work on yeast, first in recombination studies, and later in telomere studies and other aspects of yeast biology. Ray was a wonderful advisor,[3] and in addition to his scientific advice I absorbed much of his way of running a lab, which in essence was to be there when advice was needed but otherwise to let creative students and postdocs run with their ideas.

The Wu lab at Cornell.

My postdoctoral studies of recombination in yeast were enabled by the discovery, in Gerry Fink's lab at Cornell, of a way to introduce foreign DNA into yeast.[4] These pioneering studies of yeast transformation showed that circular plasmid DNA molecules could on occasion become integrated into yeast chromosomal DNA by homologous recombination. Rod and I began to search for ways of increasing the frequency with which transformants were recovered. Increasing the target size for recombination seemed like a good possibility, and indeed when I transformed yeast with plasmids containing fragments of rDNA, I did recover more transformants, and these contained plasmid DNA integrated at the rDNA locus. These strains allowed me to initiate studies of unequal sister chromatid exchange in the rDNA locus, resulting in my first publication in the field of recombination.[5] Towards the end of my stay in Ray Wu's lab, Rod and I came upon the first hints of double-strand break stimulated recombination in yeast. Our preliminary experiments suggested that cutting plasmid DNA within a region of homology to yeast chromosomal DNA led to an increase in the recovery of transformants, presumably reflecting increased recombination of the input DNA with the homologous chromosomal locus. The idea that you could increase transformation frequency by cutting the input DNA was pleasingly counter-intuitive and led us to continue our exploration of this phenomenon.

My first independent position was at the Sidney Farber Cancer Institute (now the Dana-Farber Cancer Institute). I owe a great debt to Prof. Ruth Sager, who was the main force behind hiring me. She established a terrific group of young investigators in her division, including Richard Kolodner and Gerry Rubin, creating a superb intellectual atmosphere. Ironically, I heard many years later that Ruth was only able to hire me over the objections of some of the senior clinical faculty, who did not believe that studies of yeast had any place in a cancer institute. Times have changed, and fortunately model systems are now much more widely appreciated. My graduate students came from the graduate program at Harvard Medical School, where I had an academic appointment in the Department of Biological Chemistry. These students were wonderful, and together we made rapid progress in setting up a productive yeast genetics lab.

Our initial focus was the study of double-strand breaks in DNA and their repair by recombination. This work was spearheaded by my first graduate student, Terry Orr-Weaver, who is now a Professor at the Whitehead Institute and MIT. Terry's work, and our continuing interactions with Rod Rothstein, led us to think intensively about the kinds of reactions engaged in by DNA ends.[6] There was considerable debate about different models for recombination within the wider DNA repair and recombination community, and seminars and conferences were important means for the exchange of the latest information. For many years, the major international recombination meeting was held in Aviemore, Scotland, which afforded the opportunity to sample diverse single-malts while discussing the intricacies of genetic exchange. I do recall that excessive sampling at one Aviemore meeting did make it difficult for me to present my work the next morning.

I also enjoyed attending Gordon Conferences and Cold Spring Harbor meetings, which were small and highly interactive meetings that provided wonderful opportunities for young scientists to present their work and meet and talk to people doing the best and most important current work. In the summer of 1980, I attended the Nucleic Acids Gordon Conference, expecting to hear the latest advances in DNA synthesis, sequencing and repair. However, for me the high point of the meeting was hearing Liz Blackburn talk about her work on telomeres in *Tetrahymena*. Our subsequent discussion led to the initiation of a collaboration in which we decided to test the ability of *Tetrahymena* telomeres to function in yeast. Those experiments are described in my Nobel Lecture; here I will just say that it was an incredibly exciting time for me. I performed the experiments myself, and experienced the thrill of being the first to know that our wild idea had worked. It was clear from that point on that a door had been opened and that we were going to be able to learn a lot about telomere function from studies in yeast. Within a short time I was able to clone bona fide yeast telomeres, and in a continuation of the collaboration with Liz Blackburn's lab we soon obtained the critical sequence information that led us to propose the existence of the key enzyme, telomerase.

With the success of the recombination and telomere projects, my lab began to grow. My second graduate student, Andrew Murray, now a Professor at Harvard, began to work on building artificial chromosomes. Andrew was a brilliant

and energetic student who was fun to talk with about any conceivable experiment; his colorful personality (and dress) enlivened the lab. My collaboration with Rod and Terry grew to include Frank Stahl, the world's leading expert on the genetics of meiotic recombination, with whom we had many detailed discussions of the genetic implications of specific physical models. I particularly remember an afternoon I spent at Frank and Mary Stahl's house in Eugene, Oregon, going back and forth with Frank about different versions of the double-strand break repair model as we worked on our manuscript.[7] It was an intense and stimulating experience that I still treasure.

After five very productive years at the Farber, a remarkable opportunity induced me to move to the fledgling Department of Molecular Biology at the Massachusetts General Hospital (MGH). Howard Goodman, the founder of the Department and a major figure in the emerging field of biotechnology, had arranged an extremely interesting and innovative academia/industry collaborative venture. In this deal, the pharmaceutical giant Hoechst AG agreed to fully support all research in the MGH Department of Molecular Biology for a period of about ten years, in return for limited intellectual property rights. This was extremely attractive to me, as it promised to allow me to pursue research in any direction that I found to be of interest, without having to worry about obtaining traditional grant support for novel and hence untried ideas. Thus, in the summer of 1984 I moved my lab from the Farber to our new home in the downtown Boston campus of MGH (humorously referred to by colleagues at MIT's Whitehead Institute as "one of the finest research institutes in downtown Boston").

At that time, I was actively exploring the possibility of moving into other fields. By 1984, I had a growing feeling that my work in yeast was becoming less significant, in the sense that other people would inevitably end up doing the same experiments we were doing in a few months or years at the most. To learn more about other fields and to prepare myself to work in a new area I audited several courses at Harvard. A delightful course by Steve Kosslyn on cognitive psychology explored the fascinating correlations between localized brain lesions and cognitive deficits, and highlighted the emerging neuroimaging technologies that were promising to revolutionize studies of brain function. I also audited an applied math course to brush up on the skills I would need should I decide to seriously enter into structural biology. Finally an outstanding course on enzymology and catalytic mechanisms by the late Jeremy Knowles stimulated my interest in catalysis. Later, when Jeremy left science to become Dean of the Faculty of Arts and Sciences at Harvard, I had the good fortune to "inherit" one of his graduate students, Jon Lorsch, who migrated to my lab and did outstanding work on ribozyme selections and mechanistic enzymology.

The combination of Jeremy's enzymology course, and the recent discovery of ribozymes by Tom Cech and Sid Altman (who shared the 1989 Nobel Prize in Chemistry for their work), ultimately led me to begin a transition to work on ribozymes. This seemed like a reasonably conservative way to switch fields, since the methods used to study ribozymes were largely a combination of molecular biology and chemistry. I was surprised that so few people were entering the field, since I thought that there were major questions to be addressed in terms of understanding the origins of biological catalysis in the hypothetical RNA World that preceded the evolution of protein synthesis.

I began to work with RNA myself, playing around with Cech's *Tetrahymena* ribozyme, which I obtained from the same piece of DNA that contained the *Tetrahymena* telomeres I had worked on just a few years earlier. The first student to join me in this new area was Jennifer Doudna. Jennifer had actually come to my lab to work on yeast genetics, but I was fortunate to persuade her that the future lay in RNA. Jennifer's energy and determination drove our efforts to convert self-splicing introns into an RNA replicase. We were soon joined by Rachel Green and several other dedicated students, techs, postdocs, and a memorable sabbatical visitor, Francois Michel, who impressed everyone with his work ethic, his uncanny ability to intuit structure from phylogeny, and his parallel career in butterfly evolution.

Even as I pushed our gradual transition to a focus on RNA, I maintained a substantial effort in yeast genetics for several years during the mid to late 80s. My interest in recombination and telomeres had not disappeared, and I wanted to bring our earlier advances to a satisfying conclusion. Recombination remained a large part of the lab, with Doug Treco, Alain Nicolas, Neil Schultes, and Hong Sun maintaining a focus on the role of double-strand breaks in meiotic recombination. Most important for the telomere story was Vicki Lundblad's ground breaking work on telomere genetics in yeast, which provided a link between telomere maintenance and senescence and aging.[8] Barbara Dunn linked the telomere and recombination realms by studying the transfer of sub-telomeric repeats between chromosomes by recombination.

By the end of the 80s, our yeast work was almost done, and the lab was increasingly focused on RNA. The RNA floodgates really opened with the work of Andy Ellington on in vitro selection,[9] which ushered in a new era of work on the in vitro directed evolution of new functional molecules. Over time we came to feel that we could evolve a binding site for virtually any target molecule, using any kind of nucleic acid. This confidence led us to try to evolve new catalysts, and, returning to the RNA World hypothesis for inspiration, we aimed for the chemistry of nucleic acid polymerization.[10] This was the basis of Dave Bartel's ground-breaking work on the selection of ribozyme ligases, which he subsequently (in his own lab at the Whitehead Institute and MIT) evolved into an RNA molecule with bona fide RNA polymerase activity. Our advances fueled my interest in the role of RNA in early evolution, and seemed to bring the resurrection of the RNA World almost within reach. Our ability to evolve new aptamers and ribozymes was so intoxicating that my lab spent most of the 90s exploring the range of possibilities and the limitations of what RNA could do. Our advances began to attract attention, leading to my election to the National Academy of Sciences, and appointment as a Howard Hughes Investigator in 1998. At the same time, the Hoechst funding of my department was winding down, making my HHMI

appointment particularly welcome as a means of enabling ventures into new scientific areas.

As other labs also started to evolve new and interesting ribozymes, the difficulty of evolving de novo proteins began to seem the greater challenge. We entered the field of protein and peptide evolution when Richard W. Roberts, a postdoc in my lab, learned how to trick the translation apparatus into covalently linking a newly translated protein to its own mRNA through the action of the antibiotic puromycin.[11] Galvanized by this advance, I encouraged several new lab members to develop and use this mRNA-display technology to address fundamental questions about the origin of protein structure. Most significantly, Tony Keefe used this method to evolve a novel ATP-binding protein from a large library of random sequence polypeptides.[12] Remarkably, this non-biological protein looks indistinguishable from any normal biologically derived small protein domain. Postdoctoral fellows John Chaput and Sheref Mansy continued to evolve this protein and study its structure over the following years.

The development of this protein evolution technology led me to co-found a startup biotechnology company, together with Rich and my colleague Brian Seed. Although the company was not a business success, it was a very interesting and educational experience. The collaborative efforts of a team of scientists ranging from protein biophysicists to people with clinical drug development experience allowed us to evolve a small protein domain with therapeutic potential; this artificially evolved protein is now in clinical trials. While I have continued to maintain a focus on fundamental questions in my laboratory, I firmly believe that small startup companies are the best way to develop more applied research to the point that it can eventually be therapeutically useful.

By the year 2000, I started to pay more attention to fundamental questions related to the origin of life. My interest in the role of compartmentalization and cellular structure in the origin of life was stimulated by discussions with Pier Luigi Luisi and David Bartel. A year of debate led to a synthesis of our views on the roles of genetics, compartmentalization and evolution, which we expressed in our 2001 *Nature* paper "Synthesizing Life".[13] This paper catalyzed my entry into the field of membrane biophysics, for I felt that having proposed a model for early cells in which bilayer membranes played a crucial role, it was incumbent on us to show that such models were physically plausible. I have to admit that I was somewhat surprised to find myself working with lipids and membranes, which are remarkably squishy and ill-defined by comparison with nucleic acids. However, in at least one way, the study of membranes composed of prebiotic building blocks such as fatty acids was perfect for me, since this field was filled with important yet technically addressable questions. When postdoctoral fellow Marty Hanczyc and graduate student Shelly Fujikawa joined the project, we were able to make rapid progress, and within a few years had demonstrated a proof-of-principle path for vesicle growth and division based solely on physical processes. I began to grow more confident that it might ultimately be possible to deduce plausible explanations for at least some of the mysterious steps in the origin of life. My enthusiasm grew when Irene Chen, a brilliant biophysics graduate student, made further progress by demonstrating a pathway for competition between protocells. We worried that our model protocells would not be able to take up nutrients, such as the nucleotides needed for the replication of their genetic material, but Sheref Mansy showed that this was not a problem. Most recently, another graduate student, Ting Zhu has come up with a very attractive pathway for spontaneous, coupled growth and division, so it is beginning to seem that the assembly and replication of protocell membranes is not as difficult as we once thought.

The dramatic progress in the identification of pathways for the self-replication of protocell membranes has encouraged us to focus on the hardest remaining problem, the replication of the genetic material. Here the big question is whether RNA was in fact the first genetic polymer, or whether RNA was preceded by some simpler, easier to make or more robust genetic material. This question has driven the most recent transformation of my lab, into a well equipped synthetic organic chemistry lab. We are synthesizing amino-nucleotides, the building blocks for phosphoramidate polymers, due to their greater reactivity than normal nucleotides. Alonso Ricardo, a postdoc, and Jason Schrum, a graduate student, have recently made very significant progress in the template-directed synthesis of 2′,5′-linked phosphoramidate DNA,[14] and we are now exploring a series of related polymers in a search for even better self-replicating genetic materials. The complexity and fragility of RNA long made it seem an unlikely candidate for the first genetic material, but this prospect has been revived by the brilliant recent work from John Sutherland's lab in Manchester.[15] With John's former graduate student Matt Powner now in my lab as a postdoc, we are eagerly exploring new avenues to the chemical replication of RNA. It's thrilling to me to see people in my lab developing new approaches to the synthesis of modified nucleic acids, but the suspense is almost unbearable as we await the results of template-directed polymerization experiments.

From our current vantage point, it is not clear whether there will be many solutions to the problem of chemically replicating genetic polymers, or just one, or none, but in any case it's an exciting quest. Encouraged by our small advances on the way, we are continuing to feel our way towards the tantalizing goal of building replicating, evolving chemical systems.

Lab photo after Nobel announcement.

2. Nobel Lecture

The contributions of my laboratory to our understanding of telomere function and maintenance by telomerase were made over a limited period of time early in the development of this story, from 1980 to 1989. What I would like to discuss here are some of the problems that we had to overcome, especially the preconceptions we had about models for telomere function and how hard it was to let go of those models. Fortunately the evidence we uncovered was strong enough to bring us to the right conclusions! Then, since I left the telomere field fairly early on, I would like to take this opportunity to briefly review some of the work that we've done since, primarily to show students who are just entering science that it is not only possible but really fun to address very different questions in different fields during one's career.

There were two well-known and long-standing puzzles associated with the nature of eukaryotic chromosome ends, or telomeres: the problem of the stability of the ends of chromosomes, and the problem of complete replication. My first introduction to these issues came when I was an undergraduate student at McGill University in Montreal. The first of those two problems, the reactivity of chromosome ends, had been a puzzle for many decades, ever since the pioneering work of Hermann Muller[16] and Barbara McClintock[17] in the 1930s. Muller used X-rays to create breaks in DNA, while McClintock used cytogenetic tricks to break chromosomes. But both came to the same conclusion, which is that the ends of broken chromosomes are very reactive and do things that normal chromosome ends never do. This is dramatically illustrated by the famous breakage-fusion-bridge cycle explored by McClintock (Figure 1). The basic observation is that the replication of a chromosome with a broken end results in two ends that can join together, generating a chromosome with two centromeres. When those centromeres are pulled towards opposite poles of the spindle during cell division, the chromosome is broken again, regenerating chromosomes with broken ends. This results in continuing cycles of fusion and breakage, a consequence of which is the formation of cells that have lost important parts of chromosomes. Not surprisingly many dead cells are generated in this process. Normal chromosomes never do this, so it was clear

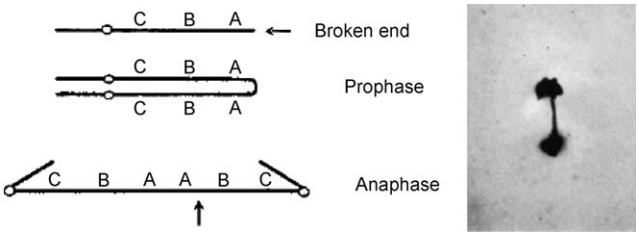

Figure 1. The chromosomal breakage-fusion-bridge cycle explored by Barbara McClintock. Left: After the replication of a broken chromosome, the two broken ends join together, creating a dicentric chromosome. When the two centromeres are pulled to opposite poles of the dividing cell, the chromosome breaks, and the new broken chromosomes continue the cycle (*Genetics*, **1941**, *26*, 234–282). Right: micrograph of a dicentric chromosome bridging the two poles of a mitotic spindle (*Genetics*, **1938**, *23*, 315–376).

that there was something very special and different going on at the ends of normal chromosomes that prevents end-to-end joining. But at the time of this work it wasn't even known that DNA was the genetic material, so they had no way to think in molecular terms about what was going on.

Much later on, long after it was recognized that DNA was the genetic material in chromosomes, an additional problem was discussed by Watson[18] and by Olovnikov,[19] who recognized that the replication of the very ends of DNA molecules posed a special problem (Figure 2). When a

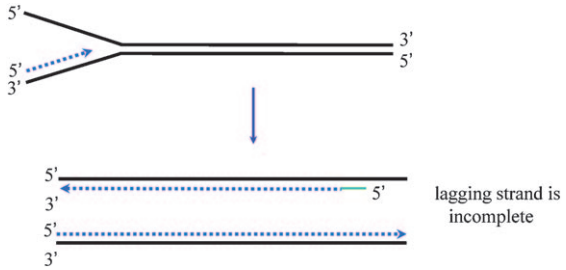

Figure 2. The end-replication problem as posed by Watson[18] and by Olovnikov.[19] When a replication fork reaches the end of a chromosome, the lagging strand will necessarily be incomplete as a result of the removal and potentially internal location of the last primer generated by primase.

replication fork heads towards the end of the chromosome, the leading strand can go all the way to the end, but the lagging strand cannot since it is generated by the extension of an RNA primer by DNA polymerase. If this RNA primer is generated at an internal site, any distal DNA will remain unreplicated; even if the RNA primer was made at the very end, after the RNA primer is degraded, a short region of unreplicated DNA would remain. In the absence of some compensatory mechanism, the ends should get shorter and shorter, and since that doesn't happen, there must be some unknown process to counterbalance the necessarily incomplete replication.

Although I learned about these problems as a student, I can't say that they made a very big impression on me and I didn't really think about them very much until years later when I began working on the molecular reactions engaged in by broken pieces of DNA. This was work that I started as a postdoc at Cornell with Ray Wu, working in collaboration with my friend and colleague, Dr. Rodney Rothstein. Means for introducing DNA molecules into yeast cells, a process referred to as yeast transformation, had just been discovered down the road from our lab at Cornell in Gerry Fink's lab.[20] The ability to do this opened up a huge number of interesting experiments. Rod and I started to examine some variations on the initial procedure, such as cutting the circular DNA molecules before putting them into yeast. Shortly thereafter, when I moved to Boston and was setting up my lab at the Sidney Farber Cancer Institute, we continued this collaboration with the additional participation of my first graduate student, Terry Orr-Weaver.[21,22]

In the course of our experiments on transformation and recombination, we observed a process that is analogous to the

fusion events studied by McClintock in maize decades earlier (Figure 3). We began with a circular DNA molecule that was able to replicate as a circular DNA plasmid in yeast because it contained a yeast origin of DNA replication.[23,24] Intact

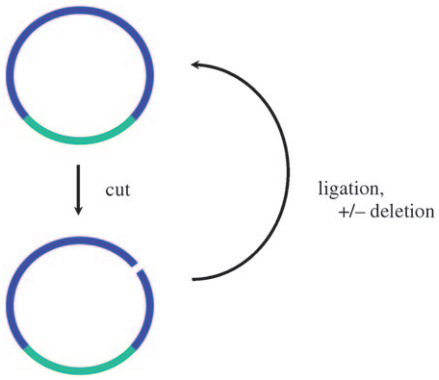

Figure 3. Non-homologous end-joining in yeast. A circular plasmid, cut with a restriction enzyme in a region of DNA that is not homologous to any yeast chromosomal DNA, can only survive and replicate in yeast if the cut ends are re-joined by ligase. Some DNA adjacent to the cut site may be degraded prior to ligation of the ends.

circular DNA of that plasmid yielded a high frequency of yeast transformants, because chromosomal integration was not required for plasmid maintenance. When we made a cut in the DNA with a restriction enzyme, in a region of the DNA that is not found in any yeast chromosome, we recovered many fewer transformants. When we analyzed the few transformants that we did recover, the cut DNA ends had been joined back together, presumably by the action of the enzyme DNA ligase.[22] In many cases some DNA was lost as the ends were chewed back by exonucleases before being joined together by ligase. As with McClintock's much earlier results, these DNA reactions are very different from anything that would happen at the ends of natural chromosomes.

Terry, Rod and I actually spent most of our effort looking at what happened when we made cuts in regions of plasmid DNA that were homologous to a segment of a yeast chromosome (Figure 4). When a circular DNA molecule containing a region of homology with a chromosome is used to transform yeast, the occasional recombination event will occur, resulting in the plasmid becoming integrated into the yeast chromosome. This was the pathway found in the Fink lab in their early studies of transformation. What Terry, Rod and I found was that cutting the DNA in this region of homology led to a greatly increased frequency of such recombination events.[21] We continued to follow this up by studying the reactions that broken DNA ends undergo (Figure 5). If a DNA molecule is broken by cutting with a restriction enzyme, then in the cell the ends can be chewed back by nucleases, and exonucleases can generate single-stranded ends that can invade a homologous sequence. Strand invasion allows repair synthesis to begin using DNA polymerases, and Holliday junctions can be formed which can branch migrate. After repair synthesis, the Holliday junctions can be resolved by special enzymes called resolvases, to yield

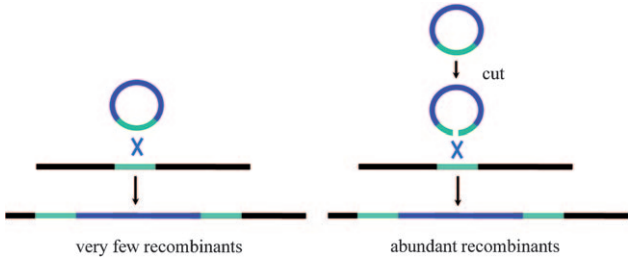

Figure 4. Double-strand breaks in DNA stimulate recombination. Intact circular DNA lacking a replication origin yields few transformants, because recombination events leading to chromosomal integration are rare. The same plasmid, when cut within a region of homology to a yeast chromosome, yields many more integrated transformants.

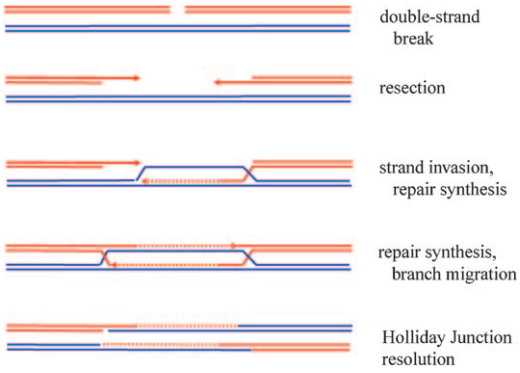

Figure 5. The double-strand break repair model for recombination. Two homologous chromosomes (red and blue) recombine when one is broken. The initial cut is further processed by nucleases, exposing single-stranded DNA, which invades the homologous duplex. Repair synthesis and branch migration generate Holliday junctions, the resolution of which generates recombinant DNA products.

crossover or non-crossover configurations. This work eventually led us to propose, along with Frank Stahl, that cells entering meiosis engage in the programmed breakage of their chromosomal DNA as a means of initiating meiotic recombination by double-strand-break repair.[25] So broken DNA ends do a lot of things, but they are all things that don't happen with normal chromosome ends. I mention them here because these are the reactions I was thinking of before I entered the telomere field.

In the summer of 1980, I attended the Nucleic Acids Gordon Conference and heard, for the first time, Elizabeth Blackburn talk about her amazing work on the stable DNA ends from *Tetrahymena thermophila*.[26] This unicellular organism is very divergent from metazoans, and has an unusual cell biology characterized by the presence of both a micronucleus with normal chromosomes and a macronucleus in which the chromosomal DNA has been chopped into thousands of small fragments, many of which become highly amplified. Liz talked about the very simple repetitive sequences, just stretches of a GGGGTT repeats, that she had found at the ends of these very abundant short DNA molecules in the large macronucleus of *Tetrahymena* (Figure 6). It was incredibly striking that these little pieces of DNA were stable ends, and were apparently fully

replicable, that is, they seemed to behave just like normal chromosomal telomeres. They clearly behaved completely differently from the DNA ends that we were studying in my

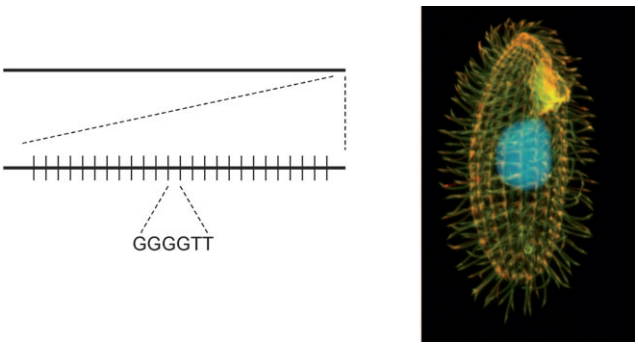

Figure 6. Telomeres from *Tetrahymena*. Left: DNA from the macronuclear fragments end in a series of tandem repeats of the hexanucleotide GGGGTT. These DNA ends are stable and fully replicated. Right: Image of *Tetrahymena*, showing the large macronucleus (blue).

lab, in yeast cells. After Liz's talk I sought her out to discuss these experiments, and we realized that there was a really simple and potentially very interesting experiment that we could do to see if the telomeric ends from *Tetrahymena* would work as stable telomeric ends in yeast cells. Neither of us thought that the experiment was very likely to work, because *Tetrahymena* and yeast are so very distantly related. On the other hand, we had all the necessary bits and pieces and technically the experiment was quite trivial, so we decided to go ahead. Liz sent me some DNA that she had painstakingly purified from *Tetrahymena*, and I took this little restriction fragment from the end of the ribosomal DNA of *Tetrahymena* and put it into yeast to see how it would function.

There is an amazing aspect of this piece of DNA from *Tetrahymena* that I would like to comment on before describing the yeast experiment (Figure 7). Right next door to the telomere sequence, just a couple of kilobases in, is the primary ribosomal RNA transcript of *Tetrahymena*. In that transcript there is a little intron, just over 400 bases long, and that intron is the first self-splicing intron ever discovered,[27] in the work for which Tom Cech was awarded the Nobel Prize in chemistry in 1989. A very nice piece of DNA indeed!

Returning to the experimental test of *Tetrahymena* telomere function in yeast, what we really wanted to do was to test the idea that the biochemical machinery underlying telomere function might have been very highly conserved. If that turned out to be true, then the mechanisms that were being learned about in *Tetrahymena* might apply broadly to eukaryotic organisms, which would make the whole process much more significant. This was the motivation for the experiment that Liz Blackburn and I collaborated on. What we had available at that time, in my lab, were circular DNA plasmids containing yeast genes[28,29] so that we could select for yeast transformants, that is, cells that had taken up the DNA. These plasmids also contained origins of replication (known then as autonomous replication sequences or ARS elements[23,24]) so that they could replicate independently of

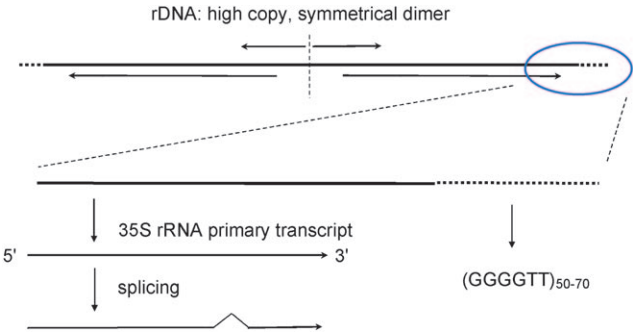

Figure 7. A very special piece of DNA. The *Tetrahymena* ribosomal DNA fragment from the macronucleus is a symmetrical dimer. The ends are telomeres and consist of GGGGTT repeats. Close to the ends is a region of the rRNA genes coding for a self-splicing intron.

integration into the chromosome. When intact circular plasmid DNA of this type is used to transform yeast cells, many transformants are recovered and they almost all contain replicating circular DNA molecules. As I explained above, if the plasmid DNA is cut with a restriction enzyme (in a region that is not homologous to yeast genomic DNA) so as to generate linear DNA with "broken ends", those ends do not function as stable telomeric ends and as a result very few transformants are recovered. The critical experiment was to take the little pieces of telomeric *Tetrahymena* DNA ending in G_4T_2 repeats, and ligate them onto each end of the linearized plasmid DNA (Figure 8). I carefully purified the ligated DNA, put that into yeast, and recovered transformants. I was then able to ask whether the plasmid DNA was replicating as a linear molecule, which would mean the telomeres were working, or whether I had only recovered standard replicating circular plasmids. I distinguished between linear and circular DNA forms by preparing DNA from a dozen or so transformants, and analyzing the DNA by gel electrophoresis. When DNA molecules are separated by gel electrophoresis, circles generate a series of bands corresponding to monomers and multimers, and relaxed and supercoiled forms, leading to a complicated pattern. Linear

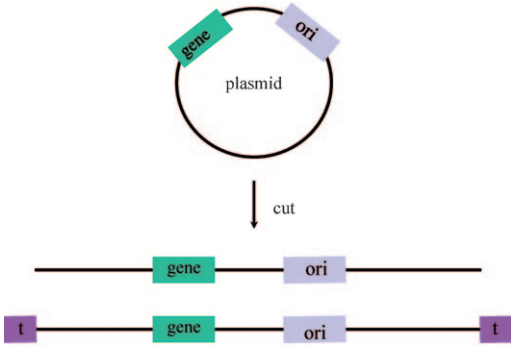

Figure 8. Moving *Tetrahymena* telomeres into yeast. A yeast plasmid vector containing selectable markers and an origin of replication was linearized by digestion with a restriction enzyme. *Tetrahymena* telomeres were ligated onto both ends, and the ligated DNA was purified and used to transform yeast cells. The resulting transformants contained replicating linear plasmids.

DNA molecules don't have any of those alternative forms, so they migrate as a single band. The two possible results of the DNA analysis were therefore quite distinct. When I analyzed the DNA from the transformants that I had recovered, about half of them contained plasmid DNA that migrated as a single band on the gel. This was perhaps the most clear-cut experiment I have ever done. It was immediately obvious that the experiment had worked, and that the *Tetrahymena* ends were able to act as functional telomeres in yeast.[30] We therefore knew immediately that the underlying biochemical machinery must be very broadly conserved because these two organisms were so distantly related to each other. It also meant that we could now use all of the tools of yeast genetics and molecular biology to study telomeres in yeast.

One of the first things that I wanted to do with the new linear plasmid with two *Tetrahymena* ends was to use it as a vector for cloning natural telomeres from the ends of yeast chromosomes. That experiment was extremely simple conceptually (Figure 9). I began with yeast chromosomal DNA,

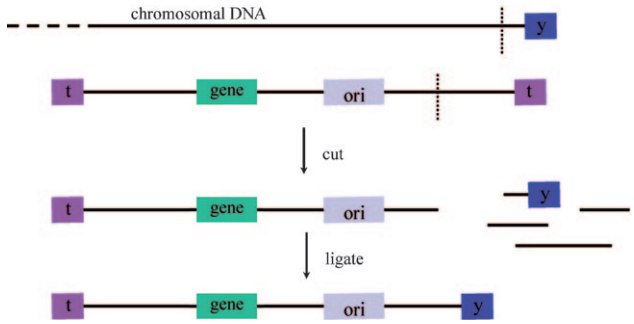

Figure 9. Cloning yeast telomeres. Yeast chromosomal DNA was digested with a restriction enzyme, as was the linear plasmid with two *Tetrahymena* telomeres. The purified vector fragment was ligated to the yeast DNA fragments, and the resulting mixture was used to transform yeast. A few linear plasmids were recovered, in which one end of the linear vector was replaced by a yeast telomere.

and cut it up with restriction enzymes into lots of pieces. Most of them were internal fragments, but the occasional fragment from the end of a chromosome would have one restriction cut end and one end derived from a yeast telomere. I then took our vector DNA, the linear plasmid with two *Tetrahymena* ends, cut off one end, and carefully purified the resulting DNA. This DNA molecule, which had one functional telomeric end and one non-functional "broken" end, could not be maintained in yeast cells. The yeast telomere cloning experiment then simply involved joining the yeast DNA fragments and the purified vector DNA together using DNA ligase. Every now and then, this would result in a molecule with a *Tetrahymena* telomere at one end and a normal yeast telomere at the other end, and those rare molecules were expected to be able to replicate as linear molecules in yeast cells. I did recover some transformants with the expected linear structure,[30] and I was able to confirm through a variety of tests that one end was indeed a yeast telomeric DNA fragment. This allowed us to start looking at the structures found in normal yeast telomeres, including the DNA sequences characteristic of yeast telomeres. We didn't expect the repeat sequences to be the same, since the *Tetrahymena* sequences didn't cross-hybridize with yeast DNA. Other hybridization experiments, done in collaboration with Tom Petes[31] showed that yeast telomeres contained stretches of alternating GT repeats. Still, when Janis Shampay, a graduate student in Liz's lab, sequenced the yeast telomeres I had cloned, we were all a bit surprised to see a somewhat irregular sequence, summarized as $G_{1-3}T$ repeats.[32] This was independently confirmed in the Tye and Petes laboratories based on the cloning of telomeric ends by hybridization with $(GT)_n$ probes.[33] While yeast did fit the general finding of a GT rich 3′-terminal strand, the absence of simple repeats was puzzling, and didn't seem to fit easily into the prevailing recombination-based models of telomere replication.[34] It was the resolution of that puzzle that would eventually lead to us to telomerase.

At this point, I would like to take a little digression to describe how we used these new telomeric DNA fragments as a tool to study the requirements for proper chromosome function in yeast. This work was done by Andrew Murray, my second graduate student. What we did was to take an engineering approach to seeing if we really understood the elements of chromosome structure. With telomeres in hand, we thought that we had all of the pieces that would be required to generate a fully functional chromosome. We had centromeric DNA, first cloned in John Carbon's lab;[35] we had various genes such as LEU2 and HIS3,[28,29] and we had origins of replication, first cloned by Kevin Struhl and Dan Stinchcomb in Ron Davis's lab.[23,24] Those were all of the elements known at the time to be important in terms of chromosomal function. We thought that it would be interesting to put them all together and see if we could make something that behaved like a natural chromosome. To do this we constructed a circular plasmid that had all of the known chromosomal elements (Figure 10), linearized it so that it had two telomeric ends, and put it into yeast. Despite the fact that this DNA molecule had all the pieces (an origin of replication, a centromere, genes, and telomeres), when we put it into yeast it didn't behave at all like a proper chromosome. During

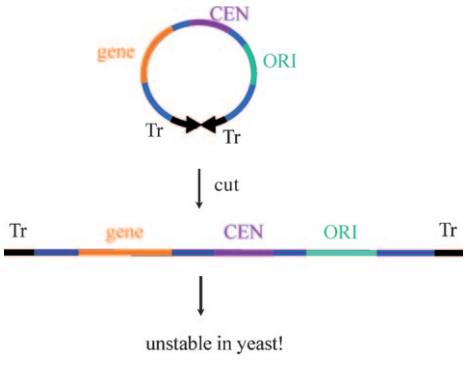

Figure 10. Our first attempt to make an artificial chromosome. We constructed a circular plasmid containing yeast genes, an origin of replication, a centromere, and telomeric DNA. This was linearized by cutting between the telomeric sequences, then introduced into yeast, where the DNA was maintained as a linear plasmid. Unexpectedly, this DNA molecule did not behave like a normal chromosome—it was mitotically unstable due to a high frequency of segregation errors.

mitosis it displayed a very high frequency of segregation errors, so that instead of being maintained over many cell cycles it was lost at a high frequency.[36] This was a very interesting result, because it said there was something going on that we didn't understand. What could be missing? What were the potential problems that prevented accurate inheritance of this mini-chromosome? We tested many possible explanations. Eventually, Andrew figured out that what was missing was just more DNA.[36,37] By simply adding enough non-yeast DNA from phage lambda to our small artificial chromosomes, he was able to make much bigger DNA molecules that now exhibited stable inheritance and behaved much more like natural yeast chromosomes (Figure 11). We

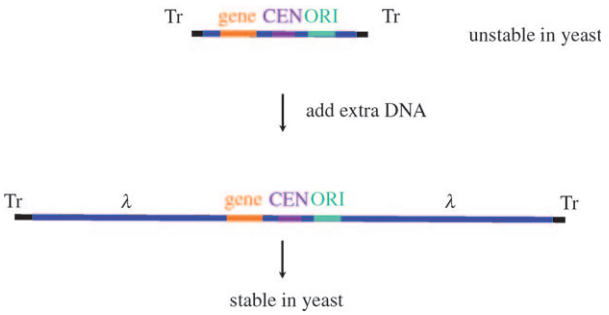

Figure 11. Successful construction of a yeast artificial chromosome. The addition of 50 to 150 kb of non-yeast DNA from phage λ greatly improved the mitotic stability of the DNA molecule, conferring improved chromosome-like behavior.

considered various models for this, and based on the observation that the linear centromeric plasmid was much less mitotically stable than a similar circular centromeric plasmid, we proposed that the intertwining of DNA after the completion of DNA replication[38] played a role in holding sister chromatids together. This was long before the modern story of cohesin and separase[39] and the complex biochemistry that underlies the adherence and separation of sister-chromatids after replication. Our artificial chromosomes were also technically useful, at least for a little while, in the early days of genomic sequencing because it turns out that they are very nice vectors for cloning extremely large pieces of DNA, up to a megabase or two in length.[40]

Returning once more to the story of telomeres and how they are fully replicated, all of our early models for thinking about this problem were based on recombination and the various kinds of reactions known to be engaged in by DNA ends. A very simple model that seemed quite attractive after Liz Blackburn's discovery of the short repetitive sequences of *Tetrahymena* telomeres was that recombination between different ends, perhaps biased in some way, could generate ends that were longer than either of the input DNA ends (Figure 12).[41] Alternatively, strand-invasion by the 3′ end of one telomere into the repeats of another telomere could lead to repair synthesis which would result in elongation of that end (Figure 12). Another model that we considered invoked Holliday junction resolution. This model was based on the idea that the very end of telomeric DNA was actually a

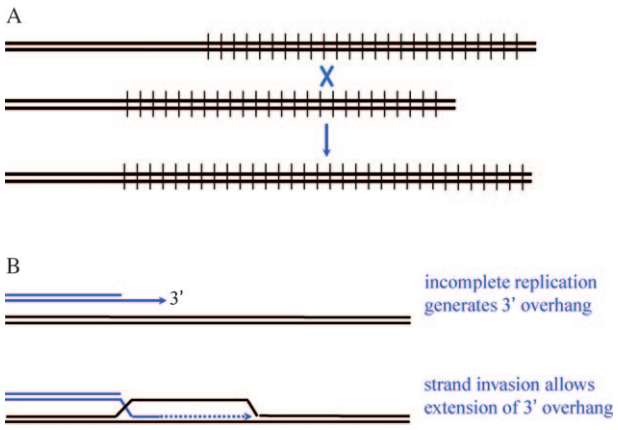

Figure 12. A) Telomere lengthening by recombination. B) Telomere lengthening by repair synthesis.

hairpin, that is, the strand loops around at the end. That was attractive because it meant that there was no actual DNA end, and a hairpin could act as a relatively inert DNA terminus. Replication would generate an inverted repeat structure, which could isomerize into a central Holliday junction, resolution of which by the corresponding recombination enzyme would generate two new hairpin terminated telomeres (Figure 13). A more complex variant of this model that

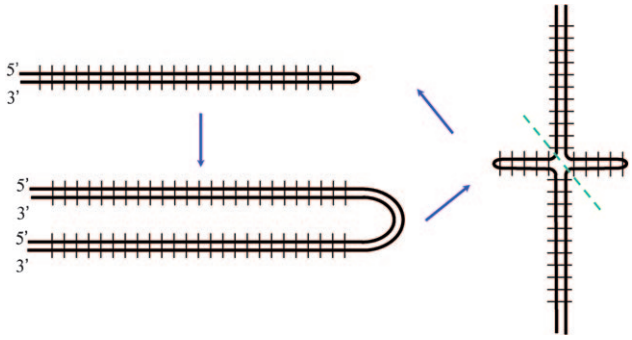

Figure 13. Telomere replication by Holliday junction resolution. Early models of telomeric DNA proposed a hairpin terminus. Replication would generate an inverted repeat, which could isomerizes to form a Holliday junction, resolution of which would regenerate the original structures.

originated in Piet Borst's lab[42] was that internal nicks within the repeats were sites of unpairing followed by gap-filling synthesis, leading to synthesis of new repeat units. These were the kinds of recombination based models that we discussed in the early years of thinking about telomere replication. How did we finally let go of these models and come to the correct explanation? Remarkably, we were driven to the answer by analyzing the sequences of *Tetrahymena* telomeres after their replication in yeast.

To understand why the replication of *Tetrahymena* telomeres in yeast was so important, consider again the linear plasmid with *Tetrahymena* ends. Those telomeric ends began as a restriction fragment of a certain size, but we noticed that after their maintenance in yeast, they had grown

longer, by as much as a few hundred base-pairs, as well as becoming heterogeneous in size. We didn't know where this extra DNA had come from, but there were several possible explanations. It could have been, for example, a result of recombination between *Tetrahymena* ends on different molecules, or a result of strand-invasion and repair synthesis. Eventually, we cloned some of these lengthened *Tetrahymena* ends and, in a continuation of the collaboration with Liz, sent those DNA samples to Liz's lab where once again Janice Shampay did the actual sequencing. To our complete shock, we found that the actual structure consisted of G_4T_2 repeats from the *Tetrahymena* ends joined directly to the irregular $G_{1-3}T$ repeats that were characteristic of yeast telomeres (Figure 14).[32] Thus the reason the DNA had become longer

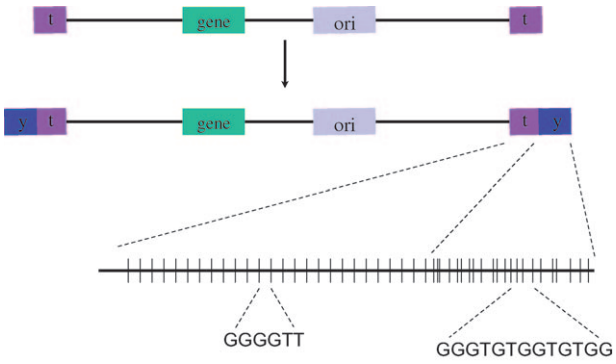

Figure 14. Yeast adds new DNA to *Tetrahymena* telomeres. Cloning and sequence analysis of *Tetrahymena* telomeres after replication in yeast (as the telomeres of a linear plasmid) revealed the addition of yeast telomeric sequences.

was that the yeast-specific sequence had become appended to the *Tetrahymena* ends. This new DNA seemed to have just dropped out of the sky. Such a different and irregular sequence couldn't possibly have been generated by any recombinational process, so we immediately knew that all of our early models were wrong. The new sequencing data led directly to the idea that there must be a specific new enzyme that adds extra DNA to chromosomal ends. Shortly after these results and our prediction of this new enzyme, of course, Carol Greider went on to identify the predicted enzyme activity biochemically.[44] Characterization of the purified enzyme, later named telomerase, showed that it is a ribonucleoprotein enzyme that contains an RNA template that specifies the telomeric repeat sequences, which are synthesized by a reverse transcriptase component of the enzyme.[45] We now know that the different telomeric repeats found in different organisms are specified by the RNA templates of their particular telomerase enzymes. A great deal of work has been done to characterize telomerase in many organisms, including *Tetrahymena*, yeast and humans, by Elizabeth Blackburn, Carol Greider, Tom Cech, and many other people.

It is interesting to revisit the end replication problem in light of the activity of telomerase. As mentioned above, one of our early models was that the actual end was a hairpin structure. Of course that also turned out to be wrong, and the proper structure is a 3′-end overhang consisting of GT-rich repeats (Figure 15). This was originally worked out in a different ciliated protozoan, *Oxytricha*, in the lab of David Prescott,[43] and then found to be a universally conserved

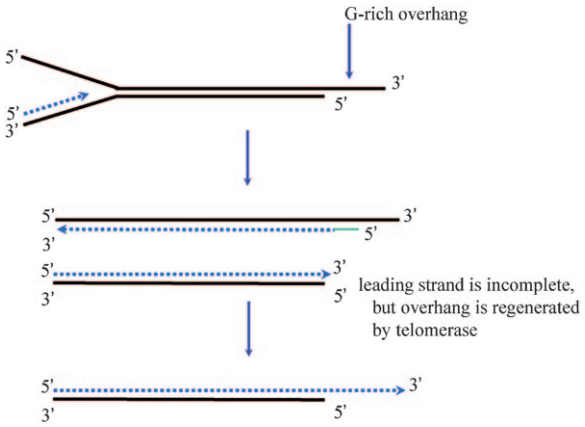

Figure 15. New model for telomere shortening, and the role of telomerase in telomere maintenance. When a replication fork reaches the end of a DNA duplex, the leading strand cannot regenerate the 3′-overhang. This is done by telomerase.

aspect of telomere structure. If we consider the replication of DNA with a 3′ overhang, the end-replication problem is actually a little bit different from that noted earlier by Watson[18] and by Olovnikov.[19] A replication fork heading towards this kind of end retains the previously noted problem of incomplete replication of the 3′ end strand, but a much worse problem is that the leading strand can go to the end, but can't regenerate a 3′-overhang. The 3′-overhang will therefore be lost in every cycle of replication, unless there is a compensatory process. This, of course, is the role of the telomerase enzyme, which adds extra repeats to telomeric ends and thereby on average maintains the proper telomeric length and structure. The regulation of proper telomere length and structure has turned out to be quite elaborate, and the biochemistry of the corresponding protein-DNA interactions is remarkably complex and interesting.[46]

The activity of telomerase and its associated regulatory machinery in controlling telomere length turns out to have important biological consequences. Cells with high levels of telomerase activity can divide without limit, because they maintain functional telomeres. In contrast, cells with insufficient telomerase activity cannot maintain telomere length, and as a result have limited division potential. This prediction was initially verified by Vicki Lundblad, who came to my lab as a postdoc and decided to address this issue genetically in yeast.[47] What Vicki did was to set up a large and actually quite difficult screen for mutants that would be unable to maintain telomeres at their proper average length. She was able to recover mutants that had the property we were looking for, namely that telomeres would get shorter and shorter over an increasing number of cell divisions (Figure 16A). The first mutation with that property was named *est-1*, for "*ever shorter telomeres*". The most interesting property of this mutation (and similar mutations recovered

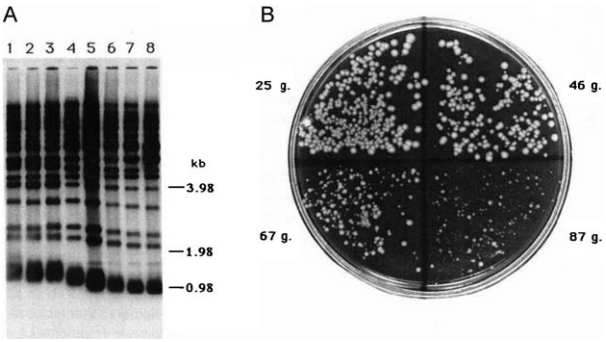

Figure 16. Senescence of yeast EST-1 cells. A) Telomeric yeast DNA fragments from an EST-1 mutant strain are visualized by Southern blotting. Lanes 1 through 8 represent increasing numbers of generations of growth. B) A mutant EST-1 strain streaked out on an agar plate after 25, 46, 67, and 87 generations of prior growth.

later) is that it confers a delayed senescence phenotype, just as predicted (Figure 16B). This phenotype is visually apparent in colonies of the mutant strain of yeast that have been grown for different numbers of generations. After 25 generations, the mutant colonies look just like wild-type colonies. After 46 generations the colonies are a little more irregular, and there are some small colonies; by 60 to 70 generations they are quite small and irregular, and after 80 to 90 generations the mutant strain can hardly grow at all. There are many dead cells in the small colonies, and there is a very high level of chromosome loss. Because the telomeres are getting shorter and shorter, eventually proper telomeric structure isn't maintained. As a consequence, ends are getting joined together leading to chromosome breakage and loss, so that cells are generated that are missing big chunks of their DNA. This was the first experimental demonstration that an inability to maintain normal telomere length would lead to a senescence phenotype, and therefore this inability to maintain telomeres might have an important role in problems of cellular senescence in higher organisms. At about the same time very similar experiments were done in Liz's lab, using *Tetrahymena*, and led to the same conclusion.[48] We thought this was a potential explanation for the senescence seen during repeated passage of primary cells in tissue culture, and by extension perhaps to problems of ageing related to a gradual decline in tissue renewal, perhaps due to limited cell division potential. The shortening of telomeres during passage of fibroblasts was soon demonstrated by Carol Greider,[49] and the causal role of this shortening in cellular senescence was later proven.[50] Of course, this has turned out to be a very important aspect of our growing understanding of ageing and age-related diseases.[51] The complementary aspect of this has turned out to be very important for our understanding of cancer. In the vast majority of cancer cells, which have unlimited division potential, the telomerase gene has been up-regulated and functional telomeres are maintained indefinitely.[51,52]

At that point in my career it became clear that many people would soon be exploring the roles of telomeres and telomerase in cancer and aging. I felt that the main questions were clear, and that they would be addressed whether or not I remained active in the field of telomere biology. I therefore began to look for other interesting questions that could be addressed experimentally, but where there were not too many people trying to look at the same issues.

Even as Vicki was doing her genetic work on telomere maintenance in yeast, I was already becoming interested in ribozymes, because Tom Cech's discovery of the self-splicing introns was very new and exciting.[27] I thought there were many interesting questions, and I was surprised that more people weren't entering that field. In particular, I was attracted by the RNA World hypothesis[53] and the idea that RNA might be able to catalyze its own replication without protein enzymes. Since the experiments were largely molecular biology in nature, I thought that we might be able to make some contributions to that nascent field. For several years we studied the group I introns and tried to use various molecular techniques to force them to catalyze RNA replication reactions. Several of my students including Jennifer Doudna[54] and Rachel Green[55] worked on that problem, with some success. But eventually we came to the conclusion that the ribozymes available from nature were not good enough. Those ribozymes were doing jobs that they had evolved to do in modern organisms, and what we were primarily interested in were questions about what RNA could have done much earlier.

In the late 1980s we started to think about ways of evolving new RNA molecules that would do things that we were interested in. The basic idea was simple: prepare huge collections of random sequences, and then isolate the rare functional molecules that did what we wanted. The technology for doing this in vitro selection, or directed evolution, was worked out by Andy Ellington when he was a postdoc in my lab,[56] and independently by Craig Tuerk in Larry Gold's lab.[57] We spent most of the 90s applying this kind of selection technology to the laboratory evolution of RNA and DNA molecules that could do all kinds of interesting things. For example, an RNA molecule isolated by Mandana Sassanfar when she was a postdoc in the lab folds up into a three-dimensional shape that contains a binding site for ATP (Figure 17).[58] Subsequently, we and others were able to show that it is possible to evolve, in the laboratory, RNA and DNA sequences that will fold into defined shapes that can bind almost any target molecule of interest. Ongoing studies in several different labs and companies are aimed at exploring potential therapeutic uses of these target binding RNA molecules, known as aptamers, perhaps doing some of the things that we use antibodies to do today.

Once we were able to evolve aptamers routinely we turned our attention to evolving RNA molecules that could catalyze interesting reactions. Dave Bartel, when he was a graduate student in the lab, isolated a surprisingly intricate RNA molecule that catalyzes a joining reaction between two adjacent RNAs aligned on a template (Figure 18).[59] It uses the same chemistry that RNA and DNA polymerases use, that is, the 3′-hydroxy group of one RNA substrate attacks the α-phosphate of the triphosphate of the other RNA substrate, generating a new phosphodiester bond. The ribozyme has an intricate folded secondary[60] and three-dimensional structure.[61] This was a very exciting demonstration that RNA

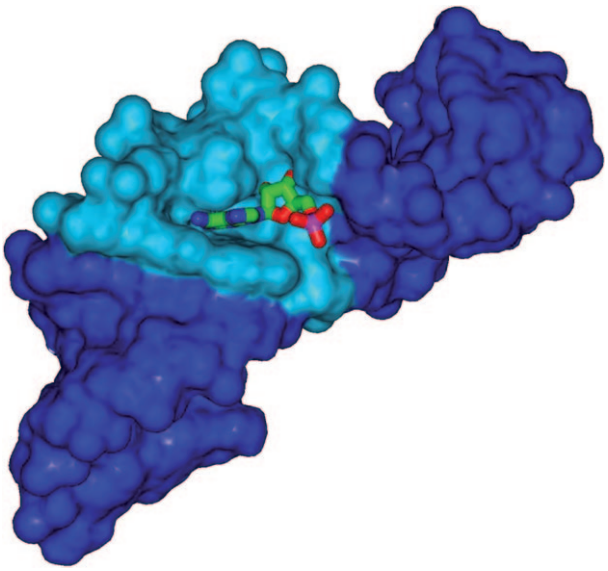

Figure 17. An ATP binding RNA molecule. This RNA was evolved from an initially random population of sequences. Dark blue: double-helical regions; light blue: folded recognition loop; stick Figure presents bound AMP.

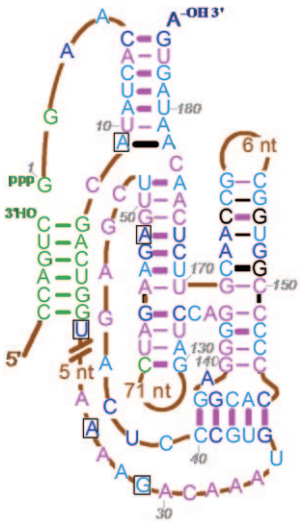

Figure 18. Secondary structure of the class I ribozyme ligase. This ribozyme catalyzes template-directed RNA-RNA ligation. It was evolved from an initially random population of RNA sequences.

could catalyze the chemistry of RNA replication. Subsequently, in his own lab at the Whitehead Institute at MIT, Dave Bartel further evolved this ribozyme into an actual RNA polymerase that can copy RNA templates using nucleoside triphosphates as substrates.[62] This is a marvelous "proof-of-principle" of the plausibility of the RNA World hypothesis. Unfortunately the current versions of this RNA polymerase are not yet good enough to copy themselves and exhibit full cycles of replication, so there is plenty of scope for additional evolutionary optimization.

More recently we have applied RNA in vitro selection to the analysis of human genomic sequences, in work done by Kourosh Salehi-Ashtiani and Andrej Luptak when they were postdocs in my lab (Figure 19).[63] Kourosh began this project by generating a large library of pieces of human DNA. He then transcribed them into RNA, and selected for molecules that could cut themselves at a unique site. He recovered four distinct self-cleaving RNAs or ribozymes. One of these is found in the CPEB3 gene, which has been implicated in memory,[64] possibly through a role in controlling localized protein translation at synapses. There are two interesting things about this self-cleaving human genomic ribozyme. One is that it turns out to have exactly the same structure as a well known viral ribozyme, the HDV ribozyme of the hepatitis delta virus. The fact that there is a version of this ribozyme in the human genome suggests that the viral ribozyme may be derived from the genomic copy. Another potentially very interesting observation is that there is a polymorphism in the human population at a position within this ribozyme that affects its activity. A recent genetic study done by a group in Switzerland[65] has found an association between this polymorphism and performance on a word-recall memory test. A lot more work needs to be done on this, but the possibility that a self-cleaving catalytic RNA may play a role in human memory is fascinating.

In the 1990s we extended our work on RNA and DNA directed evolution by developing methods for evolving proteins. Rich Roberts developed a clever means of tricking the ribosome into chemically linking a nascent peptide or protein chain to its own mRNA,[66] so that selection for a functional protein would also enrich the corresponding coding mRNA. This approach was used in later work done by Tony Keefe who isolated a small ATP-binding protein from a library of completely random protein sequences.[67] This little protein domain looks indistinguishable from any natural biological protein domain. These kinds of laboratory evolution experiments showed that it is relatively easy to evolve functional RNAs, DNAs, and even proteins out of completely random collections of sequences.

The above experiments showed very directly that Darwinian evolution, applied to populations of molecules, is a powerful means of generating functional sequences. That led us to deeper questions: how did evolution get started? How did the transition from chemistry to Darwinian evolution first happen on the early earth? These are the central questions concerning the origin of life, and addressing these questions has become the main focus of my laboratory. The approach that we are taking is essentially a synthetic or engineering approach. We have a simple model for what we think early cells might have looked like (Figure 20).[68] This is not by any means a universally accepted model, but it is our view of what a very primitive cell might have looked like, and we are trying to construct such systems in order to define possible pathways from chemistry to biology. We think that a primitive cell would have two critical components, the first of which is a cell membrane. In our experiments we make these membranes out of simple molecules that might have been around on the early earth, such as fatty acids. The cell membrane has to be able to grow spontaneously and divide to make daughter cells. The other important component of a primitive cell would be a polymer that could mediate the inheritance of genetic

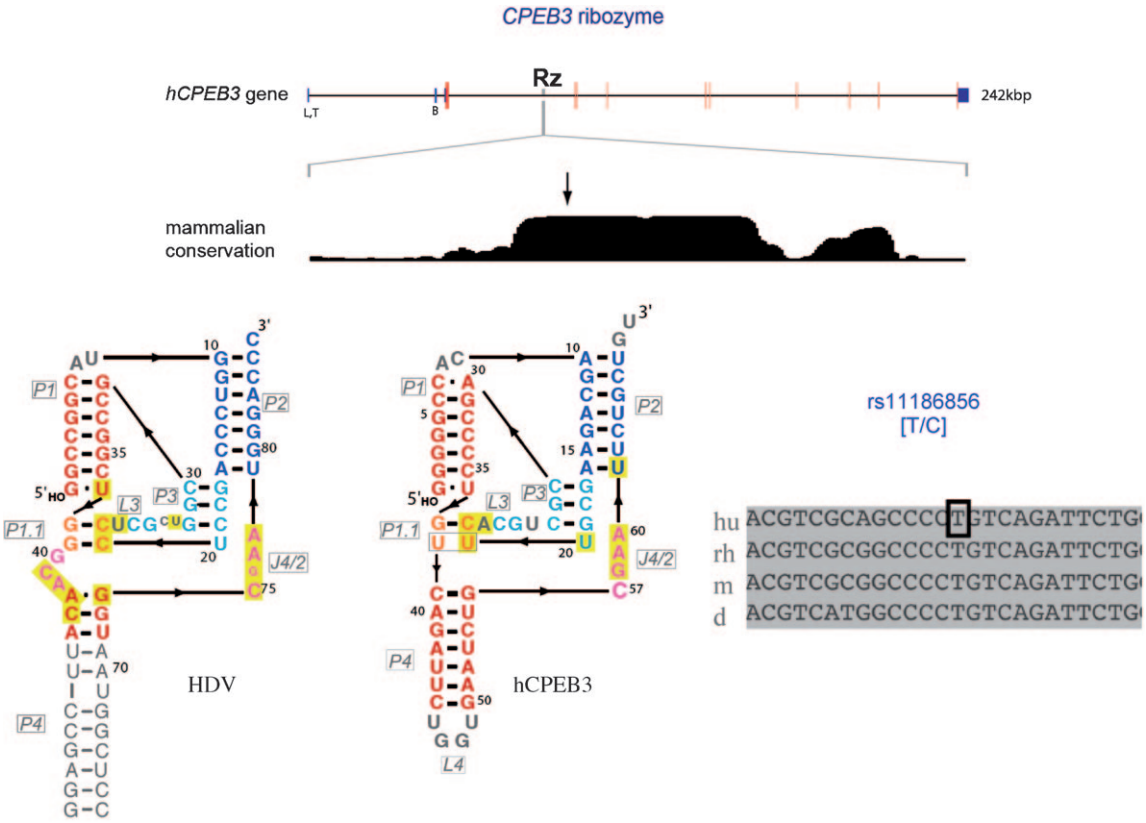

Figure 19. An HDV ribozyme in the human genome. Top: The self-cleaving ribozyme is located within the second intron of the CPEB3 gene. The ribozyme sequence is highly conserved relative to flanking intron sequences. Bottom left: The secondary structures of the human genomic ribozyme and the HDV ribozyme are virtually identical. Bottom right: A polymorphism with the ribozyme sequence affects ribozyme activity, and may affect human memory.

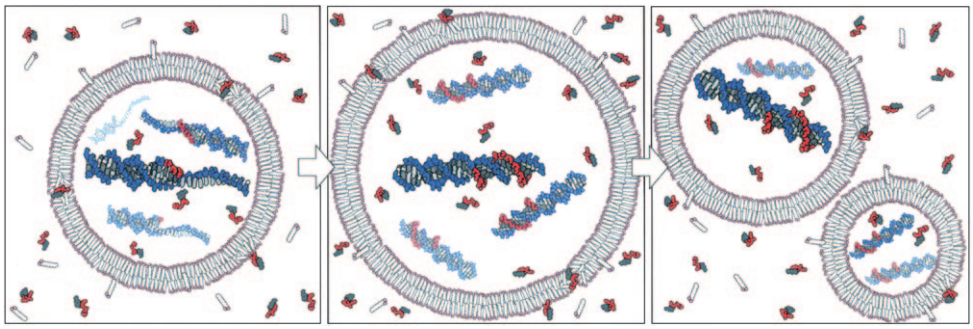

Figure 20. Model of a protocell. A simple cell might be based on a replicating vesicle for compartmentalization, and a replicating genome to encode heritable information. A complex environment provides nucleotides, lipids and various sources of energy. Mechanical energy (for division), chemical energy (for nucleotide activation), phase transfer and osmotic gradient energy (for growth) may be used by the system.

information. Here the big question is whether this could be RNA itself, or is more likely to be some simpler progenitor material that was subsequently replaced by RNA? In either case, this material has to be able to replicate spontaneously without any of the highly sophisticated evolved machinery that is used by modern biology. The key question is therefore: how could both cell membranes and early genetic materials replicate prior to the evolution of complex biological machinery? The approach that we are taking is to try to divide this big problem up into simpler pieces that can be addressed separately. I will briefly describe a few of the experiments that we have done in the last six or seven years.

About six years ago Marty Hancyzc, a postdoc, and Shelly Fujikawa, a graduate student in the lab, became interested in how protocell-like assemblies could be formed. They found that a common clay mineral, formed from volcanic ash and seawater, can facilitate this assembly process in a surprising way.[69] This clay mineral is well known in the prebiotic chemistry community because it had been shown several years previously by Jim Ferris and Leslie Orgel to catalyze the assembly of RNA from activated nucleotides.[70] Marty and Shelly showed that the same mineral could catalyze the assembly of membranes. Moreover, it can bring genetic polymers, such as RNA, into the vesicles it helps to assemble (Figure 21). Thus a common mineral can help to make genetic materials, help to assemble

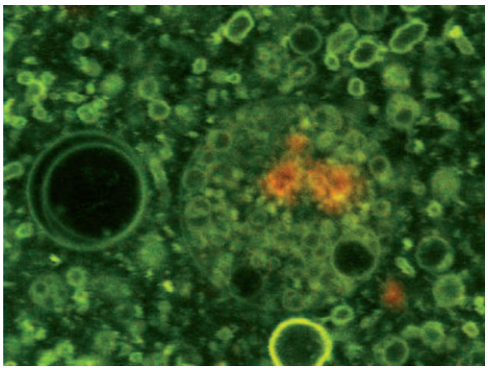

Figure 21. Montmorillonite can bring RNA into vesicles. Fluorescently labeled RNA (orange) on the surface of a clay particle is trapped inside a large vesicle (green) along with numerous small vesicles, all assembled as a result of the catalytic activity of the clay particle.

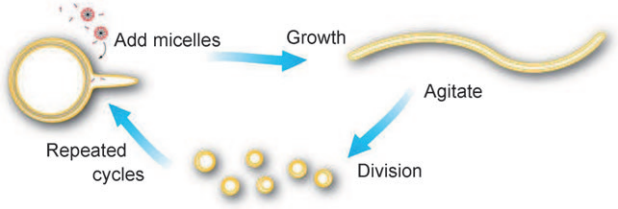

Figure 22. Cycles of growth and division of a model protocell membrane. Large multilamellar vesicles grow into long hollow vesicles following the addition of excess fatty acids. The filamentous vesicles are fragile and fragment in response to mild shear stress. The smaller daughter vesicles can grow and repeat the cycle.

membranes, and bring them together,[69] all of which is very attractive in terms of the assembly of early cellular structures.

The replication of protocell-like structures is much more difficult than their assembly. However, the growth and division of the protocell membrane, which looked like an almost impossible problem just a few years ago, has actually turned out to be relatively simple. Our current model for what an early cell cycle might have looked like with respect to the cell membrane is based on the work of Ting Zhu, a graduate student in the lab.[71] We prepare large multilamellar vesicles, and feed them with new fatty acids. Remarkably, they grow into long filaments, which are quite fragile; in response to gentle agitation, such as might result from waves on a pond, they break up into daughter cells (Figure 22). That generates a robust cycle that can be carried out indefinitely. Thus, the spontaneous growth and division of membrane compartments appears to be a relatively straightforward process.

What about the replication of genetic information? At the moment, this still seems to be difficult, because we don't understand how to accomplish this step. The RNA World hypothesis is based on the idea of RNA catalyzing its own replication,[53] but that has turned out to be a harder problem than we thought. Could genetic replication have begun as a chemical, that is, non-enzymatic, process? Almost twenty years ago, Leslie Orgel, one of the giants of prebiotic chemistry, proposed that chemical means of replicating genetic polymers should be found fairly easily by chemists, and that the solution to that problem would be relevant to the origin of life.[72] That hasn't happened, perhaps because it's a harder problem than anybody thought, but also perhaps because there are not that many people working on this problem. I think that makes it a perfect problem to tackle because it is important, interesting and there are many reasonable experimental approaches. What we are doing is making synthetic nucleotides that are modified so as to become more reactive (Figure 23). For example, changing the hydroxy nucleophile to an amine results in nucleotides that spontaneously extend a primer in a template-directed manner, without any enzyme.[73] We do not yet have a robust and general replication system, but that is our goal.

There is an interesting aspect of the problem of chemical replication that we have just recently started to think about, namely, how can the very ends of our sequences be copied in the absence of telomerase? This turns out to be very interesting. Chemical replication results in spontaneous template-directed primer-extension, but once the end of the template is reached, the reaction slows down but often doesn't stop entirely. Depending on the conditions, we sometimes see chemical extension beyond the end of the template, generating a 3′-overhang (Figure 24).[73] Thus complete replication of a template does not seem to be a problem, and in fact this process generates new sequences. It is interesting to speculate that this spontaneous chemical

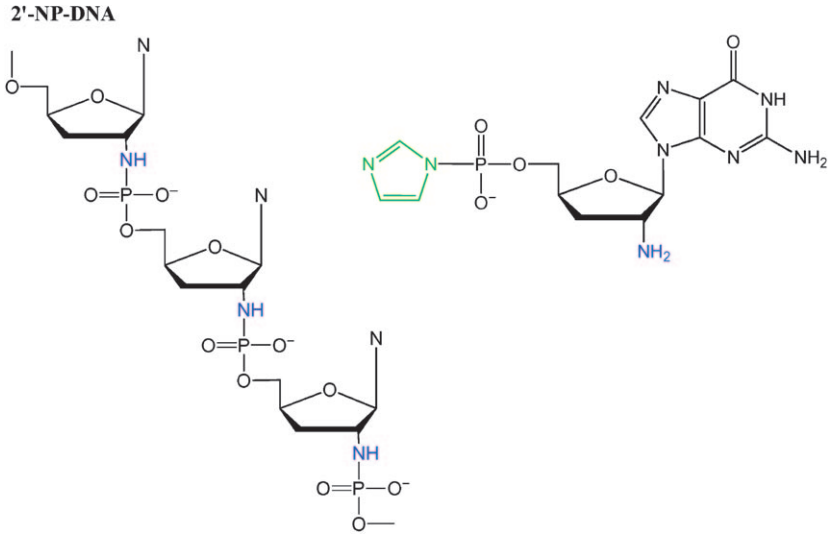

Figure 23. Typical monomer for spontaneous nucleic acid synthesis and the corresponding polymer. Left: 2′,5′-linked phosphoramidate DNA. Right: The activated 2′-amino monomer. Note the 2′-amino nucleophile (blue) and the imidazole leaving group (green) on the 5′-phosphate. The combination of a good nucleophile with a good leaving group allows for rapid non-enzymatic polymerization of this class of monomer when aligned on an appropriate template.

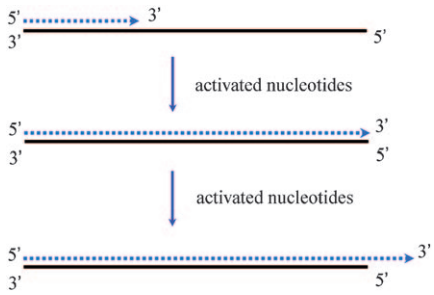

Figure 24. Origin of telomerase in spontaneous copying chemistry? Under certain conditions non-enzymatic primer-extension proceeds past the end of the template, generating a 3′-overhang. Enzymatic control and elaboration of this chemical process could provide an evolutionary path towards telomerase.

reaction might have something to do with the eventual emergence of genetically encoded catalysts that would control and exploit this process, eventually leading to the telomerase enzyme that has been the main subject of my lecture.

I would like to thank all of the many brilliant students, postdocs, friends, and collaborators who contributed to this work.

Received: February 1, 2010
Published online: September 3, 2010

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