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Genetic Approaches to the Characterization of the Yeast Telomerase Complex

Introduction

Telomeres protect the ends of eukaryotic chromosomes from shortening due to nucleolytic degradation and incomplete DNA replication. Telomere shortening, if unregulated, can lead to cellular senescence. The conventional eukaryotic DNA replication machinery is unable to completely replicate the 3' end of chromosomes, a phenomenon termed the "end replication problem." Although the leading strand of a newly-made DNA molecule can be continuously synthesized in a 5' → 3' manner, the lagging strand must be discontinuously synthesized as Okazaki fragments. After the RNA primers used to synthesize these fragments are removed and DNA polymerase and DNA ligase have filled in the gaps left by this removal, there is a small 5' gap that remains at the end of the chromosome. This 5' gap – or 3' overhang – is thought to serve as a recognition site for binding proteins that help protect the DNA. It serves as the substrate for telomerase. However, the leading strand, which is synthesized continuously lacks this 3' overhang and it must be created through exonuclease action at the 5' end of the molecule. This strand resection leads to the loss of DNA with successive rounds of replication. Over time, this would not only lead to the loss of crucial genetic information, likely diminishing cell viability, but also would promote damage to the existing DNA. These unprotected telomeres are recognized by cellular repair machinery and are fused together. These fusions create chromosomes with two centromeres that, during anaphase of mitosis, are pulled apart forming structures called anaphase bridges. When these anaphase bridges are eventually broken, the DNA

between them breaks, causing massive chromosomal breakage and genomic instability [Hartwell, et al. 2008].

In most eukaryotes, telomere length is maintained by telomerase, a ribonucleoprotein that uses a short region of its RNA subunit as a template for reverse transcription. In the yeast *Saccharomyces cerevisiae*, an important model organism for many studies of telomeres and telomerase, this RNA subunit (*TLC1*) and a reverse transcriptase (RT) (*EST2*) constitute the catalytic core of telomerase [Eugster et al. 2006]. In a 2006 *Nature Structural and Molecular Biology* essay entitled "Telomeres in the '80s: a few recollections," scientist Vicki Lundblad describes the research that led to the discovery and characterization of telomerase in the late 1980's and early 1990's as an "eclectic mix of many projects involving the yeast genome." [Lundblad, V. 2006] This mix of biochemical experiments, spearheaded by researchers Elizabeth Blackburn and Carol Greider, and genetic analyses, fueled by the work of Lundblad and her then-advisor Jack Szostak, initially led to the proposal that telomere extension was mediated by a terminal transferase-like enzyme. It is the genetic analysis pioneered by Lundblad and Szostak that led to one of the first studies of a component of telomerase complex – *EST1* – and paved the way for subsequent work by Lundblad and colleagues to uncover genes in this complex.

"A Mutant with Defects in Telomere Elongation Leads to Senescence in Yeast"

The work done by Lundblad and Szostak identified a new gene in yeast, which they called *EST1* for "ever shortening telomeres." [Lundblad, V. and J.W. Szostak, 1989] Although the discovery of this gene was paramount, its importance was secondary to the general assay they developed to "detect mutants of yeast that are defective for any of several aspects of telomere function." These protocols enabled the detection of mutations that were associated with cellular senescence due to the gradual shortening of telomeres. This focus on a senescence phenotype

represented a departure from the previous methods used to identify genes associated with telomere function – these genes(including *CDC17, CDC18, CDC21, TEL1,* and *TEL2)* when mutated either render cells inviable or cause telomeres to become shortened, but stable. The senescence phenotype screened for by Lundblad and Szostak came to be known as an *EST* phenotype, and became an instrumental marker in characterizing the function of subsequent *EST* genes. By seeking mutations that inhibited telomere addition, Lundblad and Szostak were able to find genes with contributions to telomere function without prior knowledge or assumptions about these genes.

To identify mutants of *S. cerevisiae* that were defective for telomere maintenance, Lundblad and Szostak created a centromeric (single-copy) plasmid containing *LEU2* and inverted telomeric sequences from a related yeast species, *Tetrahymena thermophila*, flanking the *URA3* gene. This plasmid was transformed into an *ura3Δleu2Δ* strain. *URA3* encodes orotidine 5-phosphate decarboxylase, a product used in the synthesis of uracil; however, in the presence of the drug 5-fluoroorotic acid (5-FOA) this compound is converted to the toxic 5-fluorouracil, killing any cells with this gene. *LEU2* encodes a product required for the synthesis of leucine and was used to select for cells that had received the plasmid. In very rare cases, the plasmid breaks in the *URA3* sequence, converting the plasmid from a circular to linear molecule with the telomerase-mediated addition of DNA to the *Tetrahymena* sequences. Such telomere addition is required to protect the linear DNA from degradation. Thus, they screened for cells that showed a reduced frequency of colony formation on medium lacking leucine and containing 5-FOA. They hypothesized that in some mutants, this reduced ability to form colonies would be due to defects in the telomere-mediated extension event required to maintain the linear plasmid. They also recognized that mutants with altered frequencies of plasmid linearization or with

defects in proteins that influence telomere structural stability (thereby compromising the integrity of the linear plasmid) might also be isolated.

After mutagenizing cells with ethyl methane-sulfonate (EMS) and growing them in liquid cultures, they spotted approximately 7,000 colonies on the –leu 5-FOA plate. From these, they found several heat-sensitive mutants and performed crosses to determine that these mutations were not only recessive, but also chromosomal. They hypothesized that, if these mutants were unable to initiate telomerase-mediated telomere-extension, they should also exhibit altered telomere length. This proposal was confirmed by Southern Blot analysis – they found two strains with alterations in telomere length, one of which had telomeres approximately 220-250 bp shorter than wild-type length. This mutant was named *est1-1*. Through further crosses, Southern blot analysis, and observation of growth over several generations, they determined that mutations in this gene are associated with a progressive decrease in telomere length and accompanying cell senescence. With this information, they began the vast undertaking of identifying *EST1* through cloning and sequencing. To clone *EST1*, Lundblad and Szostak screened a yeast library for complementation of the temperature sensitivity and senescence of their mutant. Lundblad recalls that, after isolating a 2.6 kb fragment of DNA, Szostak inquired as to whether or not she had obtained its sequence [Lundblad, V., 2006]. Six months later, Lundblad's sequence contained a 689 codon open reading frame, but lacked important sequence motifs, indicative of DNA or RNA binding domains. Unable to directly correlate *EST1* function from its structure, they created *est1Δ::HIS3* deletion mutants. These strains, like the *est1-1* strains, showed a progressive loss of telomeric DNA and cell senescence, indicating that slow telomere loss is the null phenotype of this gene. The authors also noted that the *est1Δ* strain had an increased frequency of chromosome loss, as assayed by the loss of a genetically marked artificial chromosome.

The protocols employed by Lundblad and Szostak were instrumental in leading to the characterization of other *EST* genes and the telomerase complex. However, the work of their 1989 paper was published when many of the work-horse tools of today's molecular genetics – the polymerase chain reaction (PCR), the internet and its collections of manuscripts and of databases, such as the Basic Local Alignment Search Tool (BLAST), and refined DNA sequencing technology were non-existent or in limited usage. Advents in these areas, when combined with the insights from Lundblad and Szostak, enabled further probing into the genome. Experiments could be designed on a larger scale and executed more efficiently and, with the tools of the internet age, allowing them to interpret their results with more information. In light of these improvements, Lundblad recognized the limitations of this work and was able to refine the techniques she and Szostak employed, allowing her to identify other *EST* genes.

The *TLC1* RNA Template for Telomerase and Further Characterization of the Telomerase Complex

The intervening years between Lundblad and Szostak's identification of *EST1* in 1989 and the discovery three other genes that displayed the *EST* phenotype – cellular senescence resulting from the progressive shortening of telomeres – in 1996 by Lundblad and her group yielded many advances that allowed Lundblad to repeat her screen and identify more genes. Most importantly, since she and Szostak had published their seminal work, another essential component of the telomerase complex had been discovered: its template for catalysis. In 1994, "*TLC1*: Template RNA Component of *Saccharomyces cerevisiae* Telomeres," a paper by Miriam S. Singer and Daniel E. Gottschling characterized telomerase as a reverse transcriptase – an enzyme that synthesizes DNA through an RNA intermediate – and identified its RNA template, *TLC1* [Singer, M.S. and D.E. Gottschling, 1994]. Singer and Gottschling performed a screen to find genes that would, if overexpressed, suppress the ability of telomere DNA to

silence neighboring genes. They hypothesized that such genes having the ability to suppress this telomere silencing might contribute to telomere maintenance. Using a cDNA library containing inserts controlled by a *GAL1* promoter (overexpression of these genes results from the strong induction of *GAL* in the presence of galactose), they isolated genes that affected telomere silencing with the use of *URA3* and *ADE2* markers artificially integrated near telomeres. *ADE2* encodes a product required for adenine biosynthesis, and *ADE2* deficient colonies appear red, rather than white. When *ADE2* is inserted near a telomere, colonies are mostly red because transcription of the gene is repressed. After induction with galactose, they screened for white colonies and found 48 plasmids that suppressed telomeric silencing and were able to grow on media lacking uracil and adenine. From these 48 mutants, 10 different genes were isolated. One of the genes, *TLC1*, was found to have a telomere-specific effect – it did not suppress silencing at the *HML* locus, a transcriptionally silent gene that encodes an unexpressed copy of the *Saccharomyces cerevisiae* mating type gene. Overexpression of *TLC1* not only showed telomere-specific suppression of gene silencing, but also altered telomere length. In the presence of *TLC1* on its endogenous promoter, telomere length averages 330 bp; in their overexpression assay, telomere length was dramatically decreased. This result suggested to Singer and Gottschling that *TLC1* was specifically involved in controlling some aspect of telomere structure.

To test this hypothesis, they sequenced *TLC1*-containing inserts, determining that this gene was 1301 bp and that it did not contain a large open reading frame (ORF). This observation indicated that, although they knew *TLC1* was transcribed, it was not translated into a protein. Based on the interpretation that *TLC1* might encode an RNA component, they performed a Northern blot, determining that a wild type *Saccharomyces cerevisiae* strain contained RNA that hybridized to a *TLC1* probe. These results led them to speculate that *TLC1* encoded an RNA

template for telomerase. If *TLC1* encoded this template, Singer and Gottschling reasoned, it would contain a short region complementary to the telomeric repeat. Indeed, a 13-bp motif (GTGTGTGGGTGTG) in *TLC1* served as evidence of a template region for *TLC1*. To verify that *TLC1* was acting as the template for telomere synthesis, they first demonstrated that a complete disruption of *TLC1* yields the *EST* phenotype that Lundblad and Szostak described – cellular senescence, the result of a progressively shortening telomere. To directly determine that the *TLC1* gene product was the template RNA, they constructed a *TLC1* allele that incorporated two base pair changes, creating a *Hae III* restriction site. This mutation was incorporated into yeast to create a *TLC1/TLC1-1(Hae III)* diploid strain. Earlier work with *Tetrahymena* had shown that a mutated RNA template causes the incorporation of complementary altered sequences into telomeres when introduced into a cell. Singer and Gottschling's result was that telomere DNA from wild-type strains was not cleaved by *Hae III*, as expected. However, in all of their diploid strains, digestion with *Hae III* resulted in cleavage, indicating that these mutations had been incorporated into newly synthesized telomere DNA. This result confirmed that *TLC1* sequence encoded telomere repeat sequences.

Using the information that *TLC1* encoded the template for telomerase, technological advances, and the insights of almost five years of additional research, Lundblad was able to screen for mutants with the *EST* phenotype exhibited by both *est1* and *tlc1* to identify the genes *EST2, EST3,* and *EST4* [Lendvay, T.S, et al., 1996]. Because the plasmid linearization assay that she and Szostak employed was extremely labor-intensive, they had originally only screened 7,000 mutant colonies. However, for her later work, she used a four-tiered screen, looking for mutants that exhibited all of the characteristics of *est1*: increased frequency of chromosome loss, loss of plasmid linearization, reduction in telomere length, and cellular senescence. Increasing

the number of processed mutants 50-fold, from 7,000 to 350,000, and using the four-tiered

screen described above, Lundblad essentially repeated the assay that she and Szostak had used to

find mutants defective for telomere maintenance. After testing 19 mutants with the *est* phenotype

and three mutants with short but stable telomeres, she found that all of her mutants

complemented a *tlc1*Δ strain but 11 mutants failed to complement an *est1*-Δ strain, indicating

that these mutants contained mutations in *EST1* and not a novel gene.

  She proceeded to cross the remaining eight mutants with one another, finding three new

complementation groups within this subset of mutations. These complementation groups were

later named *EST2, EST3*, and *EST4*. Her crosses showed that mutations in these genes gave the

same phenotype as *est1* and *tlc1* mutants in all cases. She also demonstrated that cells lacking

any one of these genes – *EST1, EST2, EST3, EST4*, or *TLC1* – can maintain telomeres

independently of telomerase through the same recombination-mediated pathway. This work

indicated that all of these genes function in the same pathway, a pathway controlling telomere

synthesis. She verified this proposal by showing that multiply mutant strains – any strain

containing mutations in two or more of the aforementioned genes – exhibit no enhancement of

phenotype of over single mutant strains. For instance, an *est3 tlc1* strain has the same phenotype

as an *est1* or *tlc1* strain, indicating that these genes function in the same pathway.

The End is Only the Beginning: The Emergence of Telomere Research and the Discovery of
***EST* genes**

  The work by Lundblad and Szostak in 1989, Singer and Gottschling in 1994, and the

Lundblad group in 1996 showcase developments in the telomere and telomerase research during

a time when this field was just beginning to establish itself. Lundblad and Szostak's

characterization of *EST1* was truly remarkable when we consider how little they (and everyone

else) knew about telomerase at the time of their work. However, just as their discoveries were driven by that of Blackburn and Greider before them, the discovery of *EST1* was a catalyst for further work in this area. Though Lundblad and Szostak had identified this gene, their work said nothing about it is function. Hence, the 1994 work Singer and Gottschling was a milestone in elucidating the telomerase complex, as it established the function of an important gene in this complex. By this point, technological innovations and advances in the field enabled Lundblad to perfect the basic protocol she and Szostak established for the identification of *EST* mutants, which she used to identify *EST2, EST3,* and *EST4.* The significance of these studies is apparent: this work was instrumental in establishing several important components of the telomerase complex. These studies form a solid foundation upon which the work of today's geneticists is based, studying the function and interactions of the components Szostak, Lundblad, Singer, and Gottschling identified.

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