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Telomere Length in Meiotic Cells of *Aspergillus nidulans*

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Abstract

Telomeres are nucleoprotein complexes at the ends of chromosomes and consist of non-coding, tandem repeats. They serve to protect chromosomal ends as well as ensure the complete transmission of genetic information from one generation to another. In a wide range of organisms, including humans, telomeres in germline cells are believed to be significantly longer than those in somatic cells. We are interested in developing a simple model organism to study this phenomenon. Our lab uses the fungus *Aspergillus nidulans* due in part to its exceptionally short telomeres observed in vegetative cells. The telomeres in sexual cells have not been studied. We hypothesize that in this fungus, the sexual spores have longer telomeres than the vegetative cells, analogous to what is seen in other eukaryotes. The initial steps of testing this hypothesis involved opening the ascospores and subsequently isolating intact DNA from them. Though I encountered many failed experiments in attempting those initial steps, I was eventually able to develop a protocol for extracting intact DNA from ascospores to use in a PCR assay to determine telomere length. No such extraction procedure was previously available. After obtaining DNA from ascospores for PCR, I used the anchored telomere PCR assay to determine the telomere length in the sexual spores of *A. nidulans*. Though I was not able to do so, I have discovered differences in telomeres between various strains of *A. nidulans* that rendered our anchored telomere PCR assay effective in one strain while ineffective in others in the process.

Overview

This chapter provides a review on the sources of fuel and migratory strategies used by New and Old World migrants in order to fulfill the energy demands to complete their journey. Although fat is the densest energy source, migrants crossing harsh geographic barriers encounter difficult fueling at stopover sites *en route* due to their unavailability or poor quality. If glycogen and fat stores become depleted and cannot be readily replenished, a bird must then use protein as a source of fuel. Of migration, functional tissue is utilized as an energy source. Thus, it is no surprise that after the discovery of telomeres, scientists have devoted enormous amounts of effort to studying telomeres and applying the knowledge gained to the treatment of age-related diseases and cancer. Telomeres are nucleoprotein complexes at the ends of chromosomes; they are composed of non-coding, tandem repeats of nucleotides and specific binding proteins. Since the characterization of the Tetrahymena (a ciliated protozoan) telomere by Elizabeth Blackburn in 1979, scientists have sequenced telomeres in many different organisms. Some species share a homogeneous telomeric sequence; for example, *Aspergillus*, humans, and trypanosomes all carry the tandem array of TTAGGG repeats at their chromosomal ends (de Lange et al., 1990). Other species such as budding yeast, fission yeast, and budding yeast, fission yeast, and budding yeast, fission yeast, and budding yeast, fission yeast, and budding yeast, fission yeast, and budding yeast, fission yeast, and budding yeast, fission yeast, and budding yeast, fission yeast, and budding yeast, fission yeast, and budding yeast, fission yeast, and budding yeast, fission yeast, and budding yeast, fission yeast, and budding yeast, fission yeast, and budding yeast, fission yeast, and budding yeast, fission yeast, and budding yeast, fission yeast, and budding yeast, fission yeast, and budding yeast, fission yeast, and budding yeast, fission yeast, and budding pear, eukaryotes have at least one telomere sequence; for example, *Aspergillus*, humans, and trypanosomes have irregular repeats (Blackburn, 2001). Another interesting characteristic of telomeres is that they do not have blunt ends. Instead, the terminus of a telomere is a single-stranded 3' protrusion rich in G nucleotides; it is called the G overhang (Verdun & Karlseder, 2007).

Telomeres have many important functions in the biological processes of cells, and one function is to protect chromosomal ends. Broken DNA ends, once detected by the cell, often lead to the activation of the DNA repair machinery and arrested growth of the cell (reviewed in McEachern et al., 2000). Another deleterious consequence of broken DNA ends is the direct joining of two nonhomologous ends. This could lead to a loss of genes and random chromosomal fusion (reviewed in McEachern et al., 2000). Telomeres prevent such abnormalities by “capping” the ends of chromosomes so they are not processed the same way as double-stranded DNA breaks.

Another way telomeres contribute to the survival of the cell is it solves the so-called “end replication problem.” A brief description of this problem is as follows: in the process of cell division, due to the incomplete lagging strand synthesis, the distal primer cannot be replaced (reviewed in McEachern et al., 2000). Genetic information could be lost in each round of cell division as the chromosome gets progressively shorter. The presence of telomeres at the ends of chromosomes protects the organism from losing important DNA information; the tandem repeats in telomeres do not code for proteins, so it was believed at first to be harmless if telomeres are shortened (reviewed in Blackburn, 2001).

Telomerase

Telomeres act as a buffer for the chromosome during DNA replication. However, if they shorten dramatically after multiple cell divisions, the telomere length may cross a critical threshold, eliciting an irreversible cell cycle arrest (Bekaert et al., 2004). The enzyme telomerase can prevent this from happening. Telomerase is a ribonucleoprotein complex that adds telomeric repeats to the 3' overhang (Cech, 2004). It is consisted of an essential RNA portion that serves as a template for the synthesis of the telomeric sequence and a protein called telomerase reverse

Introduction

Aging and cancer are two of the most important topics in the biological sciences today.

*This author wrote the paper as a senior thesis under the direction of Dr. Karen Kirk.
transcriptase (TERT). TERT acts as the enzymatic component that works in similar ways to DNA polymerase (Cech, 2004), thus regulating the length of the telomere.

**Telomere Length Regulation**

The length is an important characteristic of the telomere because of its implications for senescence and carcinogenesis (reviewed in Blasco, 2005b). Thus it is essential that there are mechanisms to regulate it at many levels. One way in which telomere length can be regulated involves tertiary structures consisted of the double-stranded telomeric DNA and/or the single-stranded telomere terminus. The 3' tail of the telomere can fold back and invade the double-stranded region of the telomere, forming a lasso-like structure known as a telomeric loop (t-loop) and generating a displacement loop (D-loop) (Grandin & Charbonneau, 2007). These structures protect the chromosomal end and prevent the telomerase from acting on the telomere terminus (reviewed in Cech, 2004). Single-stranded telomeres can also form quadruplex structures in vitro that may protect them from telomerase extension (reviewed in Cech, 2004). These structures are maintained in vitro through the triple hydrogen bonds formed by the GC-rich sequence in the telomeric repeat (reviewed in Cech, 2004).

Telomere length is also regulated by many DNA-binding proteins; one major protein complex that accomplishes this task is shelterin. The components of shelterin are TRF1, TRF2, TIN2, Rap1, TPF1, and POT1 (de Lange, 2005). TRF1, TRF2, and Rap1 act by binding to the double-stranded telomeric DNA, thus aiding in the formation of the t-loop which prevents telomerase from elongating the telomere (Cech, 2004). During cell division, the telomeres shorten progressively due to lagging strand synthesis, causing the number of protein-binding sites to decrease; this restores the accessibility of telomeres to telomerase (Cech, 2004). POT1 in mammals regulates telomere length by binding to the single-stranded G overhang where telomerase attaches. The 3' tail of the telomere typically functions as the region of alignment for the telomerase RNA; when bound by POT1, the single-stranded DNA is no longer available to telomerase's enzymatic actions (Cech, 2004). POT1 also interacts with TRF1, and this relationship allows POT1 to relay information about the length of the telomere to the G overhang (Loayza & de Lange, 2003). Orthologues of POT1 have been found to be associated with telomere length regulation in a diverse group of organisms, including chickens, Trypanosoma brucei (a protozoan), and Aspergillus nidulans (a filamentous fungus) (Grandin & Charbonneau, 2008; Pilt et al., 2003).

**Telomeres, Aging and Cancer**

Telomerase is not active in all cells to extend telomeric ends. In fact, only germ cells and cancer cells have active telomerase; somatic cells either lack telomerase or exhibit a low level of telomerase activity (Shay & Wright, 2007). In somatic cells, when telomeres shorten to below the critical length, the cells enter an irreversible state of growth arrest; this is known as replicative senescence where the cell is metabolically active but no longer able to divide (Shay & Wright, 2007). At this point, cells produce a different array of proteins compared to when they were in a quiescent state (Shay & Wright, 2007). This leads to changes in the cells' homeostatic environment that contribute to the loss of cell viability and eventually the aging of the organism (Shay & Wright, 2007). In fact, telomeres are often called "molecular clocks" in that they can act as a measure for the replicative history of a cell. A study done on human fibroblasts demonstrated that the telomere length shortened by about 2 kb when they were grown until senescence; this is one of the first experiments showing that telomeres shorten continuously through the lifetime of a cell (Harley et al., 1990). However, due to the complexity of organismal aging, replicative senescence induced by telomere shortening can only be seen as one aspect of the aging process.

One might question the usefulness of a biological process such as replicative senescence that submits an organism to the inevitable fate of aging in the context of natural selection. However, the other side of the argument is that this mechanism can limit the number of times a cell can divide in its lifetime, which can in turn reduce the chance of carcinogenic mutations accumulating (Shay & Wright, 2007). In other words, replicative aging protects the organism from cancer.

In the normal process of replicative senescence, the telomeres in somatic cells shorten until they reach the threshold for growth arrest, at which point they stop dividing and lose viability (Shay & Wright, 2007). However, cells with genetic mutations that ignore the signal to stop cell division; this usually leads to the state of crisis characterized by genomic instability and cell death (Greider & Blackburn, 1996). If telomerase is activated at this point, tumor cells can arise because cells can escape death even with an accumulation of detrimental mutations that causes the genome to be unstable (Vermeulen & Kirkland, 2004). Not only does telomerase activation induce tumorigenesis by rescuing cells with critically short telomeres, it can also promote tumor growth in the presence of sufficiently long telomeres (Blasco, 2005a). Thus telomerase plays a crucial role in the formation of cancerous tissues.

An important animal model used extensively to study the effects of short telomeres is the telomerase-deficient mouse model (Blasco, 2005a). These mice were generated by deleting the Terc gene which codes for the RNA component of telomerase, and the viability of these mice was severely compromised (Blasco, 2005a). The same concept can be applied to humans as well. Some human age-related diseases are caused by shortened telomeres and telomerase defects, and they have symptoms similar to those found in telomerase-deficient mice (such as heart failure, infertility, digestive tract atrophies) (Blasco, 2005b). In humans, there are also premature aging syndromes like ataxia telangiectasia and Werner syndrome; they are the result of mutations in certain genes that cause an accelerated rate of telomere degradation with age (Blasco, 2005b). One specific example is dyskeratosis congenita (DC), a disease caused by genomic instability and telomeres and proteins associated with telomerase (Mitchell et al., 1999). These mutations result in telomerase instability and shortened telomeres that lead to pathologies such as short stature, infertility, bone marrow failure, and premature death (Blasco, 2005b). An important difference between the telomerase-deficient mouse model and DC patients is that the mouse model shows increased resistance to cancer while DC patients exhibit elevated susceptibility to spontaneous cancer (Gonzalez-Suarez et al., 2000). One explanation for this disparity is cells in DC patients still have low levels of telomerase activity, and that could have been upregulated, leading to tumor formation (Blasco, 2005b).

**Telomeres and Meiosis**

Apart from being important in aging and cancer, telomeres also play a special role in meiosis. This was first proposed when a diverse group of organisms were found to have longer telomeres in germ cells than in somatic cells; this is a phenomenon that we are especially interested in. In humans, the telomeres in sperm cells have been shown to be 15 times longer than those in somatic cells (de Lange et al., 1990). The telomeres in the micronucleus (which provides the genetic information for the germ line cell) of Tetrahymena are...
approximately seven times longer than those in the macronucleus (which provides genetic information for vegetative growth) (Kirk & Blackburn, 1995). The current explanation for this phenomenon is as follows: in an organism, the function of the germ cell is to propagate the entire set of genetic information from one generation to the next. Thus it is extremely important that chromosomes are replicated fully without losing nucleotides in the cell division process. Maintaining long telomeres in germ cells would potentially ensure the complete transmission of the genome.

The mechanism by which telomeres become relatively long in germ cells is telomerase activation (Bekaert et al., 2004). As explained before, telomeres in somatic cells shorten progressively in the process of cell division and eventually undergo replicative senescence when they reach critically short lengths (Bekaert et al., 2004). However, in order to pass on the complete genome to the progeny, telomerase is activated in germ cells to act against telomere attrition by virtue of its enzymatic activities (Bekaert et al., 2004). Telomerase activity has been demonstrated in fetal, newborn, and adult ovaries and testes in human (Fujisawa et al., 1990). Also, consecutive generations of a group of telomerase knockout mice showed a dramatic decrease in fertility, as illustrated by reduced litter size, testis size, and number of primary spermatocytes: eventually the progeny mice became completely sterile (Liu et al., 2002).

Apart from acting as a buffer for genetic material during cell division, telomeres also play other special roles in meiosis; namely, they contribute to meiotic chromosome architecture and meiotic spindle integrity (Siderakis & Tarsounas, 2007). When the cell enters the early stages of the meiotic prophase I, telomeres attach to the nuclear envelope in a polarized configuration, thereby anchoring the chromosome firmly to the inner nuclear membrane (Siderakis & Tarsounas, 2007). The telomere/nuclear envelope (NE) attachment has been shown to be conserved across many species (Scherthan, 2007). Not only does the cell require the presence of telomeres to accomplish NE attachment, the telomeres need to be of a certain length as well (Siderakis & Tarsounas, 2007). Liu et al. (2004) generated telomerase-deficient mice with short telomeres, and those telomeres were shown to be lacking the perinuclear distribution characteristic of NE attachment. More severely, telomere erosion in these mice led to defects in meiotic homologous recombination and chromosome segregation which eventually resulted in meiosis breakdown (Liu et al., 2004).

As the meiotic cell continues on to leptotene-zygotene transition, NE-attached telomeres reposition along the inner nuclear envelope to temporarily cluster around the cytoplasmic microtubule organizing center (MTOC) of animals and fungi; this is termed the bouquet formation which may be a universal characteristic of meiosis (Scherthan, 2007; Siderakis & Tarsounas, 2007). The exact function of the bouquet formation during meiosis has yet been revealed. But scientists have evidence that both NE attachment and bouquet formation aid in the recognition and recombination of homologous chromosomes (Bekaert et al., 2004). In Schizosaccharomyces pombe, genetically modified telomeres caused pairing of homologous chromosomes during meiosis to fail, incorrect chromosome segregation, and problems in the production or release of spores (Nimmo et al., 1998). Caenorhabditis elegans has been shown to fail in segregating meiotic chromosomes when its telomeres are shortened (Ahmed & Hodgkin, 2000).

Aspergillus nidulans as a Model Organism
To learn more about telomere length regulation in meiosis, we decided to use Aspergillus nidulans as our model organism. Aspergillus nidulans is a filamentous fungus that has been long established as an excellent genetic model (Oakley & Morris, 1980). Its entire genome has been sequenced and made available to the public via the Broad Institute (Galagan et al., 2005), and many techniques have been developed to carry out genetic analysis on this fungus. Biological processes such as spore development, cell cycle, metabolism, pH control, and signaling have all been studied extensively using A. nidulans as a model organism (Todd et al., 2007). There are several reasons why A. nidulans is a tractable model for studying telomere length regulation in meiosis. First, the fungus maintains extremely short telomeres (120 base pairs) throughout its asexual development (Bhattacharyya & Blackburn, 1997). Compared to many other organisms such as budding yeast (300 bp), mice (20-150 kb), and human (5-15 kb), A. nidulans keeps its vegetative telomeres very tightly regulated (Bekaert et al., 2004). Secondly, the overall length of the telomeres in the fungus is kept relatively homogeneous during vegetative growth (Bhattacharyya & Blackburn, 1997). This is very different from many other microbes such as Tetrahymena which has very heterogeneous telomeric length (Petcherskaia et al. 2003, Kirk et al. 2008). Thirdly, A. nidulans has a complete well-characterized sexual cycle that permits genetic analysis. It has ten different cell types that ultimately form the fruiting body (cleistothecium) containing the sexual spores (ascospores) (Kirk & Morris, 1991; Todd et al., 2007). Figure 1 depicts the cleistothecium containing the ascospores, the Hule cells that aid in the formation of the cleistothecium, and the asexual spores (conidia).

**Figure 1. Different Cell Types of Aspergillus nidulans.** This diagram represents the distinctive cell types of A. nidulans, including the reproductive structures conidia and cleistothecium. Conidia contain the vegetative cells of the organism while cleistothecium contains the sexual spores (also known as ascospores) (modified from Kirk & Morris, 1991; Genes & Development, 5, 2014-2023).

**Novel Anchored Telomere PCR Assay**
Mithaq Vahedi’s 08 successfully designed a novel anchored telomere PCR assay that can detect and measure telomere length in A. nidulans. Traditionally, telomeres are detected using a Southern hybridization assay, an assay that works by first producing telomere restriction fragments and then probing the fragments with complementary radio-labeled telomere probe (Baird, 2005). However, there are several disadvantages to Southern hybridization assays. First, it requires at least microgram quantities of DNA to complete the analysis; this severely limits the method in its effectiveness when analyzing small samples (Baird, 2005). Secondly, the assay has a telomere length threshold below which it will not be able to detect telomeres so it has limited
Figure 2: Principle of novel anchored telomere PCR assay. Chromosome II-L of A. nidulans has a unique subtelomeric region, thus its telomere end was chosen for amplification. The steps of the telomere PCR assay are as follows: Step 1-C nucleotides (blue nucleotides) are added to the end of the telomere at the 3’ end with the enzyme terminal deoxynucleotidyl transferase (Tdt). Step 2-The DNA is heat denatured, and the telomeric primer (black nucleotides) and the subtelomeric primer (orange arrow) are added. Step 3-PCR is performed on the PCR reaction mixtures. Step 4-The amplified telomeric DNA sequences are separated by size via gel electrophoresis. Figure 2A shows a chromosome with only one telomeric repeat at the 3’ end, while in Figure 2B there are three telomeric repeats. Thus, when the same PCR is carried out on both, the gel confirms that product A is at a lower size than product B due to its shorter sequence.

sensitivity (Baird, 2005). Also, Southern hybridization cannot track changes in telomere length in individual chromosomes as it only provides the telomere length for all chromosome ends (of which there are 16 in A. nidulans).

Thus, the anchored telomere PCR assay was designed to overcome all the aforementioned problems and ultimately be able to detect telomere changes in an individual chromosome in A. nidulans. Apart from being very efficient and inexpensive compared to Southern hybridization assay, this PCR assay only requires 0.3 ng of genomic DNA for it to be effective (M. Vahedi, personal communication, April 25, 2008). Also, it can detect telomere lengths as little as five telomeric repeats (M. Vahedi, personal communication, April 25, 2008), making it much more sensitive than the traditional method. These advantages make the anchored telomere PCR assay a very useful tool for detecting small changes in telomere length in minute amounts of A. nidulans DNA.

The process of the anchored telomere PCR assay is as follows (also shown in Figure 2). First of all, DNA is isolated and purified from the organism (M. Vahedi, personal communication, April 25, 2008). The second step is to add C nucleotides to the 3’ overhang of the telomere using the enzyme terminal deoxynucleotidyl transferase (Tdt). A primer is designed to complement not only the C tail but also the last telomeric repeat; this way, the primer is "anchored" to the end of the telomeric array and not bound just anywhere on the C tail. This primer, along with a more internal primer, amplifies the telomeric region. They are called the telomeric primers. Another set of primers, the subtelomeric primers, is designed to amplify a DNA sequence just before the start of the telomeric sequence. Because the left arm of chromosome II has a unique subtelomeric region in A. nidulans, both sets of primers are designed for chromosome II-L. PCR is then performed on the tailed DNA with these two sets of primers.

Afterwards, the PCR products are separated based on size by gel electrophoresis. The sizes of the telomeric bands will be determined by comparing with the DNA ladder that ran with the PCR products (M. Vahedi, personal communication, April 25, 2008).

Thesis Aim

The aim of my thesis is to determine if there is a difference in telomere length between the vegetative cells and the meiotic cells of A. nidulans using the novel anchored telomere PCR assay developed by Mithaq Vahedi. The hypothesis is telomeres are longer in the meiotic cells than in the vegetative cells despite the fact that this fungus does not require sexual reproduction to propagate the species. If they are longer, it suggests that telomeres play a fundamental role in meiosis, be it aiding in meiotic recombination or contributing to the bouquet formation. If there is no difference in the telomere length between the two cell types, the implications are that long telomeres are not required for the meiotic process and these telomeres are extremely
tightly regulated in all stages of asexual and sexual development.

Results

Our hypothesis for this experiment is telomeres are longer in the meiotic cells of *A. nidulans* than in the vegetative cells despite the fact that this fungus does not require sexual reproduction to propagate the species. In order to test this hypothesis, we needed to first isolate DNA from the ascospores of *A. nidulans*. However, there was no previously published method for such a procedure. Thus, much literature research was needed to design an experiment for DNA extraction from ascospores. The first step to extracting DNA from ascospores was isolating the ascospores, and this was an established method. Cleistothecia, the fruiting bodies that contain ascospores, were isolated and cleaned by rolling around on a 3% agar plate (Todd et al., 2007). The ascospores were then released from the cleistothecia by crushing with a glass pipette (Todd et al., 2007).

The next step was opening up the ascospores themselves. However, the process of finding the effective method for this purpose was long and difficult. I first tried physical methods such as bead beating and crushing after liquid nitrogen freezing. An enzymatic approach was also taken, and it involved chitin degradation with the enzyme chitinase (chitin being a known component of the spore cell wall) (Yamazaki et al., 2007). Microscopy results (not shown) indicated that none of those methods yielded satisfactory results. After more extensive literature research, I found a high-speed, efficient homogenizer called FastPrep®-24 that has been shown to be able to lyse the mycelia of *A. nidulans* as well as tissue samples from a variety of organisms (Todd et al., 2005). The following results were those obtained after this breakthrough was made.

Success at Opening Ascospores: FastPrep®-24

To compare the telomere length in ascospores and conidia, we must first isolate intact DNA from the ascospores of *A. nidulans*. FastPrep®-24 (the closest to us was at University of Chicago) was recruited to do this job. As seen in Figure 3A, the ascospores were round, whole, and naturally red before lysis. The lysis process itself was carried out with two different matrices: matrix A with garnet and a ceramic sphere and matrix C with silica beads. The red broken pieces seen in Figure 3B confirmed that ascospores were indeed lysed in FastPrep®-24.

Before carrying out PCR, we wanted to determine the degree of conidial contamination in the ascospore samples with the current level of enrichment. We wanted to do this because if telomeres in ascospores are of the same length as those in conidia, we needed to be certain that the PCR product came from just ascospores and not conidia. Thus, a hemocytometer was used to count the ascospores and conidia in our samples at a 1/10 dilution before lysis. 43 ascospores were counted along with 2 conidia, making the concentration 7.6 X 103 ascospores/µL and 3.0 X 102 conidia/µL. Since ascospores are binucleate (Adams et al., 1998), though the number of ascospores was about 25 times that of the number of conidia, the genetic material in ascospores was actually 50 times of that in conidia. Thus, the concentration of ascospore DNA was calculated to be 18.1 ng/µL, and the concentration of conidia was calculated to be 0.36 ng/µL.

Nuclease Degraded DNA Isolated From Untreated Ascospores

After the initial isolation of DNA from ascospores, we wanted to ensure that DNA was present in the samples. Both DNA isolated from ascospores lysed with matrix A and ascospores lysed with matrix C were used in the subsequent PCR. Hyphal DNA from FGSC A773 (GR5) was used as the positive control since *A. nidulans* hyphae and conidia were found to have telomeres of the same length (Bhattacharyya & Blackburn, 1997). Two sets of primers were used for all three samples of DNA: POT1 primers and subtelomeric primers. Figure 4 shows the positions of the regions of DNA amplified by these two sets of primers and the telomeric primers (used later on in another experiment) relative to each other on the chromosome. The POT1 primers are more internal in position compared to the subtelomeric and telomeric primers. This set of primers serves to confirm the presence of *A. nidulans* genomic DNA as the pot1 gene has been found in the genome of the organism (Pitt et al., 2003). The subtelomeric and telomeric primers detect and measure the subtelomeric and telomeric regions of the chromosome.

As it can be seen in Figure 5, lanes 2-5 did not yield any bands, while the positive control in lanes 6 and 7 showed bands at the expected sizes (536 bp and 585 bp). Ascospore DNA could not be detected. Thus, it can be concluded that there was no DNA in the lysed ascospore samples.

Nuclease degradation was hypothesized to be the reason behind the absence of DNA in the broken ascospores. To determine if this was the case, hyphal GR5 DNA was incubated with the lysed ascospore samples, and a PCR reaction similar to the one described above was carried out on the two samples along with just hyphal GR5 DNA as positive control. Figure 6 showed that hyphal GR5 DNA was degraded when incubated with either samples of ascospores as no bands were seen in lanes 2-5 while the same DNA yielded bands at the expected sizes in lanes 6.
and 7. A conclusion can be made that nuclease degraded the ascospore DNA in the process of ascospore lysis.

**Phenol Chloroform Can Prevent Nuclease Degradation**

Phenol chloroform extraction is a widely accepted method in biochemistry for isolating DNA, RNA, and proteins (Madisen et al., 1987), thus we attempted to use phenol chloroform to prevent nuclease degradation in our lysed ascospore samples. Three DNA samples were prepared. Sample 1 contained untreated lysed ascospores. Sample 2 contained untreated lysed ascospores incubated with hyphal GR5 DNA. Sample 3 contained lysed ascospores first treated with phenol chloroform and then incubated with hyphal GR5 DNA. PCR was run on the three samples as well as hyphal GR5 DNA as positive control, all with POT1 primers. We expected samples 1 and 2 to yield no PCR product, while sample 3 should yield a PCR product since it was treated with phenol chloroform.

As seen in Figure 7, lanes 2 and 3 showed the expected lack of ascospore DNA in samples 1 and 2. Lane 4 contained sample 3 and yielded a band at the expected size of 536 bp, same as the positive control in lane 5. We can conclude that phenol chloroform can prevent nuclease degradation in the lysed ascospore samples, thus this information is useful for intact DNA extraction in future ascospore lysis.

**Inability to Detect Telomeres in Strain A92 with Anchored Telomere PCR Assay**

After confirming the extraction of intact DNA from ascospores, we proceeded to carry out the anchored telomere PCR assay to detect telomeres in ascospore DNA. C tailing was first carried out on the ascospore DNA as well as hyphal GR5 DNA as a positive control. For PCR, telomeric, subtelomeric, and POT1 primers were used on all samples of tailed DNA.

![Figure 6: Nuclease degradation was the cause for the inability to detect DNA in the ascospore samples.](image)

![Figure 5: Ascospore DNA could not be detected using POT1 or subtelomeric primers.](image)

In Figure 9, we can see the characteristically telomeric band in lane 5 for the tailed hyphal GR5 DNA. However, no telomeric band was obtained for the tailed ascospore DNA and in lane 2. The tailed ascospore DNA in lane 3 did not yield any bands with the subtelomeric primers while the tailed hyphal GR5 DNA in lane 6 yielded a band at the expected size with the same primers. Lane 3 did have a smeary band at a very low size; it could be a non-specific low molecular weight product such as primer dimers.

Though the telomeric and the subtelomeric primers did not yield any PCR products with the tailed ascospore DNA, the PCR reactions carried out with the POT1 primers yielded bands at the expected sizes for both the tailed ascospore DNA and the positive control. One unexpected occurrence was that though lane 8 in Figure 9 had no DNA template, it yielded a low, smeary band similar to lane 3. We do not know what could have caused it, but it could again be a non-specific low molecular weight product.
such as primer dimers. Thus, although an internal region of the chromosome (pot1) is detected, we could not detect telomeres in the ascospore DNA of strain A92 with the anchored telomere PCR assay.

We needed to determine the reason behind our inability to detect telomeres in strain A92 with the anchored telomere PCR assay that has been shown to be successful in strain GR5. One plausible explanation could be the telomeric sequence in ascospores is different from that in conidia. Another explanation could be that different strains of *A. nidulans*, namely A92 and GR5, have different telomeric sequences. To narrow down the possibilities, we decided to examine telomeres in hyphal A92 DNA with the anchored telomere PCR assay.

![Figure 7: Phenol chloroform can prevent nuclease degradation in lysed ascospore samples. Lane 1 had the 50-3000 bp DNA ladder. The following are the DNA in lanes 2-4 respectively: untreated DNA extracted from ascospores, untreated DNA extracted from ascospores incubated with hyphal GR5 DNA, DNA extracted from ascospores treated with phenol chloroform and then incubated with hyphal GR5 DNA. Lane 4 produced a band at the expected size of ~536 bp (blue arrow). Lane 5 contained hyphal GR5 DNA as positive control. The same primers were used throughout, namely the POT1 primers (Outer ForwardP nimU and Outer ReverseP nimU).](image)

Hyphal A92 DNA was isolated and tailed with C nucleotides. In the subsequent PCR assay, tailed ascospore A92 DNA, tailed hyphal A92 DNA, and tailed hyphal GR5 DNA (positive control) were amplified with telomeric, subtelomeric, and POT1 primers. The resulting gel as seen in Figure 10 shows that neither tailed ascospore A92 DNA nor tailed hyphal A92 DNA yielded bands with telomeric primers (lanes 2 and 5) and subtelomeric primers (lanes 3 and 6). However, both ascospore and hyphal A92 DNA yielded bands at the expected sizes in lanes 4 and 7 with the POT1 primers. The tailed hyphal GR5 DNA yielded bands at the expected sizes in lanes 8, 9, and 10 for all three sets of primers. Thus, we can conclude that there is so much difference in telomeric sequence between strain A92 and GR5 such that the anchored telomere PCR assay is not effective for A92 though it is for GR5. However, the results with the POT1 primers also indicate that the internal regions of the chromosome are similar between the two different strains of *A. nidulans*.

**Discussion**

**Evidence for Strain Variability in Telomeres**

I have successfully developed a novel protocol for isolating intact DNA from *Aspergillus nidulans* ascospores. This is important because DNA from the sexual spores of this organism has never been isolated for PCR amplification before. Further research could be conducted on such topics as telomere length regulation and the function of telomeres during meiosis in *A. nidulans* with this tool in hand. However, in terms of the initial hypothesis for this project, that is to determine whether there is a difference in telomere length between the asexual and sexual spores of the fungus, I was unable to use the anchored telomere PCR assay to detect telomeres and the subtelomeric regions in ascospores. I conducted all my experiments on strain FGSC A92 while the assay was designed for FGSC A4 and FGSC A773 (GR5). Thus, we suspect that the differences in telomeres between the different strains of *A. nidulans* caused us to be unable to use the anchored telomere PCR assay to detect telomeres and subtelomeric regions in both the ascospores and the hyphae of A92.

There is abundant evidence from the experiments I have conducted to support my conclusion. After the DNA was isolated from ascospores, the first step taken was to determine if it was intact. I was able to confirm this by carrying out PCR on all the ascospore DNA samples with POT1 primers. The next step in the experiment was to measure telomere length in A92 ascospore DNA using the anchored telomere PCR assay. However, my results indicated that A92 ascospore DNA did not yield any telomeric PCR products. To get a better idea of how A92 ascospore DNA compared to hyphal DNA of the same strain, A92 hyphal DNA was extracted, and the anchored telomere PCR assay was carried out on DNA from both cell types. We could not detect telomeres in either A92 hyphal DNA or A92 ascospore DNA though both proved to be intact with the amplification of the internal pot1 gene. Thus, I was able to conclude the telomere PCR assay was ineffective on *A. nidulans* strain A92.

**Why Are There Differences in Telomeres Between Different Strains of *A. nidulans***

One explanation for the inability of the anchored telomere PCR assay to detect telomeres in strain A92 is that there are no telomeric repeats at the end of the chromosome in A92. However, that is an unlikely scenario because telomere loss in most organisms has catastrophic consequences; it often leads to loss of cell viability and chromosomal instability (Blasco, 2005b). This phenomenon was first shown in the model organism *Saccharomyces cerevisiae* (Sandell & Zakian, 1993). Sandell & Zakian (1993) directly eliminated telomeric DNA from a single chromosome end in vivo by inducing a specific break in the double-stranded DNA. This was done by inserting the recognition site for the HO endonuclease immediately adjacent to the left telomere of chromosome VII, thus causing the telomere to be eliminated by galactose-induced expression of HO (Sandell & Zakian, 1993). When a single telomere was eliminated in wild type cells, a RAD9-mediated cell division arrest was induced (Sandell & Zakian, 1993); this is the typical response to DNA damage as demonstrated by Weinert & Hartwell (1988) in *S. cerevisiae*. This result demonstrated that telomeres help cells distinguish broken DNA ends from intact chromosomes (Sandell & Zakian, 1993). The loss of a telomere also
increased chromosome loss in *S. cerevisiae*, indicating that telomeres are essential for maintaining chromosome stability (Sandell & Zakian, 1993).

To study the effects of telomere loss in mouse models, telomerase-deficient mice were generated by elimination of the Terc gene, Terc being the RNA component of telomerase in mice (Blasco et al., 1995). Without the elongation of the telomerase, the mice are viable for only a few generations before telomere loss and chromosomal end-to-end fusion start manifesting themselves in the form of severe health issues (Blasco, 2005a). These pathologies include loss of fertility, heart failure, various tissue atrophies, and decreased tissue regeneration ability, compromising the long term viability of the organism (Blasco, 2005b).

The same phenomenon occurs in the model organism Drosophila melanogaster. Titen & Golic (2008) detected cell death through Chk2- and Chk1-controlled p53-dependent apoptotic pathways in these organisms when telomeres were eliminated by breaking off an induced dicentric chromosome. However, there was also a small fraction of cells that escaped apoptosis even with telomere loss (Titen & Golic, 2008). These cells continued to divide repeatedly, thus accumulating karyotypic abnormalities characteristic of cancer cells such as aneuploidy (Titen & Golic, 2008). Thus, telomere loss causes genomic instability in cells that manage to evade death, ultimately leading to carcinogenesis (Blasco, 2005b).

Copious evidence point to the lethal effects of telomere loss in an organism. Thus, seeing that FGSC A92 is a wild type strain that exhibits normal characteristics of *A. nidulans* development, we can conclude that telomere loss is not occurring in this particular strain.

We believe that the reason we could not detect telomeres in strain A92 with our anchored telomere PCR assay is due to differences in telomeres between different strains of *A. nidulans*. One such difference could be variability in telomeric sequences between strains. If the difference in sequence encompasses both that in the telomeric region and near the telomeric region, we can account for the lack of PCR amplification in A92.

Prior studies in the fungus *Candida albicans* show that such is the case in this pathogenic organism. Sadhu et al. (1991) discovered that DNA fragments containing telomeric repeats are highly variable among different strains while looking for a method to distinguish between various strains of *C. albicans*. Those repeats included telomeres and a type of subtelomeric repeat adjacent to the telomere (Sadhu et al., 1991). In fact, three out of the nine strains tested lacked that specific subtelomeric repeat sequence entirely (Sadhu et al., 1991).

**Making Use of Telomeric Differences Between Various Strains**

One major concern with using PCR to detect the difference in telomere length between sexual and asexual spores is conidial contamination in the ascospore sample. When cleistothecia, the structures containing ascospores, are collected, they are covered in mitotically grown Hulle cells and conidia. To get rid of the Hulle cells and conidia, the cleistothecia are rolled around a 3% agar plate with a glass pipette. This process is only partially effective. With the current level of enrichment, I know that 1 in 26 spores is conidium. That means that 1 nucleus in 51 nuclei is vegetative (since ascospores are binucleate). If the PCR results indicate that telomeres in the ascospore samples are longer than those in the conidia samples, we would be able to differentiate between the telomeres of the two cell types. However, if the telomeres in ascospores are of the same size as those in conidia, we would be unable to distinguish between them in PCR. With PCR’s ability to detect minute...
Figure 9: Ascospores from strain A92 do not seem to be yielding telomeric bands using the anchored telomere PCR assay. Lane 1 had the 50-3000 bp DNA ladder. Lanes 2-4 contained the tailed DNA extracted from ascospores treated with phenol chloroform. Lanes 5-7 held hyphal GR5 DNA as positive control whereas lane 8 contained the negative control. Telomeric primers (Ch2 Long Forward Prim1 and the 4th permutation primer) were used in lanes 2 and 5. Lanes 3, 6, and 8 used the subtelomeric primers (Ling Outer For Prim-Ch2 and Ling Outer Rev Prim-Ch2) while lanes 4 and 7 used the POT1 primers (Outer ForwardP nimU and Outer ReverseP nimU). Lane 5 yielded a telomeric PCR product with hyphal GR5 DNA at the expected size of ~332 bp (green). For the ascospore samples, only lane 4 yielded a band at the expected size of ~536 bp (blue), same as lane 7 which contained hyphal GR5 DNA (blue).

Figure 10: The anchored telomere PCR assay is ineffective on strain A92. Lane 1 had the 50-3000 bp DNA ladder. Lanes 2, 3, and 4 contained the tailed DNA extracted from ascospores treated with phenol chloroform. Lanes 5, 6, and 7 held tailed hyphal A92 DNA while lanes 8, 9, and 10 contained tailed hyphal GR5 DNA. Telomeric primers (Ch2 Long Forward Prim1 and the 4th permutation primer) were used in lanes 2, 5, and 8. Subtelomeric primers (Ling Outer For Prim-Ch2 and Ling Outer Rev Prim-Ch2) were used in lanes 3, 6, and 9 while POT1 primers (Outer ForwardP nimU and Outer ReverseP nimU) were used in lanes 4, 7, and 10. For A92 ascospores and hyphae, only lanes 4 and 7 yielded bands at the expected size of ~536 bp (blue), same as in lane 10 containing hyphal GR5 DNA (blue).
amounts of DNA, even small amounts of contamination from conidia could compromise the telomere PCR result for ascospores.

With the discovery of the ineffectiveness of the telomere PCR assay on strain A92, we now have a method for ensuring that the telomere PCR result for ascospores is valid. More specifically, we can find the point at which we can detect contamination from conidial DNA in PCR if the telomeres in ascospores and conidia are of the same size. We will first isolate A92 ascospores and GR5 conidia (schematic shown in Figure 11). In order to determine whether 1 conidium in 26 spores would cause noticeable contamination in PCR, GR5 conidia would be added to A92 ascospores in a 1:25 ratio. Cell lysis, DNA extraction, and C-tailing will be carried out on the sample. For the anchored telomere PCR, telomeric primers will be used, and the final telomeric PCR products will be separated based on size on an agarose gel. At this point, we will be able to determine whether this level of contamination will yield a PCR product that can be detected. If we do see contamination, we will then make dilutions of conidia in the sample to find the concentration of contamination that will not produce a visible PCR product.

![Figure 11: Schematic of the experiment conducted to verify validity of the telomere PCR assay.](image)

Further dilution of conidia in sample

**Figure 11:** Schematic of the experiment conducted to verify validity of the telomere PCR assay. First, GR5 conidia and A92 ascospores are extracted in a 1:25 ratio. FastPrep®-24 is then used to lyse the spores. Subsequently, DNA is extracted from both types of spores, and C-tailing is carried out afterwards. The telomere PCR assay is performed on the DNA samples from GR5 conidia and A92 ascospores with the telomeric primers. The amplified telomeric DNA sequences are separated by size on an agarose gel. If the contamination level is too high, we will be able to detect the PCR product, thus further dilution of conidia in the sample is needed. If the contamination level is not high enough to produce a PCR product, we will be assured that with the current level of ascospore enrichment in the anchored telomere PCR assay, the PCR product will only be from ascospores.

**Future Studies**

Though I was unable to confirm my initial hypothesis of telomeres being longer in the vegetative cells of *A. nidulans* than the vegetative cells, I was successful in developing a novel method for extracting DNA from the ascospores of this organism for PCR. I also discovered strain differences in telomeres in *A. nidulans* that could prompt other research questions in this fungus. Making use of this strain difference in telomeres, I could determine whether the current level of enrichment will be sufficient for the anchored telomere PCR assay which would enable us to be able to detect and measure the telomere length of ascospores with confidence. However, for future studies, I would like to answer my initial question and determine if my original hypothesis can be supported. I will detect and measure the telomere length of ascospores in strain GR5 and compare my results to the telomere length of conidia in GR5. Three different kinds of results could occur. Telomeres in the ascospores of GR5 could be longer than, shorter than, or of the same length as telomeres in the conidia of GR5. One way the cell could control the length of telomeres is by regulating the expression of genes coding for DNA-binding proteins. POT1 is one such DNA-binding protein that regulates telomere length. It acts by binding to the 3' overhang of the telomere, thus controlling the enzymatic actions of telomerase (Cech, 2004). This protein can also interact with another telomeric DNA-binding protein, TRF1, to transmit information about the telomere length to the telomere terminus where telomerase regulation takes place (Loayza & de Lange, 2003).

Thus, the different telomere lengths in ascospores and conidia could be partly due to the different expression levels of the pot1 gene. One way of measuring gene expression is Northern blotting, which quantitatively measures levels of mRNA. If telomeres are found to be longer in ascospores than conidia, then the hypothesis for this experiment would be that the pot1 gene is expressed at lower levels in ascospores than in conidia. If telomeres in ascospores are found to be shorter than those in conidia, then the hypothesis would be the pot1 gene is expressed at higher levels in ascospores than in conidia. If the telomere length for both cell types is the same, then the expression levels of the pot1 gene should be the same as well. The results of this study would take us one step further in understanding the regulation of telomeres in the meiotic and vegetative cells of *A. nidulans*.

**Materials and Methods**

**Isolation of Hyphal Genomic DNA**

Hyphal genomic DNA was isolated from two different strains of *A. nidulans*, FGSC A92 and FGSC A773 (GR5). A92 is wild type while GR5 is a strain of *A. nidulans* with genotypes pyrG89, wa3, and pyrA4 (Osherov & May, 2000). Lyophilized hyphae of GR5 were graciously given to us by Dr. Peter Mirabito from the University of Kentucky. It was ground up using a mortar and pestle to a fine powder of an off-white color. The DNeasy Plant Minikit from Qiagen (Cat no: 69104) was used to isolate genomic DNA from the ground hyphae. For the procedures, a modified version of the Maxi protocol developed by Sandeep Mallareddy from Dr. Pete Mirabito’s lab was used. The ground hyphae were put in a 1.5 mL Eppendorf tube. 700 µL of buffer AP1 and 7 µL of RNase A (100 mg/mL) were added to the hyphae and vortexed vigorously. The mixture was incubated at 6°C for 10 minutes; during incubation, the mixture was mixed two or three times by inverting the tube. Then, the tube was spun at 13,000 rpm for 5 minutes. 400 µL of the supernatant was transferred to a new 1.5 mL Eppendorf tube. 130 µL of buffer AP2 was added to the lysate, and the entire mixture was mixed and incubated on ice for 5 minutes. After this period of incubation, the lysate was applied to the Qiashredder Mini spin column and centrifuged for 2 minutes at 13,000 rpm. The flow through was transferred to a new 1.5 mL Eppendorf tube without disturbing the cell debris/pellet (if formed). 1.5 volumes of AP3/E was added to the now cleared lysate and mixed by pipetting (about 675 µL of AP3/E was added to 450 µL of lysate). 650 µL of the mixture prepared from the last step was applied to the DNeasy spin column and centrifuged at >8,000 rpm for 1 minute; the flow through was discarded. This step was repeated,
and the flow through was again discarded. The DNeasy spin column was then placed in a new collection tube, and 500 µL of buffer AW was added to the column and spun at >8000 rpm for 1 minute. The flow through was discarded. 500 µL of buffer AW was added to the column again, and the column was centrifuged for 3 minutes at 13,000 rpm to dry the membrane. The column was transferred to a new 1.5 mL Eppendorf tube. 100 µL of buffer AE that was heated to 65°C was added to the center of the column and incubated at 15-25°C for 5 minutes. At last, the tube was centrifuged at >8000 rpm for 1 minute to elute.

A92 was grown on A. nidulans complete media plates for three days at 37°C. The conidia were then collected with the aid of 0.2% Tween solution and a glass pipette bent into a loop. The solution with conidia was centrifuged for 3 minutes at 5,000 rpm, and the pellet formed was washed and resuspended in ~2.5 mL of sterile water. The conidia were then counted using a hemocytometer after making a 1:10 dilution. 4,602 conidia were counted, and after calculations, it was determined that the concentration of the conidia solution was 4.60 x 10^6 spores/mL. To produce hyphae, 6.5 µL of the conidia solution was added to 50 mL of liquid A. nidulans complete media and grown in a shaking incubator at 37°C and 120 rpm. After ~20 hours, the hyphae were collected by filtering with MiraCloth from CalBiochem (Cat no: 475855) and ground to a fine powder in liquid nitrogen using mortar and pestle. The DNeasy Plant Kit from Qiagen (Cat: no: 69104) was also used to isolate DNA from the ground hyphae of A92. This was done following the mini protocol for total DNA isolation from plant tissue provided by the kit.

Isolation of Ascospore Genomic DNA via FastPrep®-24

Ascospore genomic DNA was isolated from A. nidulans strain A92. The fungus was grown on A. nidulans complete media plates, and the cleistothecia were picked up using a glass pipette and cleaned by rolling on a 3% agar plate. They were stored in 10 mM Tris and 25 mM EDTA at 4°C. 1000 cleistothecia were collected and suspended in 500 µL of the said buffer. They were subsequently crushed with a glass pipette, and at this point, the ascospores were released. 250 µL of the sample was each allocated to a different matrix, namely matrix A with garnet and a ceramic sphere and matrix C with silica beads. A FastPrep® mini Kit from Qiagen (Cat: no 69104) was also used to isolate DNA from the ground hyphae of A92. This was done following the mini protocol for total DNA isolation from plant tissue provided by the kit.

To determine if phenol chloroform can prevent nuclease degradation in our samples, three samples of ascospore DNA were analyzed for nuclease degradation detection and phenol chloroform was added before or after lysis. The ascospore DNA isolation was done following the mini protocol for total DNA isolation from plant tissue provided by the kit.

To determine if phenol chloroform can prevent nuclease degradation in our samples, three samples of ascospore DNA were analyzed for nuclease degradation detection and phenol chloroform was added before or after lysis. The ascospore DNA isolation was done following the mini protocol for total DNA isolation from plant tissue provided by the kit.
Steps 2 through 4 were repeated 44 times, making a total of 45 cycles performed on those steps. For the PCR reactions with the POT1 primers, the only difference was that step 3 was set at 60°C. All but one experiment had PCR tubes with the following components: 2 µL of each of the set of primers used (25 µM), 3 µL of DNA (tailed or untailed), 18 µL of sterile water, 25 µL of JumpStart REDTaq ReadyMix. For the reaction shown in Figure 5 of results, 15 µL of DNA and 6 µL of sterile water were used instead of 3 µL and 18 µL respectively.

**Agarose Gel Electrophoresis**

For all the PCR reactions, 2.5% gels were made using agarose and 1X TBE buffer. After the electrophoresis process, the gels were stained with ethidium bromide for 20 minutes and de-stained with nanopure water for 20 minutes. Then, the gels were imaged using the BioRad Versa Doc Imaging System and Quantity One Software (version 4.6).  

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**References**


