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Sequencing a DNA Polymerase Alpha Gene in *Thermomyces lanuginosus*

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SEQUENCING A DNA POLYMERASE ALPHA GENE IN THERMOMYCES LANUGINOSUS

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Dr. Jessica Moon, Director, University Honors Program

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Introduction

Deoxyribonucleic acid, better known as DNA, encodes the blueprint that dictates the development and functions of an organism. DNA replication is an increasingly important subject to study in medical research. This is because during DNA replication mutations can occur, which may cause serious issues for the organism. These mutations could result in the loss of control over starting and stopping the process of DNA replication. The ability to start and stop DNA replication is directly related to starting and stopping cell division; if the DNA is not replicated, the cell will not divide. On the other hand, the DNA could successfully replicate but may have significant mutations, resulting in cells that will not function normally.

DNA polymerases are the enzymes that build DNA by putting together nucleotides, the building blocks of DNA. Mutations in the polymerases, which could cause a failure to stop cell reproduction, may result in diseases such as cancer. There are three forms of DNA polymerase – alpha (α), delta (δ), and epsilon (ε)-- which are instrumental in cell reproduction. DNA polymerase α works as a priming enzyme, which means that it is involved in getting the process of building DNA started. This makes it an important target to sequence and study in organisms in order to better understand its role in cell reproduction.

Problem

DNA polymerases are present in all living organisms and it is expected that there should be a reasonable degree of conservation, or similarity in how the polymerases function, between species. This makes the possibility of developing treatments using DNA polymerase more likely, as the treatment would not necessarily depend on using human DNA as the base material, which would be more expensive. However, this cannot be known unless there is a reasonable number of DNA sequences available from multiple species to analyze. This is a growing field of

research, but the majority of sequences known are from mammals. Currently there is very little research on DNA polymerases in fungi. Dr. Ira Simet, Chelsea Meier, and I worked on this project to add to research literature. The fungus that we worked with, *Thermomyces lanuginosus,* has been studied very little in scientific literature. Although this meant that we had little prior research to base our methods on, any methods that we developed are significant additions to what is known about the fungus. We had to develop techniques to successfully grow large quantities of it, extract DNA, and find primers to begin sequencing a DNA polymerase α gene.

Purpose

Our goal in this project is to find and sequence a DNA polymerase α gene in *Thermomyces lanuginosus.*

Literature Review

DNA polymerases are the enzymes that are needed for the replication (copying) of DNA prior to mitosis, for the repair of damaged DNA, and for a few other minor functions for eukaryotic cells. DNA polymerases alpha, delta, and epsilon participate in DNA replication by assembling the nucleic acids from nucleotides (adenine, thymine, guanine, and cytosine).[1] These enzymes have been studied in several organisms, (chicken, human, rat, etc.), but little is known about their fungal counterparts. *Thermomyces lanuginosus* (TL) was chosen as the target organism because it is thermophilic, meaning that it grows well in warmer conditions; its enzymes would be expected to hold up under more rigorous conditions that often occur in experiments. It grows quickly, making it practical to use because there will not be a long wait time if more fungus is

[1] Clark, David P. "Molecular Biology: Understanding the Genetic Revolution", (Burlington, MA: Academic, 2005), 107-108

needed. It also changes in appearance when it is actively growing versus when it is dormant.[2] The fact that TL changes color depending on what stage of growth it is in is especially beneficial because it allows for a qualitative prediction of the amount of polymerase that is active. Actively growing fungus should have higher amounts of polymerase present. It appears white when it is dormant and is dark brown or black when actively growing, (Figure 1). It grows best from 45-50°C, over the course of two to three days.[3]

Figure 1. Photo of *Thermomyces lanuginosus*

Enzymes are proteins, which are typically very sensitive to heat and will break apart if they are heated too much. This is known as denaturation and is very undesirable because it incapacitates the protein, rendering it unable to carry out its normal functions. Enzymes in thermophilic organisms are often more stable, which allows for more rigorous testing. This is due to their

[2] Suren Singh, "Thermomyces Lanuginosus: Properties of Strains and Their Hemicellulases."
[3] Khucharoenphaisan, K. and K. Slnma, 2011. Effect of signal sequence on the β-xylanase from *Thermomyces lanuginosus* SKR Expression in *Escherichia coli.*

preference to grow in warmer conditions, which means that their enzymes are structured to hold up under higher temperatures. Humans are not thermophilic organisms, so it is unknown how much in common human DNA polymerase will have in common with the DNA polymerase from TL. Gene sequencing is the first step in being able to better identify the other differences between typical and heat stable polymerases. Gene sequencing is the procedure of determining what order the nucleotides go in to make up an organism's DNA.[4] This is important because it is the first step in being able to decode how an organism is formed and how its systems work together. Gene sequencing is comparable to writing down a message that still needs to be decoded. It does not result in any immediate answers, but it is a necessary step in moving one step closer to being able to find them.

A nucleotide contains a nitrogenous base, a sugar, and one or more phosphate groups.[5] The bases that make up DNA are adenine, thymine, cytosine and guanine. When two bases bind to each other, a base pair is formed. Adenine and thymine will bind to each other and cytosine and guanine will bind to each other. It can be seen in Figure 2 how the base pairs are formed. This is especially important in the designing of primers, which is the first step in gene sequencing. A primer is usually a short piece of RNA that attaches to the DNA to start synthesis. In gene sequencing, a primer is specifically designed to be able to reproduce the section of DNA that is being targeted. Because the primer is made of the same bases as the DNA, which is why it will bind to the DNA, it could also bind to itself. This complicates the designing of primers as they must have a high likelihood of binding to the DNA, but a low likelihood of binding to themselves.

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[4] "What's A Genome?" *Genomenewsnetwork.org*, last modified January 15, 2003, http://www.genomenewsnetwork.org/resources/whats_a_genome/Chp2_1.shtml.
[5] David L. Nelson and Michael M. Cox, *Lehninger: Principles of Biochemistry.* (New York: W.H. Freeman, 2008), 71.

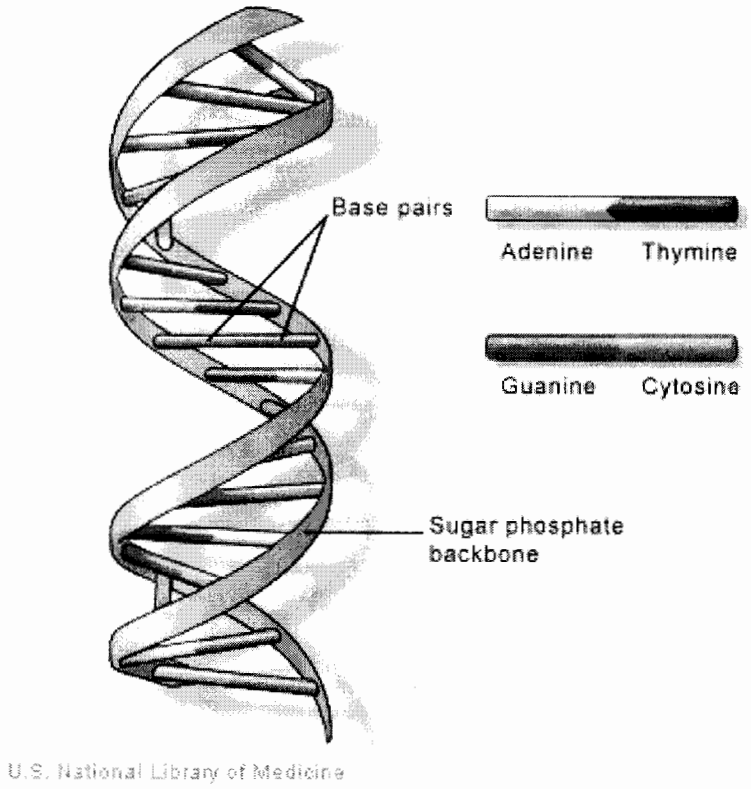

Figure 2. Double stranded DNA, it can be seen how the bases bind to each other.[6]

Of all the DNA polymerases, DNA polymerase α (DNAPα) was particularly interesting because it is involved in forming the RNA primers that are used to begin DNA synthesis. DNAPα is composed of four subunits. The priming subunit was chosen for our research because it had the most partial sequences available in the databases, which raised the likelihood of discovering a conserved sequence that could serve as a primer. A primer is a short piece of DNA that is designed to start the replication of small targeted areas of DNA. Figure 3 shows the different units that make up DNAPα. In the photo below, the priming subunit is referred to as "B-subunit" and "p86", all three names can be used interchangeably.

[6] Genetics Home Reference. *DNA double helix*.

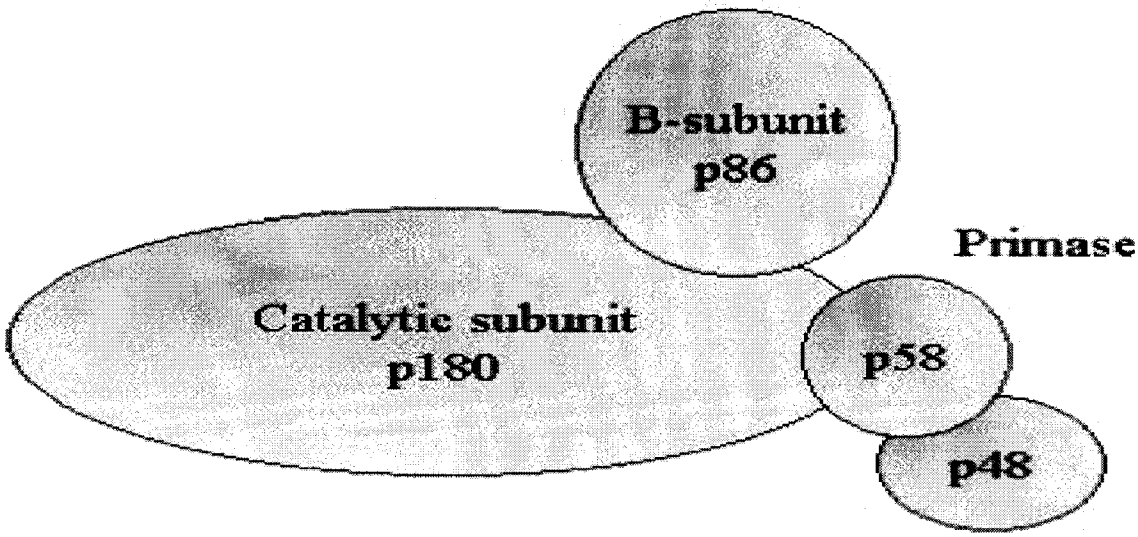

Figure 3. Subunits of DNA polymerase α[7]

PCR testing has been shown to be the most efficient way to identify if a small section of DNA is being successfully replicated.[8] This is important in gene sequencing, as it is impossible to transcribe the entire sequence of DNA at once. PCR experiments test for and result in the production of large amounts of short DNA segments.

As DNAPα is vital to cell reproduction, inhibiting it will inhibit cell growth. It has been seen that DNAPα inhibitors can decrease tumor size.[9] Tumors are made of cells that, due to mutations, have uninhibited growth. DNAPα inhibitors curb this growth and as the cells die off naturally and are no longer growing out of control, the tumor size decreases. The more that can be understood about how the presence of DNAPα , the more possibilities that alternative treatments to diseases, like cancer, could be developed. In addition, learning how cells turn cell

[7] Replication Machinery. *DNA polymerase-α.*.
[8] Morange, Michel. *A History of Molecular Biology*. (Cambridge, MA: Harvard UP, 1998), 241-242.
[9] Maeda, Naoki, et al. "Anti-tumor Effects of Dehydroaltenusin, a Specific Inhibitor of Mammalian DNA Polymerase α." *Biochemical and Biophysical Research Communications* 352 (2007): 390-396

reproduction on and off with DNA polymerases could also lead to different techniques of gene therapy. Cells that have cut off points in regenerating themselves, such as nerve cells, etc., could be stimulated to divide when an injury occurs. This would be a significant development in treating conditions such as paralysis and multiple sclerosis.

Research Methods

We followed the procedure that was used to find an expressed sequence tag (EST) for DNAPα in embryonic chicken brain in order to find a similar EST for DNA polymerase α in TL fungus. The procedure that was developed with embryonic chicken brain was started by a prior student researcher and carried on by Rebecca Krewer. Rather than identify the whole gene, an EST allows for the identification of a partial sequence that is unique to that gene. DNA polymerases have been found to have been highly conserved; there is minimal change in the genetic sequence among eukaryotic organisms.[10] This allowed us to utilize the genetic sequences that have been found in other eukaryotes to identify primers and guide our search for this sequence in TL.

We used National Center for Biotechnology Information to look up DNAPα sequences in different organisms. Unfortunately, few fungal systems are complete, so three to four organisms were selected arbitrarily for each analysis. Their FASTA sequences were analyzed by the Clustal W2-Multiple Sequence Alignment. This resulted in a document that displayed the sections of the gene sequences that were identical, correlated, or unrelated. At least five bases of exact correlation between the analyzed sequences was the main identifier for primers. If such a pattern was seen and appeared to have a reasonable amount of corresponding bases around it, we then searched for another section, 300-600 bases away, of at least five base pairs that matched precisely between the specimens. This second primer served as the reverse primer, allowing us

[10] Baker, Tania A. "Polymerases and the Replisome: Machines within Machines" *Cell* 92.3 (1998): 295-305.

to isolate a small, specific fragment of DNA. If another five base pair was found, the primers were extended to 15-24 bases and analyzed. They were analyzed using the OligoAnalyzer[11] to determine if their melting points were compatible and whether their bonding, both to each other and themselves, allowed for successful binding to DNA.

In Figure 4, the PCR process is detailed and it can be seen how the double stranded DNA is separated using heat, the denaturation step. The forward primer will bind to one strand and the reverse primer will bind to the other. This is important because DNA replication always occurs from the 5' end to the 3' end.[12] That is why there needs to be two primers to attach to the DNA and build in different directions.

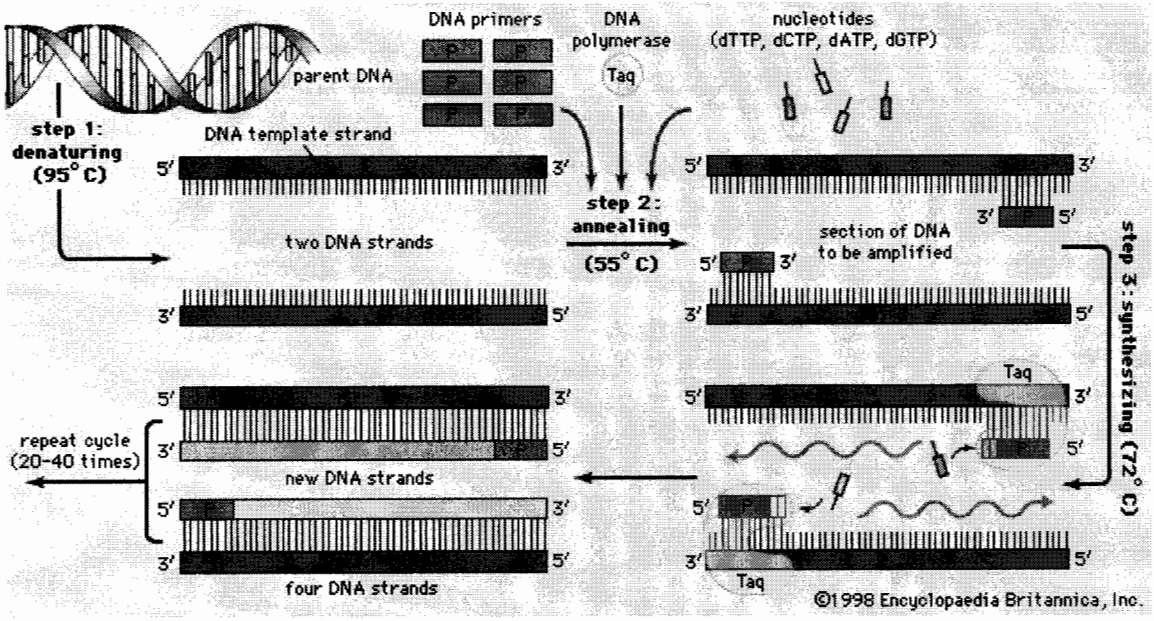

Figure 4. PCR replication, the primers attach at the 3' end of the DNA strand that they replicate.[13]

[11] http://www.idtdna.com/analyzer/applications/oligoanalyzer/

[12] David L. Nelson and Michael M. Cox, *Lehninger: Principles of Biochemistry.* (New York: W.H. Freeman, 2008), 992.

[13] PCR Lab. *PCR replication.*

A hairpin analysis detected the possible ways that the primer could bind to itself. If the primer has too many base pairs, it is less likely to bind to the DNA. Fewer base pairs result in a weaker bond and even if the hairpins form, they will break when presented with the opportunity to bind to the larger strands of DNA. Because we wanted to keep the chances of forming unbreakable hairpins as small as possible, we decided that four base pairs would be the most we would tolerate in the analysis.

For example, if the primer analyzed was 5'-GGGAAGGGTTCCC-3', the potential hairpins are shown in Figure 5.

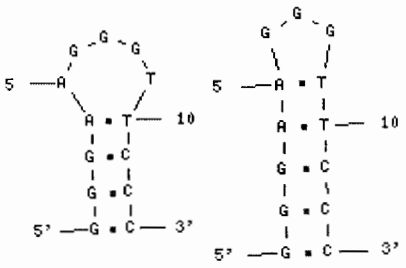

Figure 5. , The potential hairpins of 5'-GGGAAGGGTTCCC-3'

The hairpin on the left has a four base pair hairpin, but the hairpin on the right has a five base pair hairpin, making this primer unsuitable.

If the primer is changed to 5'-GAAAAGGGTTCCC-3', the potential hairpins change, as shown in Figure 6.

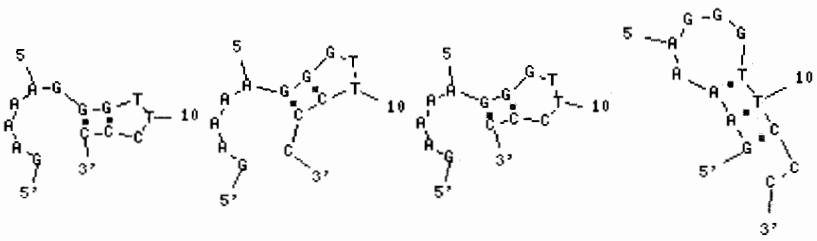

Figure 6. The potential hairpins of 5'-GAAAAGGGTTCCC-3'

Although there are more potential shapes for the hairpin to take, there is only one hairpin that has three base pairs. The rest are all two base pair hairpins, which are usually not significant. This primer can then be analyzed for possible homodimerization and heterodimerization.

Homodimerization analysis detects the number of base pair bonds that could be formed between two copies of the same primer, shown in Figure 7. Heterodimerization analysis detects the number of base pair bonds that formed between the forward and reverse primer.

```
5' GAAAAGGGTTCCC
       |||  :::
3'     CCCTTGGGAAAAG


5' GAAAAGGGTTCCC
       ||   ::
3'    CCCTTGGGAAAAG
```

Figure 7. Homodimer example

Again, these base pairs are not a cause for concern, because the most base pairs formed is three. In Figure 7, the straight lines indicate base pairs formed and the dotted lines show that there is attraction between those base pairs, but they do not form solid bonds. Lastly, it must be checked whether the forward and reverse primers will bind to each other significantly, forming a heterodimer. Again, this will result in a failure to bind to the DNA and replication of the desired segment.

Our ideal criteria for the primers were for them to have no more than four base pairs in a homodimer, heterodimer, or hair pin. Later on in the semester we discovered a different primer designing website[14], which would not only analyze sequences, but would also pick out primers that were most likely to bind well to DNA. This was a very important discovery, as it greatly

[14] http://primer3.wi.mit.edu/

reduced the amount of time needed to analyze different combinations. This particular website also formatted the forward and reverse primers automatically, reducing the chances of error.

Another criteria in analyzing primers is their melting point. Because PCR works by heating the DNA and primers to separate them and then cooling for binding, a high melting point is desirable. Because cytosine and guanine are bound together by three hydrogen bonds and adenine and thymine are held together by two hydrogen bonds, a high number of cytosine and guanine will lead to a higher melting point.[15] The forward and reverse primers must also have similar melting points in order to be successful.

The fungi that were used in primer analysis were *Aspergillus oryzae, Aspergillus niger, Aspergillus flavus, Aspergillus clavatus, Trichophyton rubrum, Leptosphaeria maculans, Kluyveromyces lactis, Ustilago maydis, Penicillium marneffei,* and *Talaromyces stipitatus.* These were analyzed in various combinations to ascertain which appeared to display more conservation. No more than two fungi from the same genus were used in an analysis, which all included either three or four different kinds of fungi.

We grew and harvested large amounts of the fungus by pouring a pan of agar and placing a Teflon sheet over it and then seeding the pan with fungus. The agar solution was prepared according to the following recipe and directions:

15g of Bacto-Agar (Difco) and 15g of soluble starch (Fisher Scientific) were measured into one 1L Erlenmeyer flask. 5g of yeast extract powder, 1g of dibasic potassium phosphate (J.T. Baker), 0.5g magnesium sulfate (Fisher Scientific), AERWand 100µl of Vogel's trace elements

[15] David L. Nelson and Michael M. Cox, *Lehninger: Principles of Biochemistry.* (New York: W.H. Freeman, 2008), 272.

solution (10-19-10) were measured into another 1L Erlenmeyer flask. Then 500ml of distilled water was added to each flask and they were swirled to mix. The mouths of the flasks were covered with a piece of tin foil and autoclaved to ensure sterility.

The contents of the first flask were poured into the second flask and then swirled to mix. If the agar had solidified on the bottom of the flask, it was warmed over the flame of a Bunsen burner. It was important that the flask was swirled continuously and that heat protective gloves are worn during this step. When the solution appeared to be completely mixed and there are few to no lumps of solid agar, the plates were poured using sterile technique, flaming the mouth of the flask whenever the foil was taken off or put on again. If Teflon was being used to make harvesting easier, it must be wrapped in tinfoil and autoclaved. The sterile Teflon can then be laid over the agar after it has solidified somewhat, it should not sink when placed on the agar.

The seeded plates were placed in the oven and monitored over the next few days. They were harvested when the fungus was actively growing, indicated by the color. Beakers of water were kept in the oven to ensure that the humidity in the oven remained constant. Upon harvesting, the fungus was put into sterile test tubes in approximately five gram portions. The test tubes were then stored in the -80°C freezer. This was later found to be poor way to store the fungus as it led to lower quality DNA.

The Vogel's trace elements solution used was prepared by a prior student according to the following recipe.[16] In a beaker, measure out 95 ml. distilled water and dissolve the following elements successively while stirring at room temperature:

[16] "Stanford Neurospora Methods" *fgsc.net* last modified September 2004, http://www.fgsc.net/methods /stanford.html.

Citric acid, 1 H20	5. 00 grams
ZnSO4, 7 H20	5. 00 grams
Fe(NH4)2(SO4)2, 6 H20	1. 00 gram
CuSO4, 5 H20	0. 25 gram
MnSO4, 1 H20	0. 05 gram
H3BO3, anhydrous	0. 05 gram
Na2MoO4, 2 H20	0. 05 gram

The resulting total volume will be about 100 ml. 1 ml of chloroform is added as a preservative and the mixture can then be stored at room temperature.

The methodology that we followed for DNA extraction was originally developed for yeast, but we have adapted it to be used with TL.[17] Different amounts of fungus were used in this extraction, it was found that about 0.01 grams of fungus per 1.5ml centrifuge tube seemed to have the best results.

Approximately 0.01g of TL is placed into a sterile 1.5ml centrifuge tube. 100µl of LiOAc is added to each tube and then vortexed to mix. The tubes are then incubated in a water bath for five minutes at 70°C. The tubes are then removed from the water bath and 300µl of absolute ethanol is added. They are vortexed to mix and then centrifuged for three minutes at 14,000RPM. After centrifuging, the tube contained a pellet at the bottom and a clear, colored layer of supernatant on top. The supernatant of each tube was pulled off using a micropipette and discarded. 300µl of 70% ethanol was added to each tube and then the pellet was re-suspended by vortexing. The tubes were then centrifuged for three minutes at 14,000RPM. Again, there was a pellet in the bottom of the tube and a clear, colored layer of supernatant. This layer of supernatant was generally lighter than the first layer. The supernatant was pulled off using a micropipette and discarded. The tubes were then placed on their side while open to allow the last

[17] Looke, Marko, Kersti Kristjuhan, and Arnold Kristjuhan. "Yeast genomic DNA extraction with LiOAc-SDS." *Biotechniques* 51.6 (2011): 325-327, 395.

of the ethanol to evaporate. When the pellets appeared dry, but not crusty, 300µl of 1xTE solution was added to each tube. They were then vortexed to re-suspend the pellet. The tubes were then centrifuged for a final time for 30 seconds at 14,000RPM. The supernatant contains the extracted DNA and was pulled off and kept.

Ultraviolet-visible light absorbance spectroscopy (UV-Vis) at 254nm was used to confirm the presence of DNA. Although the fungus used was harvested during the rapid growth phase and has a slight color, it does not absorb in a range that complicates the quantitation of DNA. All samples were run using 10µl of the DNA extract and 990µl of the distilled water. Cuvettes were rinsed out using distilled water in between each run.

The presence of DNA was further confirmed through agarose gel electrophoresis. The reference DNA used was Promega Benchtop 1kb DNA Ladder. A Liberty 1 High-Speed Gel System was used to run all gels and all were visualized on the UVP Benchtop UV Transilluminator using the BioDoc-It Imaging System. Figure 8 shows the image that was taken, the band of DNA is light, but can be seen as a line near the top of the gel. This indicated that there were large strands of DNA present as they did not move through the gel at all.

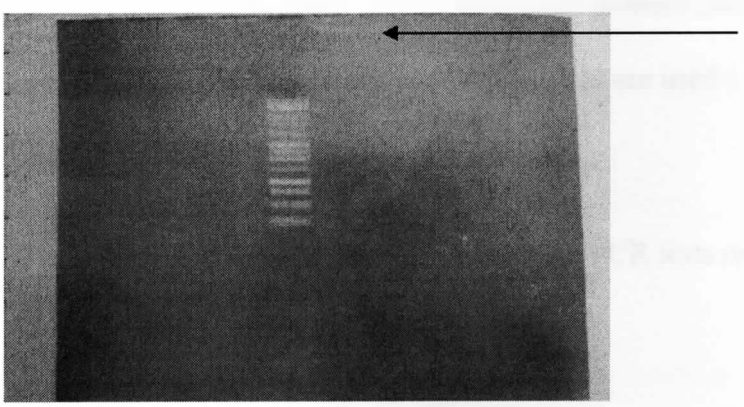

Figure 8. Agarose gel run of DNA extraction from 3/27/12. The arrow points to a band in well 8 that indicates the presence of DNA.

All agarose gels were prepared by measuring 0.7g of high grade agarose into a 250ml Erlenmeyer flask. 2ml of 50x TAE was added along with ~60 ml of distilled water. The mixture was then heated in the microwave in 30 second intervals until the agarose was fully dissolved, swirling the flask thoroughly after each interval. When the agarose had dissolved, ~40ml of distilled water was added to bring the total volume to 100 ml. The flask was again swirled to mix and then cooled to room temperature.

When the flask was cool enough to be handled comfortably, 5µl of Ethidium Bromide (EthBr) was added by micropipette. Because EthBr is a known carcinogen, take care to wear gloves during the addition and placing tips into the designated waste jar. The flask was again swirled to mix and poured. The comb with the needed number of teeth was inserted and the gel allowed to set.

When it was determined that DNA had been isolated, the next step taken was to run PCR tests using the primers that had been identified. PCR testing works by heating the DNA that is to be amplified to separate it and then cooling it enough so that designed primers will attach to the separated DNA strands. After the primers have been elongated by the DNA polymerase, the solution is heated to dissociate the elongated primers from the DNA, and the process is repeated several times. The temperatures and times that are used for the different cycles can be changed to find the ideal circumstances for the DNA.

The temperatures that were used in all of the PCR tests run were as follows: 50.2°C, 51.3°C, 52.4°C, 53.5°C, 56.0°C, 59.0°C.

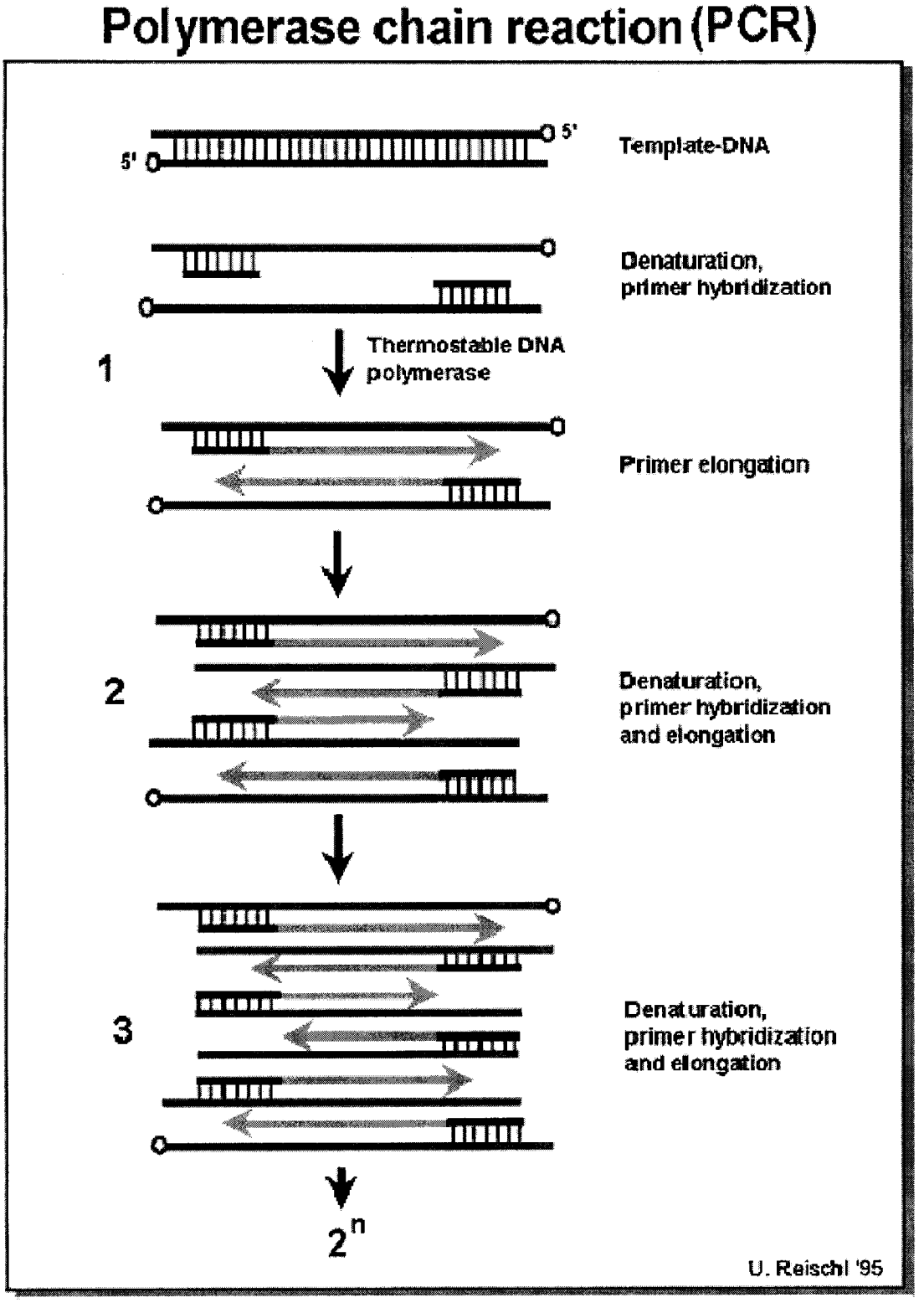

Figure 8. Process of PCR[18]

[18] Reischel, Udo. *Polymerase Chain Reaction (PCR).*

The mixes to prepare the PCR samples were determined by a basic formula. Each PCR tube contained:

- 1 µl of Left primer (50µM)
- 1 µl of Right primer (50µM)
- 1 µl of dNTPs (Quanta Biosciences 10mM dNTP mix)
- 1 µl of Mg^{2+} buffer (later switched to using DMSO buffer (3.5mM) from Dr. Jurgenson)
    - (low: 1mM, medium: 10mM, high: 30mM)
- 1 µl Taq polymerase
- 5 µl of DNA

The magnesium buffers already contained xylene cyanol dye, which helped in keeping the sample in the well when the gel was loaded. When we later switched to DMSO, a small amount of dye was added to the mix as it did not already contain dye. Taq polymerase is commonly used in PCR tests to extend the DNA primers. This was also recommended by Dr. Simet and sourced from Dr. Jurgenson's lab.

Primers were reconstituted with nuclease free water (Ambion) to yield 50 µmolar solutions. The tubes were inverted a few times to ensure that the primers had dissolved and were evenly distributed in the solution. The primer was then frozen at -18°C unless it was needed to run PCR tests. If a DNA dilution was used in running the PCR tests, nuclease-free water (Ambion) was used in the dilution.

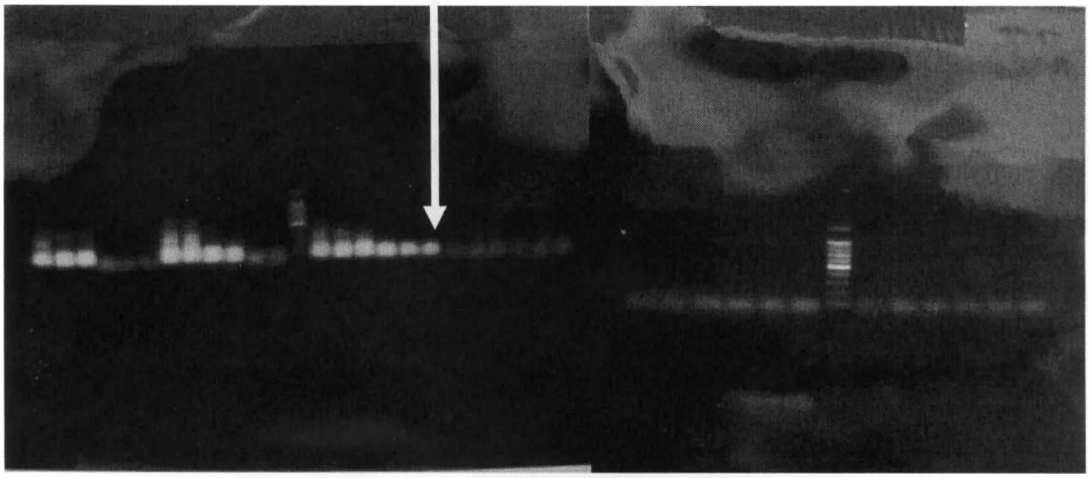

Figure 9. Gel images of PCR that was run at different DNA concentrations. The arrow points to the separation between the low DNA concentration samples on the left side and the high DNA concentration samples on the right side. There is a clear difference in the appearance of the bands.

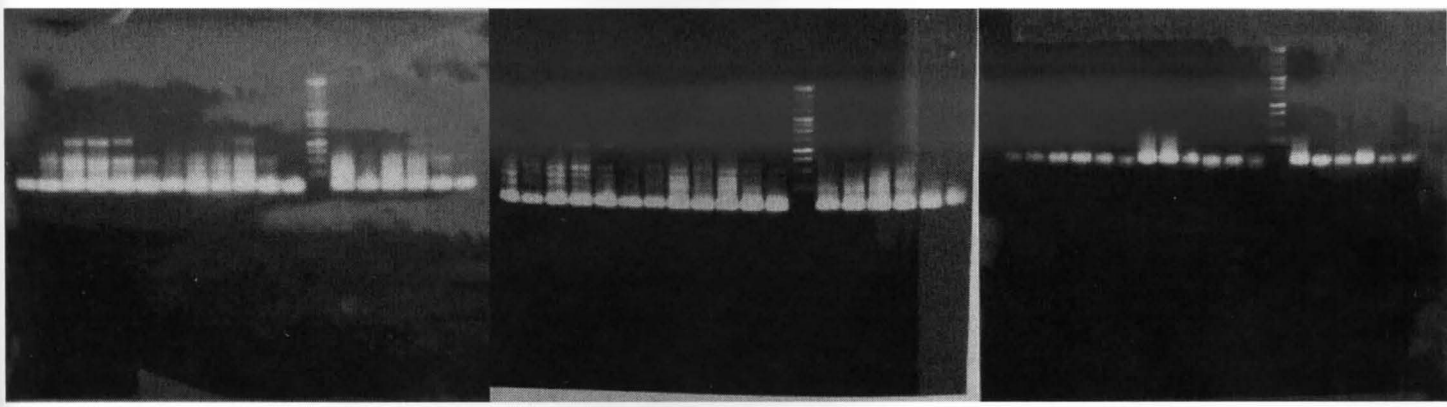

Figure 10. Agarose gel images of PCR tests that were run using new DNA extractions that rise in concentration from left to right.

The images in Figures 9 and 10 demonstrate that lower DNA concentration results in much clearer PCR results. The clear difference in results from using different concentrations of DNA are encouraging. This indicated that our DNA extraction method was successful and that smaller amounts of fungus could be used to successfully extract DNA in a concentration that could be used in PCR tests.

A successful method was finally discovered to isolate DNA from TL. It involved a phenol extraction, which is more aggressive in breaking apart cells than the previous methods tried. This approach is necessary because TL possesses melanocytes. Melanocytes are the cells that produce pigment in an organism. Although its color changing properties were part of the reason that TL was chosen as the target organism, it was not taken into account that melanocytes have thicker cell walls. This is why our earlier methods were unsuccessful as they were not powerful enough to break through the cell walls and extract the DNA.

The successful DNA extraction followed the procedure described below:

A 1.5ml microcentrifuge tube was filled two thirds full of powdered fungus, this fungus was ground using liquid nitrogen and a mortar and pestle. The fungus was suspended in 750ml of extraction buffer (50mM Na_2EDTA, 50mM Tris-Cl, pH 7.8, 3.0%SDS, 1% 2-mercaptoethanol). The tube was then incubated in a water bath at 65°C for one hour. 1.5 g of phenol was then mixed with 1 ml of $CHCl_3$, resulting in a light brown solution. After the incubation, half of the fungus mixture was placed into another 1.5 ml microcentrifuge tube and 375 µl of phnol:CHCl3 was added to each.

The tubes were then vortexed until a gray/black slurry formed and were then centrifuged for ten minutes at 12,000 ref. Three layers were visible, a lower organic layer, a middle layer with all the fungal material, and an upper aqueous layer. The organic and aqueous layers were light brown and clear while the fungal layer was gray black.

325 µl of the aqueous layer was removed from each tube and combined in a new sterile 1.5ml microcentrifuge tube. 700µl 1:1 CHCl3:isoamyl alcohol mix was added to the tube and it was vortexed until a creamy white/orange mixture formed. Then it was centrifuged for ten minutes at

12,000 ref. 20 µl of 3M NAOAc was added and the tube was then filled with IPOH, the mixture immediately turned cloudy.This was centrifuged for 30 seconds at 12,000 ref, forming a dark pellet. The tube was then centrifuged for an additional nine minutes and thirty seconds.

The supernatant was poured off and 300 µl of TE was added. It was warmed by hand for two minutes then incubated at 65°C for eight minutes in the water bath. After incubation, the pellet was re-suspended by flicking. 10µl 3M NaOAc was added and then the tube was filled to the 1.5 ml mark with 70% EtOH and then centrifuged for five minutes at 10,000 rpm.

The supernatant was collected in a sterile 1.5ml tube and the pellet rinsed with 70% EtOH. The EtOH was poured off and saved in another sterile 1.5ml tube. The tube with the pellet was inverted and placed into a dry well of the heat block set to 42°C. The tube was left there for 15 minutes. After the pellet had dried, 100µl of TE was added to dissolve it. The pellet did not immediately go into solution and so it was left in the refrigerator. A gel was later run using this extraction, as seen in Figure 11.

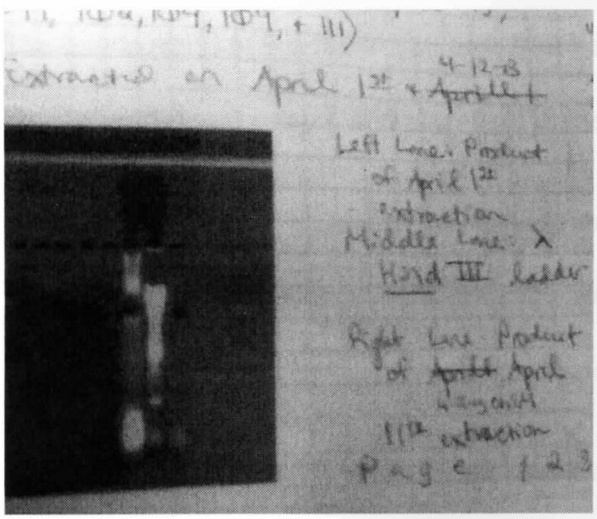

Figure 11. The image of the gel that displays successful DNA extraction, visible in the left lane, the middle lane is the λ Hind III ladder, and on the right is another unsuccessful extraction method.

Findings

As of right now, we have not yet identified a primer to begin sequencing the DNA polymerase α gene. Unfortunately, we have run into several issues in DNA extraction that have not allowed us to determine whether our primers are successful or not. We have, however, discovered a method that will successfully and consistently extract DNA.

We did learn that it is always important to run a preliminary gel testing each DNA extraction for the presence of DNA. Although our initial DNA extractions were successful, another one was started and left in the freezer for four weeks before it was completed. The power in the building was shut off and this may have allowed the DNA to disintegrate. This extraction was found to have no DNA after some PCR tests were run with no results.

We also discovered that our initial procedure for DNA extraction was isolating DNA, but not in good condition for PCR. One of the issues was how we were storing the fungal cells. After harvesting they were stored in sterile test tubes in approximately five gram amounts in the -80°C freezer. This allowed water to collect in the bottom of the test tube, which led to diluted DNA. The new method of storing it is to harvest it and place it on a small square of non-sterile aluminum foil and wrap it and place that in the -80°C freezer.

In our search for a DNA extraction method, we also tried a different procedure that we received from Dr. Jurgenson, but no DNA was extracted. This method relied on liquid nitrogen to freeze the dry frozen fungus and then using a mortar and pestle to lyse, or break down, the cells.

We did use the initial DNA that we had isolated to run some PCR tests using the primers that had been identified. Because conditions most conducive to successful amplification vary depending on the DNA used, several conditions were tested. Initially we used both low and high DNA

concentrations to identify which would yield better results. It was found that lower DNA concentrations appeared to work better. Because we do not know exactly what the DNA concentration is in our extraction, it appeared that our initial extraction was sufficiently successful to produce DNA that, even without dilution, was enough to overload the tests. All that was visible on the graphs were large smeared bands at the bottom of the gel.

Similarly, different amounts of magnesium were utilized. Low, medium, and high concentrations were studied in order to pinpoint what is necessary for this DNA. In the initial PCR runs, it appeared that medium or high magnesium concentrations were preferred. However, Dr. Jurgenson brought it to our attention that magnesium buffer is less successful in running PCR tests with this specimen and provided a different buffer to use in our experiments. Unfortunately, we ran out of good DNA and no successful PCR tests have been run using the new buffer.

Summary

Although we did not get as far as we had hoped in this project, we did discover several techniques that will benefit those who will take up the project next. This project was highly instructional in teaching the many ups and downs of chemical research and the necessity of constantly double checking and ensuring that results are reliable. Especially in research that involves DNA, it is very easy to destroy one's results by improper handling or storage of the sample.

Future researchers have a long road ahead, but we have been able to lay down a solid foundation that they can build on. We have successfully identified a DNA extraction method that works with TL. There is now a small library of primers that can be tested using the DNA to begin the process of genetic sequencing. We have found that the fungus appears to prefer lower

temperatures for successful PCR amplification, which will allow for much more focused experiments. It has also been found that a fairly low concentration of DNA is needed in PCR testing. All of this is important information that can be used in future research.

This is research that we hope will contribute to better understanding how DNA polymerase α works, not only in TL, but in other organisms. The potential for the medical applications of this information is tremendous and far reaching. Thorough understanding of this system could lead to treatments for diseases that are a result of unchecked cell growth, cancer, and disabilities due to a lack of cell growth, paralysis. It is an exciting possibility that drives this project forward towards a brighter future in treatment possibilities.

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