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Expressed sequence tag for DNA polymerase alpha in embryonic chicken brain

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EXPRESSED SEQUENCE TAG FOR DNA POLYMERASE ALPHA IN EMBRYONIC CHICKEN BRAIN

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Table of Contents

Introduction

Replicative DNA polymerases are enzymes located in the nuclei of cells. They have a role in the control of DNA synthesis in all cells, and problems with their function and control affect cell growth. One of these polymerases, DNA polymerase alpha ("pol α"), is a possible point of control for cell growth because it initiates DNA synthesis. The ability to selectively turn on or shut off this polymerase in cells could provide answers to problems ranging from cancer's unregulated growth to regeneration of tissues. However, control mechanisms for DNA pol α are not fully understood at this time. The central theme to be addressed is to learn more about the growth-related transcriptional control of DNA pol α. This project is the preliminary step looking into DNA pol α in embryonic chicken brain. Our approach is to quantify the amount of mRNA for the DNA pol α catalytic subunit in embryonic chicken brain cells at different developmental stages.

Background and Literature Review

DNA is the biological structure that encodes the genetic information of organisms and is contained in the nucleus of a cell. RNA is very similar to DNA as they are both nucleic acids, however it is found in both the nucleus and cytoplasm of cells. A single nucleic acid is made of a sugar phosphate backbone and a base (Figure 1).

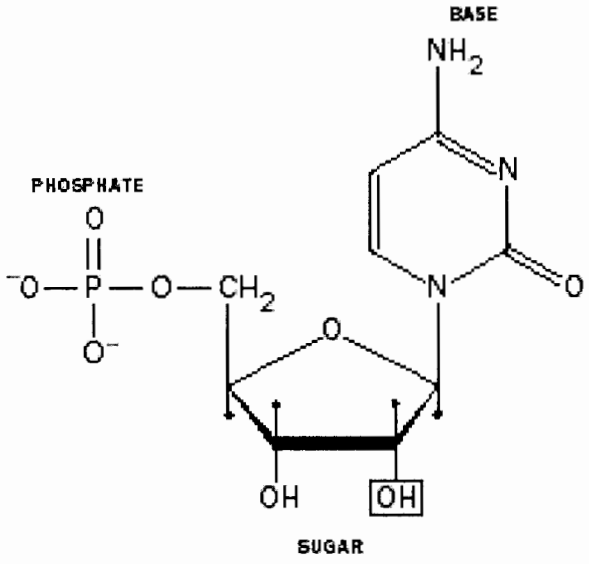


Figure 1. Sugar-phosphate base structure of a nucleic acid.

Individual nucleic acids have a polarity, which is labeled according to the carbon atom of the sugar from 1' to 5' (Figure 2).

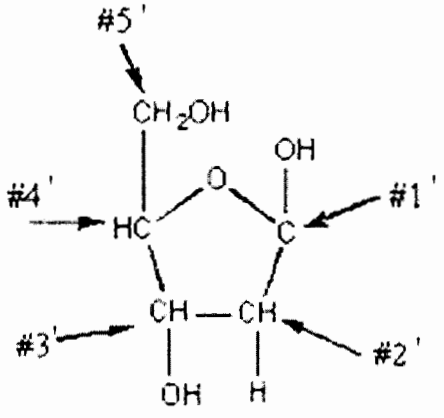


Figure 2. Nucleic acid sugar with numbered ring.

With RNA, the sugar is ribose and has a hydroxide group on the 2' carbon, while with DNA, it is deoxyribose on the 2' carbon (Figure 3).

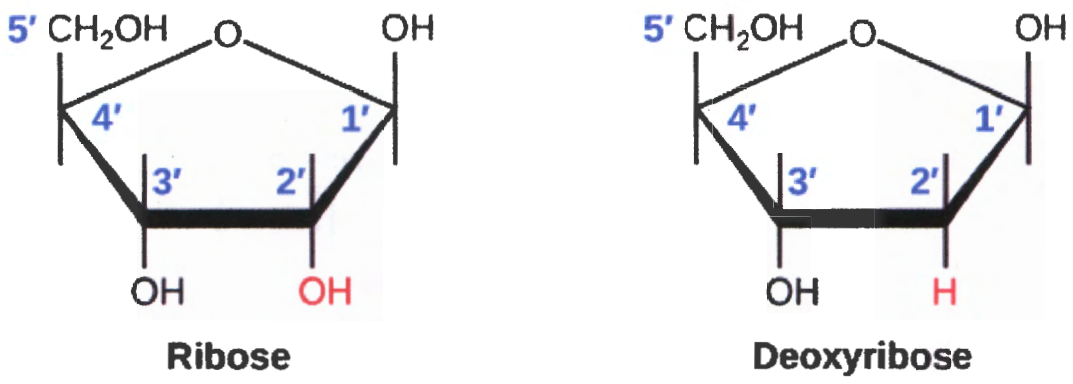


Figure 3. Pentose sugars in DNA and RNA.

There are five bases: adenine, cytosine, guanine, thymine, and uracil, and they are commonly abbreviated A, C, G, T, and U. Adenine, cytosine, and guanine are found in both RNA and DNA. Thymine is only found in DNA and uracil is only found in RNA; they are structurally similar and are homologous. Cytosine pairs, or forms bonds, with guanine, while adenine pairs with thymine or uracil. Individual nucleic acid chains are formed by linking sugar phosphate backbones, as can be seen in Figure 4. This form of DNA is called single-stranded DNA.

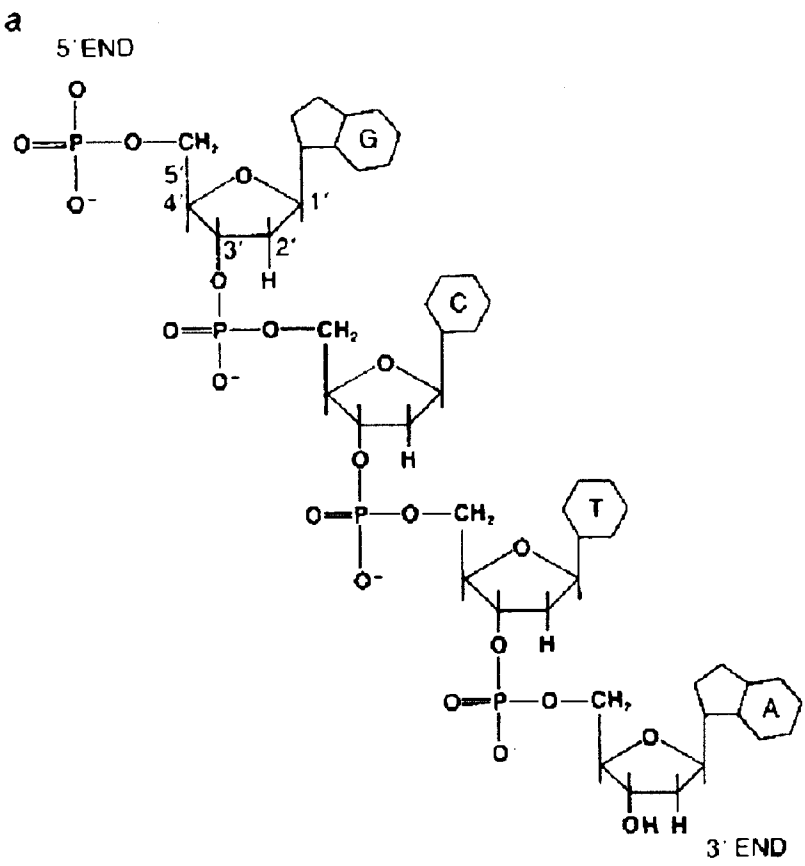


Figure 4. Single-stranded DNA.

These chains have a directionality to them; the end with the phosphate is labeled the 5' end and the end with the hydroxide group is labeled the 3' end. Two single-stranded chains of nucleic acids can associate together and are linked through hydrogen bonding of the C-G and A-T/U base pairs. However, the two strands do not point in the same direction, they point in opposite directions (see Figure 5).

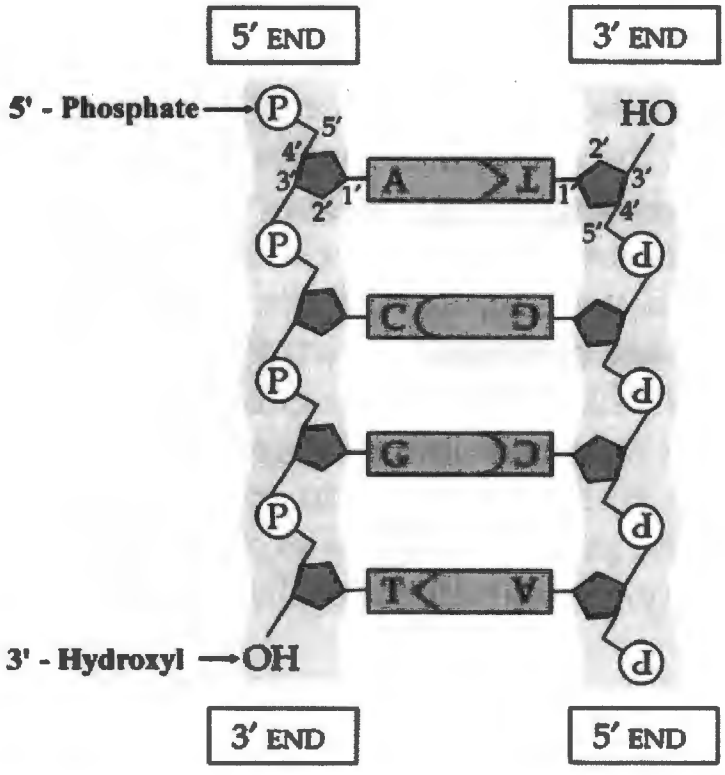


Figure 5. Individual strands point in opposite directions in double-stranded DNA.

This form curls naturally to form a double helix and is called double-stranded DNA (Figure 6). DNA most often exists in its double-stranded form, but single-stranded DNA can also exist as short lengths for a short time.

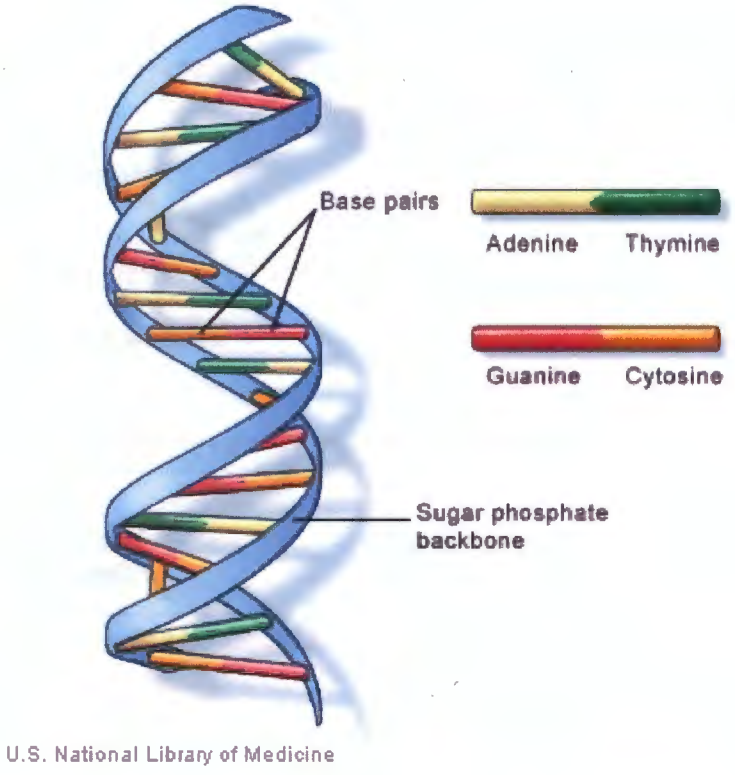


Figure 6. Schematic representation of double stranded DNA.

Before a cell can divide, it must create another copy of its DNA, so DNA replication, or copying, is required every time a cell divides. DNA replication is done by a complex of enzymes, one of which is DNA pol α, and is started by unwinding the two strands. Next, each single-stranded DNA is primed with RNA, creating a short double-stranded section where replication will start. Replication occurs in only one direction, however as stated earlier the two strands of DNA point in opposite directions. Therefore, one strand, the "leading" strand, can be primed once with RNA and the primer can be extended with DNA continually. The other strand, the "lagging" strand, must be primed multiple times and is replicated in short pieces (Figure 7). Thus, DNA pol α is very important because it primes DNA to be replicated.

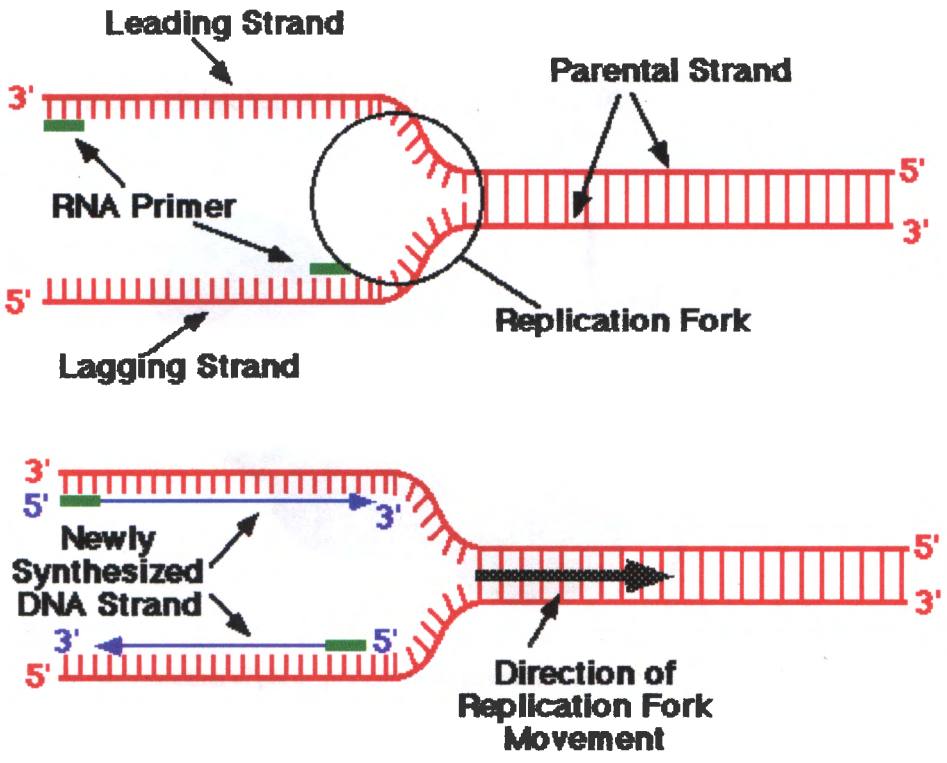


Figure 7. DNA replication and priming.

Pol α was discovered in 1975 and is distinct from other polymerases because of its ability to create primers of RNA to start DNA replication, and also the ability to extend the primers with DNA (Hubscher, Maga & Spadari, 2002, 134). Because of these functions it is the "only enzyme that can initiate DNA chains *de novo,"* or by itself (Foiani, Lucchini & Plevani, 1997, 424). Pol α creates primers of approximately 10 nucleotides and extends them with DNA for about 20-30 nucleotides, but it is not the polymerase that copies most of the DNA nor is it involved in DNA repair (Hubscher et al., 2002, 141 and Kornberg & Baker, 2005, 201).

Pol α is made up of 4 polypeptide chains: a large catalytic subunit that extends DNA, a B-subunit that provides support and possibly control, a small primase subunit that extends RNA, and a large priming subunit that provides support, as shown in Figure 8 (Johansson & MacNeill, 2010, 344). The catalytic subunit, which is the subunit in question, forms into a 3-dimensional conformation that resembles a right hand; the fingers position the template DNA and the

nucleotides to be added, the thumb binds to the template DNA, and the palm catalyzes the addition of nucleotides (Hubscher et al., 2002, 136 & 138-9).

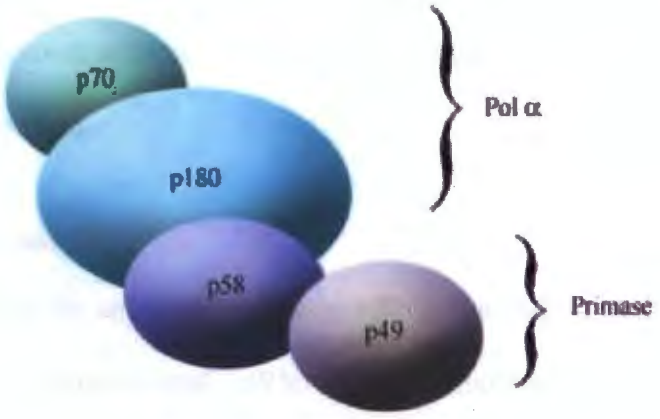


Figure 8. Schematic representation of pol α. p180 is the large catalytic subunit, p70 is the supportive B subunit, p58 is the supportive priming subunit, and p49 is the small primase subunit.

Cells have many ways to control their functions, one of which is by using checkpoints; certain mechanisms have to occur before a cell is allowed to proceed with an action. Although we do not fully understand the mechanisms of these checkpoints, pol α is implicated in the regulation and inhibition of DNA synthesis (Foiani et al., 1997, 426). Thus pol α is expected to be a check point for growth. Pol α and other polymerases have also been shown to be regulated by phosphorylation, the addition of phosphate groups (Foiani et al., 1997, 425). Beyond this control by small molecules, not much is known about the transcriptional control of pol α, which determines if mRNA of the pol α gene is being made or degraded. Thus there is still research to be done looking at the growth-related transcriptional control of pol α.

The chosen system for this research is the embryonic chicken brain. The chicken genome is one third the size of a typical mammal and has a low amount of repetitive DNA: only 11% in chickens compared to 40-50% in mammals (Burt, 2005, 1692). Although the chicken genome is much smaller than the human genome, 75% of coding regions and 30-40% of regulatory

elements are conserved between chickens and humans (Burt, 2005, 1695). The smaller size, less repetition, and high amount of conserved DNA make the embryonic chicken brain a very good but simpler model for study. These advantages, along with the availability of incubated eggs at various stage of development and ease of manipulation, make embryonic chicken brain a very good choice for this research.

Methods Background

This project will measure the amount of messenger RNA (mRNA) present in embryonic chicken brain at different developmental stages. mRNA is the medium through which the genetic instructions of the DNA are taken from the nucleus and utilized. Each cell only has one copy of DNA, but it can have multiple copies of mRNA of a gene. The mRNA is able to leave the nucleus and enter the cytoplasm, where proteins are constructed using its information (see Figure 9).

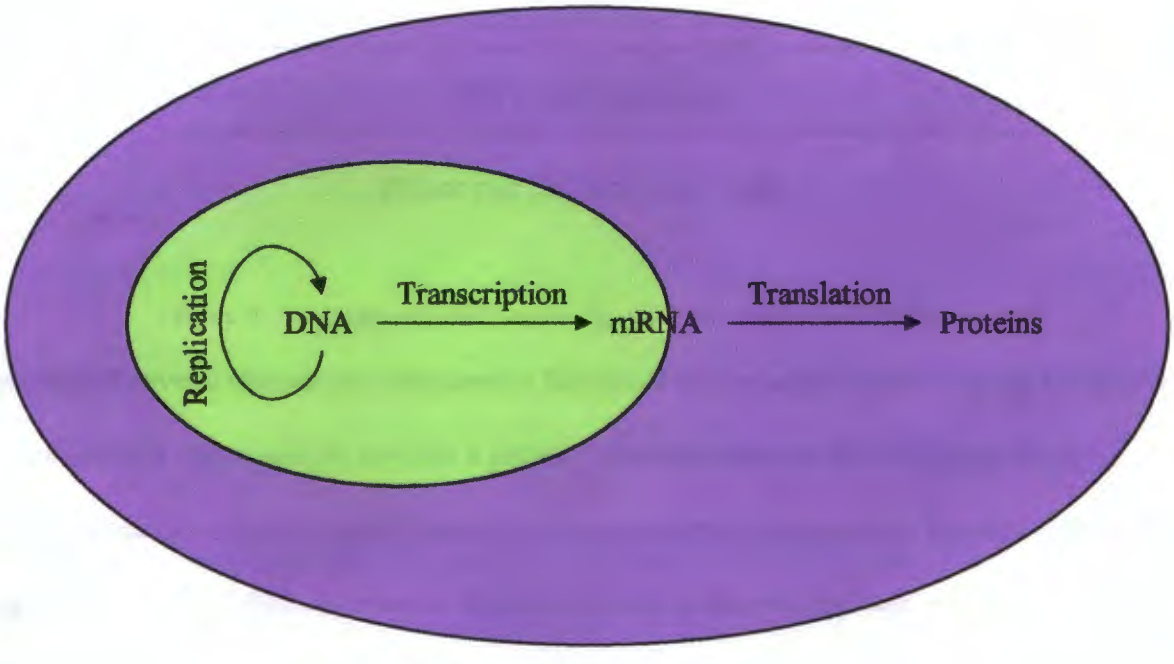


Figure 9. Relationship of DNA, mRNA, and proteins.

The sequence for the DNA pol α catalytic subunit will be the same in mRNA and DNA, except for substituting Us for Ts in RNA. The sequence of DNA that codes for the DNA pol α catalytic subunit must be isolated from the embryonic chicken genome (the entire DNA sequence). To do this an expressed sequence tag (EST) will be used. An EST is a short section (approximately 600 base pairs) of single-stranded DNA that will associate with its complementary section of single-stranded DNA. Since it should only pair with its complementary segment, an EST will be unique and can be used to isolate a single section of DNA. Thus, there are six basic steps in this project: primer pair design, polymerase chain reaction, agarose electrophoresis, cloning, sequencing, and reverse polymerase chain reaction.

First, to create an EST even smaller sections (approximately 20 base pairs) of DNA must be designed to function like bookends around the EST. These bookends, called primer pairs, are unique to the ends of the EST (see Figure 10).

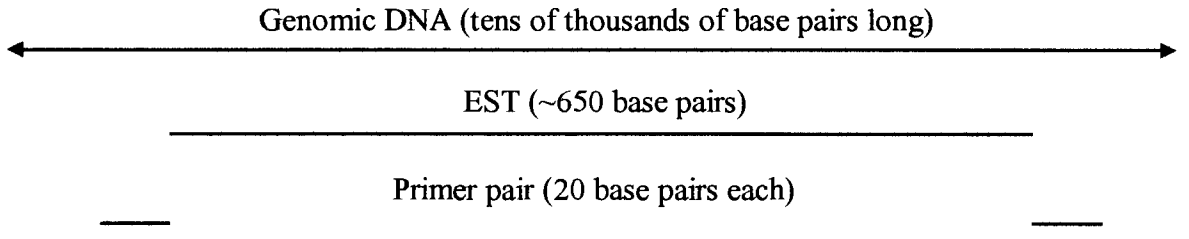


Figure 10. Comparative size and location of genomic DNA, an EST, and a primer pair.

Genomes of several species are compared to find areas of consensus, which suggest a conserved section of DNA that would be suitable a primer. Websites such as BLAST (basic local alignment search tool) are used to find consensus among many sequences. Primers should be long enough "so that their sequence is virtually unique in the genome" and will not match the genomic sequence in more than one place (Arnheim & Erlich, 1992, p. 135). Primers and ESTs

should also be checked so they do not fold upon themselves to create hairpins (folding and pairing), associate with themselves to create homodimers (associating with another primer), or associate with the other primer to create heterodimers. If any of these were to occur, the primer would not be able to associate with the template DNA as it is supposed to. Also, primers with higher G and C pair content will bind more strongly to the template, which increases the temperature at which the primer and template will dissociate, which is important for polymerase chain reaction.

Second, polymerase chain reaction (PCR) is a laboratory technique used to create a large amount of a specific section of DNA very quickly with a simple reaction (Arnheim & Erlich, 1992, p. 132). As the name suggests, PCR is a chain of reactions, or cycles, repeated over and over. Each cycle consists of denaturing the DNA, which separates the two intertwined DNA strands, followed by an annealing step, which allows the primer to associate with the DNA, and an extension step, when the DNA is replicated (Figure 11).

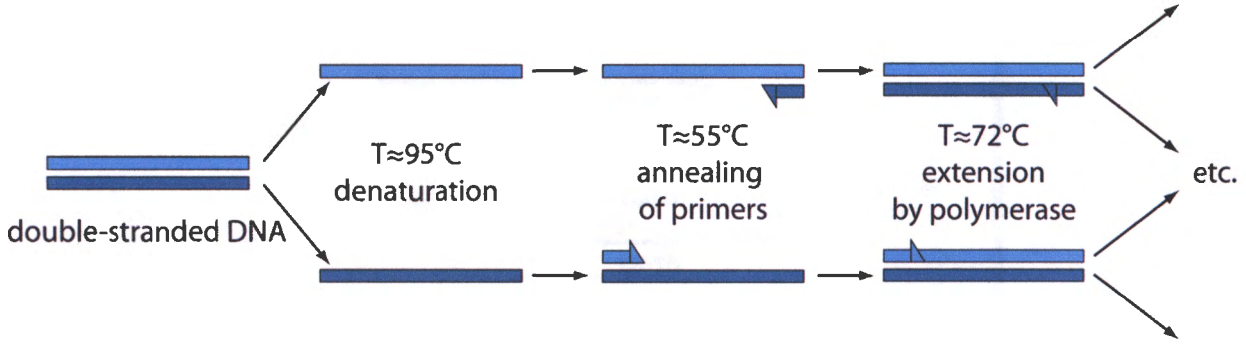


Figure 11. Schematic representation of PCR cycles.

For PCR to be successful it requires DNA containing the specific section, primers to isolate the section, an enzyme to copy the DNA, nucleotides to use as building blocks, and appropriate buffers and salts. The DNA can be purified or lysed (split open) cells can be used, which makes this technique very flexible. The enzyme used to replicate the DNA is a "heat-resistant DNA

polymerase from *Thermus aquaticus* (Taq)", commonly called Taq, which can withstand the high temperatures required for denaturing the DNA (Arnheim & Erlich, 1992, p. 133). The beauty of PCR is that each cycle creates new templates, causing exponential copying of the EST (the PCR product), as shown in Figure 12.

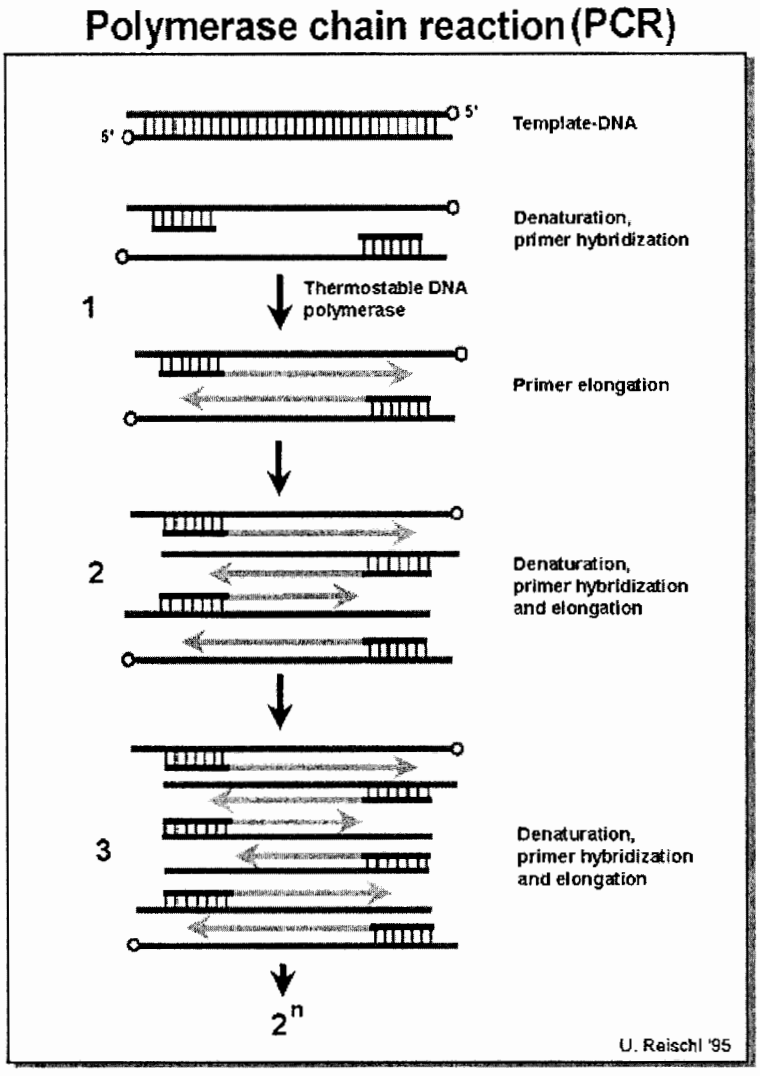


Figure 12. Creation of new templates and exponential copying of product (the EST).

Third, agarose gels are used to conduct electrophoresis, a process by which DNA and RNA are separated based on the number of base pairs (i.e. size) using electricity. The sugar-phosphate backbone in DNA and RNA is negatively charged, so when an electric current is applied to the

agarose gel, they will move toward the positive electrode. The agarose gel serves as a physical barrier to the DNA by creating a matrix with pores for the DNA and RNA to move through; the higher the percentage of agarose, the more difficult it is to move through. Thus, the smallest pieces travel furthest through the gel because they are able to move through the pores more easily than the larger pieces. Standard solutions with DNA of known size are run so that the approximate size of DNA and RNA in other lanes can be deduced (see Figure 13).

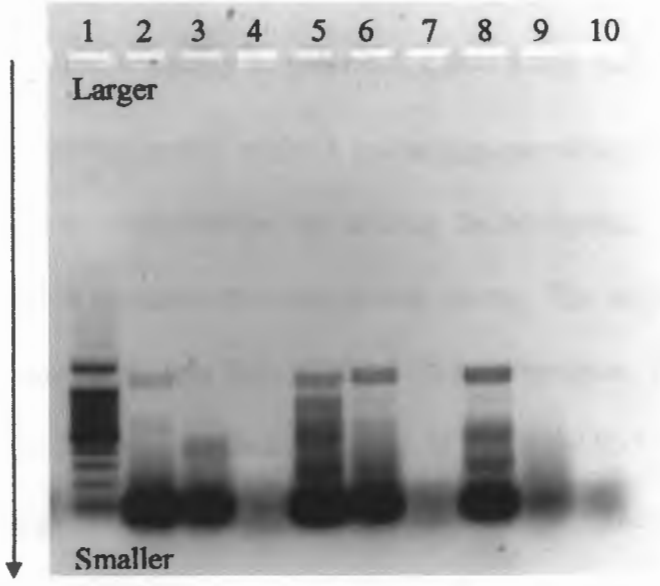


Figure 13. Sample agarose after electrophoresis has been run. Lane 1 contains the standard and lanes 2-10 have different conditions with a primer pair.

In Figure 13 the dark blots at the bottom are the primer pairs, which are only 20 base pairs long and run through the gel quite easily. The other bands in lanes 2, 3, 5, 6, and 8 are where the primer pair associated with the template DNA. Multiple bands were produced, so the primer pairs associated in multiple places and thus amplified multiple sections. Using this technique, PCR products will be run to see if the primer pair was successful at isolating a single, correct sized EST because a single band will be produced.

Fourth, the EST can be cloned using a pGEM vector, a small piece of DNA into which foreign DNA can be incorporated, and highly competent *Escherichia coli (E. coli)* cells, which take up DNA easily. The EST, which was amplified by PCR, can be incorporated into the pGEM vector, which can then be added to a culture of the *E. coli* cells. The cells take up the vector and replicate it very efficiently, creating many more copies of the vector and the EST. Then restriction endonucleases, enzymes that cut DNA in specific places, can be used to isolate the section of DNA after lysing the cells. This is a much faster more efficient way to create the number of copies of the EST necessary for sequencing than doing multiple runs of PCR.

Fifth, the cloned and amplified section of DNA can be sequenced with a modified Maxam & Gilbert method using an infrared-sensitive dye as a tag. In this method, the EST is cut after As, Cs, Ts, and Gs in the DNA sequence and marked with the tag. The sequence can be assembled by an instrument that detects the tags from all the different fragments. The sequence could then be checked by using the dideoxy sequencing method, in which the EST is primed and replicated in the presence of a tagged nucleotide with a dideoxyribose sugar. The dideoxynucleotide serves as a chain terminator, creating many shorter fragments that are tagged as ending with A, C, T, or G and can be run on an agarose gel to determine the sequence.

And finally, after the EST has been confirmed, real time reverse transcriptase PCR can be used to see if the EST reveals differences in DNA pol α catalytic subunit mRNA levels. In this technique lysed cells are run through PCR with Taq enzyme and a reverse transcriptase enzyme, which can create DNA from an RNA template. Thus, the mRNA of the pol α catalytic subunit can act as a template for PCR to amplify the EST section within it. Once the PCR products are run on an agarose gel the concentration of the EST can be quantified and the relative amounts of the pol α catalytic subunit mRNA can be deduced. If there is a difference in the levels of the

mRNA across different developmental stages, then the exact stage at which the DNA pol α gene is turned on or off can be found. Then, the search for how it is controlled can begin examining embryonic chicken brains from that stage.

Materials and Methods

Primer Pair Design

The National Center for Biotechnology Information website was used to locate the DNA pol α catalytic subunit gene in several organisms: dog (Canis lupus familiaris; NCBI XM_537985), frog (Xenopus laevis; NCBI AF202992), chicken (Gallus gallus; NCBI XM_426792, and turkey (Meleagris gallopavo; NCBI XM_003203031.1). The gene sequences for these organisms, in FASTA format, were put through the Clustal W website sequence aligner to find consensus sequences for potential use as primer pairs. These potential primer pairs were then evaluated using the Integrated DNA Technologies Oligo Analyzer website. Each was evaluated for its percent GC content, melting temperatures, hairpin formation, homodimer formation, and each pair was evaluated for heterodimer formation. Primers were ordered from Integrated DNA Technologies and constituted with Nuclease-Free Water (Ambion) to a concentration of 50μM.

RGK Primer Pair 1 ~300 Base Gap

Left Primer
- 5' GATGATGAGGACTTTGATGA 3'
- % GC = 40.0%
- Melting temperature = 49.4°C
- Hairpins = 7 with maximum base pairing of 2
- Homodimers = 7 with maximum base pairing of 2

Right Primer
- 5' TTTTATTGGCTGGATGCTTA 3'
- % GC = 35.0%
- Melting temperature = 49.7°C
- Hairpins = 1with maximum base pairing of 2
- Homodimers = 9 with maximum base pairing of 2

Heterodimers for pair = 8 with maximum base pairing of 2

RGK Primer Pair 2 ~600 Base Gap

Left Primer
5' CAGGATGATGACTGGATTGT 3'
% GC = 45.0%
Melting temperature = 52.0°C
Hairpins = 3 with maximum base pairing of 2
Homodimers = 10 with maximum base pairing of 3
Right Primer
5' GATGAACCTATGGAAGCAGA 3'
% GC = 45.0%
Melting temperature = 51.7°C
Hairpins = 3 with maximum base pairing of 2
Homodimers = 9 with maximum base pairing of 2
Heterodimers for pair = 15 with maximum base pairing of 3

RGK Primer Pair 3 ~600 Base Gap

Left Primer
5' AGGACAATGATGGGTGGACG 3'
% GC = 55%
Melting temperature = 57.2°C
Hairpins = 2 with maximum base pairing of 2
Homodimers = 8 with maximum base pairing of 2
Right Primer
5' AAGGGAGAGAGATTTTGATG 3'
% GC = 40.0%
Melting temperature = 49.4°C
Hairpins = 3 with maximum base pairing of 2
Homodimers = 6 with maximum base pairing of 2
Heterodimers for pair = 7 with maximum base pairing of 3

Isolation of DNA

Originally, Chicken Genomic DNA (Novagen, 249µg/mL) was used for PCRs, but this product was discontinued by the company for an unknown reason. Next, Control Genomic DNA: Chicken Female (Biochain, 0.38µg/µL) was ordered but arrived too degraded to yield results. Finally, chicken DNA was extracted from chicken liver using a DNeasy Blood and Tissue kit (Qiagen).

Polymerase Chain Reaction (PCR)

A Veriti 96 Well Thermo Cycler (Applied Biosystems) was used to run PCRs. For the general schematic used for PCR reactions see Figure 14.

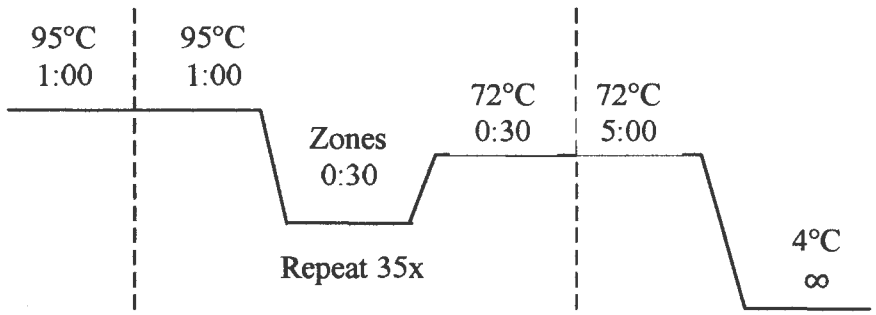


Figure 14. General PCR Schematic. Denaturing occurs at 95°C, followed by annealing at a variety of temperatures (marked "Zones"), and finally extension at 72°C.

The area marked "Zones" allows a gradient of temperatures so that primers may be tested around their melting temperature to determine what temperature is optimal for amplifying the EST.

PCR Mixes

Each PCR tube contained 10μL of mix, which consists of:

- 1μL of left primer
- 1μL of right primer
- 1μL of dNTPs (Quanta Biosciences 10mM dNTP mix)
- 1μL buffer
- 1μL Taq enzyme (from Dr. James Jurgenson)
- 5μL of chicken genomic DNA (diluted with Ambion Nuclease-Free Water)

Several different buffers were used: a set of low, medium, and high magnesium buffers with concentrations of 1mM, 2.5mM, and 4mM respectively, and a DMSO buffer obtained from Dr. Jurgenson with a magnesium concentration of 3.5mM. The concentration of the chicken genomic DNA was also varied: 10ng/μL, 5ng/μL, and 1ng/μL.

Agarose Electrophoresis

Electrophoresis was done with the Biokeystone Co. Liberty 1 High-Speed Gel System and a 0.7% agarose gel using the following recipe:

- 0.7g agarose (MP Biomedicals Inc. Agarose electrophoresis grade)
- 2mL of 50x TAE buffer (24g Tris, 3.7g EDTA, 5.7mL glacial acetic acid, H_2O to 7mL)
- 5μL of 10mg/mL ethidium bromide
- H_2O to 100mL

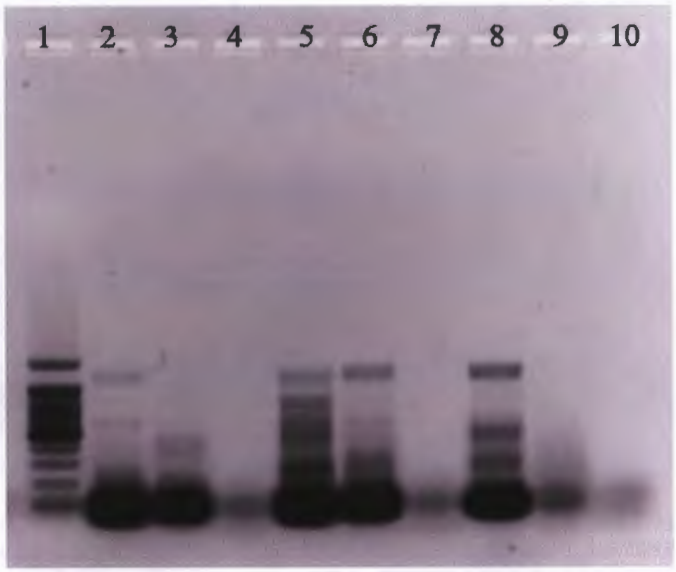
The agarose gel chamber was filled with 350mL of distilled water and each buffer chamber with 80mL of 1x TAE (diluted from the 50x stock). Each agarose gel was run at 220V for 15 minutes. A Promega BenchTop 1Kb DNA Ladder was used to judge the size of DNA fragments produced by PCR. The magnesium buffers contained xylene cyanol (XC), a coloring agent, and 5μL of the PCR product was used per lane in the gel (gels had 13, 19, or 26 lanes each). For the DMSO buffer, XC was used to dilute the DNA/H_2O mixture instead of the Ambion Nuclease-Free Water to obtain a concentration of 1μL XC per PCR tube and 5μL of the PCR product was used per lane in the gel.

Agarose gels were photographed using a UVP Benchtop UV Transilluinator with Bio Doc-It Imaging System in Dr. Nalin Goonesekere's lab.

Results and Discussion

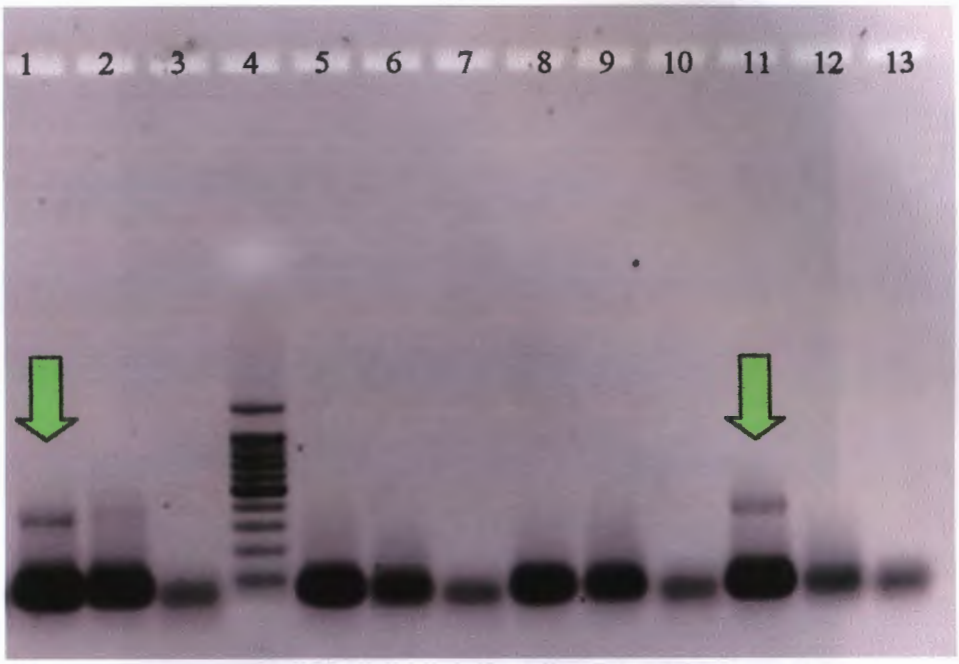
In September 2012, PCR mixes were run using all three primer pairs, three magnesium concentrations, and three DNA concentrations over a temperature gradient to determine the best conditions for amplifying the EST. Comparing all of the results for primer pair 1, it was most successful at 48°C at high and medium magnesium concentrations for all concentrations of DNA (see PCR 1). However, as the PCR image shows, this primer pair created multiple bands ranging

in size from 100 to 400 base pairs. Thus, the primer pair was not unique and did not create a single EST, and therefore was not appropriate for this research.



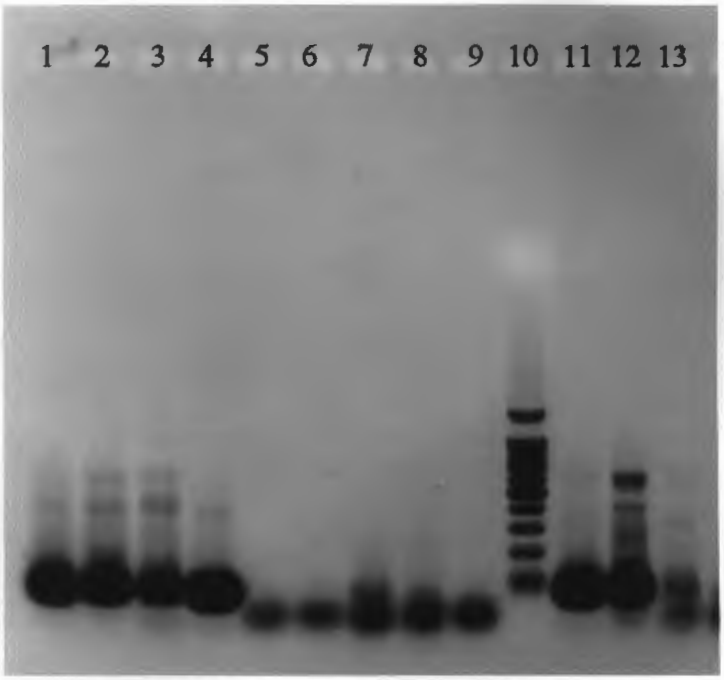
PCR 1. Sept 11, 2012. Lane 1, 1Kb DNA ladder; lane 2, low DNA high Mg+2; lane 3, low DNA medium Mg+2; lane 4, low DNA low Mg+2; lane 5, medium DNA high Mg+2; lane 6, medium DNA medium Mg+2; lane 7, medium DNA low Mg+2; lane 8, high DNA high Mg+2; lane 9, high DNA medium Mg+2; lane 10, high DNA low Mg+2; all at 48°C

Comparing all of the results for primer pair 2, it was most successful at 48°C and 50°C at high and medium magnesium concentrations for all concentrations of DNA (see PCR 2; marked with green arrows). This primer created one band of approximately 650 base pairs, just as predicted, indicating this primer as a good possibility for further investigation.



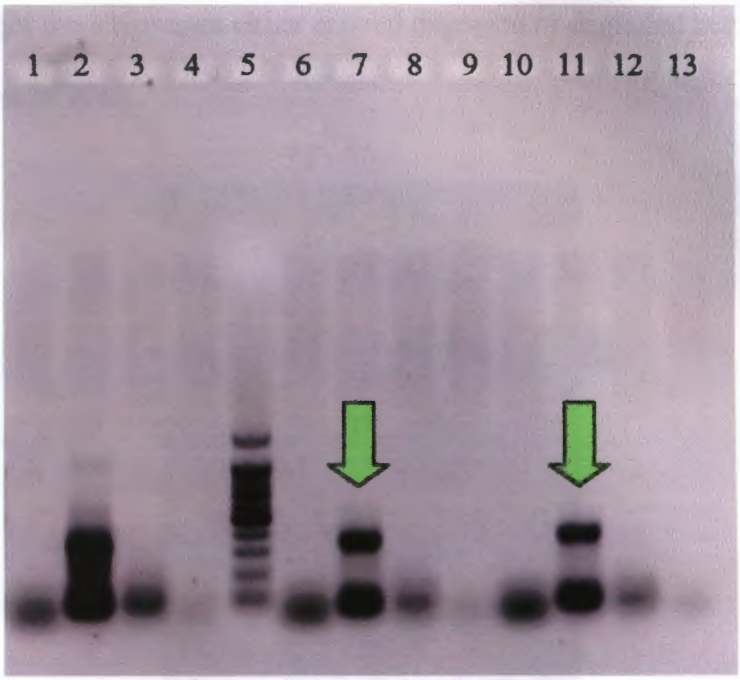
PCR 2. Sept 13, 2012. Lane 1, high DNA high Mg+2; lane 2, high DNA medium Mg+2; lane 3, high DNA low Mg+2; lane 4, 1Kb DNA ladder; lane 5, low DNA high Mg+2; lane 6, low DNA medium Mg+2; lane 7, low DNA low Mg+2; lane 8, medium DNA high Mg+2; lane 9, medium DNA medium Mg+2; lane 10, medium DNA low Mg+2; lane 11, high DNA high Mg+2; lane 12, high DNA medium Mg+2; lane 13, high DNA low Mg+2

Comparing all the results for primer pair 3 it was most successful at 48°C and 50°C at high and medium magnesium concentrations for all three concentrations of DNA (see PCR 3). However, as the PCR image 3 shows, this primer created multiple bands ranging in size from 100 to 500 base pairs and thus was unsuitable for further study.



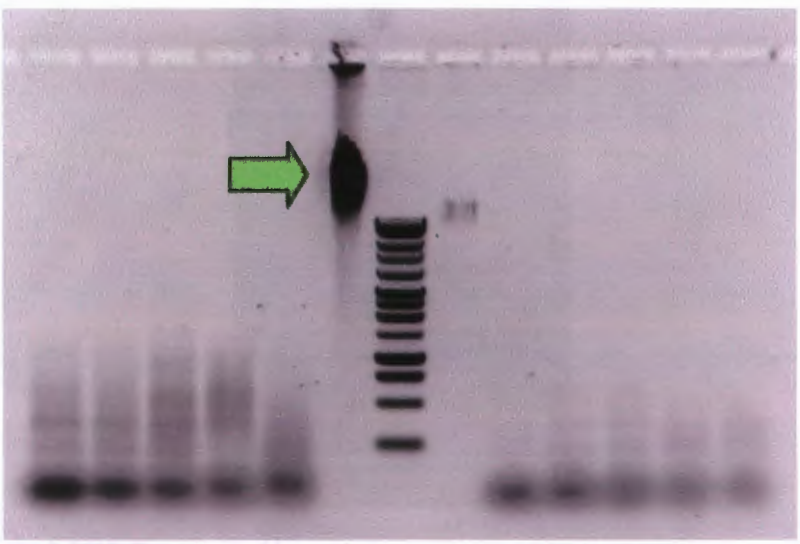
PCR 3. Sept 20, 2012. Lane 1, high DNA high Mg+2; lane 2, high DNA medium Mg+2; lane 3, high DNA low Mg+2; lane 4, low DNA high Mg+2; lane 5, low DNA medium Mg+2; lane 6, low DNA low Mg+2; lane 7, medium DNA high Mg+2; lane 8, medium DNA medium Mg+2; lane 9, medium DNA low Mg+2; lane 10, 1Kb DNA ladder; lane 11, high DNA high Mg+2; lane 12, high DNA medium Mg+2; lane 13, high DNA low Mg+2

Further investigation into the conditions for primer 2 (see PCR 4) revealed that it produced the cleanest band at 50°C and 51°C, with a high concentration of magnesium (4mM) and a high concentration of DNA (10ng/μL).



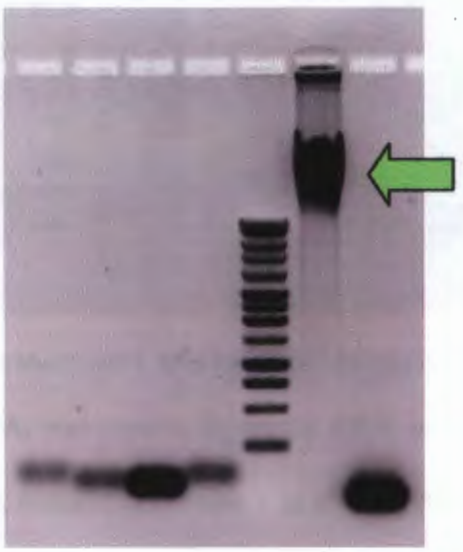
PCR 4. Oct 4, 2012. Lane 1, high primer; lane 2, medium primer, lane 3, low primer; lane 4, very low primer; lane 5, 1Kb DNA ladder; lane 6, high primer; lane 7, medium primer; lane 8, low primer; lane 9, very low primer; lane 10, high primer; lane 11, medium primer; lane 12, low primer; lane 13, very low primer. Lanes 1-4 were at 49°C, lanes 6-9 were at 50°C, and lanes 10-13 were at 51°C

During the gap between Oct 4, 2012 and Nov 1, 2012, the Novagen genomic chicken DNA degraded (see PCR 5).



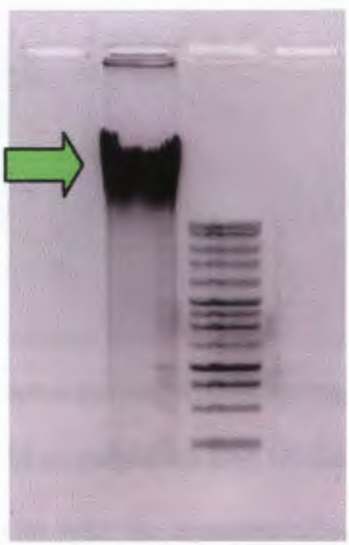
PCR 5. Nov 16, 2012. Smear marked by arrow shows genomic DNA has degraded into small pieces.

The next batch bought from Novagen either arrived degraded or degraded between Dec 6, 2012 and Jan 31, 2013 (see PCR 6).



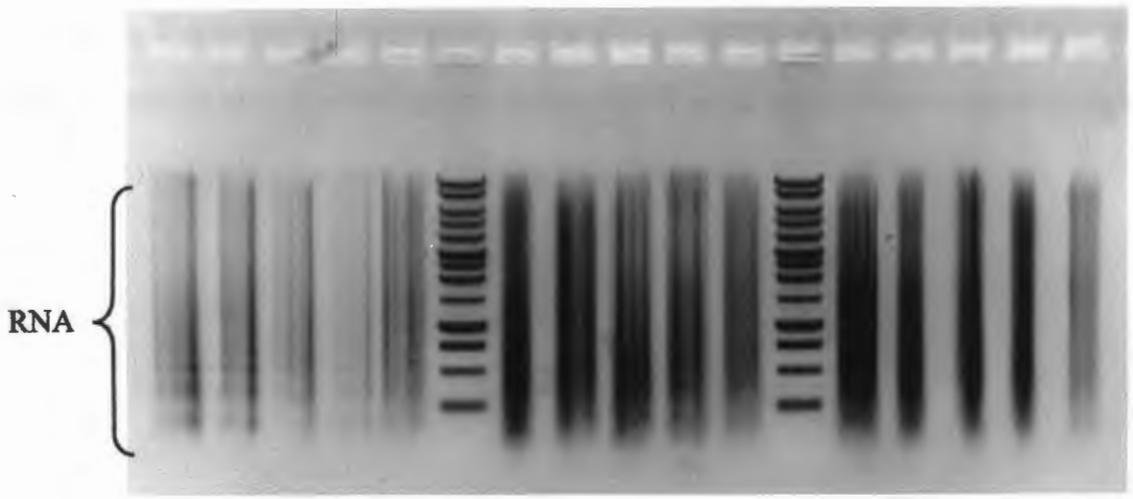
PCR 6. Feb 14, 2013. Smear marked by arrow shows genomic DNA has degraded into small pieces.

Novagen discontinued making genomic chicken DNA, so the next vial was bought from Biochain but was shown to be degraded upon arrival on March 1, 2013 (see PCR 7).



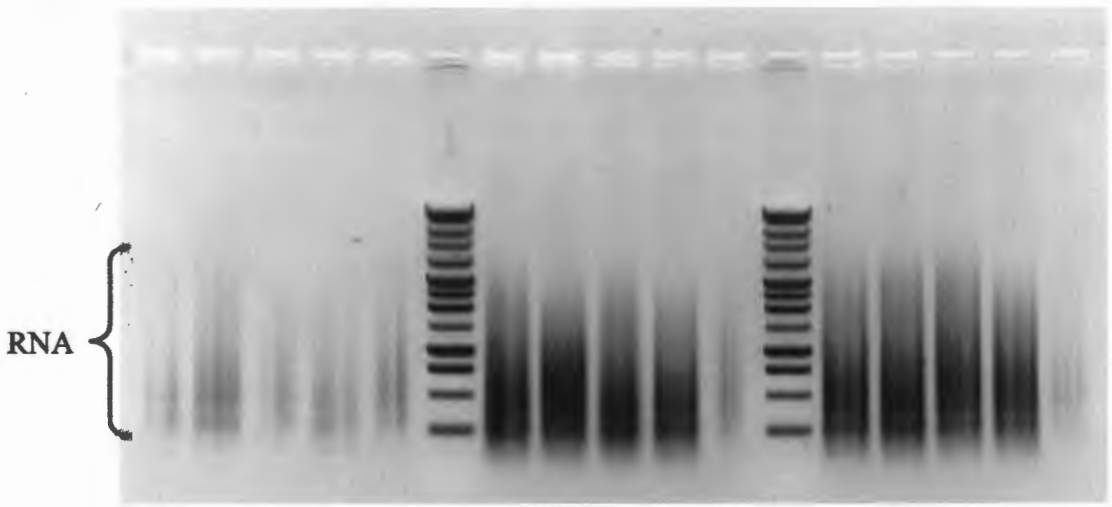
PCR 7. Mar 1, 2013. Smear marked by arrow shows genomic DNA has degraded into small pieces.

It was then decided to extract our own chicken genomic DNA from chicken liver. The results of our extraction showed that the DNA contained large amounts of RNA (see PCR 8).



PCR 8. Mar 27, 2013. RNA smears from 50bp to 1500bp.

RNase, an enzyme that cuts RNA, was used to digest the RNA, however the stock preparations on hand were ineffective. Then, sodium hydroxide was used to break down the RNA, which did have some effect (see PCR 9).



PCR 9. Apr 12, 2013. RNA has been broken into smaller pieces, resulting in smaller fragments and less smear.

In conclusion, primer pair 2 did yield an amplified EST of the expected size, however further analysis was interrupted by difficulties with the commercially-obtained genomic DNA and extracting our own chicken DNA. Future research on this project should be aimed at finding a reliable method to extract and purify chicken DNA, and then continue with the steps laid out in

this paper. This is the first step toward being able to measure the levels of mRNA for DNA pol α catalytic subunit in embryonic chicken brains, which could lead to learning more about the control of the DNA pol α gene and how to control the growth of cells.

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