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Preparation of MyoD mRNA for the Differentiation of Stem Cells into Muscle Cells

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Preparation of MyoD mRNA for the differentiation of stem cells into muscle cells

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Abstract

Embryonic stem cells (ESCs) are characterized by their two special properties: pluripotency and self-renewal. Pluripotency is the ability of ESCs to differentiate into any cell type upon expression of specific proteins called transcription factors. In order to induce differentiation, transcription factors specific for the particular cell type have to be introduced and expressed in the ESCs. The introduction of transcription factors can be achieved by using either DNA, RNA, or protein. According to the Central Dogma of Molecular Biology, DNA is first transcribed into messenger RNA (mRNA), which is then translated to make a functional protein molecule. Among the available methods, direct introduction of mRNA is the safest and most efficient approach for achieving the expression of transcription factors. However, the methods in mRNA transfection has to be tested first. First, the MyoD gene was inserted downstream from the T7 promoter in a plasmid. The plasmid was then cloned in *E. coli*. We then extracted the plasmid DNA and amplified the MyoD gene by polymerase chain reaction (PCR). The amplified MyoD DNA was concentrated and purified by ethanol precipitation. MyoD mRNA was prepared by *in vitro* transcription catalyzed by T7 RNA polymerase using the recovered MyoD DNA as the template. Finally, the resulting RNA was purified by using 100 kDa molecular weight cutoff spin columns and quantified by UV spectroscopy. This method of RNA transcription had produced 145.16 μg RNA, comparative to the standard 160 μg RNA produced by a longer template. Using this method, modified mRNA can be produced and used to transfect the embryonic stem cells in order to induce differentiation into myocytes.

Key Words: Embryonic stem cells, mRNA synthesis, MyoD, stem cell differentiation

Dedication

I would like to dedicate this work to my mom and dad, for always being there and supporting me.

Acknowledgements

I would like to thank my thesis adviser, Dr. Faqing Huang, for guiding me throughout the process of completing this project. This project would be impossible had it not been for his guidance. I would also like to thank Krishna Sapkota and Joe Spangler, the graduate students in our lab, for helping me to understand the many reactions in the project, and to learn from the many failed attempts in the process. Thank you for everything.

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List of Abbreviations

ATP	Adenosine triphosphate
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
ECM	Extracellular matrix
ESC	Embryonic stem cell
EtBr	Ethidium bromide
5mCTP	5-methylcytidine-5'-triphosphate
GFP	Green fluorescent protein
GTP	Guanosine triphosphate
iPSC	Induced pluripotent stem cell
kb	Kilobase
mRNA	Messenger Ribonucleic acid
NTP	Nucleotide triphosphate
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
UTP	Uridine triphosphate
UTR	Untranslated region

Chapter 1: Introduction

Stem cells have been extensively studied by scientists as they have the ability to differentiate into different somatic cells, or diploid cells, and carry out different functions. However, somatic cells could also be induced to differentiate back into stem cells and then be differentiated into other cell types. The differentiation of somatic cells and stem cells is triggered by transcription factors. The transcription factor could be a protein or a piece of RNA. To induce cell differentiation in a lab, the targeted cell will have to be transfected with the transcription factor. Through a number of studies, it is found that transfecting the cell with mRNAs is the better transfection method, since mRNAs are the templates for translation to make proteins and there is little concern for their possible integration into the genome, which would be a complication of the DNA transfection method. By transfecting the cell with mRNAs, the cell will produce the proteins that the mRNAs code for, which then could direct cell differentiation[1]. However, cells often have innate immunity mechanisms to protect themselves from being attacked by viruses and bacteria. The innate immunity often will recognize particles that are foreign and target them for clearance[2]. This innate immunity response is great for the cells to have, however, it makes the transfection with mRNA difficult, since the transfected mRNA is very likely to be recognized as a foreign molecule, and will be cleared by the cell. Other than problems with innate immunity, the half-life of mRNA is relatively short, which means the mRNA is easily degraded and a reduced amount of protein is translated.

This project focuses on testing the protocol of synthesizing the mRNA for the transcription factor MyoD. MyoD is a protein that plays an important role in initiating myocyte differentiation[3]. It was chosen due to the easily visualized myotubule formation. Due to the Central Dogma of Molecular Biology, DNA serves as the template for RNA transcription, and RNA serves as the template for protein synthesis. A cloning vector is used to insert the RNA polymerase promoter upstream of the MyoD gene. To isolate or amplify the MyoD DNA with the promoter from the cloning vector, there are two methods. One method is to use two restriction enzymes to cut the MyoD gene out of the plasmid, and then use it directly as the template for RNA preparation by *in vitro* transcription. The second method is amplifying the MyoD gene by PCR using two primers (Figure 1). This project uses the second method, since it allows us to avoid using restriction enzyme digestion, and we do not have to isolate the DNA segment resulting from the restriction enzyme digest. The MyoD DNA has 1176 base pairs, including the T7 promoter region, a 5' untranslated region (5' UTR), the open reading frame for MyoD protein, and a 3' untranslated region.
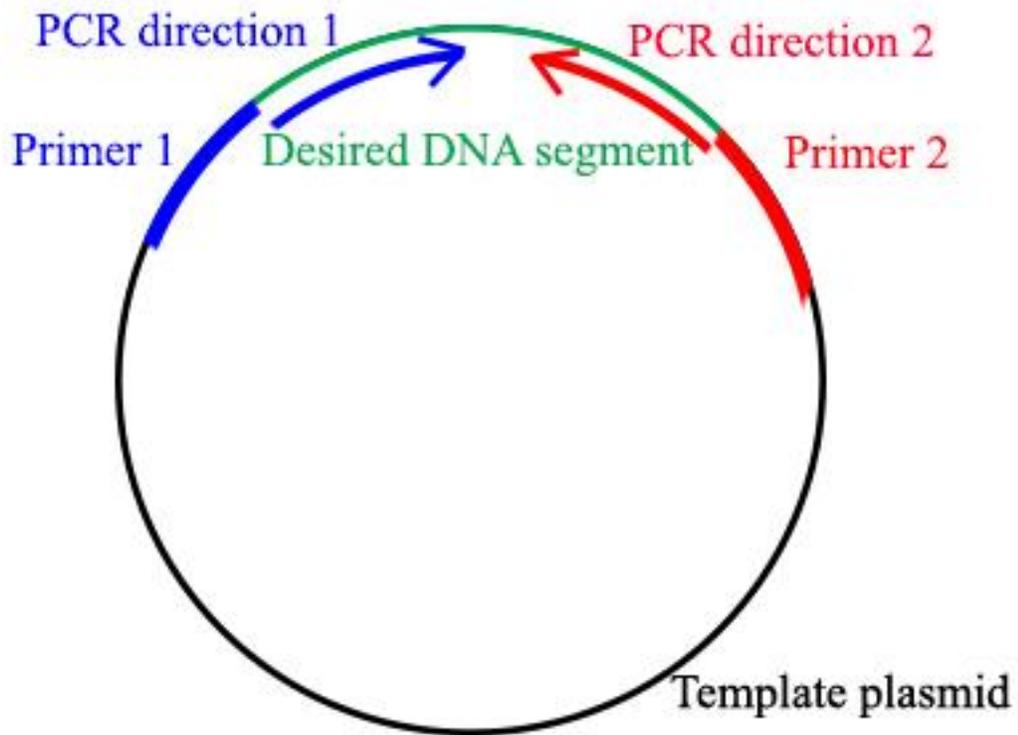
Figure 1: PCR amplification using two primers on a template plasmid.

Once the DNA template is ready, *in vitro* RNA transcription can take place to

synthesize the mRNA for the MyoD gene. As mentioned previously, the MyoD DNA

has 1176 base pairs, including the T7 promoter region. During transcription, however,

the T7 RNA polymerase binds to the promoter region, and transcribes the DNA

downstream of the promoter region, resulting in an mRNA that has fewer bases than the

DNA. The resulting MyoD mRNA is about 1150 nucleotides long. The structure of

eukaryotic mRNA should include a 5' end methylated cap, and a 3' end poly [A] tail

(Figure 2). The synthesized mRNA must have these two features in order to be

recognized by the translation machinery, the ribosome, in the cell. In addition, the cap

and poly [A] tail serve to prevent mRNA degradation by enzymes. The structure of the

5' methylated cap consists of a guanosine that is methylated on the 7-nitrogen. The

methylated guanosine is linked to the first mRNA nucleotide in a 5' to 5' linkage (Figure 3).
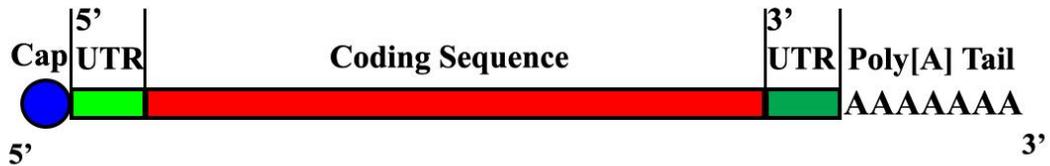
Figure 2: The structure of a eukaryotic mRNA. The 5' methylated cap, 5' untranslated region, coding sequence, 3' untranslated region, and the 3'-Poly [A] tail are labeled.
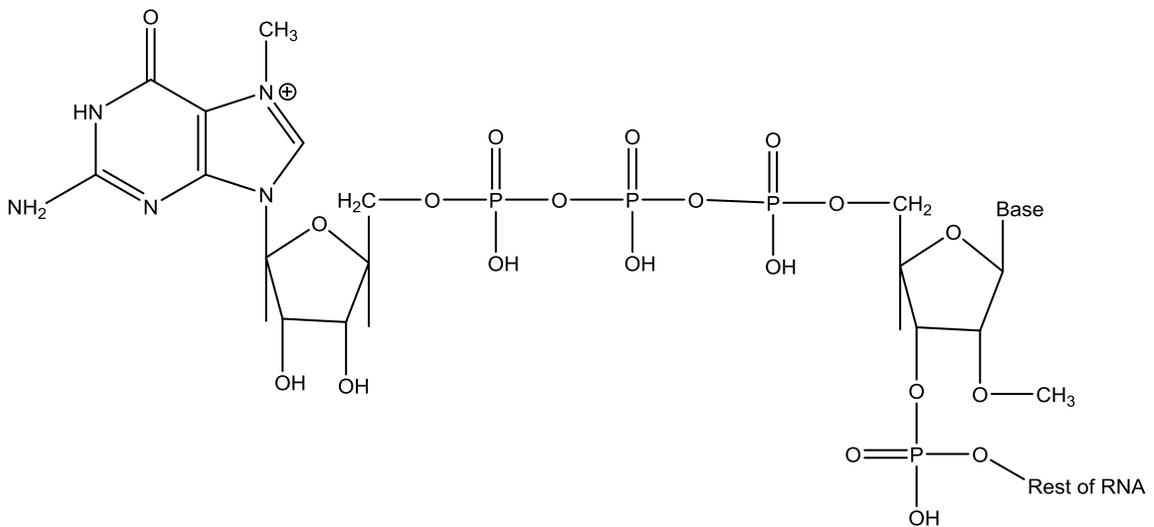
Figure 3: Five prime methylated cap on eukaryotic mRNA.

A recent study showed that incorporating 5-methylcytidine-5'-triphosphate, 5mCTP, and psudouridine-5'-triphosphate, pseudo-UTP, in the mRNA increases protein expression[1]. The structure of 5mCTP consists of a cytosine that is methylated at 5-carbon (Figure 4). The structure of pseudo-UTP contains a C-C bound linkage between the sugar and the base instead of the typical C-N linkage (Figure 5). These two modified

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NTPs could be used instead of the unmodified version, CTP and UTP, during transcription. Ethanol precipitation and spin column were used to concentrate the DNA and RNA products and to filter any excess reagents, such as NTPs, out of the product solutions.

Figure 4: 5-methylcytidine-5'-triphosphate, 5mCTP, structure.

Figure 5: Pseudouridine-5'-triphosphate, pseudo-UTP, structure.

Chapter 2: Literature Review

Stem cells

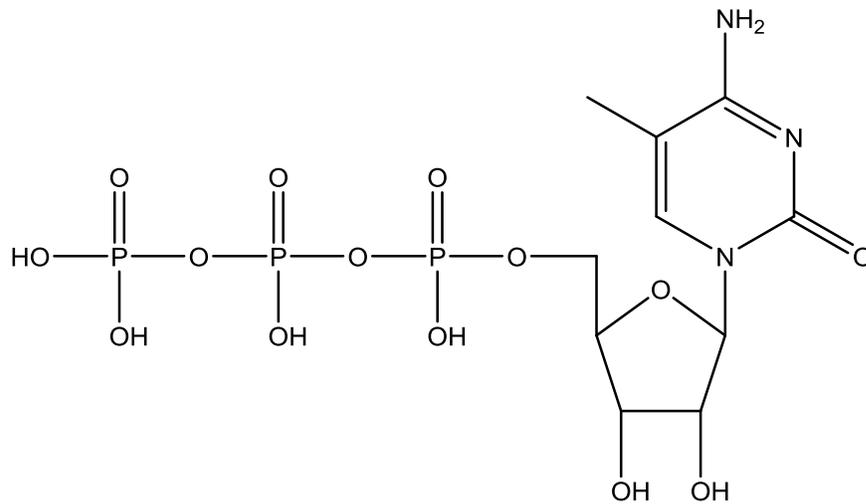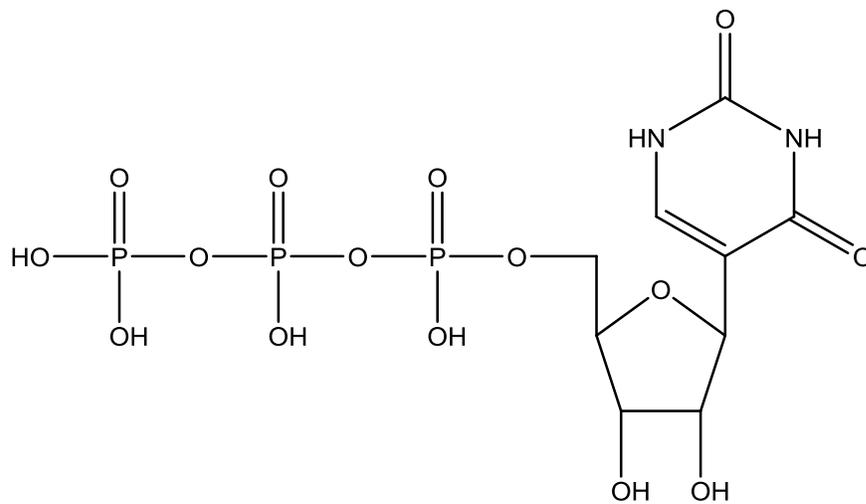Stem cells have been widely studied as they have the ability to differentiate into other cell types or cell lineages and to perform self-renewal. These abilities of stem cells are often used in synthesizing new tissues, treating autoimmune diseases, and performing genetic therapies. However, the differentiation of stem cells does not happen randomly during the cell cycle. In order for the cell to differentiate, a transcription factor must be present inside the cell to trigger differentiation. It is believed that the differentiation process has several different pathways and can be triggered by different types of transcription factors[1].

Transcription factors

Transcription factors are typically proteins that bind to the enhancer region of DNA to initiate or increase the transcription of a specific gene. The presence of a transcription factor can change the level of expression of a specific protein in the cell, or induce cell differentiation, as mentioned previously. Alves et al. used an aluminum-gallium-arsenide diode laser to compare the expression levels of mRNAs that encode transcription factors, such as MyoD, myogenin, and IL-6. They found that MyoD plays an important role in inducing the initial differentiation step in myogenesis. Further studies showed that with a decreased amount of MyoD mRNA expression, there was a decrease in myotubule formation[3, 4]. Since myotubules can be easily visualized using a

microscope, and we are at the early stage of research on this topic, we have decided to use MyoD to induce stem cell differentiation.

RNA in Cell Differentiation

Several other studies revealed that RNAs can also act as a transcription factor in cell differentiation. MicroRNAs (miRNAs), which are small noncoding RNAs, can affect and guide the RNA for transcription. Furthermore, the presence of small interfering RNAs (siRNAs) can create an environment similar to an extracellular matrix (ECM) around the stem cell to affect the shape of the cell[5]. While cell differentiation can be affected by small pieces of RNAs, it can also be affected by long noncoding RNA pieces. In human bone marrow mesenchymal stem cells (MSCs), a long noncoding RNA (lncRNA) is found to participate in very important roles in the differentiation of MSCs[6].

Other than siRNAs, miRNAs and lncRNAs, mRNAs can also take part in cell differentiation. Messenger RNAs (mRNAs) are transcribed from the DNA template and then used as templates for protein synthesis. Mothe and Brown's study in 2000 revealed that mRNA can be moved by an ECM molecule called SC1[7]. Messenger RNA transportation by SC1 changes the shape of the cell to further aid in cell transportation and proliferation[7]. One year later, they found that the ECM molecules, SC1 and SPARC, do not just aid in mRNA and cell transportation, they also help to regulate the expression of a particular mRNA. In this study, the mRNA was involved in the development of cochlea in the rats' inner ears. They also found that a mutation of these ECM molecules can cause several inner ear diseases[8].

Even though the studies of transcription factors regulating cell differentiation provided an overview of factors that can affect the expression of mRNA and the shape of

the cell, these studies all acquired their transcription factors from their targeted tissues. They first extracted the cells, cultured them and then studied the effects of the transcription factors acquired from these cells. This project instead focuses on synthesizing the mRNA that codes for transcription factor, MyoD.

mRNA Transcription

In the lab, the mRNA will be transcribed using *in vitro* transcription of the MyoD DNA template. *In vitro* transcription can use several different RNA polymerases, such as T7 and SP6. In order for the RNA polymerases to transcribe the DNA, a polymerase specific promoter region has to be present on the DNA strand upstream to the segment being transcribed[9,10]. There are two classes of T7 RNA promoters, φ2.5 and φ6.5 (Figure 6). The two classes of promoters differ in the transcription start site; φ2.5 initiates with adenosine, A, and φ6.5 initiates with guanosine, G. The φ2.5 promoters have a higher efficiency in labeling the transcription start site with an R group. Since eukaryotic mRNAs have a 5' methylated cap and the cap, as mentioned previously, consist of 7-methylated guanosine linked to the first transcribed nucleotide in a 5' to 5' linkage, the 7-methylated guanosine can be added to label the first transcribed nucleotide, adenosine, from φ2.5 promoters. Different RNA polymerases require different conditions for optimal enzymatic activity. T7 RNA polymerase requires a reducing environment, since it contains a sulfhydryl group that is essential for its enzymatic activity[11].
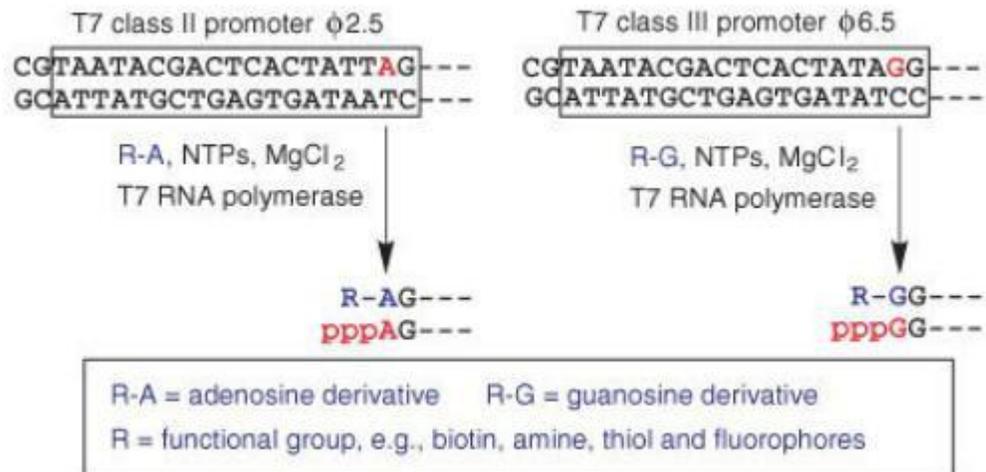
Figure 6: φ2.5 and φ6.5 T7 promoter sequence comparison[12]. The transcription initiator, or the first transcribed nucleotide, is highlighted in red.

mRNA Modifications

Warren et al. developed a new method to reprogram the mRNA directing cell differentiation and the transfection of the target cells with the resulting mRNA[1]. They used induced pluripotent stem cells (iPSCs), since iPSCs have potential for use in genetic therapies if the transfection does not alter the cell's genome. They synthesized a modified mRNA coding for transcription factor MyoD. Upon continuous transfection of the targeted cells with the modified mRNA, they found that the targeted cells did differentiate, and that the second generation of the targeted cells after transfection did not have any point mutations (insertions), which would happen if the method was DNA based instead of RNA based. While modifying the mRNA, the features of mRNA must be maintained, which include a 5' methylated cap, an open reading frame or coding sequences, and a 3' poly [A] tail. Warren et al. also used the mRNA with these basic features[1]. However, eukaryotic cells have innate immunity, which would recognize foreign particles, in this case, an *in vitro* synthesized mRNA, and attack it in an attempt

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to remove it. To reduce innate immunity responses, Warren et al. introduced modified nucleotide bases into the synthesized mRNA. They completely substituted cytidine bases with 5-methlycytidines (5mC), and uridine bases with pseudouridines (psi), and found that they obtained the best results when both of these substitutions are introduced into the mRNA coding for green fluorescent protein (GFP) (Figure 7). To further reduce innate immunity, they also used interferon inhibitors[1].
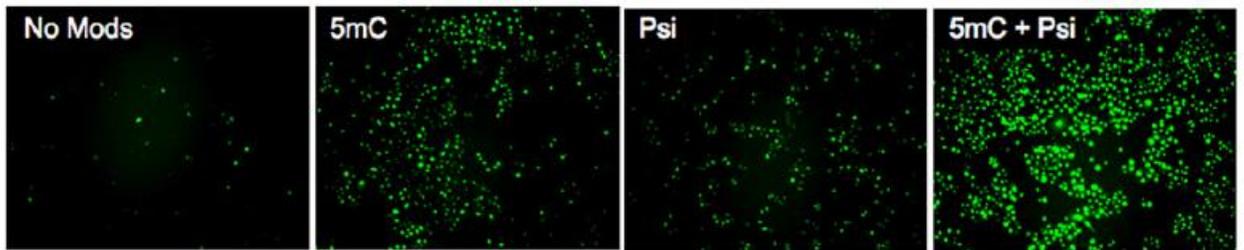

Figure 7: Pictures of cell cultures with modified nucleoside bases. The modified gene codes for green fluorescent protein, GFP[1]. The higher intensity of color signifies the higher protein expression.

Although the technique developed by Warren et al. showed that somatic cells can be induced to differentiate to iPSCs, and that modified nucleotide bases in synthetic mRNA leads to high protein expression, it does require continuous transfection of the stem cells with modified mRNA because of immune responses and the fast degradation of the mRNA. Also, a lower protein expression was seen upon second and third transfections due to the adaptive immune response[1]. This project therefore aims to prepare a modified eukaryotic mRNA that is designed to further reduce immune responses and increase the life span of the mRNA inside the transfected cell.

Chapter 3: Research Methods

Amplification of MyoD DNA

Testing optimal cycle number

Two plasmids, pMD4 and pD4, were used in this project. They were both extracted from *E. coli* and were used as templates to amplify the MyoD gene with two primers. The primers used for the pMD4 were M13f/Ma3r, and the primers used for pD4 were P1/P2. The plasmids were prepared by Dr. Huang. Since pD4 yielded the best results, this plasmid was chosen to proceed. The PCR used 1X buffer, 2.5 mM magnesium solution, 0.2 mM dNTP, 0.5 µM P1/P2 (primers solution), 1 ng pD4 (DNA template) and 0.05 u/µL Taq polymerase per 10 µL of reaction solution. The Taq polymerase was kept at -20ºC, and was the last reagent added to the reaction solution. The optimal PCR cycle number was tested first; the batch PCR reaction solution was 50 µL. After adding all the reagents in the order described above in a PCR tube, the batch solution was separated into three test tubes, marked as 20, 25, and 30, respectively. The tubes were placed in a thermal cycler with its program set to initial denaturation at 95 ºC for 2 minutes, second denaturation at 95 ºC for 30 seconds, annealing temperature at 54 ºC for 30 seconds, extension at 72 ºC for 80 seconds, and final extension at 72 ºC for two minutes. The program was set for 30 cycles. One test tube each was taken out of the machine when 20 and 25 cycles were completed, respectively.

Gel electrophoresis

To analyze the optimal cycle number, the samples from each test tube were analyzed on an ethidium bromide stained 1% agarose gel at 85 V for 45 minutes. The gel

was then scanned using the BioRad Molecular Imager FX to visualize the bands and to determine the optimal cycle number.

Amplification

After the optimal cycle number was determined, another PCR solution was prepared using the same protocol as described above for 150 µL reaction solution. The PCR solutions were kept under 50 µL per PCR tube. The samples after PCR were analyzed using gel electrophoresis as described above.

Ethanol Precipitation

After the DNA template was amplified using PCR, and taken out of the thermal cycler, ethanol precipitation was used to concentrate and precipitate the MyoD DNA template. Thirty microliters of distilled water, 20 µL 3 M sodium acetate, and 600 µL ethanol were added to 150 µL PCR product. The solution was then vortexed for a few seconds, and kept at -20 ºC for 30 minutes. The solution was then centrifuged at maximum speed for 8 minutes, and 100 µL supernatant was removed at a time until there were about 50 µL supernatant left. It was then centrifuged at maximum speed for a quick pulse, and the rest of the supernatant was removed carefully to not disturb the pellet. The pellet was then re-suspended in 20 µL distilled water. To analyze the precipitation process, 0.5 µL aliquot was diluted with distilled water to 5 µL. Two microliters of the diluted sample was analyzed with the same protocol as above for gel electrophoresis.

RNA transcription

The concentrated DNA was used as the template for RNA transcription *in vitro*. The transcription reaction used 1 μL 1X buffer, 7.5 mM NTP, 10 mM DTT, and 1X DNA template per 10 μL reaction, along with 1 μL T7 RNA polymerase and 0.5 μL RNase inhibitor. The reagents used for RNA transcription were manufactured by Thermo Fisher. The stock solutions used were all RNase-free, including water. The NTP solution was a mixture of ATP, GTP, CTP, and UTP. After proper mixing, the solution was kept in an incubator at 37 ºC for 2 hours.

Spin column

The solution was taken out of the incubator after 2 hours of RNA transcription, and 80 μL RNase-free water was added to the solution. The diluted solution was added onto a 100k DA molecular cut-off spin column and spun at 14, 000 g for 5 minutes. A hundred microliter of RNase-free water was added to the spin column, and spun at the same speed for 7 minutes. The spin column was checked periodically until there were about 5 μL solution left on the spin column. Fifteen microliters of RNase-free water was added to the column and vortexed for a quick pulse. The column was then inverted into another tube and spun at 2, 000 g for 1 minute. From the RNA sample collected in the tube, 0.5 μL was taken out, and mixed with 500 μL distilled water in a cuvette. It was then analyzed using UV-Vis. The absorbance at 260 nm and 320 nm was measured, and the micromolar concentration of RNA and mass of RNA produced was calculated.

Amplification of MyoD DNA
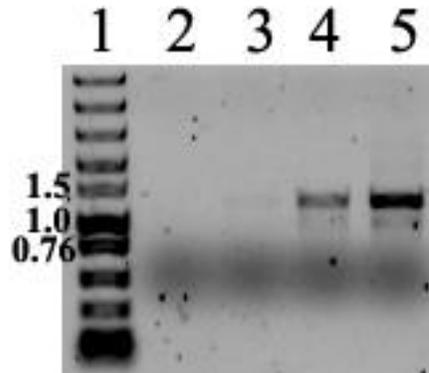
Testing optimal cycle number

Figure 8: EtBr stained 1% agarose gel of pMD4 PCR optimal cycle number testing. Lane 1: Fast DNA ladder with three standard sizes labeled (kb), lane 2: unreacted PCR solution, lane 3: 15 cycles sample, lane 4: 20 cycles sample, lane 5: 25 cycles sample.
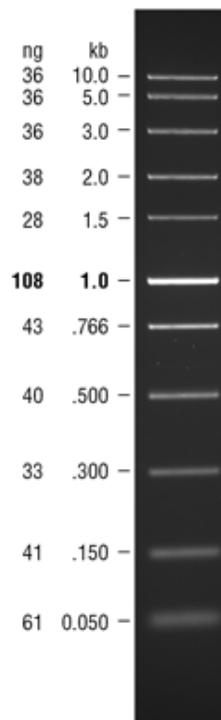

Figure 9: Fast DNA ladder by NEB for molecular size (kb) reference.

Figure 8 shows the MyoD sample amplified from pMD 4 after 15, 20, and 25 PCR cycles, with Fast DNA ladder. The molecular size corresponding to each band on the Fast DNA ladder can be seen in Figure 9. It is important to know that the darker the band, the more concentrated the sample. The expected MyoD DNA has 1176 base pairs, which should be in between the 1.0 kb and 1.5 kb bands on the ladder, which was what was shown in Figure 9. This gel data allows us to visualize the relative concentration of the DNA samples after 15, 20, and 25 PCR cycles. However, there was a second band that appeared on the gel for the samples after 20 and 25 PCR cycles. Although the second band was very light, it suggested that the DNA sample was not pure, since there should only be one band present.
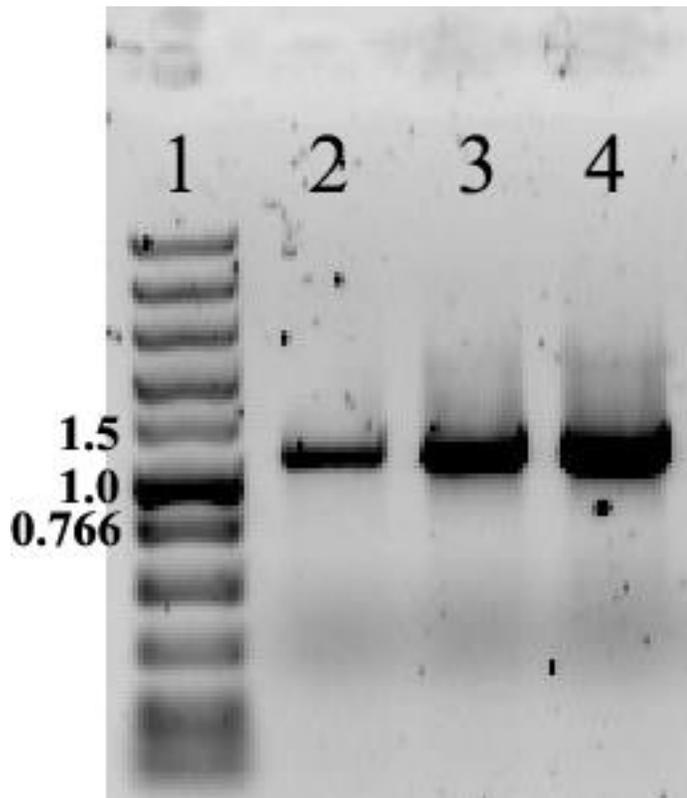

Figure 10: EtBr stained 1% agarose gel of pD4 PCR optimal cycle number testing. Lane 1: Fast DNA ladder with three standard sizes labeled (kb), lane 2: 20 cycles, lane 3: 25 cycles, lane 4: 30 cycles.

Due to the possible impurity of pMD4, another plasmid containing the MyoD gene, pD4, was obtained. Figure 10 shows the MyoD sample amplified from pD4 after 20, 25, and 30 PCR cycles, with Fast DNA ladder. As with the gel for pMD4, the darker the band, the more concentrated the sample. The optimal PCR cycle number would be when the band is dark, but not too dark where it will begin to smudge on the gel, since the volumes of aliquots and standard DNA ladder were the same.
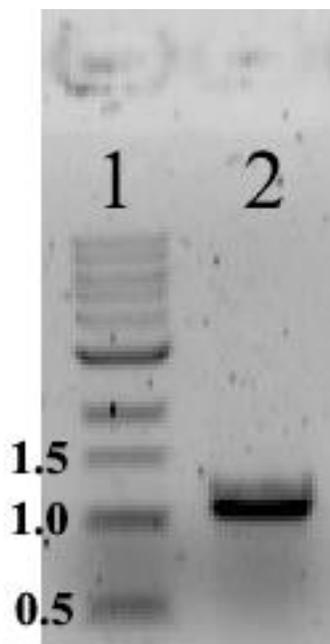
Amplification

Figure 11: EtBr stained 1% agarose gel of pD4 DNA after PCR amplification for 20 cycles. Lane 1: 1 kb DNA ladder with three standard sizes labeled (kb), lane 2: DNA amplified from pD4.
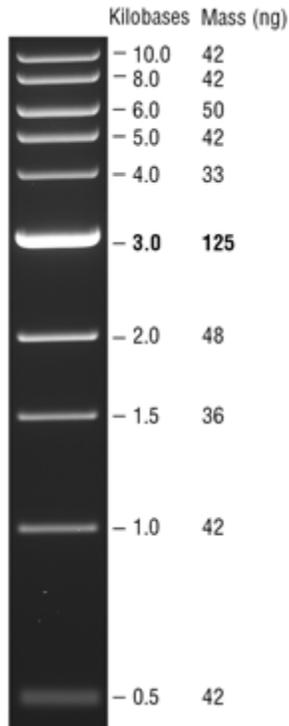
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Figure 12: 1 kb DNA ladder from NEB for molecular size reference.

Figure 11 shows the MyoD DNA sample amplified from pD4 after amplifying for 20 PCR cycles, with 1 kb DNA ladder. This gel allows us to ensure that the PCR reaction worked, and the amplified DNA template can be used to proceed. Figure 12 shows the molecular size references for the 1 kb DNA ladder.

Ethanol Precipitation
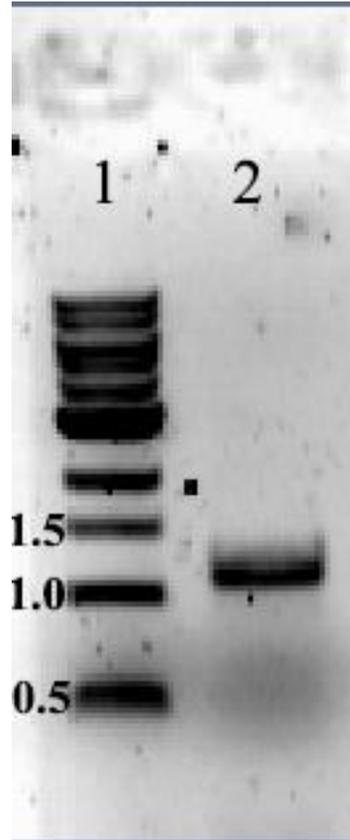
Figure 13: EtBr stained 1% agarose gel of MyoD DNA after ethanol precipitation. Lane 1: 1 kb DNA ladder with three standard sizes labeled (kb), lane 2: DNA sample after ethanol precipitation.

Figure 13 shows the MyoD DNA sample after performing ethanol precipitation, ran against 1 kb DNA ladder, for molecular weight references, refer to Figure 12. The volume of ladder used in this gel was doubled the amount of ladder used for the PCR gel (Figure 11). This gel allows us to ensure that we did not lose any DNA during the process of precipitating, and that we have a sufficient amount of MyoD DNA to proceed to transcription.

RNA transcription
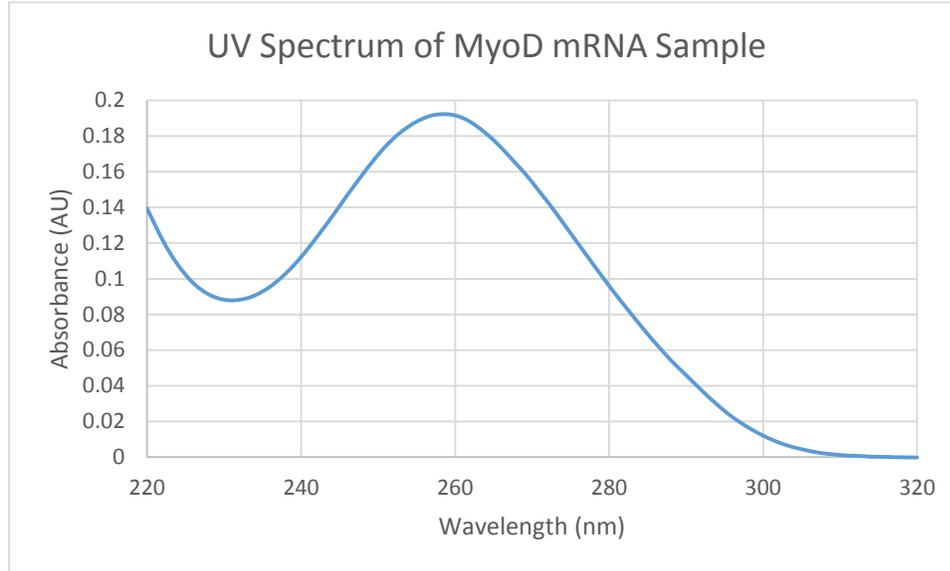
Figure 14: UV spectrum of MyoD mRNA sample after spin-column purification.

Figure 14 shows the UV-Vis graph of the MyoD mRNA sample after spin column purification. The absorbance was taken using 0.5 µL mRNA in 500 µL water. The absorbance at 260 nm is 0.191 AU, and the absorbance at 320 nm is 0 AU. The concentration was calculated to be 23.2 µM and the mass of RNA produced was calculated to be 145.16 µg. This yield is comparable to the standard yield of 160 µg of RNA produced from a DNA template with 1300 bases (our MyoD DNA template is 1176 bases long).

Chapter 5: Discussion and Conclusions

Both the plasmids, pMD4 and pD4, were prepared by Dr. Huang. As seen in

Figure 8, the first plasmid, pMD4, after PCR amplification, had an additional band

underneath the MyoD gene. This could be caused by an impurity of the DNA sample, or

the primers had another binding site on the plasmid. In consideration that the impurity of

the MyoD DNA sample could affect the mRNA product, another plasmid containing the

MyoD gene, pD4, was obtained. After testing, the optimal cycle number for pD4 was

determined to be 20 cycles, since the bands for 25 and 30 cycles began to smudge with

the same amount of aliquot from each sample and the same amount of DNA ladder

(Figure 10). The volume of 1 kb DNA ladder used for gel electrophoresis after ethanol

precipitation of MyoD DNA (Figure 13) was doubled, since the bands of 1 kb DNA

ladder in the gel for pD4 amplification (Figure 11) was very light. The mass of the RNA

after the spin column was calculated to be 145.16 μg, comparative to the standard yield of

160 μg RNA produced from a longer DNA template. Therefore, this approach was

concluded to be successful. However, this MyoD mRNA was not usable for transfection.

The RNA produced did not contain the modified nucleotides, since they are more

expensive than the unmodified nucleotide bases, and this project is mainly focused on

testing the protocol. The RNA product also does not have a 5' methylated cap and 3'

poly[A] tail. The only way for us to put a cap on the mRNA in the lab, at this moment, is

to incorporate the methylated cap with the transcription initiator, adenosine[12] with the

φ2.5 promoter. However, the methylated cap is expensive and difficult to synthesize, and

since this project was to test the protocol for sufficient RNA yield, the methylated cap

was not used. For future studies, a cap could be incorporated during transcription with the φ2.5 T7 promoter, modified nucleotide bases can be incorporated, and the 3' poly[A] tail can be added. There is another problem that is of concern. If the 5'methylated cap—labeled adenosine is incorporated into the mixture of NTPs during transcription, the T7 RNA polymerase can use both the 5'methylated cap—labeled adenosine and the unlabeled adenosine, resulting in a product that will consist of both capped MyoD mRNA and uncapped MyoD mRNA. The uncapped mRNA could trigger immune responses in the target cell after transfection, which may cause damages to the cell before the capped mRNA can express the MyoD protein. In the future, studies focusing on separating the capped mRNA and the uncapped mRNA before transfection will be useful to reduce immune responses in the cell.

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