EXPLORATION OF THE RHO FACTOR

By

Drake Christopher Maestas

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University Honors
University of California, Riverside

APPROVED

Dr. Gregor Blaha
Department of Biochemistry

Dr. Richard Cardullo, Howard H Hays Jr. Chair
University Honors

ABSTRACT

Transcription and translation of genetic information is a crucial aspect to every living organism. It begins with RNA polymerase transcribing the template strand of DNA to mRNA. Shortly after, the mRNA is translated by the ribosome into a protein. The principles of transcription and translation remain constant in both prokaryotes and eukaryotes. However, in prokaryotes translation of the mRNA can take place during its transcription. In *E.coli* the translation has to start before transcription is completed, otherwise a protein called Rho will prematurely terminate transcription. Rho binds the mRNA and translocates toward the RNA polymerase where it dislodges the polymerase from the DNA, terminating transcription. The goal of my research was to explore the interaction between the Rho, RNA Polymerase, and the 30S small ribosomal subunit. Using electromobility shift assays, I observed that Rho forms a complex with RNA Polymerase but not with 30S subunits. Interestingly, in the presence of RNA polymerase, Rho forms a complex with 30S subunits. This complex has an electromobility that is distinct from those of the Rho bound to RNA polymerase and from RNA polymerase bound to the 30S subunit. This suggests that RNA polymerase can bind the 30S subunit and the Rho at the same time. Further research is required to understand the ramification of this observation for transcription and translation.

ACKNOWLEDGEMENTS

I am grateful to my mother, for pushing me to always try my best and to never back down from a challenge. Through her encouragement and sometimes tough love, I remained dedicated to my studies and to my ambitions.

I would like to extend my gratitude to Dr. Chetty, who sparked my interest in pursuing biochemical research when she taught Dynamic Genome in my freshman year (BIOL 020). It was through her mentorship that I was able to develop specific techniques early on that would only aid in my current research.

Finally, I would like to extend my thanks to Dr. Gregor Blaha of the Biochemistry Department, who allowed me to perform biochemistry research in his lab. This hands-on experience helped to solidify complicated concepts and allowed me to be successful in my undergraduate career.

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Background Information:

Deoxyribonucleic Acid or DNA, along with other macromolecules such as proteins, lipids, and sugars, is the principle building block of all life on Earth. DNA is naturally double stranded, forming a double helix. This double helix is formed through the base pairing of complementary nitrogenous bases Adenine with Thymine and Guanine with Cytosine. DNA itself stores genetic information. To use this information the cell employs a two-step process. In this process the information encoded on the DNA is transcribed into Ribonucleic Acid or RNA by a protein called RNA polymerase. DNA and RNA are very similar to each other, except that typically RNA is single stranded and contains a nitrogenous base called Uracil in place of Thymine. After the genetic information is transcribed from DNA to RNA, it is then translated into a functional protein that will fulfill a specific role in the cell.

The first part of the central dogma, where the genetic information encoded on the DNA is transcribed to RNA is called Transcription. This process requires the aid of a protein called RNA polymerase, which is able to unwind the double-stranded DNA to create a transcription bubble. The purpose of the bubble is to allow complementary bases of RNA to pair those of the coding DNA strand to form immature messenger RNA (mRNA). In prokaryotic cells, the mRNA that was synthesized from RNA polymerase can now proceed to the second stage of the central dogma, which is a process called translation.

Translation is accomplished through what is known as a ribosome, which binds to mRNA molecules and translates with the aid of tRNA molecules the genetic information encoded in the mRNA into a protein. The ribosome consists of a large subunit and a small subunit. Both subunits are made of one or two ribosomal RNAs (rRNA) and 20-30 ribosomal proteins. The ribosome binds the mRNA and up to two tRNAs at the same time. tRNAs are RNA molecules

that have an L-shape. The tip of one arm binds an amino acid; the tip of the other arm houses a three nucleotide sequence, anticodon, that is specific for the bound amino acid. The anticodon sequence base pairs with the complementary sequence of the mRNA, codon. All translated RNAs start with one codon sequence, AUG, which is known as the start codon. This specific codon codes for the amino acid methionine. The ribosome contains three sites, the A, the P, and the E site. These sites allow for tRNA molecules with bound amino acids to enter into the ribosome and start to build a polypeptide chain. Once the tRNA enters the A site, then the tRNA moves into the P site. After this occurs, another tRNA enters the A site, bound to its respective codon sequence. At this point, the tRNA in the P site transfers its amino acid to the tRNA in the A site. The tRNA in the A site, now with two amino acids attached to it, now moves to the P site, whilst the tRNA with no an amino acid attached moves from the P to the E site where it can dissociate from the ribosome. Once the mRNA molecule nears the end of the coding sequence of the mRNA, a stop codon appears in the A site. Three possible stop codons are known: UAG, UAA, and UGA. The reading of these codons causes the release of the newly synthesized protein bound to the tRNA in the P site.

These steps of the central dogma take place in every organism, including the model organism of my research, the bacterium *E.coli* . However, certain distinctions must be made between the process of transcription and translation in bacteria compared to eukaryotes. Eukaryotic cells contain membrane bound organelles, one of them being the nucleus. The nucleus of eukaryotic cells contains the DNA necessary for gene expression. Transcription occurs in the nucleus as well as the post-transcriptional processing of the mRNA, such as splicing and the addition of a 5’ cap and a polyA tail. Mature mRNA must travel outside of the nucleus in order to be translated into a functional protein. However, prokaryotic organisms are

different from eukaryotic cells. Being smaller in size, they do not contain membrane bound organelles. Their genetic material is present in the cytoplasm along with RNA polymerase and ribosomes; no barrier is separating the polymerase and the ribosome. Due to this close proximity, transcription and translation are able to occur simultaneously. As RNA polymerase is transcribing mRNA from DNA, the ribosome is able to attach to the mRNA that is exiting RNA polymerase and immediately translate it. That means the processes of transcription and translation take place at the same time in a prokaryotic cell.

In prokaryotes, there are two mechanisms to terminate transcription, known as Rho-dependent and Rho-independent termination. In the rho-independent termination, the formation of a hairpin loop structure in the newly synthesized RNA causes the RNA polymerase to cease transcription and release the newly synthesized RNA molecule. Rho-dependent termination requires a transcription factor called the Rho.

The Rho is 49 kDa large and forms a homohexamer. In its hexameric form, Rho can terminate transcription in prokaryotic cells. Its mechanism of action is simple: Rho binds the RNA exiting the RNA polymerase and moves, driven by ATP hydrolysis, along the RNA toward the polymerase. Once caught up to RNA polymerase, Rho proceeds to cause the polymerase to stop transcription and release the newly synthesized RNA (Banerjee, Sharmistha, et al, 2006).

Hypothesis:

It is known that Rho-dependent termination occurs as a result of Rho traveling along the RNA toward the RNA polymerase, where it causes the polymerase to terminate transcription. It was suggested that Rho binds to the polymerase to induce transcription termination. Because RNA polymerase can also bind the small ribosomal subunit, I hypothesize that the polymerase

may bind Rho and the 30S subunit at the same time, enabling Rho and the 30S subunit affect each other's function either directly or mediated via the polymerase.

Materials and Methods:

Small ribosomal subunits and RNA polymerase were purified according to Blaha et al. and Svetlov V et al., respectively.

Briefly, crude ribosomes were purified by pelleting ribosomes from a cell lysate through a sucrose cushion. Ribosomal subunits were prepared by passing the crude ribosomes over a 10-35% sucrose gradient under dissociation conditions. The isolated 30S and 50S subunits were collected, concentrated, quantified by their absorbance at 260 nm wavelength, flash frozen in liquid nitrogen and stored at -80C for further use.

For purification of the RNA polymerase, T7 Express cells were transformed with a plasmid containing RNAP-SNAP (RNA polymerase with a SNAP tag followed by a His-tag). Transformed cells were grown in LB media supplemented with Ampicillin and Chloramphenicol at 37 C at 180 rpm. Cells were induced at early log phase with 1 mM IPTG for four hours. Harvested cells were resuspended and lysed using a cell disruptor. The cell lysate was clarified by centrifugation before loading onto a Nickel NTA column. A high salt wash was performed before eluting the polymerase with imidazole. The eluted protein was dialyzed into a low-salt buffer and loaded onto an SP-Column. The polymerase was eluted from the column via a salt gradient. The fractions from the gradient were analyzed by SDS-polyacrylamide gel electrophoresis. Fractions with pure RNA polymerase were pooled, concentrated, flash frozen in liquid nitrogen and stored at -80C until further use.

Rho was prepared by growing bacteria transformed with a plasmid that allowed the overexpression of rho. A large-scale growth under optimal conditions was to be performed in which the Rho Factor would be purified from (details on the purification of the Rho Factor will be provided in the results section). The purified Rho was used to determine its interactions with RNA polymerase and ribosomes *in vitro*.

For electrophoretic mobility shift assays a vertical two-component agarose gel was prepared. The first component was a high-melting agarose gel that formed a scaffold for a low-melting agarose gel. The different mixtures of RNA polymerase, ribosome, and Rho were loaded onto the low-melting gel and separated by electrophoresis. Optimizing the preparation of the gel and the running conditions of the electrophoresis were a critical component of my thesis and are described in the results section in more detail.

Results:

Optimizing the growth conditions for optimal expression Rho:

E.coli cells were grown overnight on a LB agar plate supplemented with Kanamycin. The cells were plated using a streaking method of application. Several colonies were collected and combined for the inoculation of 50 mL of starter culture of liquid LB broth with Kanamycin. The overnight growth of the starter cultured was used to inoculate several 50 mL cultures with different growth media. All cultures were grown to an optical density of 1.0, at which time Rho expression was induced with 1 mM IPTG. I tested different media broths and times of induction to identify the conditions that would produce the highest yield of Rho. Expression levels of Rho were determined by SDS polyacrylamide gel electrophoresis. Cells grown in LB Broth with Kanamycin and induced overnight produced the highest yield of Rho.

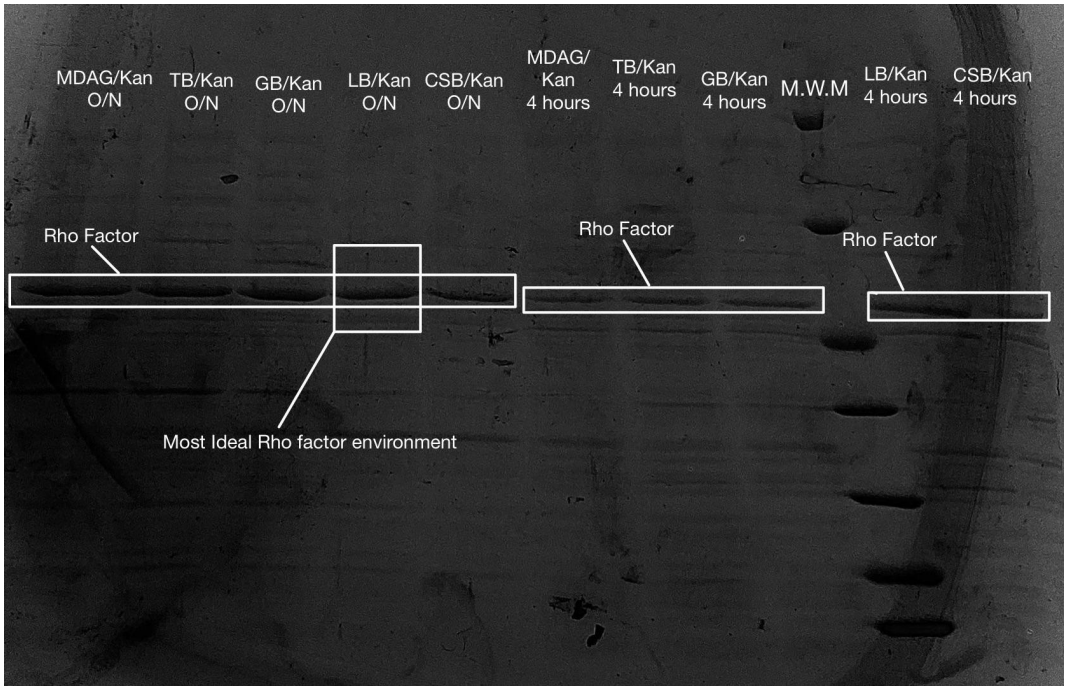

Figure 1. SDS-PAGE analysis of cells grown in different medias between hours 4-overnight

SDS-PAGE was used to determine the most optimal growth conditions. A large-scale growth was performed using six liters of LB/Kan. Another agar plate of cells was grown using the methods described previously, and another 50 mL preculture of cells using LB media was grown. Equal amounts of cells were transferred into each of the six liters and grown until an optical density of 0.8. The cells were then harvested and flash frozen in liquid nitrogen and stored in -80 C until further use.

Purification of the Rho:

A 2 Liter stock solution containing 10 mM Tris-HCl, pH 7.9, 5% pure glycerol, 0.1 mM EDTA (pH 8.0), and 0.1 mM DTT was prepared. This stock solution was used to prepare a lysis buffer, wash buffer, and elution buffer with 150 mM NaCl, 150 mM NaCl in addition to 20 mM Imidazole, and 400 mM Imidazole respectively. Cells were resuspended in the lysis buffer which was composed of the stock buffer and 150 mM NaCl with no Imidazole. To aid the lysis of the

cells lysozyme was added to the suspension. The cells were lysed by ultrasonication for 2 minute cycles to ensure complete lysis, the cells were then centrifuged. The supernatant was loaded onto a Nickel affinity column to capture the His-tagged Rho.

A His-tag consists of six to nine consecutive histidines which are placed either on the C or N-terminus of the protein. The histidines will bind to the nickel ion that is immobilized on the column through coordinate bonds formed from the lone pair of nitrogen on histidine to the nickel ion. At millimolar concentrations imidazole is able to displace the his-tag from the nickel ion (Abcam 2023). Before loading the clarified lysate onto the nickel column, the column was equilibrated with a Lysis buffer. Unwanted non-His-Tagged proteins were removed from the column with a Wash buffer containing 20 mM of imidazole. Purified Rho was eluted from the column with a buffer containing 400 mM of imidazole. The purity of the rho was determined by SDS-PAGE (see Figure 2).

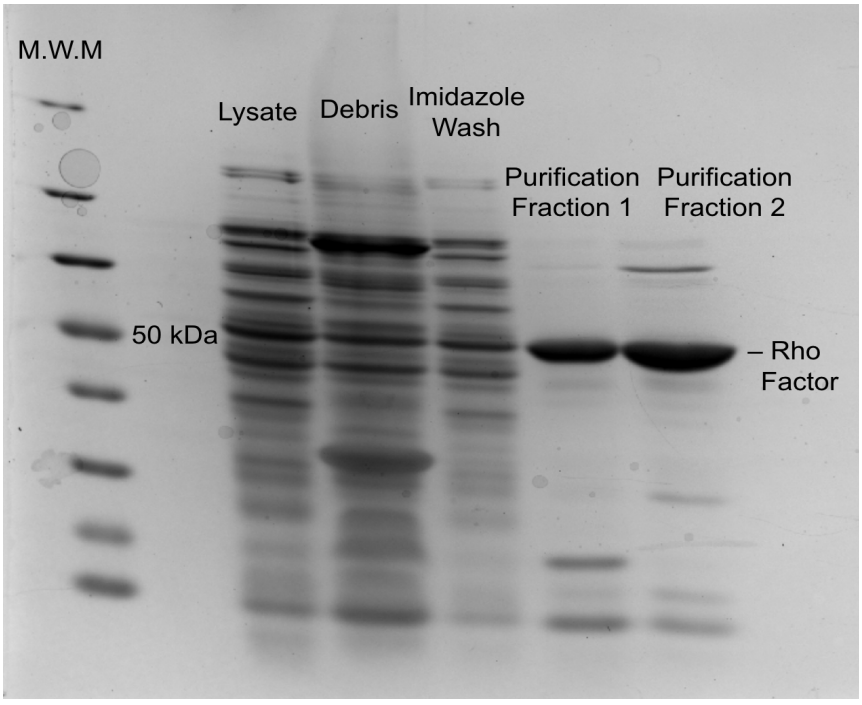

Figure 2: Purification of Rho Factor

After Rho was purified, all test tubes containing the Rho were pooled, concentrated, and buffer exchanged. Sixteen milliliters of elution containing Rho were concentrated to 1 milliliter. The concentrated protein was then aliquoted into PCR tubes, flash frozen in liquid nitrogen, and stored in the -80 C until further use.

Establishing that DNA Promotes Hexamer Formation of Rho by Electrophoretic Mobility Shift Assay:

Rho can be present as a monomer, dimer, tetramer, or hexamer; only the hexamer appears to cause transcription termination. For our analysis of Rho's interaction with the RNA polymerase and 30S subunit, I had to identify conditions that promote hexamer formation. To explore the effect of the different buffer conditions exert on the oligomeric state of rho, I employed native agarose gels. We expected that the different oligomeric states of Rho have different electrophoretic mobility. We therefore casted native gels under the different buffer conditions (T. Li and C. Arakawa, 2019). The different buffer conditions contained Tris-Base, HEPES Free Acid, Magnesium Chloride, Potassium Acetate, DTT, and EDTA all at varying concentrations. Testing different concentrations of Rho at varying salt concentrations, confirmed previously optimal conditions for hexamer formation of 5 uM of Rho in the presence of 30 mM Potassium Acetate (Geiselmann, Johannes, et al., 1992). However, our results suggest that hexamer formation was not complete. A more thorough review of the literature showed that single-stranded DNA with a high Cytosine content will promote hexamer formation (Banerjee, Sharmistha, et al., 2006). I was able to confirm the findings from the literature (see Figure 3). My native gel shows that the different oligomeric forms of Rho were converted to hexameric form when DNA is present. In all further experiments DNA was present with Rho.

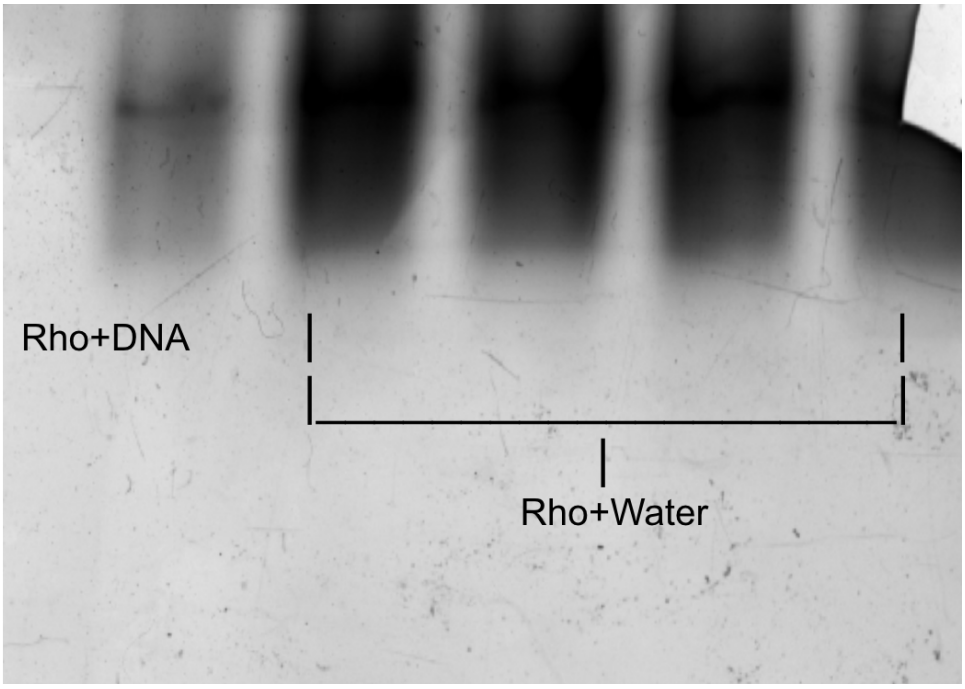

Figure 3: Native Gel denoting how DNA promotes Hexamer formation of the Rho

Rho Forms a Complex With RNA Polymerase:

Ideal concentrations of RNA polymerase were found to be between five to eight micromolar due to previously conducted experiments. Rho was able to be included with the polymerase to see their interaction. Variable amounts of RNA Polymerase were tested in decreasing amounts against a constant amount of Rho. Through the results of this assay, it can be seen that as the concentration of RNA Polymerase increases, the Rho band on the gel gets more faint, until it eventually disappears, implying that it binds to the RNA polymerase. The complex of RNA polymerase and Rho are to migrate faster than the polymerase or Rho by itself (see Figure 4).

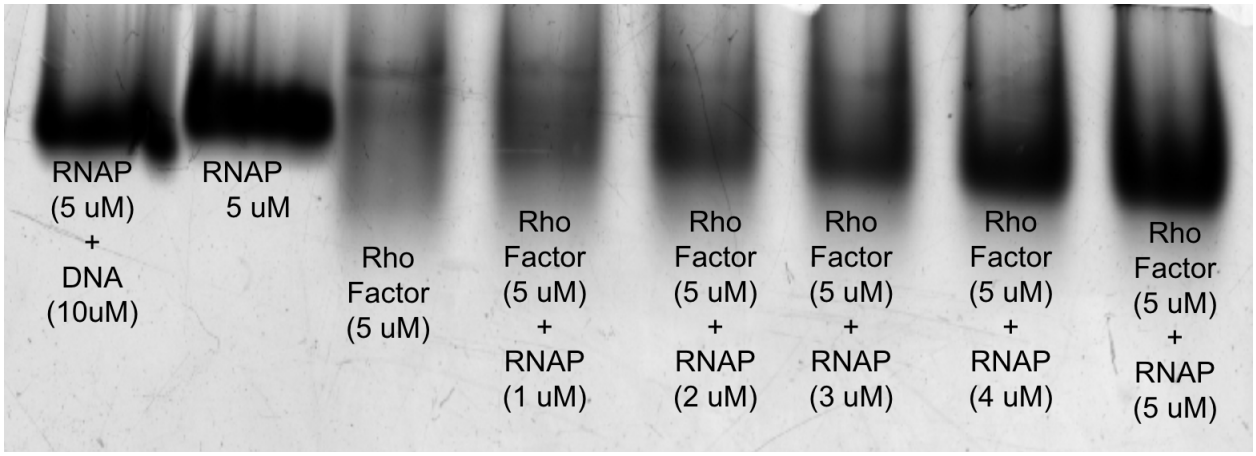

Figure 4: Native Gel result of RNA Polymerase titrated into Rho

Absence of Binding of Rho to RNA Polymerase in the Presence of ATP:

Rho is an ATPase that is able to use the energy released from the hydrolysis of the gamma phosphate of ATP to move along the RNA. In all previous experiments we omitted the addition of any nucleotides. Under those conditions, Rho can bind directly to the RNA polymerase. To test the effect of ATP on the complex formation, I included ATP during the formation of RNA polymerase-Rho complexes. The Rho Factor, being a hexamer, needs one phosphate group from ATP per monomer. Therefore, thirty micromolar of ATP combined with 5 micromolar of hexameric Rho, along with five micromolar of RNA polymerase were combined. After 25 minutes of incubation at 37C, I loaded the mixture onto the native gel. The results of the electrophoresis indicate that Rho does not bind to the RNA polymerase in the presence of ATP. The lane with the ATP in it shows that the Rho Factor is of equal intensity to the Rho Factor control lane. The lane without ATP shows that the Rho Factor is disappearing into RNA

polymerase, indicating that ATP inhibits the Rho Factor binding to RNA polymerase.

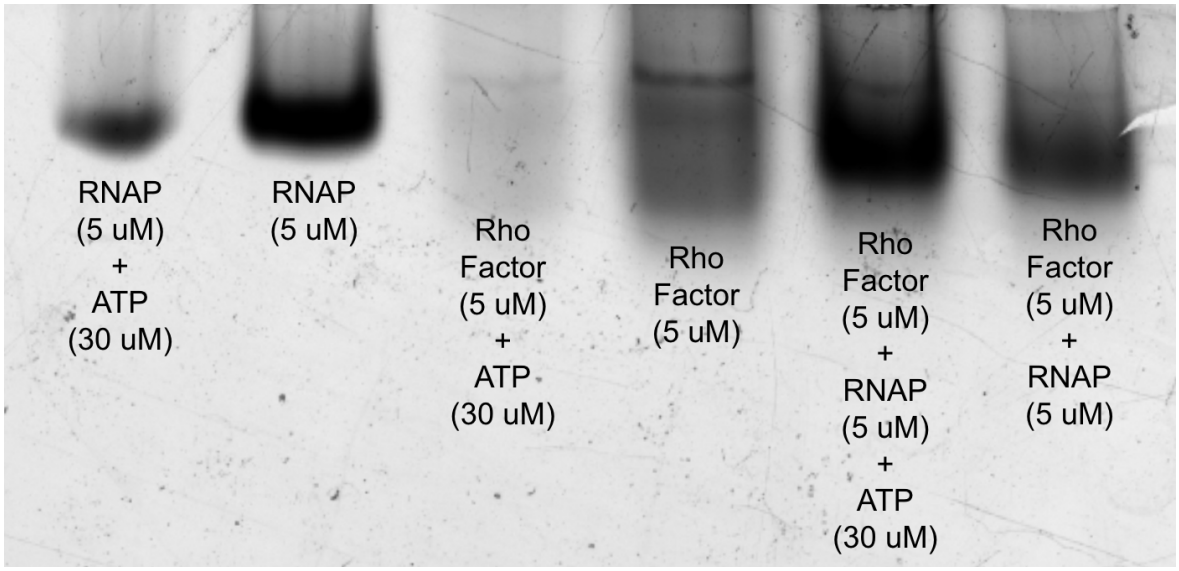

Figure 5: Native Gel showing that Rho does not bind RNA Polymerase in the presence of ATP

Binding of Rho Factor and RNA Polymerase in the Presence of ADP:

Ideally, Rho will be able to bind to RNA polymerase in the presence of ADP due to ADP not being able to activate the Rho. ADP is the unreactive version of ATP, this is due to the fact that the gamma phosphate present on ATP is hydrolyzed off and it is converted to unreactive ADP. This reaction was performed using equal concentrations of RNA polymerase and Rho (5 micromolar each), in addition to 30 micromolar of ADP so that one ADP molecule can theoretically bind to each subunit of the Rho hexamer. The results of the gel show that Rho-binding to the RNA polymerase is not affected by the presence of ADP. This is due to the fact that in the lanes that include the Rho Factor and RNA polymerase in the presence of ADP, it can be seen that the Rho Factor is binding to RNA polymerase, as it is fading away into the polymerase as it does in previous gels. This occurrence can also be seen on the control lane run without any nucleotide present. This indicates that the Rho Factor that was purified has no loss

of functionality as ATP inhibits binding to RNA polymerase and ADP does not affect the binding between these two proteins.

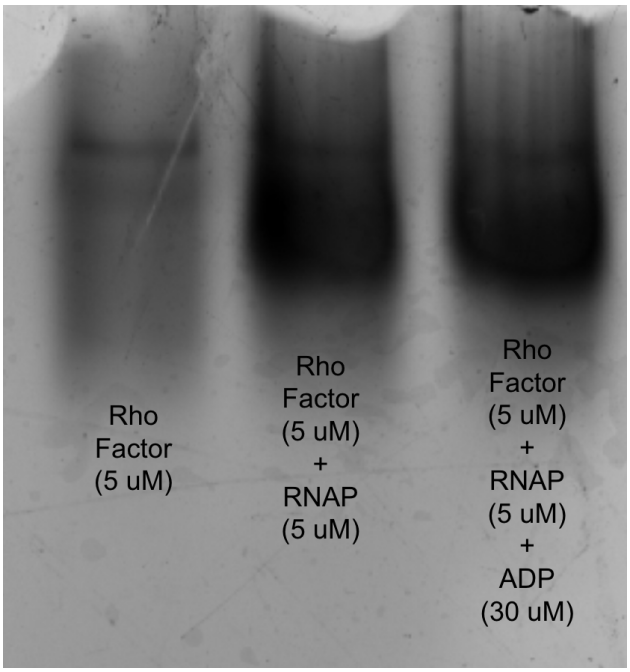

Figure 6: Native Gel showing that Rho binds RNA polymerase in the presence of ADP

RNA Polymerase Binds to 30S Subunits:

In his paper, Dr. Haitian Fan showed that the RNA polymerase can directly bind 30S subunits *in vitro* (Haitan Fan et al., 2017). Showing this once again was needed as a control for when the Rho was introduced into the mixture in addition to these two factors. 30S subunits at 7 micromolar concentration were titrated with varying concentration of RNA polymerase (from two to seven micromolar). What was found was that RNA polymerase was able to bind all of the ribosome at a 7 micromolar to 7 micromolar concentration. Any RNA polymerase concentration below 7 micromolar concentration resulted in not all of the 30S small ribosomal subunit binding to RNA polymerase. A complex can be seen forming between the two proteins on the band,

indicating a new complex of RNA polymerase and Ribosome with a new electromobility that is shown on the Native Gel.

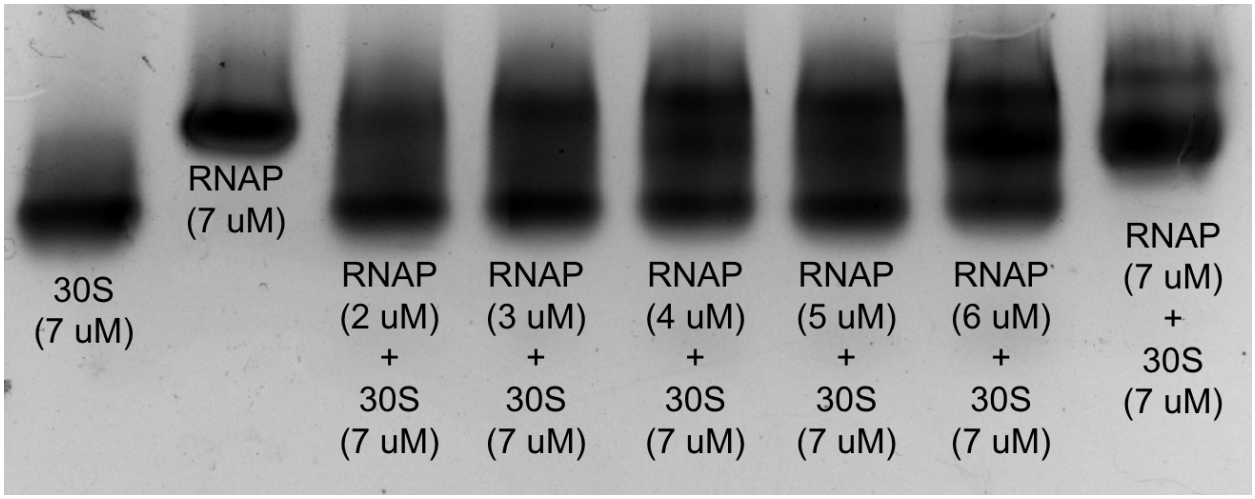

Figure 7: RNA Polymerase Titrated into Constant Amount of 30S Ribosome

Titration of 30S Subunits with Rho and RNA Polymerase:

Three lanes from Figure 8 were chosen for further study (5:7; 6:7; and 7:7). The goal was to include Rho in the 6:7 and 7:7 lanes from Figure 7 in order to see the result. The idea behind this experiment was to see a potential mega-complex form since the Rho Factor would be included in the mixture. This mega-complex was hypothesized to be formed as we saw how the Rho Factor bonded with RNA polymerase, but also how the polymerase is able to bind with the 30S small ribosomal subunit. Therefore, an experiment had to be conducted to see if this was true. Ideally, there would be a singular sharp band, denoting the presence of this mega-complex. However, since the stock concentration of 30S subunit was too low to be able to include Rho, the concentration of RNA polymerase and 30S subunit had to be lowered to a max concentration of 5 micromolar. Theoretically, this change in concentrations would have little to no effect on the binding of RNA polymerase and the 30S subunit since the lane we are most interested in is the

7:7, which is essentially a 1:1 comparison. The table below shows the concentrations of Rho, RNA polymerase, and 30S subunit in each lane. (5 micromolar had to be used instead of 7 micromolar due to final volume overload).

Lanes	9	8	7	6	5	4	3	2	1
Rho (uM)	8	5	2	8	5	2	8	5	2
RNA polymerase (uM)	5	5	5	4	4	4	3	3	3
30S (uM)	5	5	5	5	5	5	5	5	5

Table 1: Reaction Mixture per Lane of all Three Factors Tested

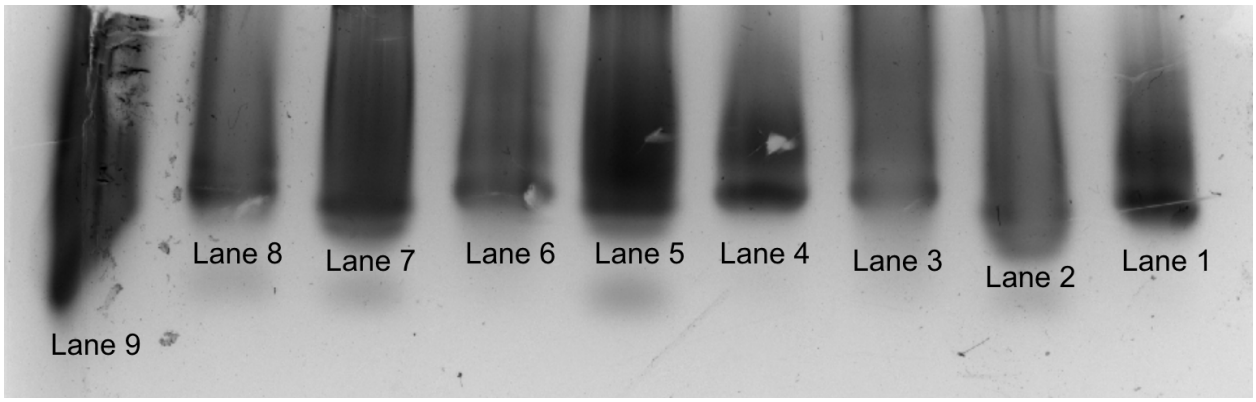

Figure 8: Native Gel Result of the Above Reaction Table

Although the results may be difficult to read, it can be seen that at 5 micromolar concentration of each component (lane 8, 5:5:5 complex in Figure 8 or lanes 1 and 2 in Figure 9) a single band is formed. This indicates to me that at five micromolar concentrations the factors all bind together to form the hypothesized mega-complex.

Different Interactions between the Factors:

Based on the above described experiments, Rho and the 30S subunit bind to RNA polymerase at the same time, suggesting a possible direct interaction between Rho and 30S subunit. The possibility of Rho directly interacting with 30S subunits, independent of RNA polymerase, has not been explored before. Since these two proteins were involved in different central dogma processes, it was unlikely that they would have any interaction. However, because of the coupling of transcription and translation in prokaryotes and how the Rho Factor and 30S subunit are in close proximity to one another, it was hypothesized that some interaction would be observed on the native gel. In terms of experimental conditions, it was decided to show the reaction with the same concentration of Rho as RNA polymerase when combined with the 30S subunit. The table below shows the different concentrations present in each lane of the native gel:

Lanes	7	6	5	4	3	2	1
Rho (uM)	X	5	X	X	5	5	5
RNAP (uM)	5	X	X	5	X	5	5
30S (uM)	3	3	3	X	X	2	3

Table 2: Reactions Per Lane for Native Page Image Below

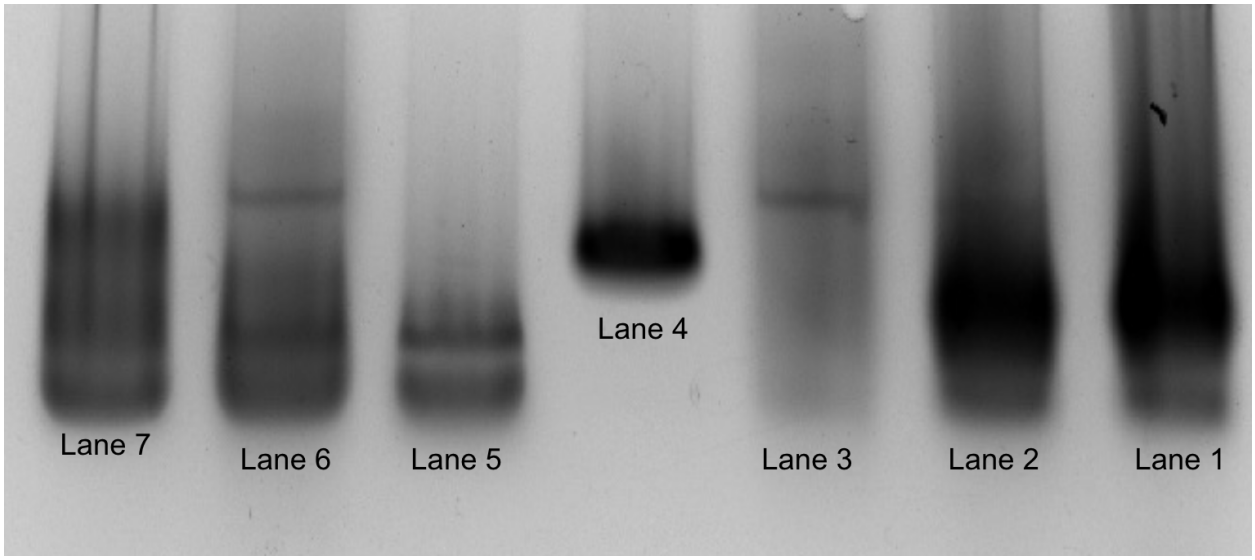

Figure 9: Native Gel showing the Mega Complex with Other Controls

The results of this gel suggests that Rho does not directly bind the 30S subunit and that RNA polymerase has to be present for a possible interaction between Rho and 30S subunit. This gel also shows the complex formed between RNA polymerase and the 30S subunit already shown in my previous titration of 30S subunits with RNA polymerase. Additionally, the "Mega Complex" of Rho, RNA polymerase, and 30S subunits can be seen clearly with an electromobility that is distinct from either component individually or in combination.

5:5:5 and 5:5:3 Mega Complexes that will Undergo Agarase Treatment:

The tendencies of Rho to bind RNA polymerase and 30S subunits were shown both together and separate, however a gel that puts my findings all in one image was needed to showcase the differences in the electromobilities between trials with differing concentrations that was found to be of significance in previous experiments. This gel would then undergo an agarase treatment so that these bands can be run on SDS-PAGE to verify the contents of the mega

complexes seen in the image. The table below displays the different concentrations of each component in each lane of the experiment:

Lanes	1	2	3	4	5	6	7
Rho Factor (uM)	5	5	5	X	X	X	X
RNAP (uM)	5	5	X	5	X	7	6
30S (uM)	5	3	X	X	5	7	7

Table 3: Reactions Per Lane for Native Page Image Below

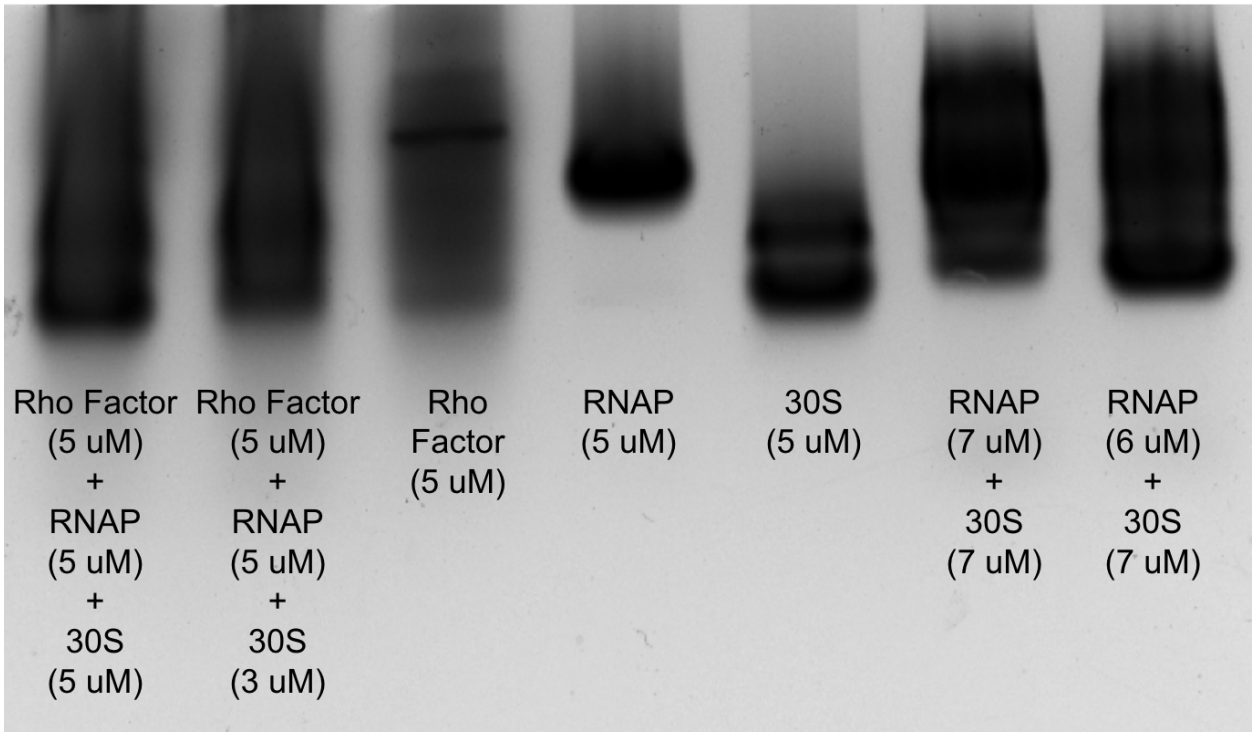

Figure 10: *Native Gel showing the 5:5:5 vs 5:5:3 Mega Complexes and the 6:7 and 7:7 RNA polymerase to 30S subunit*

Composition of the Different Complexes Identified in by Native Gel Electrophoresis:

The agarase treatment requires one to physically cut the bands so that the protein complexes can be analyzed via SDS-PAGE. The goal was to verify that Rho, RNA polymerase, and 30S subunits are present in the band identified as mega-complex in previous experiments. Portions of the bands were excised using a fresh razor blade. In reference to Figure 11, areas 2 and 9 were cut out to see if free Rho was present in those areas of the mega-complex lanes. Areas 3 and 10 were cut out to see if free RNA polymerase was present in those regions of the mega-complex lanes. Areas 1 and 8 were cut out to identify the components that make up the mega-complex. Areas 4 and 11 were cut out to see if free 30S subunits were present in the mega-complex lanes. Ideally, only areas 8 and 1 should be visible on SDS-PAGE as the majority of factors should be present at the location of the mega-complex. Areas 5, 6, and 7 were cut out and used as controls for Rho, RNA polymerase, and 30S subunits, respectively. The results show that the concentration of protein within the Native gel is too low to be seen on the SDS-PAGE (see Figure 12). However, the ribosomal proteins of the 30S subunit can be seen on lane 8 of the gel, indicating that the 30S subunit is present in the region where the mega-complex is located.

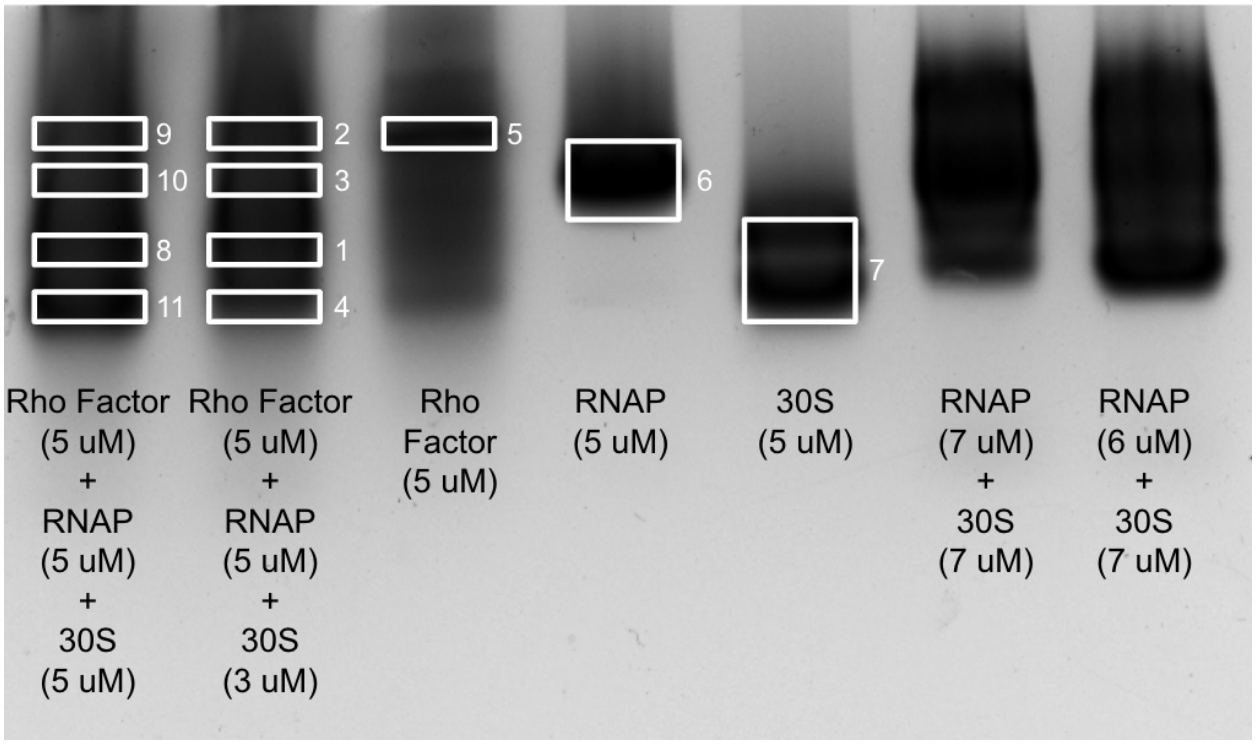

Figure 11: Native Gel demonstrating the 5:5:5 vs 5:5:3 Mega Complexes with 6:7 and 7:7 RNAP vs 30S with agarase cut sites

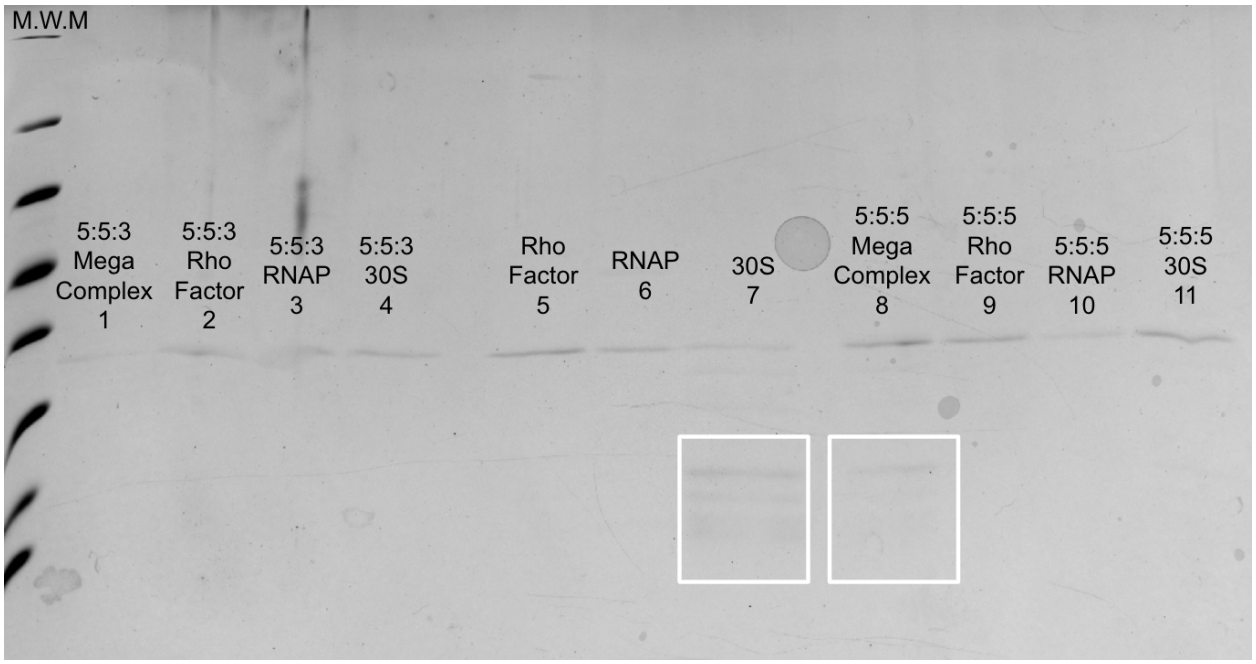

Figure 12: SDS-PAGE of the Agarase Treated Mega Complexes

Discussion:

It was hypothesized that Rho can bind to RNA polymerase in order to cause transcription termination. In previous work from my laboratory, it was shown that RNA polymerase can directly bind the 30S subunit, I hypothesized that Rho may also directly interact with the 30S subunit. Based on my experiments, I conclude that Rho does not bind directly the 30S small ribosomal subunits. This is due to the fact that the Rho Factor band is prominently present in the lane in which it was tested with the 30S small ribosomal subunit. In the course of my study, I could show that Rho can bind directly RNA polymerase in the absence of ATP. Because Rho requires ATP to translocate along an RNA transcript towards the RNA polymerase, it would ideally separate from RNA polymerase when it binds to the reactive phosphate group of an ATP molecule. Additionally, Rho does not bind 30S subunits unless RNA polymerase is present. In the presence of RNA polymerase a "mega-complex" of Rho, RNA polymerase, and 30 subunits forms, with a distinct electromobility. My attempts to verify the composition of the "mega-complex" were inconclusive due to the fact that there were no proteins present on the SDS-PAGE used in the agarase treatment. Further attempts with higher concentration of all components and larger volume are required to definitively establish the composition of the here presented "mega-complex". Current literature makes no mention of a "mega-complex" between Rho, RNA polymerase, and 30S subunits. Additional research will need to be conducted in order to see the effect of different conditions on the mega complex. The effect of adding glycerol to the buffer to try to promote protein grouping, or adding ATP into the mega-complex mixture could be future forms of study. Adding glycerol could help sharpen the bands further so that the Rho Factor can be clearer in all future experiments. However, in terms of the mega-complex location on the native gel, it does show that it is in the range of the 30S subunit binding to RNA

polymerase. This does put into question the composition of verifiability of the mega-complex from a certain viewpoint. However, as the mega complex band is consisting of the Rho Factor, RNA polymerase, and the 30S small ribosomal subunit, then if it was only consisted of the complex that forms between RNA polymerase and the 30S small ribosomal subunit, then that means the Rho Factor would still be present in the location of its control lane. However, the Rho Factor cannot be seen in the mega-complex lanes. This indicates that the mega-complex contains the Rho Factor and how it has to interact with RNA polymerase in order to form this complex with the 30S small ribosomal subunit. In terms of further steps to verify this conclusion, a fluorescent stain must be used in order to verify the Rho Factor's location in the native gel result.

Conclusion:

Rho is a critical factor for gene expression in prokaryotes. Through my *in vitro* assays, more information was gleaned about Rho's interactions with other components of the central dogma. More research is needed to fully understand the ramifications of the formation of a mega-complex between Rho, RNA polymerase, and 30S subunits for the mechanism of Rho-dependent transcription termination.

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