An Investigation of Intersubunit Bridges Important for Ribosome Stability in *Saccharomyces cerevisiae*

Honors Thesis by

Andrew Peter Hummel Ackell

Washington and Lee University

Lexington, Virginia

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To my loving family,

Acknowledgements

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Abstract

The ribosome, a large ribonucleoprotein complex responsible for protein biosynthesis, is composed of two unequal subunits that must correctly associate for proper function. X-ray crystallographic and cryo-EM studies have identified a number of specific interactions, or intersubunit bridges (ISBs), between the large and small subunits. At least two of these ISBs have been shown to be important for ribosome stability in bacteria using a technique known as modification interference. A similar modification interference approach is presented to identify ISBs that are important for ribosome stability in yeast. Native ribosomes and ribosomal subunits were isolated from yeast cells. Sucrose density gradient ultracentrifugation under a variety of conditions was investigated in order to achieve separation and purification of individual ribosomal subunits from intact ribosomes. The determination of the optimal conditions for subunit separation and isolation is a necessary step toward identifying possible ISBs through modification interference. We hope our modification interference investigations will provide insights regarding the function and conservation of ISBs.

Introduction

Ribosomes

Ribosomes are large ribonucleoprotein macromolecular machines with a complex structure that is of great interest to biochemistry. Each ribosome is comprised of two unequal subunits, a small subunit (SSU) and a large subunit (LSU). These subunits are made up of a network of interacting ribonucleic acids (RNAs) and proteins. In prokaryotes, the small subunit (also referred to as 30S) contains a ~1,540 nucleotide ribosomal RNA (rRNA) known as the 16S rRNA. The 16S rRNA interacts with at least 21 proteins, which help to organize the RNA domains and stabilize the overall structure of the 30S subunit (1,2). The large subunit in prokaryotes (also referred to as 50S) features two distinct rRNAs, a ~120 nucleotide 5S rRNA and a longer ~3,200 nucleotide 23S rRNA, which interact with approximately 36 ribosomal proteins. The overall structures of prokaryotic and eukaryotic ribosomes are very similar, with eukaryotic ribosomes being slightly larger and more complex (Figure 1). The eukaryotic small subunit (40S) is comprised of a single 18S rRNA containing ~1,900 nucleotides and at least 33 proteins. The large subunit (60S) in eukaryotes is composed of approximately 49 proteins and three different rRNAs: a ~120 nucleotide 5S rRNA, a ~160 nucleotide 5.8S rRNA, and a ~4,700 nucleotide 25S rRNA (1).

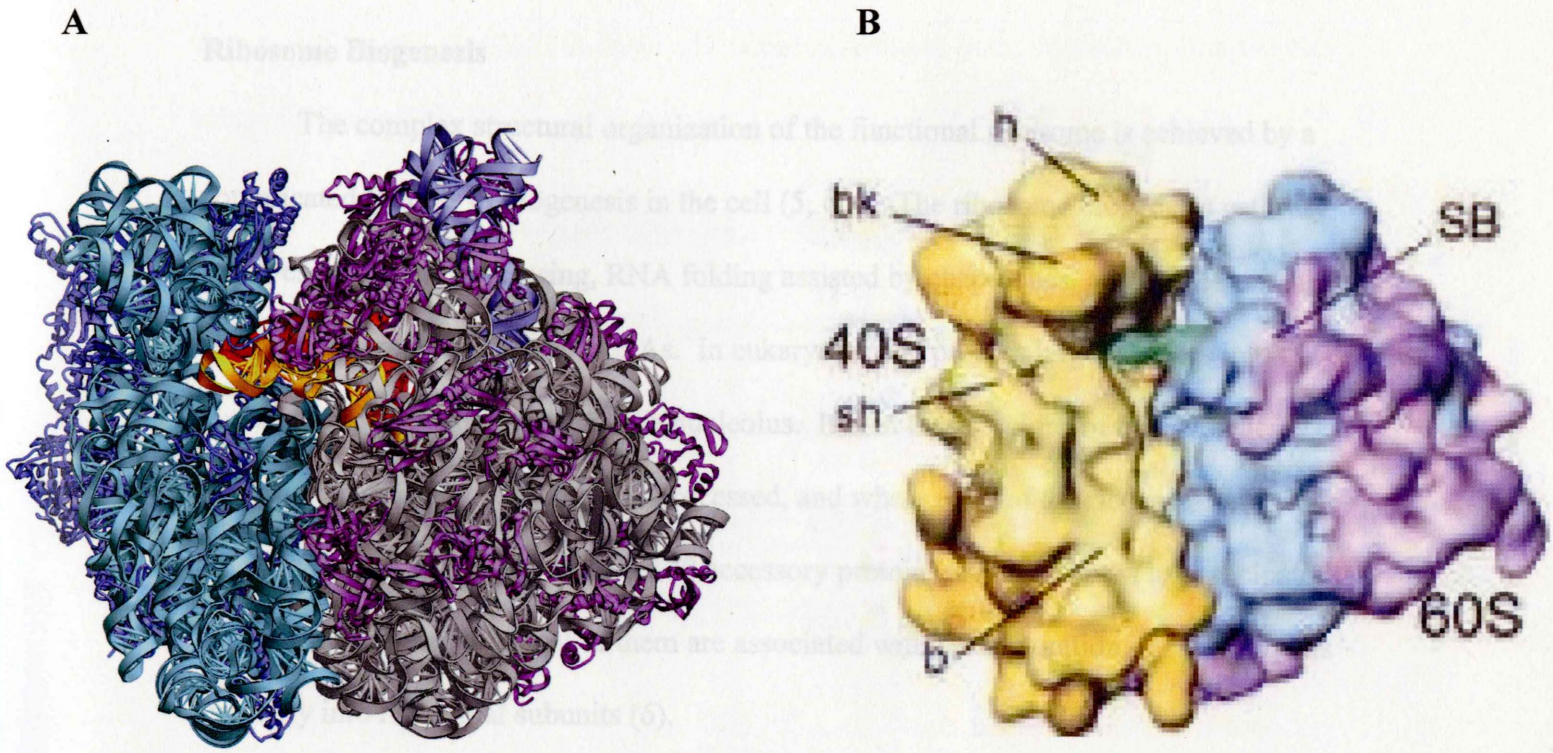


Figure 1. Prokaryotic and eukaryotic ribosome structures. (A) X-ray crystal structure of the *Thermus thermophilus* 70S ribosome, with the large (50S) subunit oriented to the right and the small (30S) subunit oriented to the left. The 23S rRNA (gray), 5S rRNA (blue, top-right), and ribosomal proteins (magenta) associated with the large subunit are visible on the right-hand side. The 16S rRNA (cyan) and ribosomal proteins (blue) associated with the small subunit are on the left-hand side. Transfer RNAs (tRNAs) (gold and orange) can be seen occupying the intersubunit cavity (3). (B) Cryo-electron microscopy structure of the yeast 80S ribosome. The small (40S) subunit is colored yellow and orange. The large (60S) subunit is colored blue and purple. A tRNA occupying the P-site of the intact 80S ribosome is shown in green. The designations "h", "bk", "sh", "b", and "SB" refer to structural landmarks known as the "head", "beak", "shoulder", "body", and "stalk base", respectively (4).

Ribosome Biogenesis

The complex structural organization of the functional ribosome is achieved by a complicated process of biogenesis in the cell (5, 6, 7). The ribosome biogenesis pathway involves pre-rRNA processing, RNA folding assisted by chaperones, and the sequential assembly of proteins onto the rRNAs. In eukaryotes, the process largely occurs in the nucleus and its subcompartment, the nucleolus. It is in these regions of the cell where rRNA is transcribed, modified, and processed, and where most of the ribosomal proteins are added to the rRNA. More than 170 accessory proteins are also known to participate in ribosome biogenesis; Most of them are associated with the maturation of rRNA and its assembly into ribosomal subunits (6).

rRNA synthesis begins with RNA polymerase I transcribing the large polycistronic 35S precursor rRNA (pre-rRNA) that contains the sequences for three of the mature rRNAs: 18S, 5.8S, and 25S. The fourth mature rRNA, 5S, is transcribed by RNA polymerase III (5). The 35S pre-rRNA is associated with 90S pre-ribosomal particles, which contain ribosomal and non-ribosomal protein factors (8). After the 35S pre-rRNA is transcribed, it undergoes exonucleolytic pruning to an intermediate 33S pre-rRNA and then a 32S pre-rRNA molecule. The 32S pre-rRNA undergoes endonucleolytic cleavage into the 20S and 27S pre-rRNAs, which are now part of the pre-40S and pre-60S particles, respectively. The 20S pre-rRNA is exported from the nucleus to the cytosol where it is then processed into the mature 18S rRNA of the 40S subunit. The 27S pre-rRNA undergoes an additional endonucleolytic cleavage in the nucleus to form the 7S pre-rRNA and mature 25S rRNA. The 7S pre-rRNA is then processed into the mature 5.8S rRNA, and both the mature 5.8S and 25S rRNAs are exported from the nucleus in

the pre-60S particles, which undergo additional processing in the cytosol to form the 60S subunit (Figure 2) (6,8).

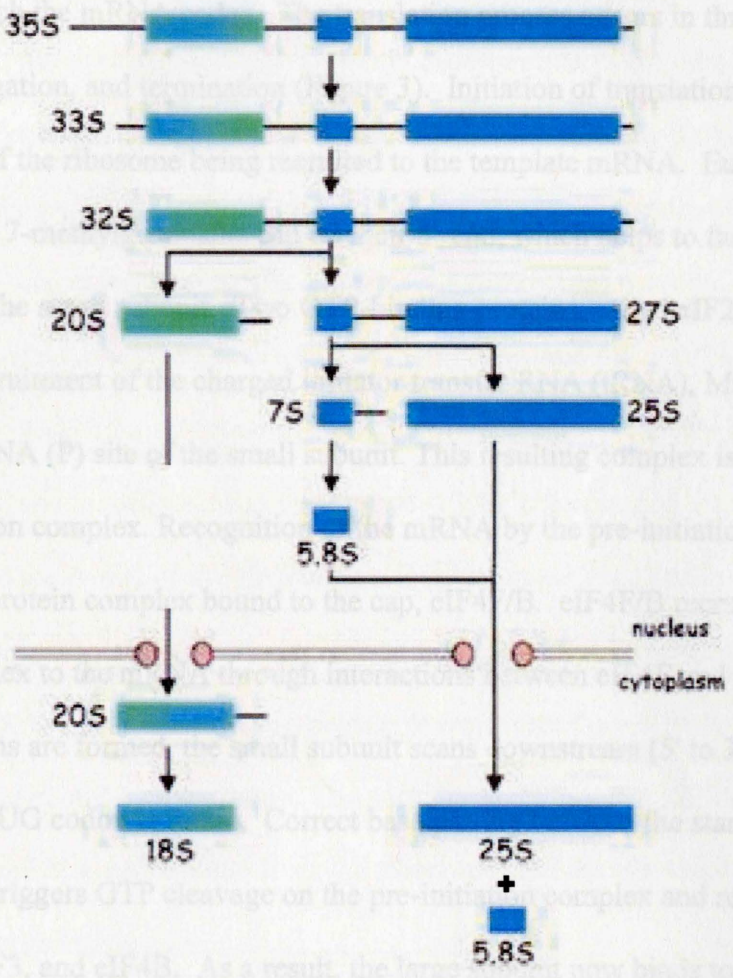


Figure 2. A pictorial overview of the steps of pre-rRNA processing. Associated proteins, small-nucleolar ribonucleoproteins (snoRNPs), and various processing and assembly factors have been omitted for simplicity. Modified from (5) and (8).

Translation

The ribosome's function is to translate a messenger RNA (mRNA) template into a protein for which the mRNA codes. The translation process occurs in three stages: initiation, elongation, and termination (Figure 3). Initiation of translation begins with the small subunit of the ribosome being recruited to the template mRNA. Eukaryotic mRNAs have a 7-methylguanosine cap on their 5' end, which helps to facilitate the recruitment of the small subunit. Two GTP-binding proteins, called eIF2 and eIF5B, mediate the recruitment of the charged initiator transfer RNA (tRNA), Met-tRNA^{met}, to the peptidyl-tRNA (P) site of the small subunit. This resulting complex is known as the 43S pre-initiation complex. Recognition of the mRNA by the pre-initiation complex is mediated by a protein complex bound to the cap, eIF4F/B. eIF4F/B recruits the 43S pre-initiation complex to the mRNA through interactions between eIF4F and eIF3. After these interactions are formed, the small subunit scans downstream (5' to 3') on the mRNA until the start AUG codon is found. Correct base pairing between the start codon and the initiator tRNA triggers GTP cleavage on the pre-initiation complex and releases initiation factors eIF2, eIF3, and eIF4B. As a result, the large subunit now binds to the small subunit, the initiator tRNA is aligned within the ribosomal P-site, and translation begins (9).

In the elongation stage, the three-base anticodon of a charged tRNA, a tRNA with its specific amino acid covalently attached, base pairs with the three-base codon of the mRNA occupying the aminoacyl-tRNA (A) site of the ribosome. The aminoacyl-tRNAs are brought to the A-site as a ternary complex of eEF1α•GTP•aminoacyl-tRNA. Following GTP hydrolysis, the aminoacyl-tRNA binds to the A-site and eEF1α•GDP is

released from the ribosome. Peptide bond formation between the growing polypeptide chain on the tRNA in the P site and the amino acid attached to the A-site tRNA is catalyzed by the peptidyl transferase center (PTC) located in the large subunit of the ribosome. Thus, the growing polypeptide chain becomes attached to the tRNA in the A-site. Next, translocation, the movement of the ribosome along the mRNA by one codon, occurs with binding of GTP-bound elongation factor eEF2●GTP to the ribosome. GTP is then hydrolyzed and, subsequently, the P-site tRNA dissociates from the ribosome, the A-site tRNA moves to the P-site, eEF2●GDP dissociates, and the mRNA moves by three nucleotides to expose the next codon in the A-site. This process repeats itself until a stop codon is reached and is positioned in the A-site (1, 10). When one of the three stop codons appear in the A-site of the ribosome, a GTP-bound release factor complex, eRF1●eRF3●GTP, binds to the codon within the A-site. After the complex binds to the A-site, GTP is hydrolyzed, and the polypeptide chain is transferred from the P-site tRNA to a water molecule, ending translation. The completed protein, the tRNAs, the release factors, and the mRNA are released from the ribosome, and the ribosome dissociates into its large and small subunits (1,11).

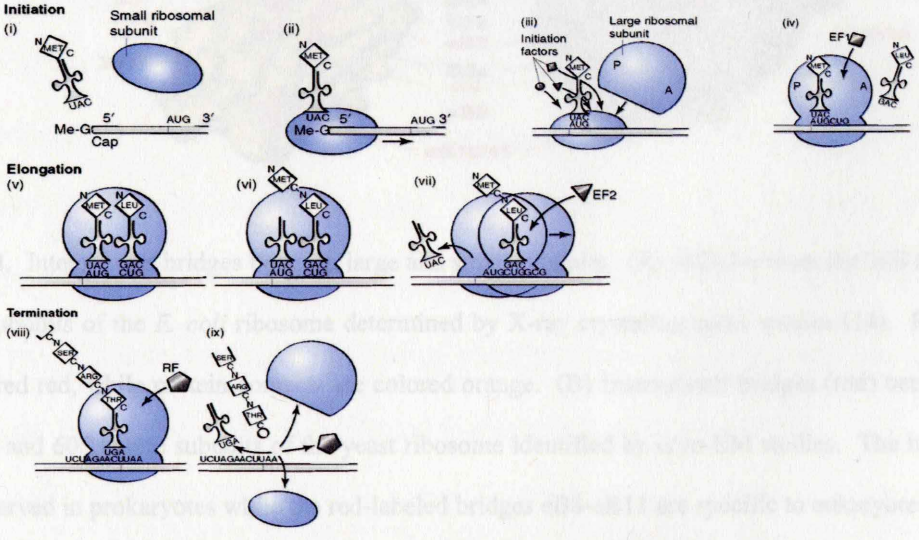


Figure 3. A summary of translation. Adapted from (12).

Ribosome Subunit Association

The two-subunit association of the intact ribosome is intimately related to its function (13). Recently, X-ray diffraction and cryo-EM techniques have revealed that there are specific interactions that exist between the two subunits of the ribosome (Figure 4) (3, 14, 15, 16). These so-called intersubunit bridges (ISBs) are areas of close contact between the two subunits, and are composed of interactions between RNA and RNA, RNA and proteins, or proteins and proteins (Figure 4).

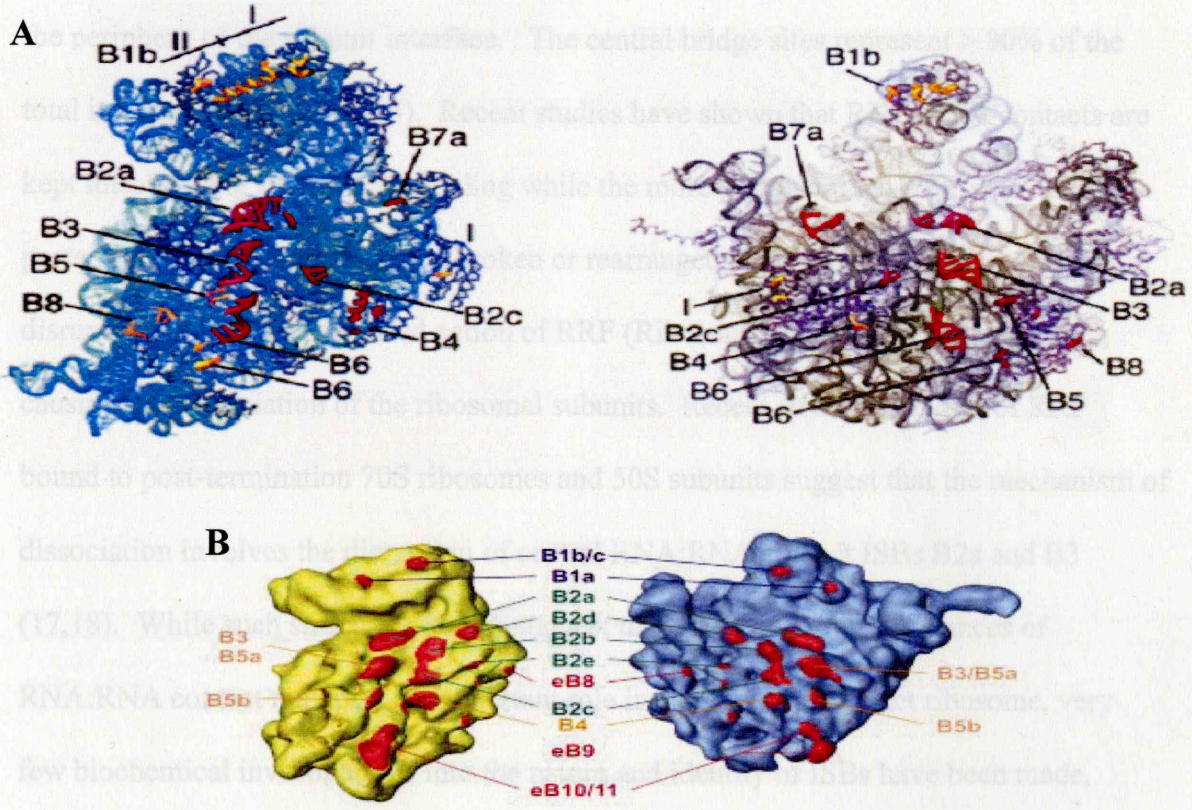


Figure 4. Intersubunit bridges between large and small subunits. (A) ISBs between the 30S (left) and 50S (right) subunits of the *E. coli* ribosome determined by X-ray crystallographic studies (14). RNA contacts are colored red, while protein contacts are colored orange. (B) Intersubunit bridges (red) between the 40S (yellow) and 60S (blue) subunits of the yeast ribosome identified by cryo-EM studies. The bridges shown are conserved in prokaryotes while the red-labeled bridges eB8-eB11 are specific to eukaryotes (16).

Intersubunit Bridges

Intersubunit bridges appear to be well conserved between all kingdoms of life (4,15). While all three classes of ISBs (RNA:RNA, RNA:protein, and protein:protein) likely perform either a stabilizing or regulating function, RNA-only bridge sites have a special significance in stabilizing the intact ribosome (3, 15). The two central areas of both subunits, the peptidyl transferase center in the LSU and the decoding center in the SSU, contain only RNA:RNA ISBs. On the other hand, protein- containing ISBs exist on the periphery of the subunit interface. The central bridge sites represent > 80% of the total intersubunit contacts (15). Recent studies have shown that RNA-RNA contacts are kept intact during EF-G•GTP binding while the more peripheral RNA-protein and protein-protein contacts are often broken or rearranged (15,17). RNA-RNA contacts are disrupted through the concerted action of RRF (Ribosome Release Factor) and EF-G, causing the dissociation of the ribosomal subunits. Recent structure studies of RRF bound to post-termination 70S ribosomes and 50S subunits suggest that the mechanism of dissociation involves the disruption of central RNA:RNA contact ISBs B2a and B3 (17,18). While such structural studies suggest that ISB's containing instances of RNA:RNA contact may play an important role in stabilizing the intact ribosome, very few biochemical investigations into the nature and identity of ISBs have been made, especially regarding eukaryotic systems. The LaRiviere Group is interested in identifying ISBs and determining their significance in eukaryotes using biochemical techniques. To date, the LaRiviere Group has identified a single ISB (B3) in yeast ribosomes using a localized site-directed mutagenesis experiment (19).

Modification Interference

While site-directed mutagenesis experiments offer a powerful tool for biochemically assessing potential ISB sites apparent from structural studies, the technique of modification interference allows for a more global search to locate ribosomal ISBs. Indeed, the first identification of rRNA participation in intersubunit interactions was accomplished using modification interference methods in bacterial ribosomes (20). Modification interference assays identify positions which interfere with macromolecular assembly via chemical modification. Chemical probes are used to modify target positions on RNA molecules (21). Based on the reagent chosen, the modification experiment can be optimized so that modifications are introduced to nucleobases at a low level (e.g. less than one modification per molecule) (22).

Recently, Remme, *et. al.* have reported modification interference assays for identifying nucleobases essential for ribosome subunit association in *E. coli* (13, 23). In one such study, native *E. coli* SSU (30S) subunits were subjected to modification using dimethylsulfate (DMS) [methylates N1 positions of adenosines, N7 positions of guanosines, and N3 positions of cytosines] or 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluene sulfonate (CMCT) [modifies N1 and N3 positions of uracils and N1 positions of guanosines] (21). These modified 30S subunits were reassociated with unmodified 50S subunits to form intact 70S subunits. Modified 30S subunits which failed to reassociate were isolated, and the 16S rRNA was extracted from both the modified 30S populations and the intact 70S populations. Using primer extension and autoradiography, the 16S rRNA was scanned for DMS- or CMCT-specific reverse transcriptase stops. Modifications which were present in the 30S subunits but

strongly reduced in the 70S ribosomes were identified as interfering with 70S formation (Figure 5) (23).

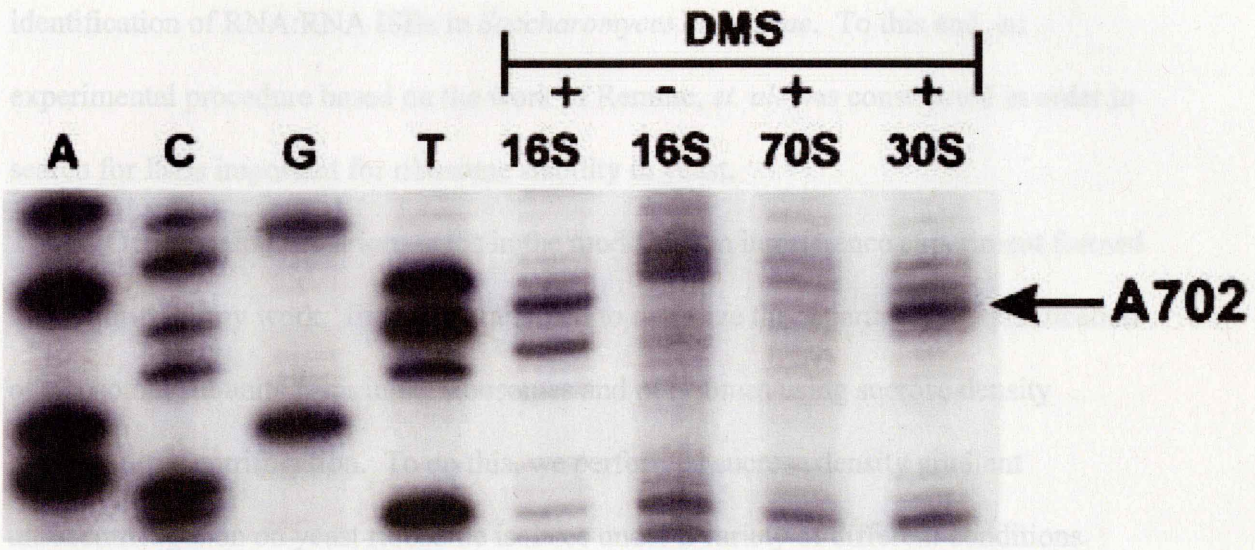


Figure 5. Analysis of the dimethylsulfate (DMS) modifications in 16S rRNA resulting in reverse transcriptase stops by Remme, *et. al.* When modified, position A702 interferes with reassociation into intact 70S. This base is located in the known ISB B7a. Adapted from (23).

Purpose of Project

My project was to begin investigating the significance of ISBs in eukaryotic systems using modification interference techniques. The project involved both the design and implementation of a modification interference experiment which would permit the identification of RNA:RNA ISBs in *Saccharomyces cerevisiae*. To this end, an experimental procedure based on the work of Remme, *et. al.* was constructed in order to search for ISBs important for ribosome stability in yeast.

Optimization of various steps in the modification interference experiment formed the majority of my work. First, we attempted to optimize the separation and purification of ribosomal subunits from intact ribosomes and polysomes using sucrose density gradient ultracentrifugation. To do this, we performed sucrose density gradient ultracentrifugation on yeast ribosome isolates under a variety of different conditions. Secondly, we also developed a protocol which allowed for the accurate assessment of subunit separation. Thus, various fractionation and UV/vis spectroscopic methods, both automatic and manual, were attempted and optimized. We hope that the optimizations of these first steps of the modification interference experiment will allow for a continuation of the protocol toward identifying RNA:RNA ISBs in yeast.

MATERIALS AND METHODS

Miscellaneous Techniques

Yeast Starter Culture Growth

To isolate ribosomes and ribosomal subunits from *Saccharomyces cerevisiae*, yeast starter cultures were grown in complete "YPD" media (1% yeast extract, 2% peptone, 2% glucose). A 50 mL conical tube containing 5 mL of autoclaved YPD was inoculated with a medium-sized colony of yeast strains BY4741 (MATa, hisΔ1, leu2Δ0, met15Δ0, ura3Δ0) or BY4733 (MATa, his3Δ200, leu2Δ0, met15Δ0, trp1Δ63, ura3Δ0) under sterile conditions. The inoculated media was placed in an incubator shaker (Excella E24, New Brunswick Scientific) at 30.0 °C and 225 rpm and allowed to grow to saturation overnight. Saturated cultures were then stored at 4 °C indefinitely.

Yeast Cell Extract Preparation

Before each cell lysis procedure, new cells were grown from a starter culture. A specific volume was removed from a resuspended starter culture and transferred into either 50 mL or 80 mL of autoclaved YPD in an Erlenmeyer flask under sterile conditions. The transfer volume was a calculated value which corresponded to a target growth level between 0.4-0.6 OD_{600} (Optical Density at 600 nm) after a set number of yeast cell doublings. Yeast strains BY4741 and BY4733 had been determined to have a growth rate consistent with a doubling in population every ~1.5 hours.

Yeast RNA Extraction

If RNA was isolated directly from yeast cells, the cells were first pelleted by centrifugation (centrifuge 5810R, Eppendorf) for 3 min at 3,500 rpm at 4 °C. The supernatant was discarded and the cells were resuspended in 1 mL of AE Buffer (50 mM

sodium acetate, 10 mM EDTA, [pH 5.2]). The supernatant discarding and resuspension steps were repeated two additional times, with the final resuspension in 450 μL of AE Buffer. 50 μL of 10% sodium dodecyl sulfate (SDS) was added, and the cells were vortexed on high for 10 seconds twice. 500 μL of saturated phenol (CAUTION: KNOWN HAZARD, SEE P. 27) [pH 4.3] was added and the solution was vortexed two additional times. The tubes were incubated at 65 °C for 5 minutes. The tubes were then placed in crushed dry ice and cooled for 30 seconds. The tubes were centrifuged (accuSpin Micro 17R, Fisher Scientific) for 5 minutes at 13,300 rpm and the aqueous layer was carefully transferred to a new microcentrifuge tube.

To these samples, or for any samples from gradient fractions from which RNA was to be isolated, 500 μL of saturated phenol/chloroform/isoamyl alcohol [pH 4.3] was added. The vortexing, centrifugation, and aqueous layer transfer steps were repeated as above. 500 μL of chloroform was then added. The vortexing, centrifugation, and aqueous layer transfer steps were again repeated as above. 50 μL of 3M sodium acetate [pH 5.2] was added to each tube followed by vortexing for 10 seconds. Two volumes (~1000 μL) of 100% EtOH was added to the samples which were then placed in a -20 °C freezer for 24-48 h to precipitate the RNA. The samples were then centrifuged at 13,300 rpm for 15 minutes, and the supernatant was discarded. The pellet was washed in 500 μL 70% EtOH. The centrifugation step was repeated and the supernatant was carefully removed. The pellet was dried for approximately 10 minutes in an automated vacuum dryer (Savant DNA 120, Thermo Electron Corporation). The pellets were resuspended in 50 or 20 μL of deionized H_2O (diH_2O) depending on whether they contained total RNA or RNA from isolated fractions, respectively.

DMS Modification

DMS modification of total yeast RNA was conducted as described (20). Total RNA was isolated from yeast cell lysates as described above. A stock solution of DMS (10.5 M) (CAUTION: KNOWN HAZARD SEE P. 26) was diluted by a factor of 1:12 with 100% EtOH to achieve a target concentration of approximately 875 mM. 1 µL of diluted DMS was added to one of two tubes containing 50 µL of 1x Mod Buffer (100 mM Tris-HCl [pH 7.5], 100 mM $MgCl_2$, 500 mM KCl, 50 mM dithiothreitol) and 10 µg of total yeast RNA resulting in a final DMS concentration of 17.5 mM. Two reactions (+DMS, -DMS) were incubated at 37 °C for 15 minutes (24). The reactions were quenched by the addition of 5 µL of 3M sodium acetate [pH 5.2] and 200 µL of 100% EtOH. The RNAs were precipitated at -80 °C for 15 minutes, followed by centrifugation (accuSpin Micro 17R, Fisher Scientific) at 13,300 rpm for 15 minutes at 4 °C. The supernatants were discarded, and the pellets were washed in approximately 200 µL of 70% EtOH. The centrifugation step was repeated and then the supernatants were carefully removed. The pellets were dried for approximately 10 minutes in an automated vacuum dryer (Savant DNA 120, Thermo Electron Corporation), and resuspended in 10 µL of deionized H_2O.

Dialysis and Reassociation of Ribosomal Subunits

80S ribosomes were reassociated from purified, unmodified 40S and 60S ribosomal subunits. The purification of ribosomal subunits is described beginning on p. 17. 40S and 60S containing fractions separated in protocols without the use of 2x Buffer A were pooled. 40S and 60S containing fractions separated in protocols using 2x Buffer A were dialyzed for approximately 48 h against 1x Buffer A (20mM Tris-HCl [pH 7.5],

16 mM $MgCl_2$, 1.0 M KCl, 12 mM β-mercaptoethanol, 0.2 mM EDTA) using dialysis cassettes (Slide-A-Lyzer Dialysis Kit - 10,000 MW capacity, Thermo Scientific) prior to reassociation. Reassociation of 40S and 60S into intact 80S ribosomes was induced by mixing 500 µL of the fractions and incubation at 37 °C for 30 minutes. Reassociation reaction products were analyzed for the presence of 80S subunits using polysome profile generating sucrose density gradient ultracentrifugation (described on p. 19 and 20).

Primer Extension/RNA sequencing

A primer extension/RNA sequencing experiment using ^{32}P-labeled primers was conducted using total yeast RNA as well as both the +DMS and –DMS reaction products described previously as substrate. Two ^{32}P-labeled primers were selected for the experiment: FL218 (5'-AATAAATACATCTCTTCCAAAG-3') and FL220 (5'-ACAACTCGGGCACCGAAGGTAC-3') which would anneal to a complementary region on the 18S rRNA and 25S rRNA, respectively (25). Twelve 5 µL volumes were made in 1x Annealing Buffer (500 mM KCl, 250 mM Tris-HCl [pH 7.5]) in thermocycler tubes. Two contained approximately 0.6 pmol of +DMS product, two contained the same concentration of –DMS product, and the remaining eight contained the same concentration of total RNA. Approximately 4 pmol of one of the two ^{32}P-labeled primers was added to each depending on whether primer extension was to be performed on 18S rRNA (FL218) or 25S rRNA (FL220). The annealing reactions were placed in a thermocycler (MG Mini, BioRad) at 95 °C for 5 minutes, followed by a step-wise decrease in temperature by -1 °C per minute to 25 °C in order to anneal the primers to the template rRNA. For primer extension, 1 µL of 1x Primer Extension Buffer and 2 µL of 1x dNTPs (100 µM each) were added to a 1 µL volume of the annealing reaction

products. For sequencing, 2 μL of either 1x ddA, ddC, ddG, or ddT (10 μM each) were added to a 1 μL volume of the annealing reaction products. 1 U of AMV-RT enzyme (Sigma) was added to each reaction and they were placed in the thermocycler for 5 minutes at 25 °C followed by 30 minutes at 42 °C. 1 μL of dNTPs (1 mM each) and 1 U of AMV-RT were then added and the reactions were heated for 15 minutes at 42 °C for further extension. 6 μL of formamide dye was added to each reaction and then they were heated for 2 minutes at 95 °C to denature the enzyme. The reactions were placed on ice prior to being loaded onto a polyacrylamide gel.

Gel Electrophoresis

The primer extension reactions were assayed using denaturing polyacrylamide gel electrophoresis and phosphorimaging. 2 μL of each reaction was loaded onto a 10% polyacrylamide / 7M urea gel. The gel was run at 20W until the leading dye had migrated nearly to the bottom of the gel (approximately 1.5 h). The gel was carefully removed, wrapped in plastic wrap, and placed on a phosphorimager screen for approximately 24 h. The screen was visualized using a PMI phosphorimager (BioRad).

Cell Lysis

Cell Lysis Procedure for Ribosomal Subunit Separation Profiles

50 or 80 mL yeast cultures grown to a target OD_{600} of 0.4-0.6 were chilled on ice for approximately 20 minutes. The cells were pelleted by centrifugation (centrifuge 5810R, Eppendorf) for 3 min at 3,500 rpm at 4 °C. The supernatant was discarded and the cells were resuspended in 2.5 mL of either Lysis Buffer (20mM Tris-HCl [pH 8.0], 140mM KCl, 5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mg/mL Heparin sodium, 1% Triton-X), Buffer X (50 mM Tris-HCl [pH 7.5], 50 mM NaCl, 1 mM dithiothreitol), 2x Buffer A

(20mM Tris-HCl [pH 7.5], 16 mM $MgCl_2$, 1.0 M KCl, 12mM β-mercaptoethanol, 0.2 mM EDTA), or Low Mg Buffer (10mM Tris-HCl [pH 7.5], 1 M NaCl, 30 mM EDTA) (19, 26, 27, 28). The centrifugation/resuspension steps were repeated two additional times, with the final resuspension in 700 µL of respective buffer. The resuspended cells were added to approximately 0.5 mL of glass beads (0.55 mm diameter) in either a 1.5 mL or 2.0 mL microcentrifuge tube and vortexed for 20 seconds, followed by chilling on ice for 100 seconds. This vortex/chill step was repeated three additional times. The lysed cells were then centrifuged (accuSpin Micro 17R, Fisher Scientific) at 4,700 rpm for 5 minutes at 4 °C. The supernatant was carefully removed and transferred to a new microcentrifuge tube. This lysate was centrifuged at 9,500 rpm for 5 minutes at 4 °C; The supernatant was carefully removed and then immediately layered onto a sucrose density gradient (19).

Cell Lysis Procedure for Polysome Profiles

50 or 80 mL yeast cultures grown to a target OD_{600} of 0.4-0.6 were treated with 0.1 mg/mL cycloheximide. Each culture was chilled on ice for approximately 20 minutes, and then the cells were pelleted by centrifugation (centrifuge 5810R, eppendorf) for 3 min at 3,500 rpm at 4 °C. The supernatant was discarded and the cells were resuspended in 2.5 mL of either Lysis Buffer, Polysome Buffer (20mM Tris-HCl [pH 8.0], 140mM KCl, 5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mg/mL Heparin sodium), or High Mg Buffer (10mM Tris-HCl [pH 7.5], 100 mM NaCl, 300 mM $MgCl_2$) (19, 24). Subsequent steps were identical to those described in "Cell Lysis Procedure for Ribosomal Subunit Separation Profiles".

Ultracentrifugation

Sucrose Density Gradient Preparation for Isolating Ribosomal Subunits Utilizing a Sorvall AH-629 Rotor

To separate 40S and 60S subunits from intact 80S ribosomes and polysomes, 25 mL of either 10-50% or 6-30% linear sucrose density gradients were prepared for each cell lysate. Solutions of varying sucrose concentrations (10%, 20%, 30%, 40%, 50% or 6%, 12%, 18%, 24%, 30%) were made in Buffer X (25). 5 mL of each solution was carefully added sequentially from lowest to highest sucrose concentration to the bottom of a 36 mL ultracentrifuge tube (Sorvall, Dupont) for use with the Sorvall AH-629 rotor. The gradients were stored undisturbed standing vertically at 4 °C and allowed to equilibrate overnight.

Sucrose Density Gradient Preparation for Generating Polysome Profiles Utilizing a Sorvall AH-629 Rotor

To separate out 40S, 60S, 80S, and polysome particles, 25 mL 10-50% linear sucrose density gradients were prepared for each cell lysate. Solutions of varying sucrose concentrations (10%, 20%, 30%, 40%, 50%) were made in Polysome Buffer (19). 5 mL of each solution was carefully added sequentially from lowest to highest sucrose concentration to the bottom of a 36 mL ultracentrifuge tube (Sorvall, Dupont) for use with the Sorvall AH-629 rotor. The gradients were stored undisturbed standing vertically at 4 °C and allowed to equilibrate overnight.

Sucrose Density Gradient Preparation for Isolating Ribosomal Subunits Utilizing a Sorvall TH-641 Rotor

To separate 40S and 60S subunits from intact 80S ribosomes and polysomes, 10 mL of either 10-50%, 10-40%, or 6-30% linear sucrose density gradients were prepared for each cell lysate. Solutions of varying sucrose concentrations (10%, 20%, 30%, 40%, 50%, or 6%, 12%, 18%, 24%, 30%, or 10%, 20%, 30%, 40%) were made in Polysome Buffer or 2x Buffer A (19, 28). 2 or 2.5 mL of each solution was carefully added sequentially from lowest to highest sucrose concentration to the bottom of a 12 mL ultracentrifuge tube (Sorvall, Dupont) for use with the Sorvall TH-641 rotor. The gradients were capped with parafilm, stored lying horizontally at 4 °C and allowed to equilibrate overnight. The gradients were turned upright approximately 1-2 hours prior to ultracentrifugation (19).

Sucrose Density Gradient Preparation for Generating Polysome Profiles Utilizing a Sorvall TH-641 Rotor

To separate out 40S, 60S, 80S, and polysome particles, 10 mL of either 10-50% or 6-30% linear sucrose density gradients were prepared for each cell lysate. Solutions of varying sucrose concentrations (10%, 20%, 30%, 40%, 50%, or 6%, 12%, 18%, 24%, 30%) were made in Polysome Buffer (19). 2 or 2.5 mL of each solution was carefully added sequentially from lowest to highest sucrose concentration to the bottom of a 12 mL ultracentrifuge tube (Sorvall, Dupont) for use with the Sorvall TH-641 rotor. The gradients were capped with parafilm, stored lying horizontally at 4 °C and allowed to equilibrate overnight. The gradients were turned upright approximately 1-2 hours prior to centrifugation (19).

Ultracentrifugation of Cell Lysates Using the AH-629 Rotor

The Sorvall AH-629 rotor, ultracentrifuge (Sorvall OTD60B, Dupont), and tube buckets were equilibrated to and kept at 4 °C prior to centrifugation. Cell lysates designated for either ribosomal subunit isolations or polysome profile generations were brought to approximately 1 mL total volume with the respective gradient buffer. The lysates were carefully layered onto the corresponding gradients, and the gradient weights were adjusted with buffer to be within 0.1 g of each other. The gradients were ultracentrifuged at varying speeds (22.0, 24.0, 27.2, 29.0 x 1000 rpm) for varying periods of time (12, 17, and 18 hours) at 4 °C depending on the experiment (see results).

Ultracentrifugation of Cell Lysates Using the TH-641 Rotor

The Sorvall TH-641 rotor, ultracentrifuge (Sorvall OTD60B, Dupont), and tube buckets were equilibrated and kept at 4 °C prior to centrifugation. Cell lysates designated for either ribosomal subunit isolations or polysome profile generations were brought to approximately 800 µL total volume with respective gradient buffer. The lysates were carefully layered onto the corresponding gradients, and the gradient weights were adjusted with buffer to be within 0.1 g of each other. The gradients were ultracentrifuged at 35,000 rpm or 37,000 rpm for 3 h at 4 °C depending on the experiment (see results).

Gradient Fractionation

Manual Fractionation of Gradients

Gradients selected for manual fractionation were carefully removed from tube buckets and held upright on a ring stand. Fractions ranging from 250 – 300 µL were pipetted carefully by hand from the very top of the gradient and placed in either microcentrifuge tubes or 96-well plates. Alternatively for some gradients, a small hole

was introduced into the bottom of the gradient and 250 µL fractions were collected dropwise into 96-well plates (Figure 6). Fractions were stored at 4 °C until analysis.

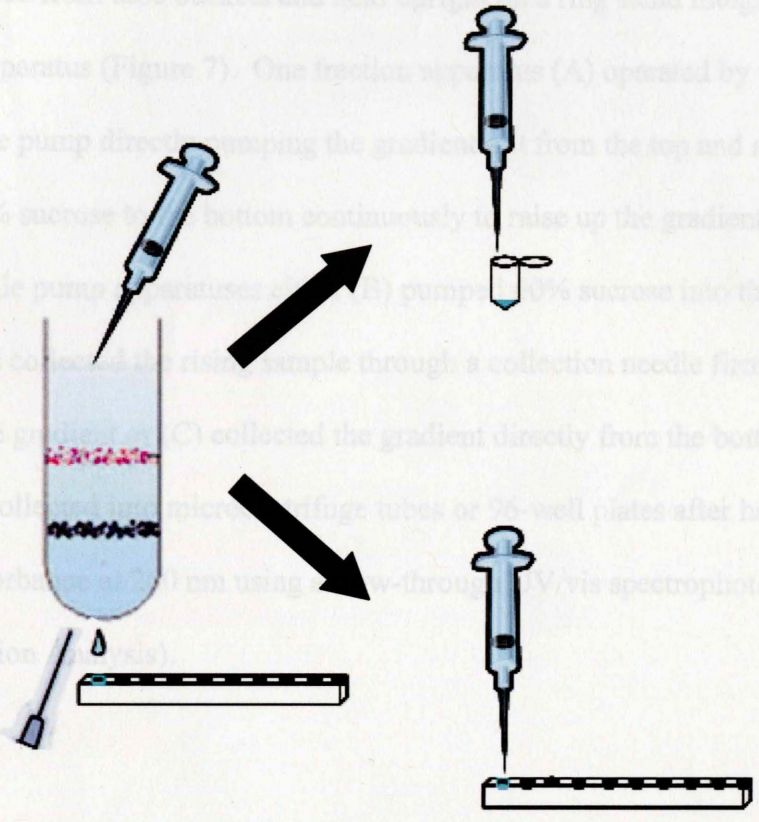


Figure 6. Manual gradient fractionation. Fractionation was either from the very top or through the bottom of the gradient. Fractions taken from the top of the gradient were stored either in microcentrifuge tubes or in 96-well plates. Fractions collected dropwise by puncturing the bottom of the gradient using a small (16 Ga) needle were stored in 96-well plates.

Semi-Automated Fractionation of Gradients

Gradients selected to be fractionated by semi-automated procedures were carefully removed from tube buckets and held upright on a ring stand integrated into a fractionation apparatus (Figure 7). One fraction apparatus (A) operated by using two pumps, with one pump directly pumping the gradient out from the top and a second pump introducing 60% sucrose to the bottom continuously to raise up the gradient for collection. Alternative single pump apparatuses either (B) pumped 60% sucrose into the bottom of the gradient and collected the rising sample through a collection needle firmly stoppered on the top of the gradient or (C) collected the gradient directly from the bottom. The fractions were collected into microcentrifuge tubes or 96-well plates after having been assayed for absorbance at 260 nm using a flow-through UV/vis spectrophotometer (see Subunit Separation Analysis).

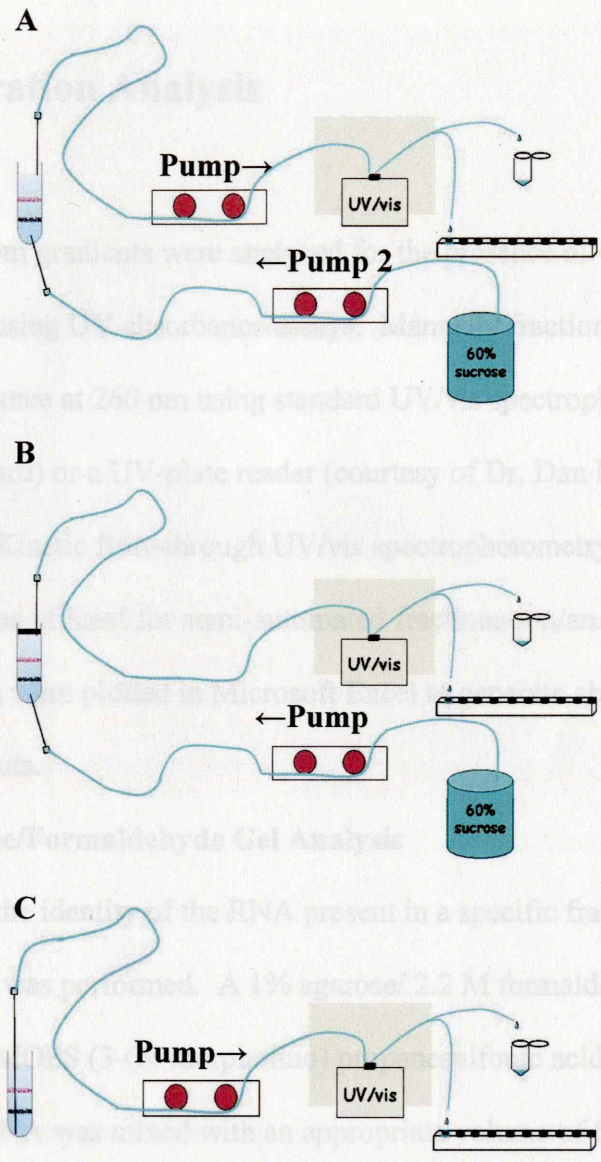


Figure 7. Semi-automated gradient fractionation. (A) One pump directly pumps the gradient out from the top as a second pump introduces 60% sucrose to the bottom continuously to raise up the gradient for collection. (B) A single pump introduces 60% sucrose into the bottom of the gradient. The top of the gradient is stoppered with a rubber stopper containing a needle which collects the rising gradient. (C) A single pump directly pumps the gradient out from the bottom. As the gradient flows through the tubing of all three setups, it is analyzed for absorbance at 260 nm in real-time before being separated into fractions by collection into microcentrifuge tubes or 96-well plates.

Subunit Separation Analysis

UV/vis Analysis

Fractions from gradients were analyzed for the presence of 40S, 60S, 80S, and polysome particles using UV absorbance assays. Manually fractionated samples were analyzed for absorbance at 260 nm using standard UV/vis spectrophotometry (Agilent 8453, Hewlett Packard) or a UV-plate reader (courtesy of Dr. Dan McCain, Virginia Military Institute). Kinetic flow-through UV/vis spectrophotometry (Agilent 8453, Hewlett Packard) was utilized for semi-automated fractionation/analysis coupled samples. The absorbance data were plotted in Microsoft Excel to generate absorbance profile curves for all gradients.

Denaturing Agarose/Formaldehyde Gel Analysis

To determine the identity of the RNA present in a specific fraction, denaturing agarose gel analysis was performed. A 1% agarose/ 2.2 M formaldehyde gel was prepared in 25 mM MOPS (3-(N-morpholino) propanesulfonic acid) [pH 7.0] buffer. 1 µg of diluted total RNA was mixed with an appropriate volume of formamide loading dye and the entire sample was loaded into the gel. The gel was run between 100-110 V until the leading dye was approximately 2/3 down the length of the gel to allow for separation of the 18S and 25S rRNA (approximately 1.5 hours). The gel was stained in 0.5 ng/mL ethidium bromide for ~15 minutes, destained in diH_2O for 20 minutes, and then visualized using a UV-camera.

Known Hazards

Dimethylsulfate (DMS)

Dimethylsulfate vapor and liquid are extremely hazardous and should only be used and handled in the hood with appropriate protections (safety glasses, double gloves, lab coat). Skin or eye contact with low concentrations of dimethylsulfate may cause analgesia (numbness). Therefore, the corrosive action of dimethylsulfate may not be immediately noticed. Skin contact with dimethylsulfate may cause skin burns or ulceration. Skin permeation can occur in amounts capable of producing the effects of systemic toxicity. Eye contact with dimethylsulfate may cause eye corrosion with corneal or conjunctival ulceration. Permanent eye damage may result The vapors and the liquid can cause severe irritation. Upon slight vapor exposure the whites of the eyes turn red.

Ingestion of dimethylsulfate may cause severe irritation of the mucous membranes of the mouth, throat, and gastrointestinal tract. Inhalation of dimethylsulfate may cause irritation of the upper respiratory passages, angioneurotic edema of larynx with hoarseness, difficulty in swallowing, temporary lung irritation effects with productive cough, discomfort, difficulty breathing, chest pain, shortness of breath or cyanosis. DMS exposure may also present modest initial symptoms, followed in hours by severe shortness of breath which requires prompt medical attention. In severe poisonings, certain central nervous system effects may occur including unconsciousness, cramps, convulsions, and paralysis. Fatality may result from gross overexposure (29).

Phenol

Phenol liquid and vapor are toxic, corrosive, and combustible. Phenol should only be used and handled in the hood with appropriate protections (safety glasses, double gloves, lab coat). Phenol is harmful if swallowed, inhaled, or absorbed through the skin. Phenol may cause severe respiratory and digestive tract irritation with possible burns. It causes severe irritation of the upper respiratory tract with coughing, burns, breathing difficulty, and possible coma. Inhalation of high concentrations may be fatal. Phenol exposure through ingestion may cause pallor, loss of appetite, nausea, vomiting, diarrhea, weakness, darkened urine, headache, sweating, convulsions, cyanosis, unconsciousness, fatigue, pulmonary edema, and coma. Phenol exposure may cause severe eye and skin burns. Exposure can result in irreversible eye damage. Direct skin contact results in white, wrinkled discolorations followed by severe burns. Phenol solutions may be absorbed through the skin rapidly, causing systemic poisoning and possibly death. Phenol exposure may also cause liver and kidney damage, reproductive and fetal effects, and central nervous system effects (30).

Results

Part 1- Experiment Design and Initial Attempts of Each Step

Overview

A modification interference protocol resulting in the successful identification of RNA:RNA ISBs has been reported for *E. coli* ribosomes (13, 23). My goal was to adapt this specific protocol to perform a similar investigation with yeast ribosomes. To this end, the discrete steps of the experiment were attempted to demonstrate that such a modification interference approach could be used to search for yeast ribosome ISBs.

The Modification Interference Protocol Design

An overview of the experiment design is outlined in Figure 8. To identify RNA:RNA ISBs in yeast ribosomes, intact ribosomes and ribosomal subunits will be isolated and purified from yeast cells using sucrose density gradient ultracentrifugation. The resulting gradients will be analyzed for the presence of 40S and 60S subunits and intact 80S ribosomes using UV/vis analysis and denaturing agarose gel analysis. The isolated 40S subunits will be chemically modified with the methylating agent dimethylsulfate (DMS). These modified 40S (40S*) will be allowed to reassociate with native 60S subunits in a reassociation reaction. The reassociation reaction will be assessed using a sucrose density gradient designed to separate 40S*, 60S, and intact 80S ribosomes which formed from a successful reassociation of 40S* and 60S. The 40S* (that did not reassociate) and 80S ribosomes will be isolated in order to determine the positions of the interfering modifications, which may correspond to the ISB site(s). This will be achieved using a primer extension assay.

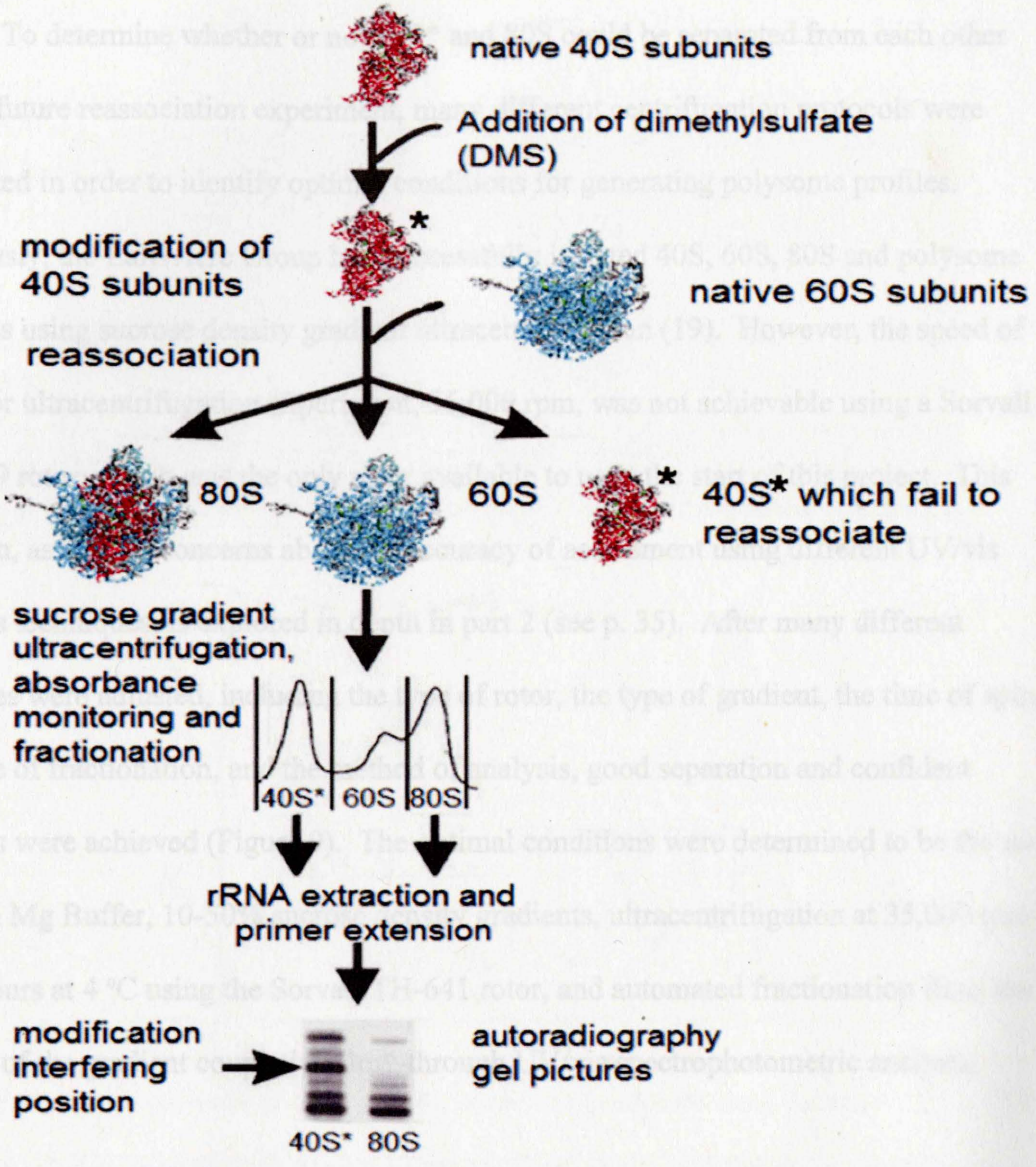


Figure 8. An overview of the modification interference protocol. All of the steps presented here were attempted as part of this project. Figure adapted from (23).

Separation of 40S subunits and Intact 80S

To determine whether or not 40S* and 80S could be separated from each other after a future reassociation experiment, many different centrifugation protocols were attempted in order to identify optimal conditions for generating polysome profiles. Previously, the LaRiviere Group had successfully isolated 40S, 60S, 80S and polysome particles using sucrose density gradient ultracentrifugation (19). However, the speed of the prior ultracentrifugation experiment, 35,000 rpm, was not achievable using a Sorvall AH-629 rotor, which was the only rotor available to us at the start of this project. This problem, as well as concerns about the accuracy of assessment using different UV/vis analysis techniques, is explored in depth in part 2 (see p. 35). After many different variables were adjusted, including the type of rotor, the type of gradient, the time of spin, the type of fractionation, and the method of analysis, good separation and confident analysis were achieved (Figure 9). The optimal conditions were determined to be the use of High Mg Buffer, 10-50% sucrose density gradients, ultracentrifugation at 35,000 rpm for 3 hours at 4 °C using the Sorvall TH-641 rotor, and automated fractionation from the bottom of the gradient coupled to flow-through UV/vis spectrophotometric analysis.

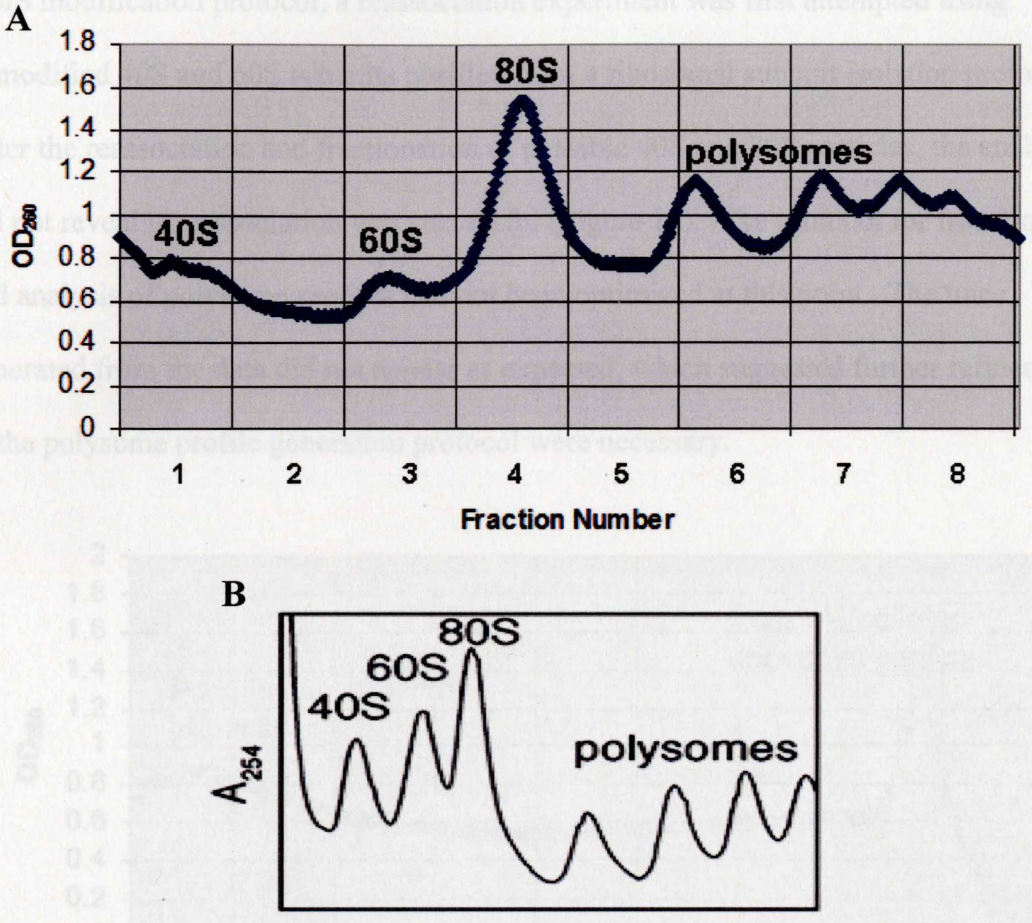


Figure 9. Optimized polysome profiles. (A) Polysome profile achieved using optimal conditions (High Mg Buffer, 10-50% sucrose density gradients, ultracentrifugation at 35,000 rpm for 3 hours at 4 °C on the Sorvall TH-641 rotor, semi-automated fractionation setup C). (B) Representative literature polysome profile adapted from (25). The trace (A) is similar to what was expected from the literature (B).

Reassociation of 40S and 60S Subunits

To determine whether or not 40S* and 60S subunits could be reassociated after a DMS modification protocol, a reassociation experiment was first attempted using unmodified 40S and 60S subunits purified from a ribosomal subunit isolation protocol. After the reassociation and fractionation of possible 40S and 80S particles, the analysis did not reveal if reassociation was successful (Figure 10). The protocol for fractionation and analysis of polysome profiles had not been optimized at this point. The trace generated from the data did not appear as expected, which suggested further refinements to the polysome profile generation protocol were necessary.

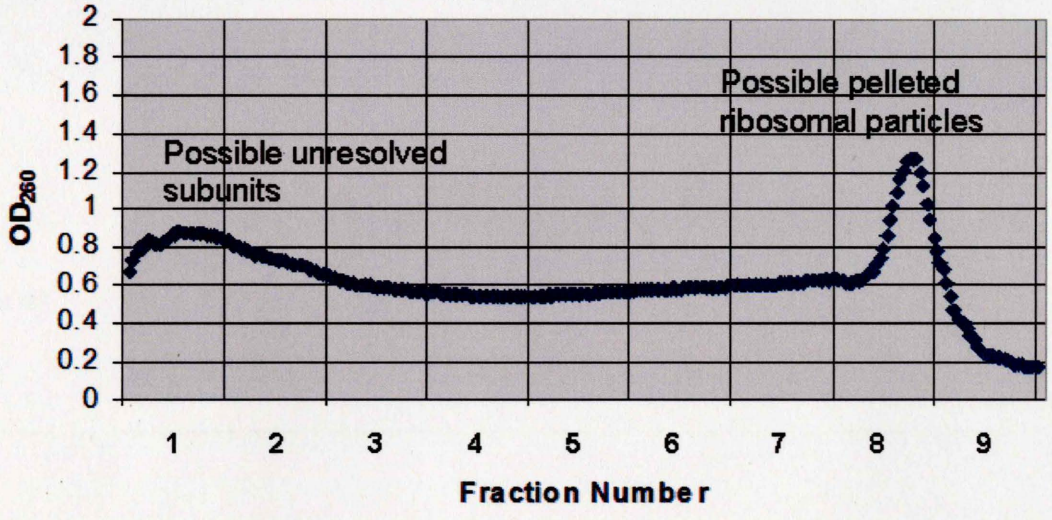


Figure 10. Inconclusive polysome profile to assess ribosome subunit reassociation. Isolated 40S and 60S subunits were allowed to reassociate in a reassociation experiment and then a fraction of these products were layered onto a gradient and analyzed (Polysome Buffer, 10-50% sucrose density gradients, ultracentrifugation at 35,000 rpm for 3 h at 4 °C on the Sorvall TH-641 rotor, automated fractionation setup A). The "peak" designations above are postulates. The lack of characteristic trace shape suggested that a refinement of the protocol was necessary.

DMS Modification, Primer Extension, and Phosphorimager Analysis

To determine if the crucial steps of our modification interference protocol (i. e., the introduction of chemical modifications and the determination of which modified positions interfere with 80S formation) were possible, a trial was performed using total yeast RNA. The RNA was modified by DMS methylation and then reverse transcribed in a primer extension experiment. The product DNA was run on a gel along with the products of an RNA sequencing reaction. The resultant gel was inconclusive as to the success of the DMS modification, the primer extension experiment, and the RNA sequencing. Significant blurring of the gel bands was apparent (Figure 11).

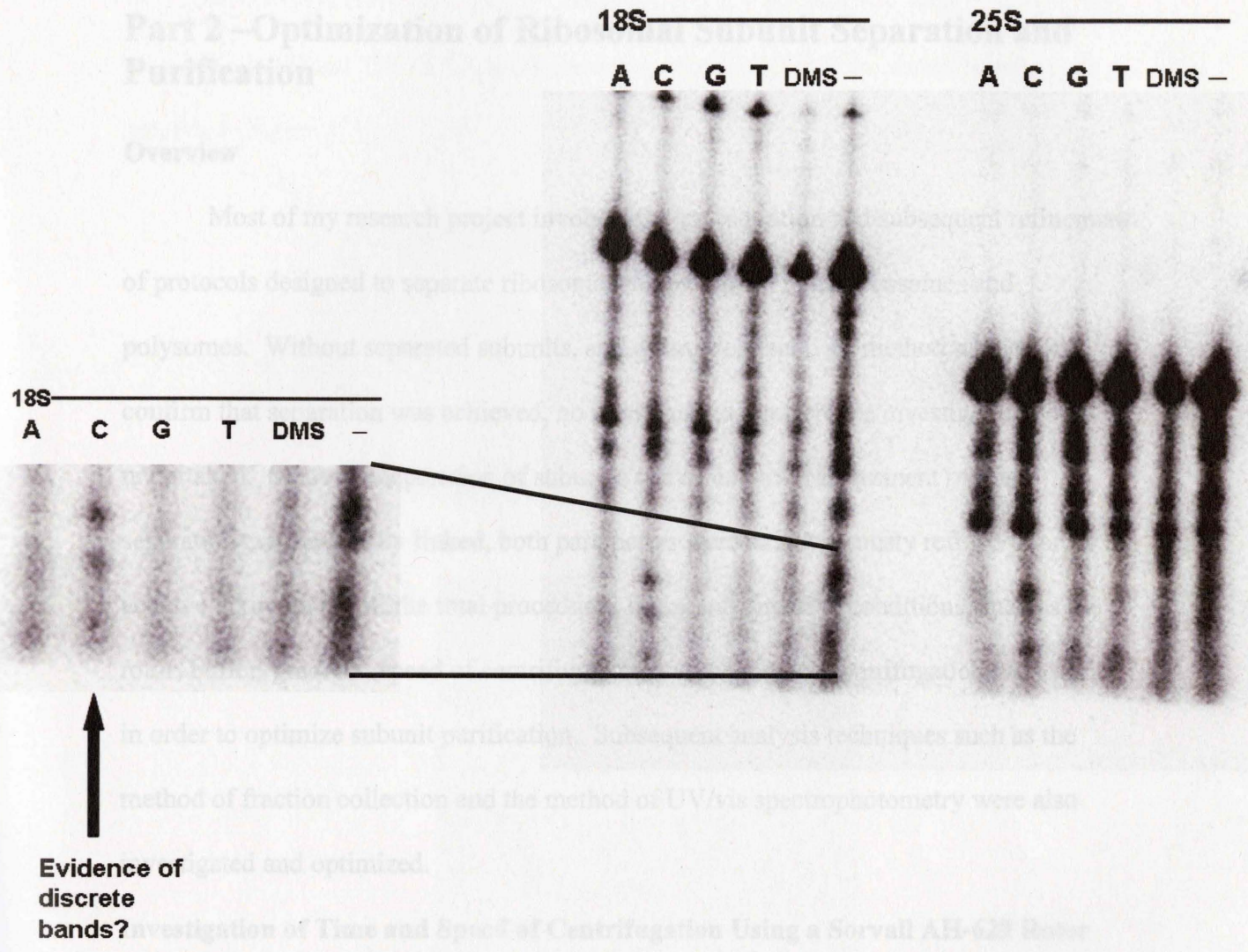


Figure 11. Primer-extension analysis of DMS-treated rRNAs. "A", "C", "G", and "T" lanes represent the sequencing reaction products. "DMS" and "—" represent DMS-treated rRNA and the control untreated rRNA, respectively. Gel banding did not appear as expected (Figure 5). Thus, the results of DMS modification, primer extension, and the RNA sequencing reactions are inconclusive. Some evidence that discrete bands formed within the gel lanes was seen upon close examination (left).

Part 2 –Optimization of Ribosomal Subunit Separation and Purification

Overview

Most of my research project involved the investigation and subsequent refinement of protocols designed to separate ribosomal subunits from intact ribosomes and polysomes. Without separated subunits, and a confident analysis method which could confirm that separation was achieved, no modification interference investigation could be undertaken. Since the separation of subunits and an accurate assessment of this separation are inexorably linked, both parameters were simultaneously refined in order to achieve optimization of the total procedure. Ultracentrifugation conditions, such as the rotor, buffer, gradient, speed of centrifugation, and the time of centrifugation were varied in order to optimize subunit purification. Subsequent analysis techniques such as the method of fraction collection and the method of UV/vis spectrophotometry were also investigated and optimized.

Investigation of Time and Speed of Centrifugation Using a Sorvall AH-629 Rotor

In order to separate ribosomal subunits from intact ribosomes and polysomes using the AH-629 rotor, a previously reported speed (35,000 rpm) used for separation could not be utilized, as the fastest spin possible on the AH-629 rotor is 29,000 rpm (19, 23). We hypothesized that an increase in the time of spin might compensate for the slower speed. However, increasing the spin times from 3 h to 12 h, 17 h, or 18 h did not result in well-separated fractions regardless of the speed chosen. Despite concerns about the method of analysis (see Investigation of Fractionations and UV/vis Analyses), it was evident by the close proximity of the peaks that good separation was not being achieved.

This observation is consistent with the results of a RNA gel analysis, which showed the presence of 18S and 25S rRNA in all fractions, confirming that the subunits were not separated (Figure 12).

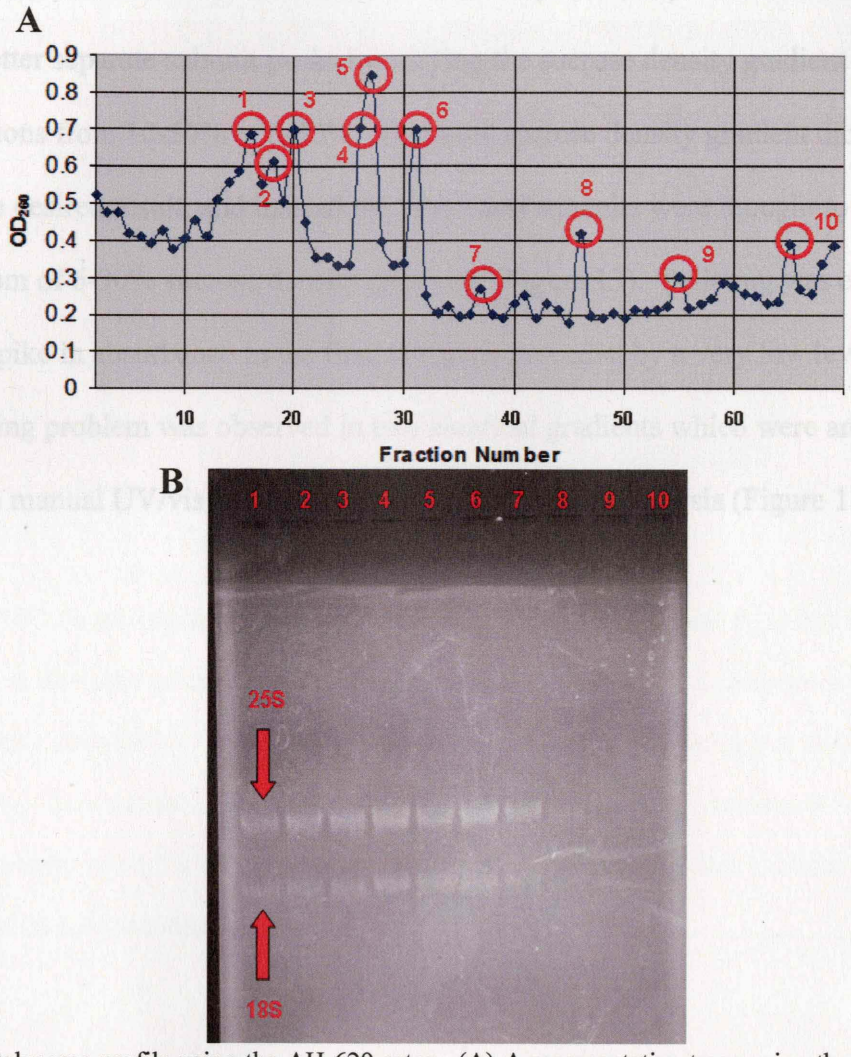
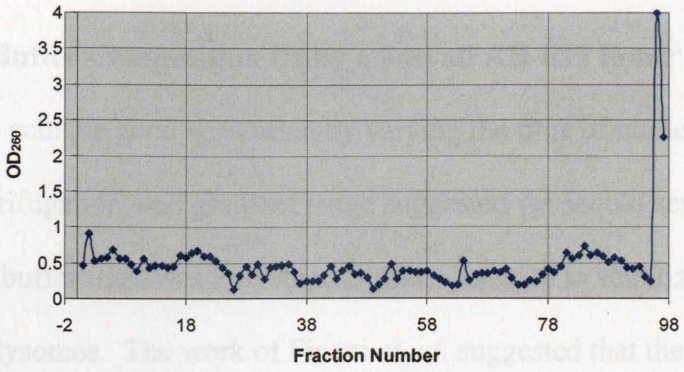


Figure 12. Polysome profile using the AH-629 rotor. (A) A representative trace using the AH-629 rotor (Polysome Buffer, 10-50% sucrose density gradient, ultracentrifugation at 22,000 rpm for 18 hours at 4 °C, manual fractionation into a 96-well plate and the use of automated UV-plate reader spectrophotometry). (B) Fractions of interest above (A) were analyzed on a 1% agarose/ 2.2 M formaldehyde gel to assess the rRNA present in each fraction. Both 25S and 18S rRNA are present in many of the early peaks (1-7), suggesting that both 60S and 40S particles are contained in these fractions. This confirms inadequate separation of subunits with these protocols.

Investigation of Sucrose Density Gradient Range Using a Sorvall AH-629 Rotor

Based on previous work in the LaRiviere lab and in the literature, ribosomal subunits usually fractionate between 6-30% sucrose (19, 25, 28). Thus, an attempt was made to better separate subunit peaks by varying the sucrose density gradient concentrations from 10-50% to 6-30%. This new sucrose density gradient did not achieve the desired result, and instead the ribosomal subunits were thought to be pelleting at the bottom of 6-30% sucrose density gradients (Figure 13). Pelleting was evident from the sharp spike in absorbance in the final fractions preceded by a very low level of OD_{260}. This pelleting problem was observed in two identical gradients which were analyzed for OD_{260} with manual UV/vis and automated UV-plate reader analysis (Figure 13).

A



B

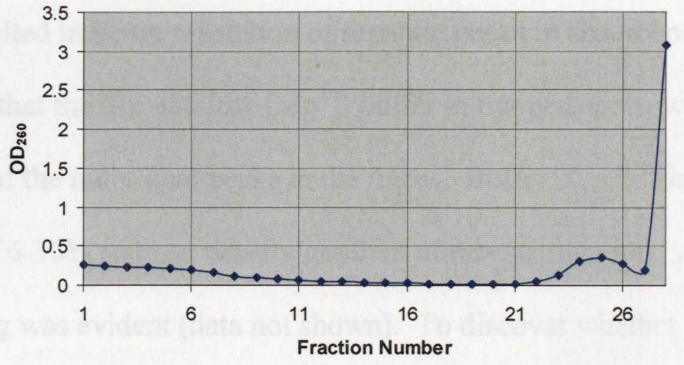


Figure 13. 6-30% sucrose density gradients with Buffer X ultracentrifugated at 29,000 rpm for 17 h at 4 °C resulted in ribosomal particle pelleting. This pelleting is indicated by a sharp spike at the end of the gradient (right) preceded by low levels of absorbance at 260 nm. The pelleting was observed in gradients analyzed by (A) manual fractionation into a 96-well plate and automated UV-plate reader spectrophotometry as well as in gradients analyzed by (B) manual fractionation into microcentrifuge tubes and standard UV/vis spectrophotometry.

Investigation of Buffer Composition Using a Sorvall AH-629 Rotor

Failures to achieve good separation by varying the time of ultracentrifugation, the speed of ultracentrifugation, and gradient range suggested we should scrutinize the buffers. The first buffer used was Polysome Buffer, designed to stabilize intact ribosomes and polysomes. The work of Fioani *et. al.* suggested that the use of buffers without Mg^{2+} resulted in better resolution of separate peaks in absorbance profiles (26). We hypothesized that the use of a low [Mg^{2+}] buffer in our protocols, would allow for better separation of the individual peaks in the traces. Buffer X, a buffer lacking Mg^{2+}, was used first in a 6-30% sucrose density gradient ultracentrifugation. As was previously observed, pelleting was evident (data not shown). To discover whether the pelleting was truly a consequence of the gradient rather than a result of the buffer, we repeated the experiment using both a 6-30% gradient and a 10-50% gradient. This resulted in the generation of the first data set which suggested that we could achieve subunit separation (Figure 14). The pelleting problem was eliminated by returning to a gradient concentration of 10–50%. The beginnings of peak resolution were visible, although further separation was desired for any subsequent isolation steps. Gel analysis confirmed that subunit separation between the 40S and 60S or perhaps a joint 60S/80S peak was achieved (Figure 14).

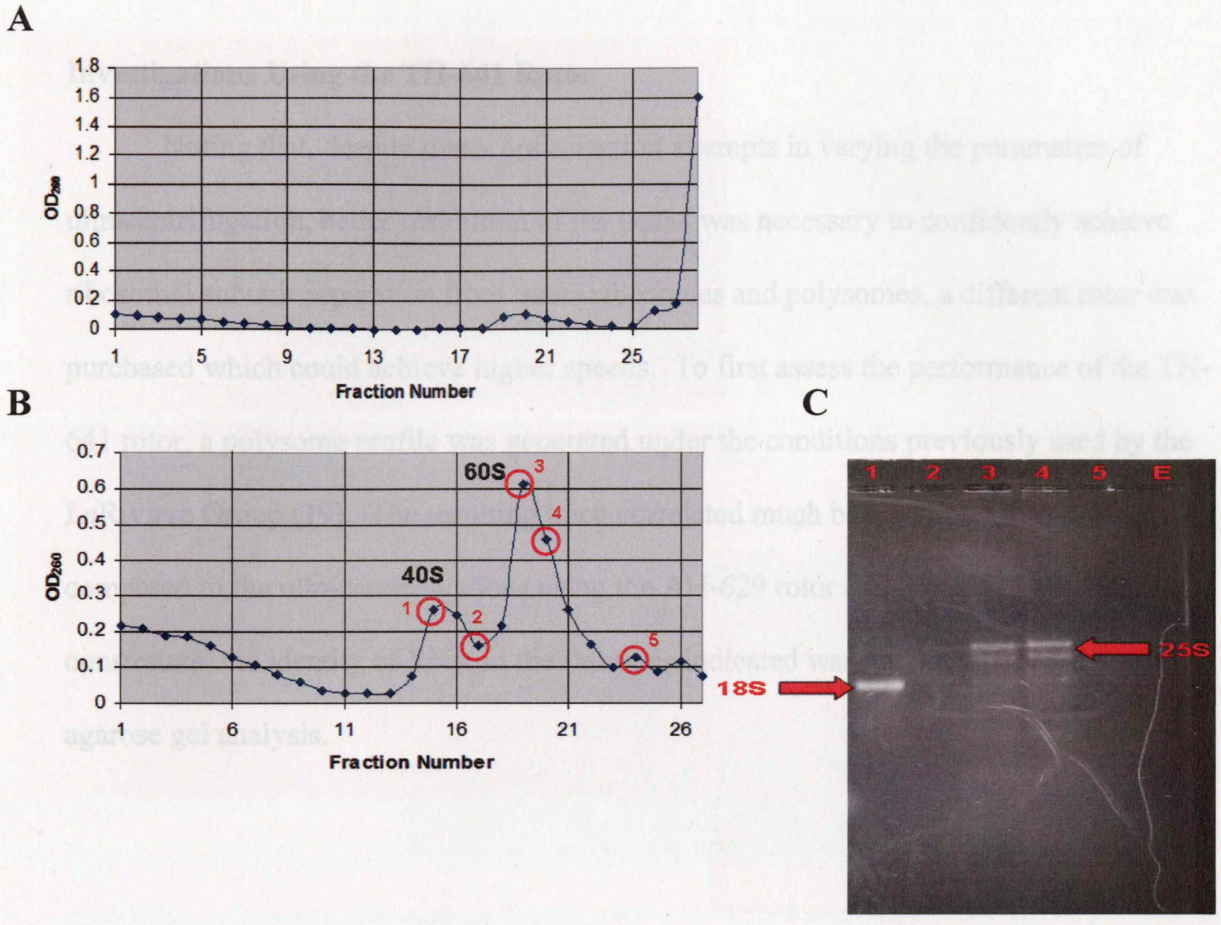


Figure 14. Ribosomal subunit separation with the Sorvall AH-629 rotor. Absorbance traces from (A) 6-30% and (B) 10-50% sucrose density gradients (Buffer X, ultracentrifugation at 22,000 rpm for 17 h at 4 °C on a Sorvall AH-629 rotor). Both gradients were fractionated manually and analyzed by standard UV/vis spectrophotometry. The 6-30% sucrose density gradient trace shows likely ribosomal particle pelleting. The 10-50% sucrose density gradient trace shows two distinct peaks. (C) Samples from the fractions indicated were analyzed for the presence of rRNA by gel analysis. The gel suggests subunit separation, with 18S rRNA appearing in the earliest peak (sample 1) as expected, and 25S rRNA appearing in the later peak (samples 3 and 4). This suggests the first peak contains the 40S subunits and the second contains the 60S subunits. Doublets in samples 3 and 4 likely are a result of minor degradation of the RNA or a gel artifact.

Investigations Using the TH-641 Rotor

Noting that, despite many optimization attempts in varying the parameters of ultracentrifugation, better resolution of the peaks was necessary to confidently achieve ribosomal subunit separation from intact ribosomes and polysomes, a different rotor was purchased which could achieve higher speeds. To first assess the performance of the TH-641 rotor, a polysome profile was generated under the conditions previously used by the LaRiviere Group (19). The resulting trace correlated much better to previous data as compared to the ultracentrifugations using the AH-629 rotor (Figure 15). Due to time constraints, the identity of RNA in the fractions indicated was not assessed by denaturing agarose gel analysis.

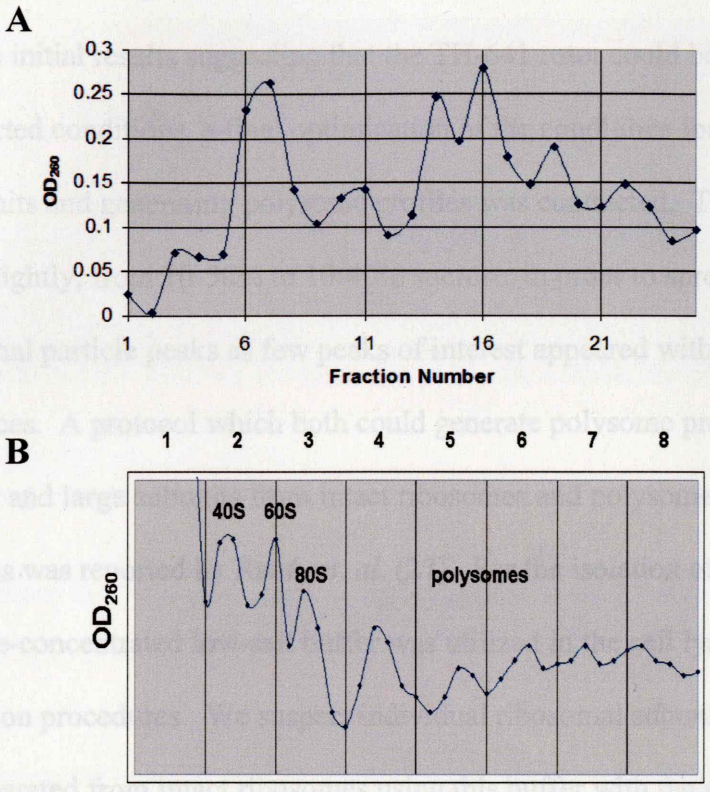


Figure 15. Polysome profile using the TH-641 rotor. (A) The polysome trace (Polysome Buffer, 10-50% sucrose density gradients, and ultracentrifugation at 35,000 rpm for 3 h at 4 °C) correlated significantly better with a (B) literature trace using the TH-641 rotor as compared to the AH-629. The gradient was fractionated manually and analyzed by standard UV/vis spectrophotometry. The first three peaks of the polysome trace likely represent the 40S, 60S, and 80S with polysome particles coming afterward similar to what is shown on the literature trace. Literature trace modified from (19).

Final Optimizations Using the TH-641 Rotor

With the initial results suggesting that the TH-641 rotor could better reproduce data under reported conditions, a final optimization of the conditions for both separating ribosomal subunits and generating polysome profiles was conducted. The gradient range was truncated slightly, from 10-50% to 10-40% sucrose, in order to spread out the relevant ribosomal particle peaks as few peaks of interest appeared within the 40%-50% area of most traces. A protocol which both could generate polysome profiles and isolate ribosomal small and large subunits from intact ribosomes and polysomes by varying the buffer conditions was reported by Raué, *et. al.* (27). For the isolation of individual subunits, a twice-concentrated low-salt buffer was utilized in the cell lysis and ultracentrifugation procedures. We suspect individual ribosomal subunits were successfully separated from intact ribosomes using this buffer with the spin conditions kept constant from the previous TH-641 rotor analysis (Figure 16). However, the RNA gel was not run to confirm this. Nevertheless, fractions 2 and 3/4 corresponding to possible 40S and 60S or a possible 40S and a 60S/80S mixture were dialyzed into 1x Buffer and a reassociation experiment was attempted in which the fractions were combined, incubated, and then run on a gradient. The experiment was inconclusive (Figure 10). The difficulty in assessing the results of the reassociation was likely a consequence of a problem with the fractionation and UV/vis analysis technique (see Investigation of Fractionation and UV/vis Analysis).

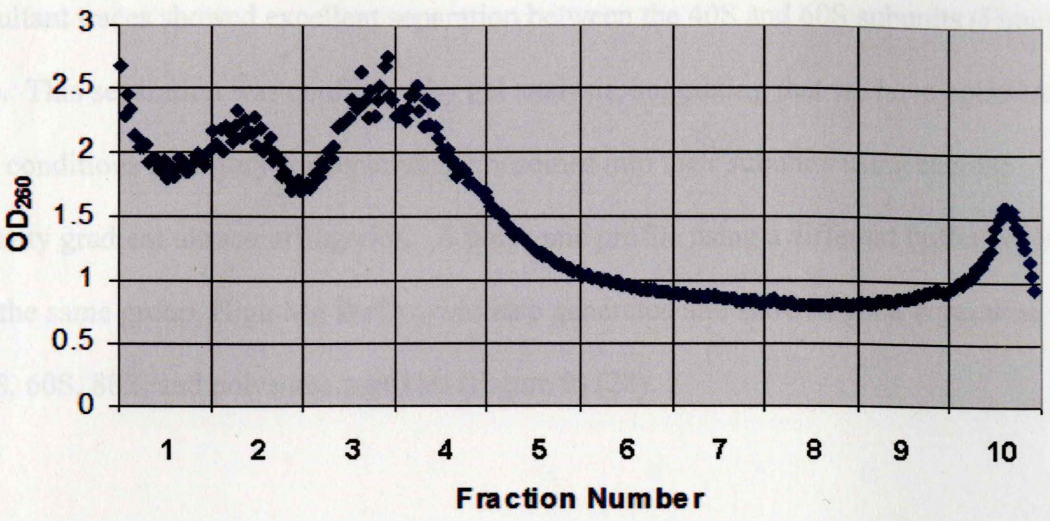


Figure 16. Absorbance trace of separated 40S and 60S/80S subunits which were subsequently used in a reassociation experiment. The conditions used are the same as in Figure 15 with the exception of the use of low-salt 2x Buffer A as the buffer and automated fractionation setup A. The first peak likely contains the 40S subunits and the second peak was thought to contain either the 60S subunits or a mixture of 60S and 80S subunits. The later classification is suggested by the second peak's slightly doublet nature.

Successful Ribosomal Subunit Separation and Polysome Profile Generation Using the TH-641 Rotor

While the previous trace was promising, we attempted additional ultracentrifugations with other buffer conditions reported in the literature. One such buffer, Low Mg Buffer, was reported to very effectively dissociate intact ribosomes into ribosomal subunits (28). Despite earlier concerns about 6-30% gradients, they were utilized in this experiment. Pelleting was thought to be unlikely because the length of the spin, 3 hours, was significantly shorter relative to previous spin times. Moreover, we continuously observed 40S- and 60S-like peaks appearing in the 6-30% sucrose range, and it was thought that better separation would result if we focused on that region. The

resultant traces showed excellent separation between the 40S and 60S subunits (Figure 17). This separation was confirmed by gel analysis, suggesting that we have optimized the conditions necessary for separating ribosomes into their subunits using sucrose density gradient ultracentrifugation. A polysome profile using a different buffer reported by the same group, High Mg Buffer, was also generated and showed good separation of 40S, 60S, 80S, and polysome particles (Figure 9) (28).

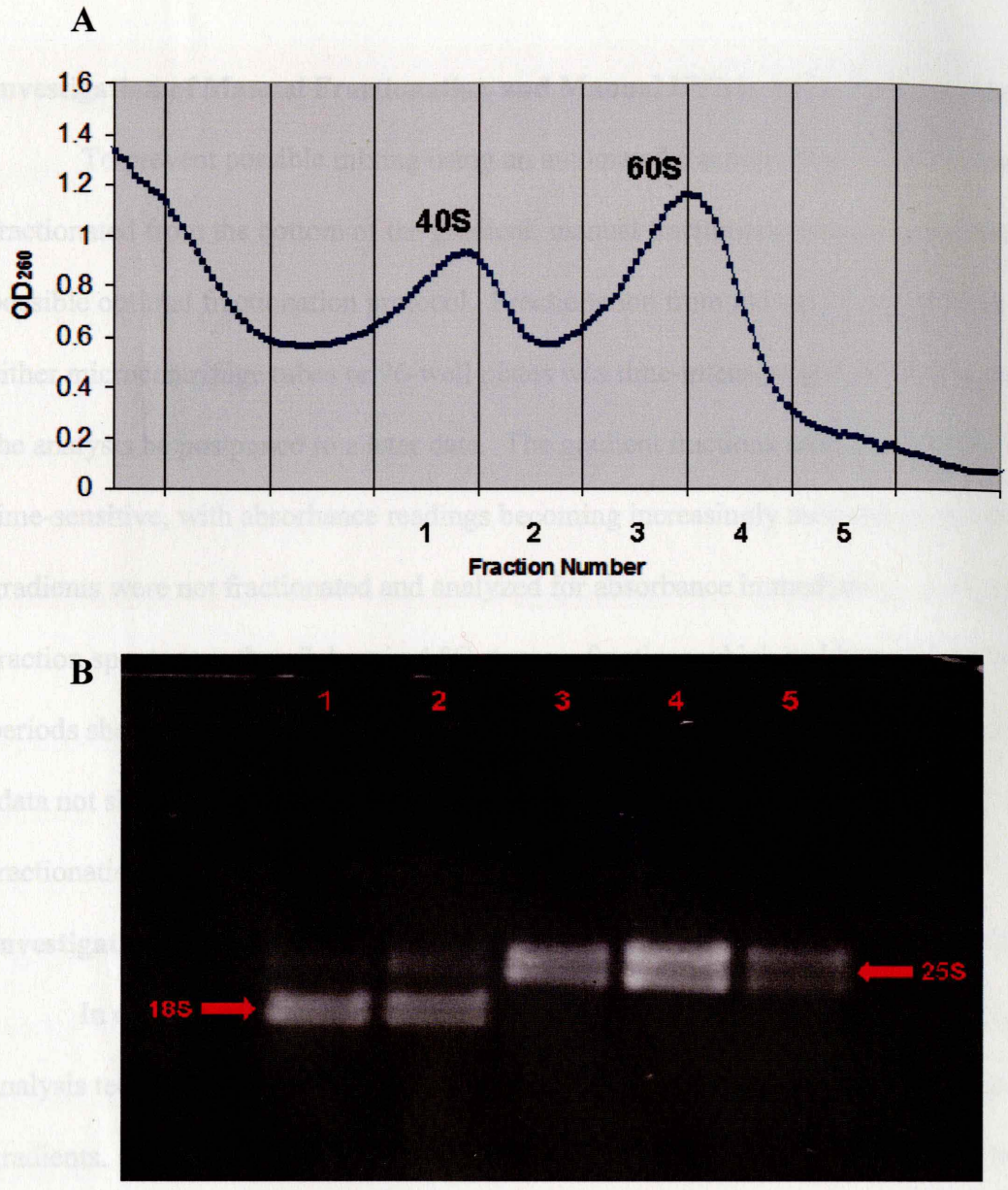


Figure 17. The optimized ribosomal subunit separation protocol. (A) Gradient fractionation trace (Low Mg Buffer, 6-30% sucrose, ultracentrifugation at 35,000 rpm for 3 h at 4 °C) obtained using the Sorvall TH-641 rotor. Automated fractionation setup C) resulted in distinctly separated peaks on the absorbance trace. Fractions of interest were assayed for the presence of rRNA. (B) The gel analysis confirms that the first peak contains 18S and therefore is likely the 40S subunit peak. The second peak contains only 25S, and therefore it is likely the 60S subunit peak. Relic bands in fractions 1 and 2 which appear to be in the 25S region likely are a result of some 60S contaminants in the 40S peak. The doublet nature of all of the banks likely is a sign of minor RNA degradation or a gel artifact.

Investigation of Manual Fractionation and Manual UV/vis Absorbance Analysis

To prevent possible mixing using an automated fraction collection device which fractionated from the bottom of the gradient, manual fractionation was investigated as a possible optimal fractionation protocol. Fractionation from the top of the gradient into either microcentrifuge tubes or 96-well plates was time-intensive and often required that the analysis be postponed to a later date. The gradient fractions were determined to be time-sensitive, with absorbance readings becoming increasingly miscellaneous when gradients were not fractionated and analyzed for absorbance immediately. Although no fraction spent more than 7 days in 4 °C storage, fractions which had been stored for long periods showed significant differences from those which were analyzed immediately (data not shown). It was therefore determined that automated or semi-automated fractionation was optimal.

Investigation of Manual Fractionation and Automated UV/vis Absorbance Analysis

In order to rule out possible UV/vis analysis abnormalities, an automated UV/vis analysis technique using a UV-plate reader was used to analyze one of two identical gradients. The absorbance data from both the automated UV-plate reader and from manual UV/vis absorbance readings correlated well, suggesting that the accuracy of UV/vis absorbance analysis was independent of the type of spectrophotometer chosen (Figure 13).

Investigation of Semi-Automated Fractionation and Automated UV/vis Absorbance Analysis

Multiple automated fractionation apparatuses that were coupled to flow-through UV/vis spectroscopic analysis were constructed and evaluated as part of this research. Fractionations by pumping the sample from the top or the bottom of the gradient were investigated. The first semi-automated fractionation apparatus we constructed fractionated from the top of the gradient while 60% sucrose was simultaneously introduced into the bottom of the gradient via a syringe. The absorbance traces generated from this set-up appeared as expected from past work in some cases, but the reproducibility of data was hindered by frequent physical limitations inherent in the apparatus design. Despite the constructed fractionator input being sealed with an epoxy and considerable attention given to achieving a tight fit of the device on the gradient, leakage was encountered regularly. Mixing of sample was observed in apparatuses which fractionated from the bottom of the gradient through a glass pipette, and this resulted in poor traces when the fractions were analyzed by flow-through UV/vis spectrophotometry (data not shown). Continued refinement of the apparatus design resulted in the optimized fractionator which successfully produced data which clearly demonstrated both ribosomal subunit separation and clearly defined polysome profiles (Figure 18).

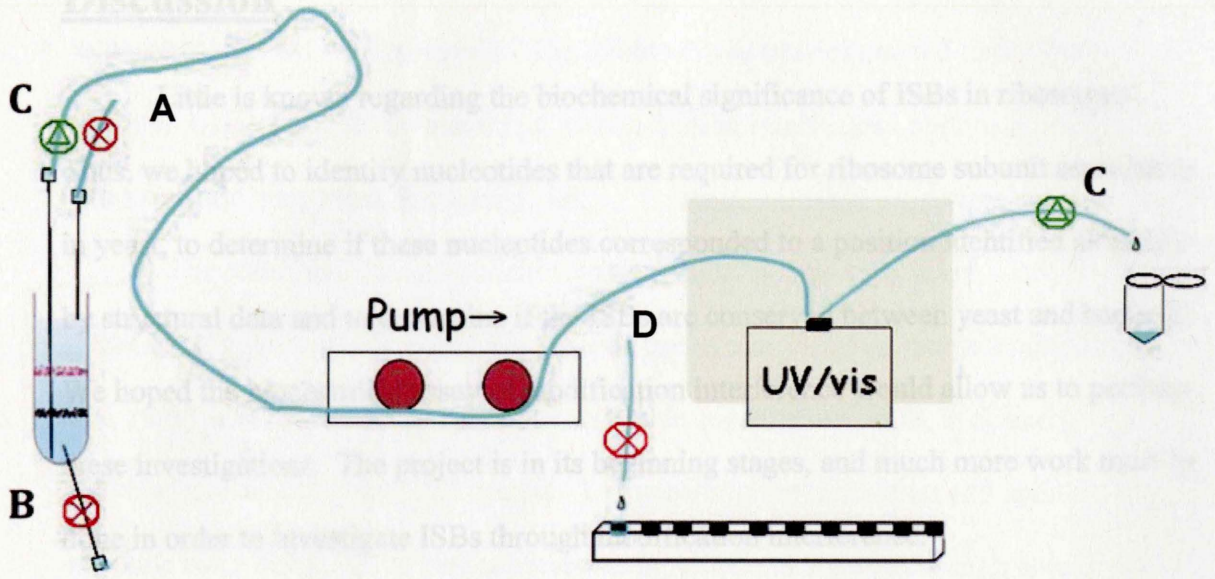


Figure 18. Final apparatus for gradient fractionation and analysis. (A) Collection from the top of the gradient required the simultaneous introduction of sucrose into the gradient (B), which proved problematic. (B) Pumping in 60% sucrose to the bottom of the gradient often would break the seal on any stoppered collection needle placed on top of the gradient. Coupling the sucrose pumping to the pump collecting the gradient proved infeasible due to inherent differences in the speed and flow consistency of any two pumps. (C) The optimized setup uses a single pump that collects the gradient directly from the bottom. A flow-through UV/vis spectrophotometer reads the absorbance at 260 nm in real-time. The gradient is collected into microcentrifuge tubes dropwise. (D) 96-well plates became superfluous without the need for the use of a UV-plate reader. Storage in 96-well plates often proved difficult due to frequent contamination of sample by other samples in adjacent wells.

Discussion

Little is known regarding the biochemical significance of ISBs in ribosomes. Thus, we hoped to identify nucleotides that are required for ribosome subunit association in yeast, to determine if these nucleotides corresponded to a position identified as an ISB by structural data and to determine if the ISBs are conserved between yeast and bacteria. We hoped the biochemical assay of modification interference would allow us to perform these investigations. The project is in its beginning stages, and much more work must be done in order to investigate ISBs through modification interference.

Our results have allowed us to optimize many different steps in our modification interference protocol. Separating and purifying ribosomal subunits and intact ribosomes from cell extracts can be done under the conditions reported herein (Figure 9). The conditions we report generate very well-defined polysome profiles, allowing for a confident identification of subunit peaks. Due to time constraints, the identification of peaks generated from our optimal conditions was not able to be correlated to gel analysis data to confirm that this step has been adequately optimized. Optimized protocols for generating polysome profiles should allow for the future purification of modified ribosomal subunits and intact ribosomes for use in subsequent primer extension experiments to identify interfering modifications.

Investigations into the reassociation of subunits and of the important DMS modification/primer extension/phosphorimager analysis were more inconclusive. The results of the reassociation experiment provide no useful information. Importantly, the optimizations of the polysome profiles reported should allow for a better analysis of ribosomal subunit reassociation in subsequent experiments. Results from the DMS

modification and primer extension experiments were unclear (Figure 11). The experiment did not work properly. This protocol was only attempted once, and it must be repeated and optimized to ensure that a modification interference approach can be taken toward identifying yeast ribosome ISBs.

The combined data presented regarding sucrose density gradient ultracentrifugation and fractionation provide optimizations of protocols to either separate and purify individual 40S and 60S subunits or to separate 40S, 60S, 80S, and polysome particles from native yeast cell lysates. The separation of individual 40S and 60S subunits from intact 80S is best achieved using ultracentrifugation at high speeds (35,000 rpm), low sucrose density gradients (6-30%) made in buffers lacking Mg^{2+}, and semi-automated fractionation coupled to flow-through UV/vis spectroscopic analysis (Figure 17). Separating 40S, 60S, intact 80S, and polysome particles is best achieved in the same manner, but with the use of buffers that contain Mg^{2+} in gradients containing higher levels of sucrose density (10-50%) (Figure 9).

The great number of different ultracentrifugation and fractionation conditions investigated as part of this project render these optimizations suggested rather robust. Great variation in the conceivable parameters of the protocols (e.g., ultracentrifugation time, speed, and rotor, gradient density and size, buffer constituency, method of fractionation, and method of analysis) was made in order to establish the optimal conditions. The protocols under conditions we report as being optimal should provide for the successful separation of 40S from native 80S ribosomes and a continuation of the modification interference experiment.

Future Work

Much more work must be done before ISBs can be identified in yeast ribosomes through modification interference experiments. What has been presented here are important optimizations for the entire modification interference protocol, especially regarding the first steps. The separation of 40S subunits from 80S ribosomes is required in order for native 40S to be modified chemically to 40S* subunits. Future work must first utilize the optimized separation and fractionation protocol presented here to successfully isolate 40S from intact 80S. Subsequently, chemical modification can be performed on these 40S to generate the modified 40S* for use in reassociation experiments. Before this can occur, however, the reassociation experiment must be first optimized as the ultracentrifugation and fractionation/analysis protocols were.

The long term goal of the entire project will be to search for ISBs in yeast ribosomes through modification interference. Nucleotides which are found by modification interference to be essential for stabilizing the intact 80S ribosome should be compared to known structural data to determine whether or not they correspond to a position known to participate in an ISB. Detailed analysis of the nucleotide identity and ribosome structure should be undertaken in order to elucidate what the ISB interaction might be (e.g. hydrogen bond base pairing, pi-pi stacking, van der Waals contact, etc.) and therefore why the modification interferes in the formation of the intact ribosome. This knowledge will greatly increase our understanding of ribosome structure and function.

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Appendix

Introduction

An additional real-time PCR experiment was conducted as part of my research. Real-time, or quantitative, PCR allows for the determination of starting concentrations of substrate through the automated monitoring of the amplification reactions in real-time using a variety of probing strategies. We hoped to perform a reverse transcription real-time PCR experiment in order to quantify the amount of rRNA transcribed from plasmid-encoded genes as compared to endogenous genes in *Saccharomyces cerevisiae*. We isolated rRNA from yeast cells and performed both reverse-transcription PCR and standard PCR in order to optimize the protocols for use in a real-time PCR experiment.

Additional Materials and Methods

Yeast Starter Culture Growth

In order to perform reverse-transcription quantitative PCR amplifications of rRNA from plasmid-expressing *Saccharomyces cerevisiae* cells, yeast starter cultures were grown in selective media. A 50 mL conical tube containing 5 mL of autoclaved ura- dropout media (1% Yeast Nitrogen Base, 1% ura- drop-out solution, 2% glucose) was inoculated with a medium-sized colony of yeast strain 10540 (MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, rad52Δ::KanMX4) under sterile conditions. The inoculated media was placed in an incubator shaker (Excella E24, New Brunswick Scientific) at 30.0 °C and 225 rpm and allowed to grow to saturation overnight. Saturated cultures were then stored at 4 °C indefinitely.

Yeast Cell Preparation

New cells were grown from a starter culture for the purposes of RNA extraction. A specific volume of a resuspended starter culture was transferred into 20 mL of autoclaved ura- dropout media in an Erlenmeyer flask under sterile conditions. The transfer volume was a calculated value which corresponded to a target growth level between 0.4-0.6 OD_{600} (Optical Density at 600 nm) after a set number of yeast cell doublings. Yeast cells of the strain 10540 were determined to have a growth rate consistent with a doubling in population every ~3 hours.

PCR Amplification

PCR amplification of DNA sequences within selected regions of yeast chromosomal or plasmid-derived DNA or from RT-PCR reaction products was conducted as follows. 20 μL reactions for amplifying DNA templates were set up containing 0.2 mM dNTPs, 100 ng of yeast template DNA, 2 mM $MgCl_2$, and 1x Taq Buffer (Fisher Scientific). 20 μL reactions for amplifying cDNA from RT-PCR products were set up containing 0.2 mM dNTPs, 1/10 of the volume of the RT-PCR reaction products, 2.25 mM $MgCl_2$, and 1x PCR Buffer (Promega). 300 nM of oligonucleotide primers complimentary to regions upstream and downstream of a region within the 5'-UTR of the 18S and 25S rRNA coding sequences were added to their respective reactions: FL307/ FL125, FL309/FL310, or FL311/FL312 were used for the 18S reactions while FL308/ FL126 or FL313/FL314 were used for the 25S reactions (Table 1). 5 U of Taq DNA polymerase (Fisher Scientific) was added and the reactions were placed in a thermocycler (MG Mini, BioRad). The reactions were subjected to the following conditions:

1. 95.0 °C for 5 minutes
2. 95.0 °C for 30 seconds
3. 50.0 °C for 30 seconds
4. 72.0 °C for 30 seconds
5. Steps 2-4 were repeated 39 additional times
6. 72.0 °C for 10 minutes
7. 4 °C indefinitely

Table 1. Oligonucleotide primers used in this study

Primer	Label	Sequence (5' – 3')
pJV12 18S upstream	FL307	CAG TGA AAC TGC GAA TGG
pJV12 18S downstream	FL125	CGA GGA TCC AGG CTT T
Endogenous 18S "short" upstream	FL309	AGT GGC CTA CCA TGG TTT CA
Endogenous 18S "short" downstream	FL310	CTT GGA TGT GGT AGC CGT TT
Endogenous 18S "long" upstream	FL311	AAA CGG CTA CCA CAT CCA AG
Endogenous 18S "long" downstream	FL312	GAC TTG CCC TCC AAT TGT TC
pJV12 25S upstream	FL308	GTA GGA GTA CCC GCT GCC CT
pJV12 25S downstream	FL126	ACT CGA GAG CTT CAG TAC C
Endogenous 25S upstream	FL313	GGG AAT GCA GCT CTA AGT GG
Endogenous 25S downstream	FL314	CTA CCC ACA AGG AGC AGA GG

Reverse Transcription-PCR Amplification Using the AMV-RT Enzyme

RT-PCR amplification of rRNA transcribed *in vivo* by pJV12 plasmid-expressing yeast was conducted as follows. 20 µL reactions designed to produce cDNA from rRNA templates were first subjected to an annealing step in 10 µL total volumes with 500 µM dNTPs, 100 ng of total RNA isolated from 10540 yeast cells as described previously, and 0.5 µM of respective downstream primer: FL312 for the 18S reaction and FL314 for the 25S reaction. The reactions were placed in a thermocycler and subjected to 70 °C for 10 minutes. The reactions were then placed on ice, and brought to 20 µL with 1x AMV-RT Buffer (50 mM Tris-HCl [pH = 8.3], 40 mM KCl, 8 mM $MgCl_2$, 1 mM dithiothreitol), 20 U of RNAse inhibitor RNasin (Promega), and 5 U of Enhanced AMV-RT (avian myeloblastosis virus reverse transcriptase, Sigma) enzyme. The reactions were placed in a thermocycler and subjected to 50.0 °C for 50 minutes. 1/10 of the reaction product volumes were then used in the PCR Amplification protocol above.

Reverse Transcription-PCR Amplification Using the ImProm-II Enzyme

RT-PCR amplification of rRNA transcribed *in vivo* by pJV12 plasmid-expressing yeast was conducted as follows. 20 μL reactions designed to produce cDNA from rRNA templates were first subjected to an annealing step in 7.7 μL total volumes with 100 ng of total RNA isolated from 10540 yeast cells as described previously and 20 pmol of respective downstream primer: FL125, FL310, or Fl312 for the 18S reactions and FL126 or FL314 for the 25S reactions. These reaction volumes were incubated at 70 °C for 5 minutes and then chilled on ice indefinitely. The reactions were then brought to 20 μL with 1x ImProm-II Buffer (Promega), 3 mM $MgCl_2$, 0.2 mM dNTP's, 20 U of RNAse inhibitor RNasin (Promega), and 5 U of ImProm-II RT enzyme (Promega). The reactions were placed in a thermocycler and subjected to the following conditions:

1. 25.0 °C for 5 minutes
2. 50.0 °C for 60 minutes
3. 70.0 °C for 15 minutes
4. 72.0 °C for 30 seconds
5. 4 °C indefinitely

1/10 of the reaction product volumes were then used in the DNA PCR Amplification protocol above.

Agarose Gel Analysis of PCR Reaction Products

To determine the identity of the DNA products from the PCR amplification reactions, agarose gel analysis was performed. A 2% agarose/ 0.5x TBE (Tris/Borate/EDTA) gel was prepared in 0.5x TBE buffer. The entire 20 μL of PCR product was mixed with an appropriate volume of loading dye and 10 uL of each sample was loaded into the gel. Two DNA ladders (100 bp and 1KB, New England Biolabs) were loaded into the gel as standards. The gel was run between 100-110 V until the

leading dye was near the end of the gel (approximately 1.75 hours). The gel was stained in 0.5 ng/mL ethidium bromide for ~20 minutes, destained in diH_2O for 15 minutes, and then visualized using a UV-camera.

Results and Discussion

Investigation into rRNA expression using real-time PCR requires that the expression of both plasmid-derived and endogenous rRNA be quantifiable through the faithful amplification of target sequences on these rRNAs. In order to assess the capability of our primers to amplify rRNA target sequences, we first assessed their ability to amplify template DNA in standard PCR experiments. A pJV12 plasmid was used as DNA template in a standard PCR amplification experiment utilizing primers which selected for a region in the 5'-UTR of either the 18S and 25S rRNA gene on the plasmid. Subsequent analysis showed that the primer pairs of FL307/FL125 and FL308/FL126 designed to target the 18S and 25S pJV12 sequences successfully amplified their target region in PCR (Figure 1).

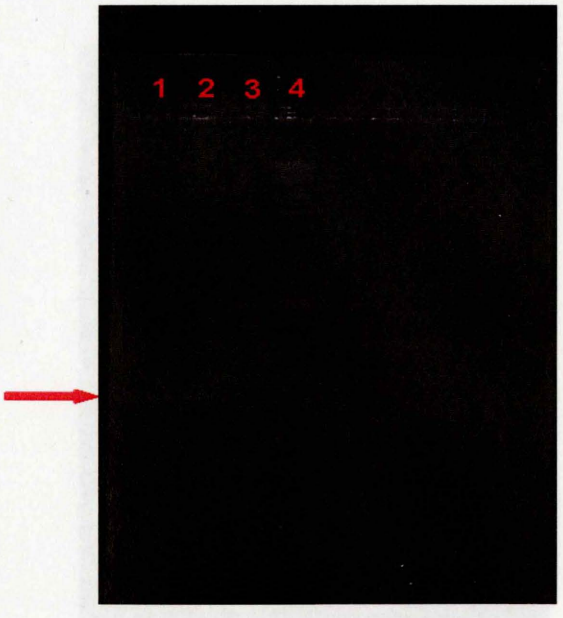

Figure 1. A 2% agarose gel analysis of PCR reaction products from pJV12 plasmid DNA template. Lanes 1 and 2 correspond to the PCR products from the 18S and 25S reactions, respectively. Lanes 3 and 4 are 100 bp and 1 KB ladders, respectively. Indicated are the PCR reaction bands which appear between 100 and 200 bp as expected.

The real-time PCR experiment requires that "endogenous rRNA" (non-plasmid derived rRNA) expression be quantifiable as well. To facilitate this, we designed primers to target endogenous rRNA sequences in the 5'-UTR of chromosomal or endogenous rRNA genes. Using primer design software (Primer 3 Input, http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), two primer pairs for the 18S and one for the 25S were chosen for further investigation as to their targeting specificity. The 18S "short" and "long" primers were designated as such, as they selected for a shorter and longer amplicon, respectively. A PCR experiment using total yeast DNA and the primer pairs was conducted and the subsequent analysis showed that the 18S "long" primer appeared to be of optimal sequence, but that the 18S "short" and 25S endogenous primer pairs may not be optimal, as the DNA products from their reactions were of a lower quality (Figure 2).

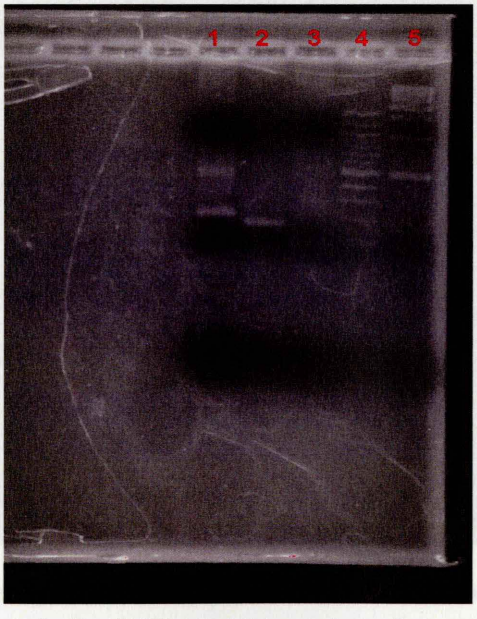

Figure 2. A 2% agarose gel analysis of PCR reaction products from endogenous yeast DNA template. Lanes 1, 2, and 3 correspond to PCR reaction products from the 18S "short", 18S "long" and 25S reaction, respectively. Lanes 3 and 4 are 100 bp and 1 KB ladders, respectively. The PCR reaction bands appear between 100 and 200 bp as expected.

The next investigation of our real-time PCR experiment was that of the reverse transcription. We conducted a reverse-transcription PCR experiment to determine whether or not optimizations of our protocol or primers were necessary. PCR amplification of template DNA or template reverse-transcription reaction product was directly compared on a gel (Figure 3). The results of this experiment indicated that the 18S "long" primer pair offered adequate targeting of both endogenous DNA template and rRNA, while the 25S primer pairs appear to be ineffective at targeting endogenous rRNA as compared to targeting endogenous DNA template. Absent significant secondary structure, the rRNA transcript should not display differing binding characteristics as compared to its DNA template for oligonucleotide primers. This led us to investigate our protocol further.

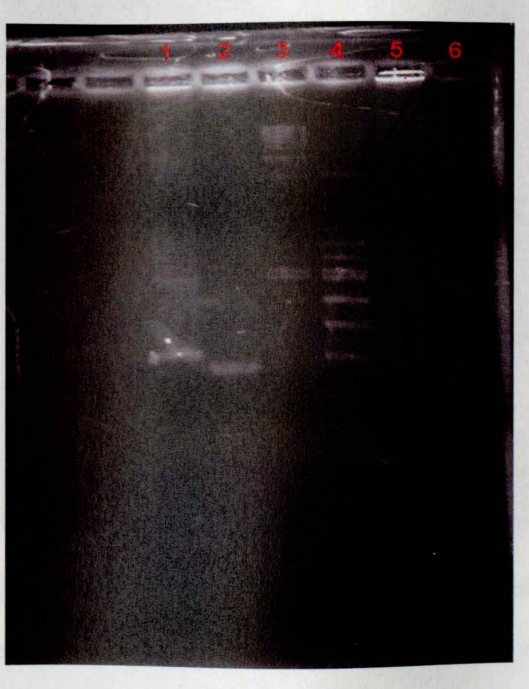

Figure 3. A 2% agarose gel analysis of PCR and RT-PCR reaction products from endogenous yeast DNA and reverse transcribed cDNA. The reverse transcription reaction utilized the AMV-RT enzyme. Lanes 1 and 2 correspond to PCR reaction products from the 25S and 18S "long" reactions, respectively. Lanes 3 and 4 are 100 bp and 1 KB ladders, respectively. Lanes 5 and 6 correspond to the RT-PCR reaction products from the 25S and 18 "long" reactions respectively.

A further investigation of the primer pairs and reverse transcription PCR was conducted with a new enzyme, the ImProm-II RT. Reverse transcription reactions were conducted with all 5 primer pairs (Figure 4). The results of this experiment were inconclusive. While the primer pairs selecting for the pJV12 18S rRNA seemed to be optimal, the other primer pairs either showed no signal (endogenous 18S "short") or multiple banding patterns, indicating possible annealing or extension difficulties. The 25S primer pairs did show targeting of endogenous rRNA, in contrast to results obtained using the AMV-RT enzyme protocol.

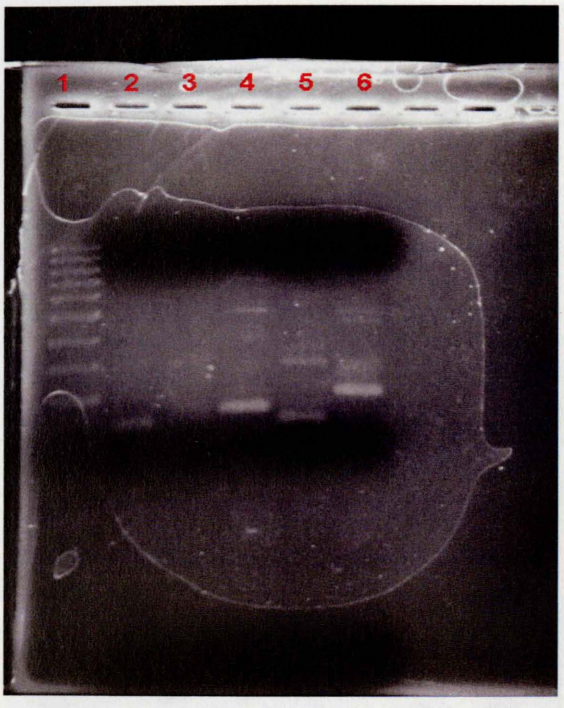

Figure 4. A 2% agarose gel analysis of RT-PCR reaction products derived from the reverse transcription of pJV12 and endogenous-derived rRNA. The reverse transcription reaction utilized the ImProm-II enzyme. Lane 1 is a 100 bp ladder. Lanes 2-4 correspond to the 18S reactions, with lane 2 representing a sequence amplified from the pJV12 derived rRNA, and lanes 3 and 4 representing a sequence amplified from endogenous 18S rRNA. Lanes 5 and 6 represent sequences amplified from the pJV12 derived rRNA and endogenous 25S rRNA respectively.

We proceeded to investigate the reverse transcription protocol further to determine if the starting template concentration was too high. Reverse transcription reactions using one primer pair (endogenous 18S "long") were conducted with a series of 10-fold dilutions of RNA template (Figure 5). All reactions showed a similar banding pattern, indicating that the problem of achieving well-targeted amplification was not template-dependent. Likely, the primers we have chosen require alteration for better specificity under our reaction conditions. Optimization of the primer pairs appears to be necessary in order to perform a reverse-transcription quantitative PCR investigation of rRNA expression.

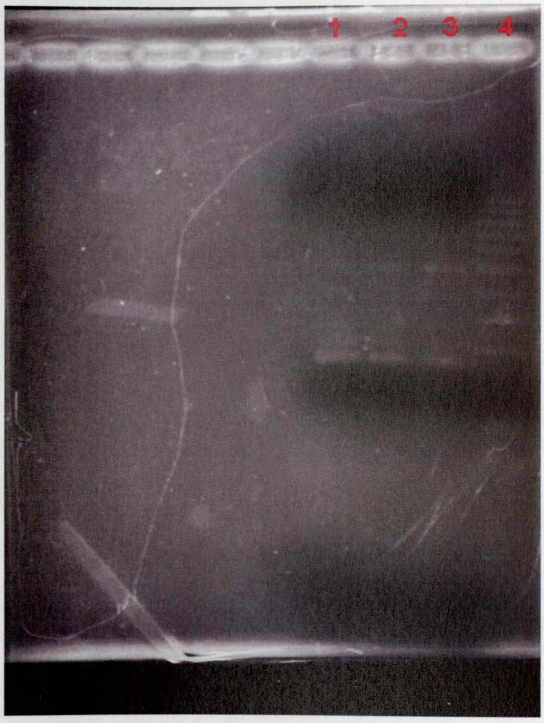

Figure 5. A 2% agarose gel analysis of a series of 10-fold diluted RT-PCR reaction products derived from the reverse transcription of endogenous-derived 18S rRNA. Lanes 1-3 represent 10-fold dilutions of template RNA of 1 ng, 10 ng, and 100 ng of template RNA in the RT reaction, respectively. Lane 4 is a 100 bp ladder.