COVER SHEET

TITLE: Attempts to Analyze the RNA-binding Specificity of the *C. elegans* RNP-8 Protein

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ABSTRACT

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(Cytoplasmic polyadenylation is a key mechanism of gene regulation in all eukaryotes. The *C. elegans* GLD-2 protein provides the catalytic activity for a cytoplasmic poly(A) polymerase, but GLD-2 must partner with another protein that provides RNA-binding activity to produce a fully functional enzyme. One such GLD-2 binding partner is RNP-8, which contains an RNA recognition motif. Here, we explored the RNA-binding specificity of RNP-8. Using the yeast-three hybrid system, we identified 34 unique RNA sequences that were bound by RNP-8, but we could not identify a consensus sequence among the 34 sequences. Therefore, RNP-8 protein may lack sequence specificity when binding to RNA.

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Attempts to Analyze the RNA-binding Specificity of the *C. elegans* RNP-8 Protein

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INTRODUCTION

Cytoplasmic polyadenylation is critical for oocyte maturation and early embryogenesis (Wickens et al., 2000). As a key mechanism of gene regulation, it is important for development and maturation of the *C. elegans.* Control of poly(A) tail length through cytoplasmic polyadenylation is imperative in regulating the stability of messenger RNA (mRNA) and translation within the cytoplasm (Wickens et al., 2000).

Cytoplasmic polyadenylation differs from nuclear polyadenylation in an important way. Nuclear poly(A) polymerases (PAPs) add a poly(A) tail to almost all newly synthesized RNA in the nucleus before being transported to the cytoplasm (Edmonds, 2002). Additional control of poly(A) tail length occurs in the cytoplasm by cytoplasmic PAPs. In general, lengthening of the poly(A) tail in the cytoplasm is connected to mRNA stabilization and translational activation, while shortening is linked to mRNA decay and translational repression (Wickens et al., 2000). In addition, cytoplasmic polyadenylation is sequence specific. The sequence specificity of cytoplasmic polyadenylation is crucial for its prominent role in gene regulation (Wickens et al., 2000).

Cytoplasmic PAPs and nuclear PAPs also differ in structure. A canonical nuclear PAP is a monomer made up of one protein that contains three domains – a central domain, catalytic region, and an RRM (RNA recognition motif)-like region, which is critical for RNA-binding (Figure 1) (Bard et al., 2000, Wang et al., 2002). By contrast, a cytoplasmic PAP contains only the central domain and the catalytic region, based on sequence comparison to the nuclear PAP. Thus, the GLD-2 regulatory cytoplasmic PAP lacks the RRM-like region, (Wang et al., 2002). The GLD-2 catalytic activity has been

hypothesized to be recruited to specific RNAs by RNA-binding partners (Kim et al., 2009; Wang et al., 2002).

C. elegans GLD-2 protein functions in germline development including meiotic entry and early embryogenesis (Kadyk and Kimble, 1998; Kim et al., 2009; Wang et al., 2002). To facilitate these roles, GLD-2 interacts with multiple distinct RNA-binding proteins. It partners with the GLD-3 protein, a member of the Bicaudal-C family of RNA-binding proteins (Eckmann et al., 2004; Wang et al., 2002) and with the RNP-8 protein, which contains an RRM (Kim et al., 2009). Both partners independently bind GLD-2 and stimulate its PAP activity in vitro (Kim et al., 2009; Wang et al., 2002). RNP-8 and GLD-3 antagonize each other genetically and compete with each other to bind to GLD-2 (Kim et al., 2009). While GLD-2/GLD-3 promotes spermatogenesis, GLD-2/RNP-8 specifies oogenesis (Eckmann et al., 2004; Kim et al., 2009).

My senior honors thesis research project focused on RNP-8. RNP-8 is an RNA-binding protein and contains an RRM in its N-terminal region of amino acids 11-79 (Kim et al., 2009). An RRM is a common motif found in many RNA-binding proteins (Cléry et al., 2008). RRMs can interact with RNA, DNA, and proteins, and can be involved in many post-transcriptional processes, such as mRNA processing, ribosomal RNA (rRNA) processing, RNA export, and stability. An RRM is approximately 90 amino acids long and forms a four-stranded β-sheet packed against two α-helices (Figure 2a) (Cléry et al., 2008). The β-sheet often serves as the primary binding surface to interact with single-stranded RNA. RNA-binding commonly occurs through the side chains of aromatic amino acids in the RNP1 (positions 1 and 3 in the β_3 strand) and RNP2 (position 2 in the β_2 strand) stretches of the RRM superfamily (Cléry et al., 2008). However, RRMs have

very versatile structures such that any portion of the RRM, including loops or α-helices, can interact with RNA, another RRM, or a completely distinct type of protein, modifying binding affinity and specificity. This structural versatility may partially account for the biologically diverse functions of RRM-containing proteins.

The goal of my senior honors research was to test the hypothesis that the RNP-8 protein binds RNA in a sequence-specific manner. To this end, I attempted to determine the RNA-binding specificity of the RNP-8 protein. As a GLD-2 partner and an RRM-containing protein, RNP-8 may be able to bind RNA and direct GLD-2 PAP activity towards its associated mRNAs. To identify the RNA-binding site of RNP-8, we used the yeast-three hybrid (Y3H) system (Bernstein et al., 2002; Hook et al., 2005; Stumpf et al., 2008). We identified 34 unique RNA sequences that bound RNP-8. However, we could not identify a consensus sequence among the 34 RNA sequences. Thus, RNP-8 may not bind RNA with sequence specificity.

RESULTS

Identification of RNP-8 Associated RNA Sequences

To characterize the RNA-binding specificity of *C. elegans* RNP-8 protein, we took an unbiased approach using the Y3H system (Bernstein et al., 2002; Hook et al., 2005; Stumpf et al., 2008). We used a library of plasmids that encoded a hybrid of RNA consisting of twelve randomized nucleotides. The library was introduced into yeast with a plasmid encoding RNP-8 fused to GAL4 activation domain (AD) (Figure 3). This first selection of colonies occurred on selection media lacking leucine, uracil, and histidine and containing 1.5mM 3-aminotriazole (3-AT); this selection allowed for the expression of the reporter gene *HIS3*. We found 397 HIS+ positive colonies from 300,000

transformants. 156 of the HIS+ positive clones were tested for β-galactosidase activity to select for expression of the reporter gene LacZ and to remove positives that only activated one of the two reporters (Stumpf et al., 2008). This test resulted in 139 positive colonies. As a negative control, we used colonies that grew on selection media lacking leucine and uracil, but could not grow on selection media lacking leucine, uracil, and histidine. Of these 139 HIS+ and LacZ positive colonies, 83 colonies were recovered and sequenced to determine the specificity of the RNP-8-RNA interaction. A total of 34 unique sequences were obtained (see Figure 4a and Table 1).

To test the strength of the RNP-8-RNA interaction, we assessed the activity of the reporter genes: LacZ and *HIS3*. The strength of the β-galactosidase activity in the Y3H system correlates with the affinity of the protein-RNA interaction (Stumpf et al., 2008). The interaction between FBF-1 protein and an FBF-binding element (FBE) was used as a positive control and as a reference point for the spectrum of strength of color change in the β-galactosidase assay (Crittenden et al., 2002). The β-galactosidase activity was weak for all 139 HIS+ and LacZ positive colonies for the RNP-8-RNA interaction, but was much stronger for the FBF-1-FBE interaction (Table 2).

Four Classes of RNA Sequences from the Y3H Transformation

The 34 unique RNA sequences identified in the Y3H assay were divided into four classes of sequence patterns. The class data and sequence orientation patterns for each class are summarized in Table 1.

Class 1 consisted of seven isolates, with no insertion of the twelve randomized nucleotides.

Class 2 consisted of 12 isolates, with six unique sequences, each consisting of a single insertion of the twelve randomized nucleotides.

Class 3 consisted of 24 isolates, with 13 unique sequences, each consisting of two consecutive insertions of the twelve randomized nucleotides running in opposite directions.

Class 4 consisted of 40 isolates, with 14 unique sequences, each consisting of three consecutive insertions of the twelve randomized nucleotides running in alternating directions.

When the RNA library was sequenced (performed by C. Stumpf, Wickens Lab), most samples contained a random single insertion. However, our results revealed various insertions ranging from zero to three copies of the twelve randomized nucleotides. Since 77.1% of the identified isolates contained more copies of insertions than expected (classes 3 and 4), perhaps RNP-8 is not a sequence-specific factor or maybe another protein is involved in the interaction. Thus, the multiple insertions might be a unique characteristic of the RNP-8-RNA interaction.

Our Positive Control: Small Scale Y3H Screen Using AD-FBF-1

To confirm that the RNA library was working properly, we carried out a small scale screen using as 'bait' the FBF protein, whose RNA-binding specificity is well-known (Bernstein et al., 2002). A plasmid encoding AD-FBF-1 was transformed into yeast with the same randomized twelve nucleotide library. The transformation resulted in 57 total HIS+ positive clones out of 9,000 transformants. 25 of the HIS+ positive clones were analyzed in a β-galactosidase assay and 20 of these tested positive for LacZ activity. 13 colonies were sequenced successfully after *E. coli* transformation. 12/13 of these

samples contained a conserved UGU sequence, which is required for FBF binding and was thus expected as an FBF-binding element (Figure 4b) (Bernstein et al., 2005). Also, the 13 isolates were unique indicating that the library is truly random (Table 1). We conclude that our RNA library is well made and working properly.

A weaker response was observed in the β-galactosidase assay for the RNP-8-RNA interaction compared with the FBF-1-FBE interaction, suggesting that the RNP-8-RNA interactions have a weaker affinity than the FBF-1-FBE interactions. AD-RNP-8 had a much larger proportion of its colonies with weaker responses (+) compared to AD-FBF-1, in which most colonies produced stronger responses (++) (Table 2).

Examining the Specificity of the RNP-8-RNA Interaction

Mutation of the RRM Region of RNP-8 to Disrupt the RNP-8-RNA Interaction

The interactions between RNP-8 and the identified RNAs were RNP-8-dependent even though their affinities were weak compared with that of the FBF-1-FBE interaction. We wanted to ask if the weak RNP-8-RNA interaction were dependent on the wild-type (WT) RNP-8 protein. If not, RNP-8 might bind to another protein, which is responsible for the interactions identified by the RNA sequences. In this case, the RNP-8-RNA interaction might be indirect.

RNP-8 contains an RRM, which is often responsible for binding RNA (Cléry et al., 2008). We hypothesized that the RNP-8-RNA interaction might be indirect or might work through some other domain of the RNP-8 protein, if RNP-8 containing mutated RRM binds the identified RNA at a similar affinity. To this end, we made four different types of substitution mutations in the RRM region of the RNP-8 protein (Figure 5).

However, due to time constraints, only the mutation at position 2 of RNP2 ($Y_{13}D$) was tested.

To compare the affinities of the WT RNP-8 and mutated RNP-8 (RRMmut), 10 of the 34 unique indentified RNA sequences were used in Y3H assays. The ten sequences tested were able to confer the strongest qualitative growth results and also represented all four classes. There was little difference in β-galactosidase activity when WT RNP-8 was compared with RRMmut 1 ($Y_{13}D$) (Table 4). Thus, the RNP-8-RNA interaction might be indirect, such as through another protein factor or another portion of the RNP-8 protein, although it is unclear that the mutation on the RRM ($Y_{13}D$) is nonfunctional. To be sure, we would need to thoroughly test the other three RNP-8 RRM mutations that were made.

Interaction Between RNP-8 and the Known Target oma-2 mRNA

To test if RNP-8 is functional in the Y3H system, the *oma-2* 3'UTR was tested in an Y3H assay. The *oma-2* 3'UTR was selected, because *oma-2* is a target RNA of RNP-8 and because RNP-8 binds the *oma-2* 3'UTR in an in vitro gel shift assay (K.W. Kim and J. Kimble, p.c.). Unexpectedly, we found that WT RNP-8 did not bind the *oma-2* 3'UTR in yeast. We also tested a different type of vector hybrid RNA: originally we used p3HR2 (Figure 6b), which contains a GC clamp, and we also tested p3HR1 (Figure 6a), which lacks a GC clamp. We found no difference. The issue may be that the RRM, which is located near the N-terminus in RNP-8, is too close to the AD and may be interfering with the RNA interaction. Therefore, we question whether the 34 unique sequences are actual binding sites. Taken together, the question of whether RNP-8 interacts with RNA in a sequence-specific manner remains unanswered.

FUTURE DIRECTIONS

Additional experiments are needed to determine the RNA-binding specificity of RNP-8. One approach would be to continue working with the Y3H system. To begin, we would test more thoroughly the other three RNP-8 RRM mutations (Figure 5), as had been done for RRMmut 1 ($Y_{13}D$). This may allow us to determine whether the RRM is the necessary domain in the RNP-8 protein for RNA-binding specificity.

A next step is to ask if the RRM region of RNP-8 is necessary and sufficient for RNA-binding. To ask if the RRM were necessary, one would remove the RRM completely and test the resultant protein for RNA-binding to test this hypothesis. If the RRM is necessary, the RNP-8 protein lacking its RRM is expected to lose its ability to bind RNA. If binding were observed after removing the RRM, we would conclude that the RRM region is not necessary for RNA-binding. To ask if the RRM were sufficient for RNA-binding, we would test if the RNP-8 RRM motif on its own were able to bind RNA. If the RRM alone was found to bind RNA, we would conclude that the RRM region was the necessary domain of the RNP-8 protein for RNA-binding. However, if RNP-8 with and without the RRM was found to still bind RNA, it could be assumed that some third protein or another part of the RNP-8 protein may be necessary to facilitate this binding and further analysis would need to be conducted. Based on the results of these experiments, we would be able to learn more about the RNA-binding specificity of RNP-8 and the involvement of the RRM region.

Given the failure of the RNP-8-*oma-2* 3'UTR interaction in yeast, a third approach would be to use a different version of the AD-RNP-8 fusion protein to continue to utilize the Y3H system. The RRM of RNP-8 is in the N-terminal region of the protein and the AD is fused to the N-terminus of the RNP-8 protein. The close proximity of the

AD to the RRM possibly interferes with the RNA-binding function of the RRM. By fusing the AD to the C-terminus of RNP-8, this possibility will be minimized. To complete this process, we would thus need to create a new RNP-8-AD fusion protein for Y3H assays and repeat the interaction tests using *oma-2* 3'UTR in yeast. If it works, we would repeat the Y3H assays to identify the RNA-binding site of RNP-8.

Another approach would be to move away from the Y3H system entirely as we have not had much success in drawing definitive conclusions when using this method with the RNP-8 protein. Instead, we would perform an in vitro RNA-binding assay, electrophoretic mobility shift assay (EMSA), to learn if RNP-8 actually binds the RNA sequences identified in the Y3H assay. We would set up an assay in which RNP-8 binds a specific target, such as the *oma-2* 3'UTR fragment in vitro. If binding is established for multiple RNA sequences, we would use it to test the interaction between RNP-8 protein and the 34 identified RNA sequences from the AD-RNP-8 Y3H screen. This would allow us to determine the RNA-binding site of RNP-8 protein.

EXPERIMENTAL PROCEDURES

Yeast Three-Hybrid (Y3H) Assay

Yeast strains were grown at 30°C. The yeast strain used in the Y3H system was YBZ-1 containing MS2 coat protein fused to LexA DNA-binding domain (Figure 3). This was obtained from the Wickens Lab (Department of Biochemistry, UW-Madison). Random DNA oligonucleotides CAAANNNNNNNNNNNAAA were cloned into the p3HR2 vector to construct the random twelve nucleotide library (performed by Craig Stumpf, UW-Madison). AD-RNP-8 (amino acids 2-583) or AD-FBF-1 was used.

p3HR2, containing a GC clamp, and p3HR1, without a GC clamp, were used separately in the Y3H system (Figure 6a and 6b). Both were obtained from the Wicken's Lab.

Three-hybrid assays were performed as previously described (Bernstein et al., 2002; Hook et al., 2005; Stumpf et al., 2008). Using RNP-8 as 'bait', 300,000 transformants were screened, and using FBF-1 as 'bait', 9,000 transformants were screened. Transformants were plated on 1.5mM 3-AT to select for activation of *HIS3*.

β-galactosidase Activity Measurements

To quantify the level of LacZ expression, a β-galactosidase (β-gal) filter assay was conducted. A grid was drawn on a Protran BA 85 (nitrocellulose) filter and placed on selection media lacking leucine and uracil. The desired colonies were picked and streaked into separate boxes of the grid. The plates were incubated overnight at 30°C. The following day, 5mL of Z buffer (60mM Na_2HPO_4, 40mM NaH_2PO_4, 10mM KCl, 1mM $MgSO_4$), 75μL of 20mg/ml X-Gal, and 3μL of β-mercaptoethanol were combined. Two pieces of Whatman paper were placed into the bottom of an empty 9cm plate and the prepared Z buffer/X-Gal solution was poured on the paper, until fully saturated. The excess liquid was disposed of. Liquid nitrogen was poured into a shallow container. Using tweezers, the filter paper containing the colonies to be tested was placed in the liquid nitrogen for 5-10s. The filter paper was removed and allowed to thaw before being placed colony-side-up on the Whatman paper and parafilming the plate shut. The plates were incubated at 30°C and the shade of blue of each of the colonies was recorded every 20min. AD-FBF-1 with FBE was used as a positive control.

Yeast Miniprep

The Promega Wizard® Plus SV Minipreps DNA Purification System was used to purify the DNA from the yeast colonies as described (Stumpf et al., 2008). Each given colony was grown overnight in 3mL of selection media at 30°C. The following day, 2mL was transferred to a microcentrifuge tube and centrifuged for 5min at 5000 Xg. The pellet was resuspended in 250μL of Cell Resuspension Solution (Promega). 5μL of Long Life Zymolase (Genotech) was added and the solution was incubated at 37°C for 1hr. 250μL of Cell Lysis Solution (Promega) was added and the tubes were inverted to mix. The solution was incubated at room temperature (RT) for 5min, 65°C for 5min, and then cooled to RT. 10μL of Alkaline Protease Solution (Promega) was added and the solution was incubated for 10min at RT. 350μL of Neutralization Solution (Promega) was added, the solution was inverted to mix, and then centrifuged at top speed for 10min at RT. The vacuum apparatus was assembled and the columns were inserted. Cleared lysate was added to the columns and the vacuum was applied. 700μL of Wash Solution (Promega) was added and the vacuum was reapplied. The samples were centrifuged for 1min at 5000 Xg. The samples were washed with 500μL of Wash Solution and centrifuged for 1min at 5000 Xg. To dry the samples, the vacuum was reapplied for 5min. The columns were transferred to the collection tubes and centrifuged at top speed for 2min. The columns were transferred to microcentrifuge tubes and 100μL of water was added to the columns. The apparatus was incubated at RT for 10min and then centrifuged at top speed at RT for 2min. The DNA collected was stored at -20°C.

Sequencing of Independent Clones

After collecting DNA from the yeast colonies that had tested positive for both *HIS3* and LacZ, it was necessary to conduct an *E. coli* transformation due to poor

sequencing results obtained directly from yeast DNA. 1μL of yeast DNA was added to 10μL of NovaBlue Singles Competent Cells (Novagen) and incubated on ice for 30min. The cells were heat shocked at 42°C for 30s and immediately transferred back to ice. 150μL of SOC Medium was added to each tube and the tubes were inverted several times. The cultures were incubated for 1hr at 37°C and then spread onto LB Amp plates. The plates were incubated overnight at 37°C. The DNA was extracted using the Qiagen Miniprep Kit and sequenced using pIIIa sequencing primer (forward).

RRM Mutagenesis

To make a mutation in the RRM region of the RNP-8 protein, the QuikChange® II XL Site-Directed Mutagenesis Kit (Stratagene) was used. Following the guidelines and protocol laid out in this kit, primers were designed (primer sequences: $Y_{13}D$ Fwd 5'-CGC TGC GAC CGA ACC GCC GAC GTT TCG GGT CTT CAG C-3'; $Y_{13}D$ Rvs 5'-GCT GAA GAC CCG AAA CGT CGG CGG TTC GGT CGC AGC G-3'; $H_{48}D/L_{50}D$ Fwd 5'-GCA ATG GAG CCG CCC GTG ACG CGG ACA TTG TTT TCA AAA CTG-3'; $H_{48}D/L_{50}D$ Rvs 5'-CAG TTT TGA CAA TGT CCG CGT CAC GGG CGG CTC CAT TGC-3'; $F_{53}D$ Fwd 5'-GGC CTT GAA CAG TTT TGT CAA CAA TGA GCG CGT GAC G-3'; $F_{53}D$ Rvs 5'-GGC CTT GAA CAG TTT TGT CAA CAA TGA GCG CGT GAC G-3') and PCR reactions were conducted. The PCR conditions were: 95°C for 1min, 95°C for 50s, 19 cycles of 60°C for 50s, 68°C for 10min, followed by 68°C for 7min. Following the PCR, each reaction was treated with 1μL of DpnI restriction enzyme and incubated at 37°C for 1hr. Each reaction was then transformed into NovaBlue Singles Cells (Novagen). Following transformation, cells were selected,

minipreped to extract the DNA using the Qiagen Miniprep Kit, and sequenced to verify that the DNA segments had undergone mutagenesis correctly.

oma-2 3'UTR-RNP-8 Interaction

Both the GC-clamp-containing p3HR2 vector and p3HR1 vector without a GC clamp were prepared by enzyme digestion with SphI and SmaI.

oma-2 3'UTR fragments (oma-2 FWD: 5'-TTT GTA AAT ATA ATA AGC TCA ATG TTC CTG TCA TAT AGA AAG CAC GCT CGC ATG-3'; oma-2 RVS: 5'-GC AGC GTG CTT TCT ATA TGA CAG GAA CAT TGA GCT TAT TAT ATT TAC AAA-3') were used as an insert. The insert was used in reactions consisting of 1μL of DNA, 2μL of 10x buffer PNK, 15μL of sterile water, 1μL of 1mM ATP (1:75 dilution of ATP solution), and 1μL of T4 polynucleotide kinase (NEB) for a total volume of 20μL. This solution was incubated at 37°C for 2hrs and then heat inactivated at 65°C for 20min. The resulting solution was heated at 95°C for 5min and immediately cooled at RT. The *oma-2* 3'UTR fragments were ligated with the prepared vector DNA using the Fast Ligation Kit (Roche) and sequenced to determine whether the oma-2 sequence inserted properly.

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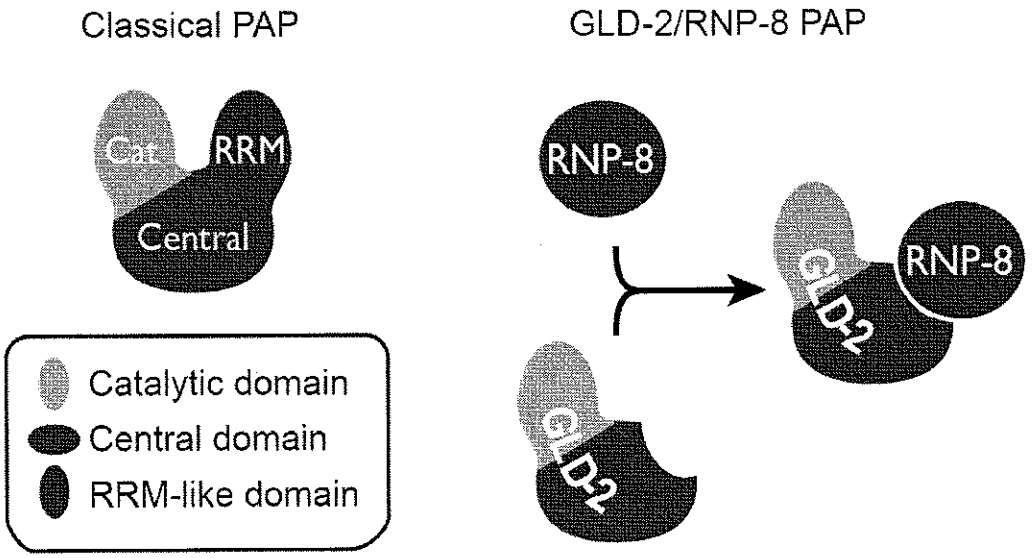
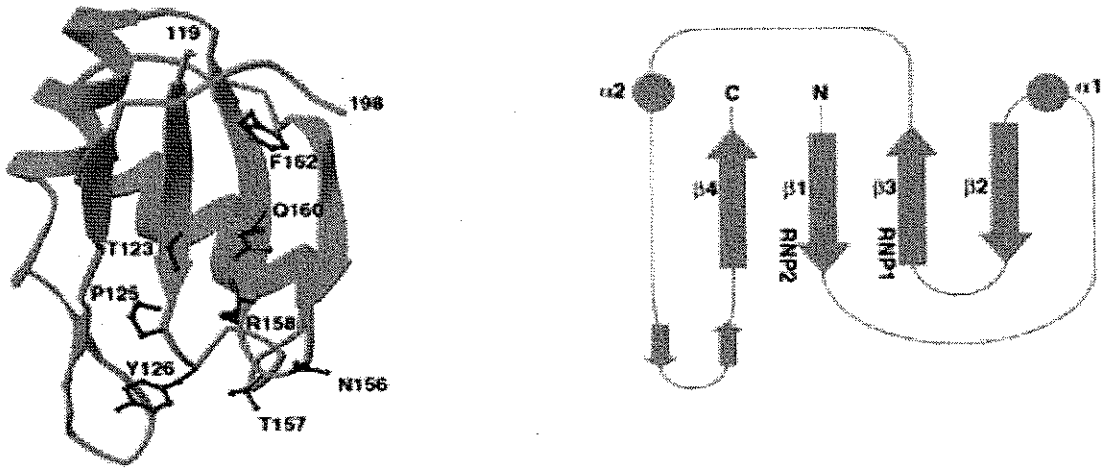


Figure 1 – Comparison of classical PAP and GLD-2/RNP-8 PAP Models
The classical poly (A) polymerase (PAP) includes a central domain, a catalytic domain, and an RNA recognition motif (RRM)-like region. GLD-2 contains both the central and catalytic domains, but lacks the RRM-like region. Alternatively, GLD-2 binds with RNP-8 in the GLD-2/RNP-8 PAP, in which RNP-8 functions as the RRM-like domain (Kim et al., 2009).

(a)



(b)

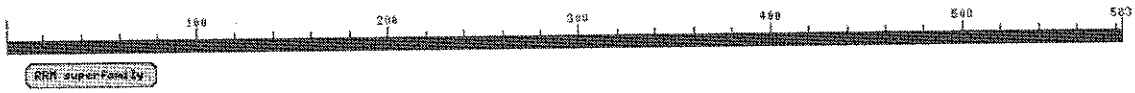


Figure 2: RRM Superfamily (a) Structure of RRM consisting of the four-stranded β-sheets with two α-helices and showing the RNP1 and RNP2 sites on the β2 and β3 strands (Cléry et al., 2008). (b) The location of the RRM domain in the RNP-8 protein.

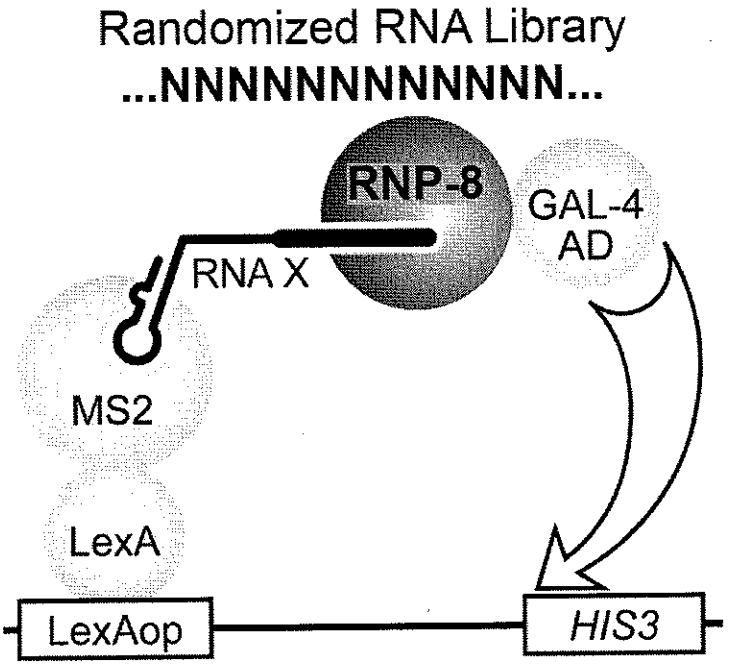


Figure 3: Yeast Three-Hybrid (Y3H) System The Y3H system was used to attempt to identify the RNA-binding site of the RNP-8 in which the yeast strain (YBZ-1) consists of DNA binding domain (BD) and MS2 protein. The protein of interest is RNP-8 which is bound to the transcription activation domain (AD). The hybrid RNA consists of the MS2 RNA and the random twelve nucleotides. Modified from Koh et al. (2008)

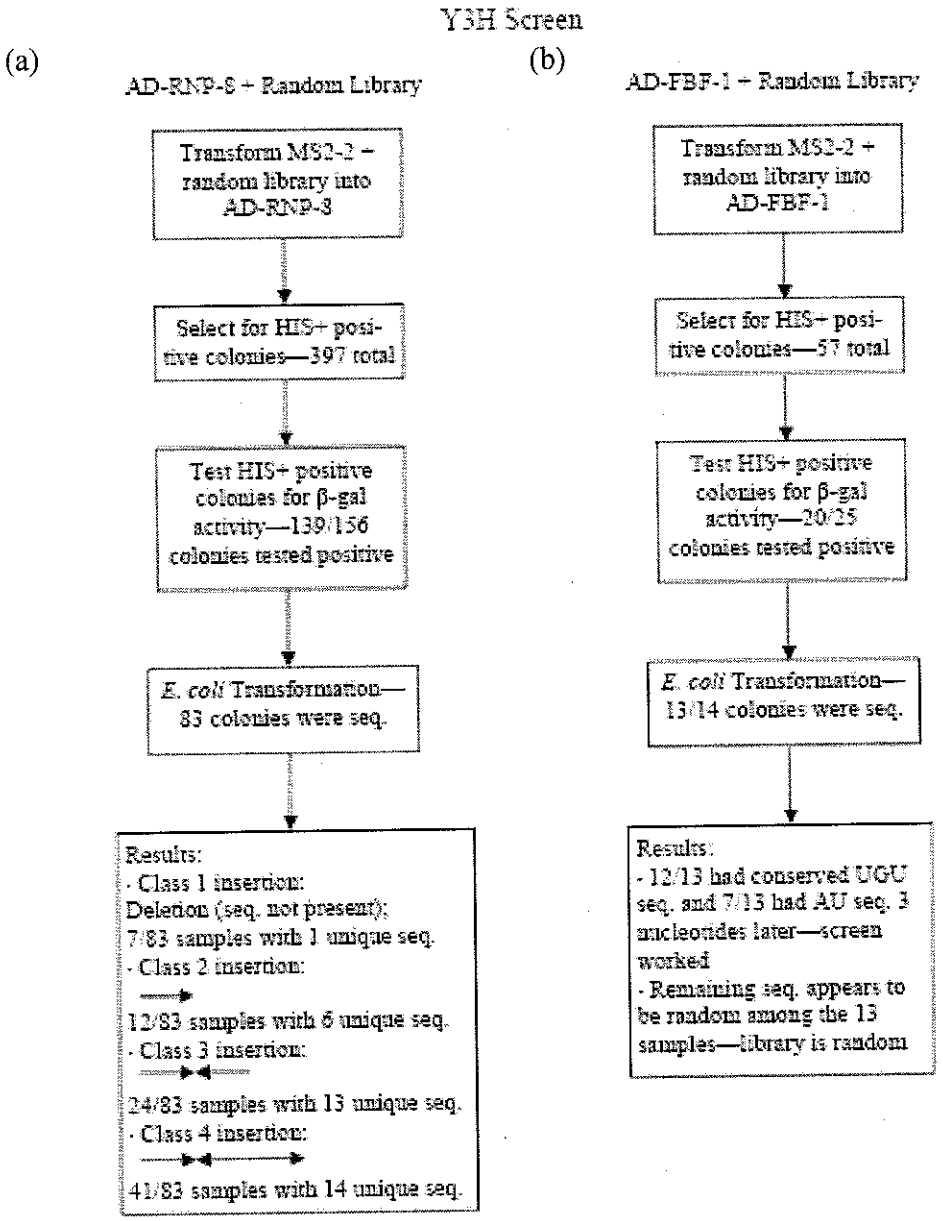


Figure 4: Flow Chart of Y3H Transformation This diagram shows the results of each step of the initial Y3H transformations as they were performed for a) RNP-8 and b) FBF-1 (positive control).

Protein	Class	# of Samples	# of Unique Sequences	Pattern of Sequence	Sequence
RNP-8	1	7	1	N/A	No Insertion
	2	12	6	→	cccggcaaaAGTAAGAGGCTCaaagcatg cccggcaaaAGTGAGTAGGCTaaagcatg cccggcaaaCGACGCGCGGATaaagcatg cccggcaaaGATTCCGATTAAaaagcatg cccggcaaaGCTGGAGGCAGAaaagcatg cccggcaaaGTAGGAAGGGTCaaagcatg
	3	24	13	→◄—	gctcgagcccgggcagcttgcatgctttAAGGAAGCAAAGtttgccggcaaaCATGCCTGCCTA gctcgagcccgggcagcttgcatgctttACTTAAGGGTACttgccggcaaaGGACCATGGCTG gctcgagcccgggcagcttgcatgctttCCCTTTAGTACttgccggcaaaGGACAGGACTAG gctcgagcccgggcagcttgcatgctttCGTTGGGTGCAGtttgccggcaaaCGAGCATCTATA gctcgagcccgggcagcttgcatgctttCGTTGGGTGCAGtttgccggcaaaGAGTGAGACATC gctcgagcccgggcagcttgcatgctttGCGCCCATATCGtttgccggcaaaCTCACGTGAGAC gctcgagcccgggcagcttgcatgctttGCTCACAGATAAtttgccggcaaaTGTCTCCACACT gctcgagcccgggcagcttgcatgctttGGTGGTCTTAACtttgccggcaaaGCATGGACCTGT gctcgagcccgggcagcttgcatgctttGTGCAGGTGTGGtttgccggcaaaTCTAGACACCCG gctcgagcccgggcagcttgcatgctttTCCCTATATCGGtttgccggcaaaCTTGGTGTCGTG gctcgagcccgggcagcttgcatgctttTGAGGAGACTGAtttgccggcaaaCCAGCCGCCCCA gctcgagcccgggcagcttgcatgctttTGCTGAGTCAGGtttgccggcaaaTCCTAAGGGAGC gctcgagcccgggcagcttgcatgctttTTGAGTGGTTCCtttgccggcaaaGGCACTACAGGT
	4	40	14	→◄—►	caaaAACCTGGCTTCCaaagcatgctttGGCGTGGGTTACttgccggcaaaGTCTCAAACCCA caaaAAGCGGCAATCGaaagcatgctttGGCCGTGGAAGTtttgccggcaaaACAGTTACACG caaaACGATGGCCAGGaaagcatgctttGATCACCTAAAGtttgccggcaaaCCACAGGGAAGA caaaAGTGCGACTGGAaaagcatgctttTACATGATACGTtttgccggcaaaACCTTTCTCGGA caaaCCCGCCGTAGATaaagcatgctttGCTTCTTTGTTGtttgccggcaaaCTATcaaaTCAA caaaGAAGGCGCGAGTaaagcatgctttTCTTCAATCGTAtttgccggcaaaCACTAGCGAAAA caaaGAGAGGGTAAAGTaaagcatgctttCCGATGGAGCAGtttgccggcaaaCACACCACGAC caaaGGGCGGGCCCAGaaagcatgctttCGGAACGGGGCTtttgccggcaaaAGCCCGCCCCCA caaaGGGGGCCCCAATaaagcatgctttCGTGTGACTACTtttgccggcaaaAGGGACACTACG caaaGGGTCGGGGTCAaaagcatgctttCATCGGGTGCAAtttgccggcaaaGTGTATTATGAG caaaTAGACCTGGCAAaaagcatgctttTAGGAGAAGGGGtttgccggcaaaCTACTGACTGTG caaaTAGGGCGCGACGaaagcatgctttGCCGCCTAACCGtttgccggcaaaCAGTTACGAGAG caaaTCACGGTCGAAAaaagcatgctttAATACAAGAGGGtttgccggcaaaTTGCCTCTGCAT caaaTGTCGGACGTAAaaagcatgctttGTGTGGCGTACCtttgccggcaaaGTGCGTGAACAA
	TOTAL	83	34	N/A	
FBF-1		13	12	N/A	AAAACA**TGT**AAATAC AGCA**TGT**CAAATA ATCA**TGT**AGAAT T**GTGT**AATAGGA ATCA**TGT**AGAAT ACA**TGT**AAATAC AAATC**TGT**GTCA**T**CT TCT**GTGT**CATCT AAAATCA**TGT**AGA**AT** GAGT**TGT**GATA**T** AAACC**TGT**TAAATAT CATC**TGTGT**CA**T**

Table 1: Sequence Data for the Four Classes of AD-RNP-8 and AD-FBF-1 This table shows the number of samples, number of unique sequences, pattern of the insertion sequence, and the sequences for the four classes of AD-RNP-8 and AD-FBF-1. These were the criteria used to initially separate the 34 unique identified RNA sequences resulting from the AD-RNP-8 Y3H transformation.

Protein-RNA Interactions	Colonies with β-galactosidase activity (Percentage of colonies)			n
	-	+	++	
FBF-1-FBE	3 (15%)	6 (30%)	11 (55%)	20
RNP-8-RNA	17 (12%)	121 (87%)	1 (1%)	139

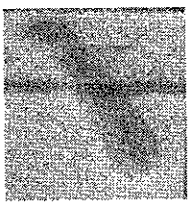
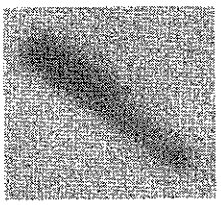
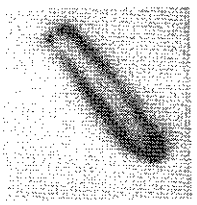
A)  B)  C) 

Table 2: β-galactosidase Assay Comparing the Response of RNP-8-RNA and FBF-1-FBE The results after the β-galactosidase assay show that the RNP-8-RNA interaction has a weaker affinity than the FBF-1-FBE interaction evidenced by RNP-8-RNA having a larger percentage of its colonies giving responses of + versus FBF-1-FBE colonies producing a majority of the responses as ++. For the β-galactosidase assay: A) "-" means no activity B) "+" means weak activity and C) "++" means high activity.

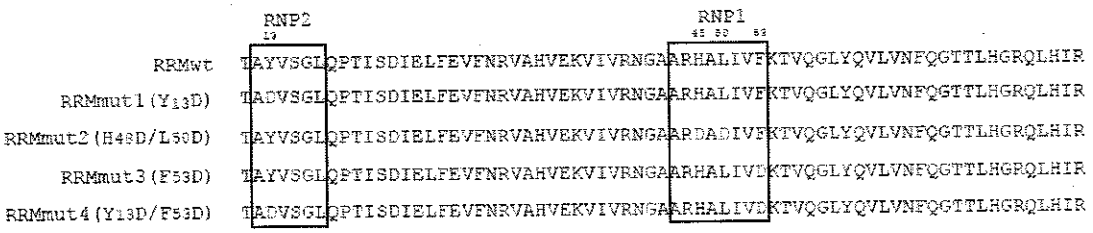


Figure 5: Four Substitution Mutations Made to the RRM Region of RNP-8
Four RRM mutations (RRMmut), compared to RRM wild-type (WT) sequence, were created by mutating specific amino acids containing aromatic side chains of the RRM region of WT RNP-8 in the RNP1 and RNP2 regions.

Y3H Screen: AD-RNP-8 Transformants			
Vector: p3HR2			
		β-galactosidase Assay Results	
RNA Classes	# of copies with same sequence	Wild-type RNP-8	RRMmut 1 ($Y_{13}D$)
1	5	++	++
	5	++	++
2	3	++	++
	3	++	++
3	8	+	++
	5	++	++
	2	++	++
4	12	++	++
	12	++	++
	17	++	++

Table 4: AD-RNP-8 Y3H Transformants versus RRMmut 1 ($Y_{13}D$) and WT RNP-8 Using p3HR2 Vector Ten samples from the AD-RNP-8 Y3H transformations were selected (those that showed the strongest β-galactosidase activity) from each of the four classes and transformed with RRMmut 1 ($Y_{13}D$) and WT RNP-8. Note: The WT RNP-8 colonies are different colonies than those used in Table 2 and thus may have been effected by extraneous variables to produce strong activity in this assay compared to weak activity in Table 2.

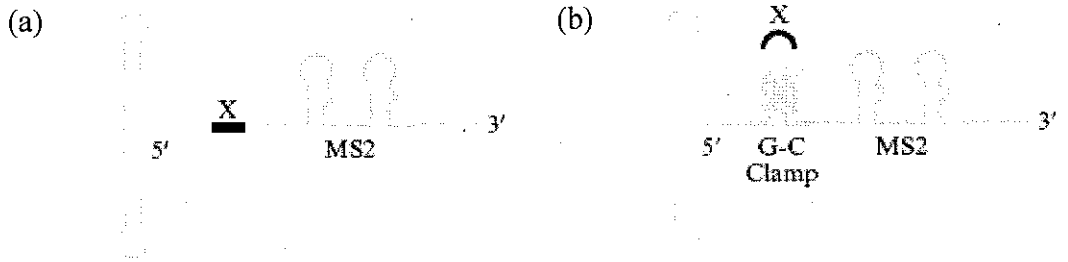


Figure 6: Vector Without a GC Clamp and Containing a GC Clamp (Stumpf et al., 2008) Image (a) shows a vector, such as p3HR1, that lacks a GC clamp while image (b) shows a vector, such as p3HR2, that contains a GC clamp. The X is where the sequence of interest can be inserted.

Protein	RNA vector	RNA	β-gal Assay	3-AT on -LUH
RNP-8	p3HR2	-	-	-
	p3HR2	*oma-2* 3'UTR	-	-
	p3HR1	-	+	-
	p3HR1	*oma-2* 3'UTR	-	-
FBF-1	p3HR2	FBE	++	+

Table 5: *oma-2* Y3H Assay Results Using *oma-2* 3'UTR as 'Bait' The transformations were analyzed using β-galactosidase assays and growth assays. For β-galactosidase assays: "-" means no activity, "+" means weak activity, and "++" means high activity. For growth assays: "-" refers to no growth and "+" refers to growth in selection media lacking leucine, uracil, and histidine and containing 10mM 3-aminotriazole.

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