The Role of G-Quadruplex RNA Motif in Fragile X Syndrome

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THE ROLE OF G-QUADRUPLEX RNA MOTIF IN FRAGILE X SYNDROME

A Thesis

Submitted to the Graduate School of Pharmaceutical Sciences

Duquesne University

In partial fulfillment of the requirement for
the degree of Master of Science

By

Yang Zhang

May 2014
THE ROLE OF G-QUADRUPLEX RNA MOTIF IN FRAGILE X SYNDROME

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ABSTRACT

THE ROLE OF G-QUADRUPLEX RNA MOTIF IN FRAGILE X SYNDROME

By

Yang Zhang

May 2014

Thesis supervised by Dr. Mihaela-Rita Mihaiescu

Fragile X syndrome (FXS), the most common cause of inherited mental impairment, is caused by the loss of expression of the fragile X mental retardation protein (FMRP). As an RNA binding protein, FMRP has been proposed to regulate the transport and translation of specific message RNA (mRNA). It has been reported that FMRP uses its RGG box domain to bind mRNA targets that form a G-quadruplex structure, structure believed to be important for FMRP recognition of at least a subclass of its mRNA targets. We have hypothesized that the interaction of FMRP with selected relevant mRNA targets occurs in a G-quadruplex dependent manner. By analyzing the structure of two FMRP \textit{in vivo} mRNA targets, Shank1 mRNA and BASP1 mRNA, and their interactions with FMRP, we showed a high-affinity interaction between Shank1 RNA G-quadruplex and FMRP. The other G-quadruplex forming mRNA BASP1, however, interacts with FMRP using other structural elements.
ACKNOWLEDGEMENTS

I’d like to acknowledge my research advisor Dr. Mihaele-Rita Mihaiescu at the first place. I appreciate that she trusted me and gave me an opportunity to join her laboratory when I really needed it. I learned how to keep being productive and manage my time from her, which benefits me in the long term. She is a very warm person who has been very patient to me. I enjoyed the short period of time I worked in her laboratory. I also appreciate her efforts on helping me start my career.

Next, I’d like to acknowledge Dr. James K. Drennen. I appreciate that he was creative and very supportive to make such an arrangement for me. I was not able to finish my program in pharmaceutics without his support and efforts. His suggestions and guidance in terms of starting my career are also extremely helpful for me. I also would like to thank Dr. Wilson Meng for being my committee member and providing me suggestions regarding my research and presentation skills.

Additionally, I’d like to thank my laboratory members, Snezana Stefanovic, Damian McAninch, Brett DeMarco and Anna Blice-Baum, for creating such a good working environment. I’d like to give a special thanks to Snezana, who taught me all the techniques and answered countless questions of mine. She also has been my motivation since I joined this laboratory. I also want to thank all undergraduates working in our laboratory, Christian Gaetano, Sara Katrancha, Allison Williams and Ayana Underwood. They have been working very hard and making our laboratory a better place.

Last but not least, I want to thank my friends in pharmacy and biochemistry for their support all the time. I couldn’t successfully earn my Master degree without them. I
appreciate all the technical support from Ian Welsh, Dan Bodnar, Ben Lauterbach and Lance Crosby. I also would like to acknowledge the National Institutes of Health grant NIH/NICHD 9R15HD078017-03A1 to M.R.M.
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CHAPTER 1: INTRODUCTION

1.1 Fragile X syndrome

Fragile X syndrome (FXS) is the most common cause of inherited mental impairment, affecting ~1 in 4000 males and ~1 in 8000 females (1). FXS patients suffer from multiple behavioral problems including cognitive impairments, impaired visuo-spatial processing, hyperactivity, anxiety, and autism in a number of cases (2). FXS is caused by the loss of expression of the fragile X mental retardation protein, due to an unstable expansion of a cytosine-guanine-guanine (CGG) trinucleotide repeat and its subsequent hypermethylation in the 5’-untranslated region (5’-UTR) of the fmr1 gene (3, 4). When the CGG repeats exceed 230, they cause the transcriptional silencing of the fmr1 gene and, consequently, the loss of the fragile X mental protein (FMRP). There is no specific treatment for fragile X syndrome.

1.2 Fragile X mental retardation protein

FMRP is produced in many tissues, being most abundantly expressed in brain and testes (5). In neurons, FMRP is present in the cell body as well as at the synapses (6). As a messenger RNA (mRNA) binding protein, FMRP contains two types of RNA-binding motifs: two K-homology (KH) domains and one arginine-glycine-glycine rich region (RGG) box (Figure 1.1). Additionally, it contains a nuclear localization signal (NLS) at the N-terminal and a nuclear export signal (NES) at its C-terminal, which allow FMRP to shuttle between the nucleus and cytoplasm (7, 8). FMRP has been proposed to act as a transport and translation regulator, functioning in downstream pathway of mGluRs and
upstream of local protein synthesis (43). FMRP binds its mRNA targets in the nucleus and transports them as part of larger ribonucleoproteins to dendrites (43). FMRP normally represses the translation of its mRNA targets, but its repression is released once FMRP is dephosphorylated upon mGluR activation, allowing the burst of local protein synthesis (Figure 1.2 A) (43). In fragile X syndrome neurons, however, a subset of dendritic proteins synthesis is constitutively elevated due to the lacking of FMRP even in the absence of mGluR activation (Figure 1.2 B).

FMRP mRNA that contains 17 exons has been shown to undergo the post-translational modifications of arginine methylation, phosphorylation and alternative splicing (9, 10, 11), resulting potentially in 20 FMRP isoforms. However, only five of them have been found in various tissues (5, 12). The three highest molecular weight FMRP isoforms correspond to isoform 1 (ISO1), isoform 2 (ISO2) and isoform 3 (ISO3). Each of them contains all 17 exons but differs in the utilization of the splice acceptor sites

**Figure 1.1** A schematic representative of the full-length FMRP, showing the nuclear localization signal (NLS), the two K- homology domains (KH1 and KH2), the nuclear export signal (NES) and the RGG box (RGG). It also illustrates the three heaviest molecular weight isoforms, ISO1, ISO2 and ISO3. The phosphorylation at serine 500 is the main phosphorylation site of FMRP (highlighted in red). Adapted from (41).
ISO1, the longest FMRP isoform, is spliced out to form ISO2 and ISO3. While all three FMRP isoforms maintain the NLS, KH1, KH2, NES and the RGG box domain, the main phosphorylation site being spliced out in the isoforms ISO2 and ISO3 (13, 14) (Figure 1.1). The investigated isoforms in this study are ISO1 and its phosphorylated mimic named ISOP. ISOP is a S500D mutant in which serine 500 was mutated to aspartic acid, as it has been shown that the phosphorylation at serine 500 is the main phosphorylation site of FMRP (15). Unphosphorylated FMRP has been shown to be associated with actively translating polyribosomes while the phosphorylated form interacts with stalled ribosomes, suggesting that phosphorylation plays a role in modulating the translation regulation function of FMRP (16). Employing these two

**Figure 1.2** Metabotropic glutamate receptor (mGluR) theory of fragile X syndrome. FMRP normally represses the translation of its mRNA targets, but its repression is released once FMRP is dephosphorylated upon mGluR activation, allowing the burst of local protein synthesis (a). In neurons lacking FMRP, however, a subset of dendritic proteins synthesis is constitutively elevated even in the absence of mGluR activation (b). Adapted from (43).
FMRP isoforms ISO1 and ISOP1, we aim to investigate if the posttranslational modification of phosphorylation modulates the FMRP translation regulator function by affecting its binding affinity to its G-quadruplex forming mRNA targets. As a regulator that controls the transport and translation of specific mRNA targets, it has been reported that FMRP uses its RGG box domain to bind mRNA targets that form G-quadruplex structures (12, 17, 19).

1.3 G-quadruplex structures

DNA or RNA sequences rich in guanine content have high potential to fold into G quartets. A G quartet is formed by four guanine residues arranged in a planar configuration by Hoogsteen-type hydrogen bonds (20, 21, 22) (Figure 1.3). Several G quartet planar structures are able to stack onto each other to fold into a G-quadruplex structure. The G-quadruplex formation is dependent on the presence of cations lying within each G quartet plane or between planes. It has been reported that K\(^+\) and Na\(^+\) ions stabilize the G-quadruplex structure (23). It has been shown that DNA G-quadruplexes are over-represented in oncogene promoters, which are viewed as an emerging therapeutic target (42). Transcriptional repression achieved by stabilizing these G-quadruplex structures located in the promoter region of specific genes could be a novel anti-cancer strategy. Given that RNA is single-stranded and that G-quadruplex formation doesn’t have to compete with hybridization to a complementary strand, RNA G-quadruplexes are generally more stable in the folded form than DNA G-quadruplexes (46). The existence of RNA G-quadruplexes in vivo is more prevalent than the existence of G-quadruplexes in DNA (44). This secondary structure has been most found in mRNAs’ untranslated region (UTR), 3’ UTR or 5’ UTR, which are known to be involved
in translational regulation, particularly for growth factors, transcription factors and onco-
proteins, implying that G-quadruplex play a role in gene expression regulation at the
mRNA level (45, 24, 25).

1.4 FMRP recognition of its mRNA targets

Though it has been shown that the FMRP participates in the post-transcriptional
control of gene expression, the exact mechanisms by which FMRP recognizes its targets
and exerts its translation regulator function are not known. It is believed that the G-
quadruplex structure plays an important role in the FMRP recognition of at least a
subclass of its mRNA targets. We have hypothesized that the interaction of FMRP with
its mRNA targets occurs in a G-quadruplex dependent manner. A crucial question
remains: is the recognition of G-quadruplex essential for fragile X mental retardation
protein function? By analyzing FMRP’s interactions with different mRNA targets, in this
study we aim to understand the principles of recognition between FMRP and its mRNA
targets. Accordingly, we selected two FMRP in vivo mRNA targets to characterize their
structures and interactions with FMRP.

Figure 1.3 A G quartet is formed by four guanine residues with Hoogsteen-type
hydrogen bonds. These planar G quartets stack on top of each other to form a G-
quadruplex structure further stabilized by K+ ions. Adapted from (42).
1.5 Shank1 mRNA

The postsynaptic density (PSD) is a protein network attached to the postsynaptic membrane of excitatory synapses. It serves to cluster cell adhesion molecules, to recruit signaling proteins, and anchor these components to the microfilament-based cytoskeleton in dendritic spines (31, 32, 33). As a translation regulator, FMRP has been reported to locally control the synthesis of proteins in dendrites, such as components of the PSD (34, 35). Specifically, some postsynaptic proteins are affected by the loss of FMRP. As one of the PSD components, SH3 and multiple ankyrin repeat domains proteins (Shank) regulate the size and shape of dendritic spines because of their capacity to directly interact with many different PSD components, and represent master scaffold proteins of the PSD (31). Due to their effect on spine morphology, altered translational regulation of Shank1 transcripts may contribute to the FXS pathology (36). Increased Shank1 levels have been found in PSD fractions obtained from FMRP-deficient mice (37). Schütt et al. (37) have also shown that Shank1 mRNA is an in vivo FMRP target. Molecular evidence has shown that FMRP represses translation of Shank1 mRNA via its 3’ UTR. In this study, we have identified G-quadruplex-forming sequences located in the 3’ UTR of human Shank1 mRNA. FMRP may repress Shank1 expression levels via its interaction with these G-quadruplex-forming sites. We hypothesize that the interaction of FMRP with Shank1 mRNA 3’ UTR is dependent upon the recognition of G-quadruplex structure. Two G-quadruplex forming sequences in Shank1 RNA 3’ UTR were predicted by QGRS mapper software (Materials and Method section), named Shank1a RNA and Shank1b RNA in our study. It’s interesting to note that these two sequences are located immediately adjacent to each other in Shank1 RNA.
1.6 BASP1 mRNA

BASP1 mRNA encodes for the brain abundant, membrane attached signal protein 1 (BASP1), which is expressed throughout the brain, with localization to the synaptic terminals, dendritic spines, and thin nerves fibers associated with synaptic vesicles, presynaptic and postsynaptic membranes and microtubes (38). BASP1 appears to be important for neuronal sprouting and plasticity, which are abnormal in FXS (39). It was reported that BASP1 was differentially expressed between fmr1 knock-out and wild type synapses in mice (40). Furthermore, BASP1 mRNA was identified as a potential FMRP mRNA target and predicted to form an intramolecular G-quadruplex recognized by FMRP RGG box (17). Accordingly, we analyzed the human BASP1 RNA by QGRS mapper to identify the sequences that most likely form G-quadruplex structures. Two selected sequences located in the coding region and 5’ UTR, respectively, were identified and were named accordingly as BASP1-Coding and BASP1-5’ UTR.

1.7 Specific aims of this study

We have hypothesized that the interactions of FMRP with its mRNA targets, Shank1 RNA and BASP1 RNA, are dependent on G-quadruplex structure. To investigate the principles of recognition between FMRP and its target mRNAs, we aim to: (i) examine the existence of G-quadruplex structure in these selected mRNA targets and (ii) analyze their interactions with FMRP. Accordingly, this study which employs biochemical and biophysical methods to analyze the structure of these two FMRP in vivo mRNA targets and their interactions with FMRP, has the following specific aims:
Specific Aim I: Characterization of the secondary structure of Shank1 mRNA and BASP1 mRNA.

Specific Aim II: Characterization of the interactions of FMRP with Shank1 mRNA and BASP1 mRNA.
CHAPTER 2: MATERIALS AND METHODS

2.1 G-quadruplex structure prediction

Quadruplex forming G-Rich Sequences (QGRS) mapper, a web-based server for predicting G-quadruplexes in nucleotide sequences, was used to identify G-rich sequences in the human Shank1 3’UTR RNA and BASP1 RNA (http://bioinformatics.ramapo.edu/QGRS/index.php). The sequences with highest G score, which indicates a high potential to form G-quadruplex structures, were selected and named Shank1a RNA, Shank1b RNA, BASP1-5’UTR RNA and BASP1-Coding RNA, respectively (Table 2.1).

<table>
<thead>
<tr>
<th>Table 2.1 The synthetic RNA oligonucleotides by in vitro transcription.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shank1a</td>
</tr>
<tr>
<td>Shank1b</td>
</tr>
<tr>
<td>BASP1-5’UTR</td>
</tr>
<tr>
<td>BASP1-Coding</td>
</tr>
</tbody>
</table>

2.2 Synthesis of RNA in vitro

Shank1a RNA, Shank1b RNA, BASP1-5’UTR RNA and BASP1-Coding RNA were synthesized using the designed synthetic DNA templates (TriLink BioTechnologies, Inc.) by T7 RNA polymerase driven in vitro transcription reactions (Table 2.1). The T7 RNA polymerase was produced in-house. The synthetic DNA templates were purchased from TriLink BioTechnologies, Inc. The produced oligonucleotides were purified by 20%
8 M urea denaturing polyacrylamide gel electrophoresis and electrophoretic elution, followed by extensive dialysis against 10 mM cacodylic acid, pH 6.5.

The fluorescencently labeled single stranded oligonucleotides were constructed by replacing one of the adenines in the original RNA sequences with its fluorescent analog 2-aminopurine (2AP), and were chemically synthesized by Dharmacon, Inc. The adenine replaced in each RNA sequence is indicated in red in Table 2.2.

<table>
<thead>
<tr>
<th>Table 2.2</th>
<th>The fluorescently labeled RNA oligonucleotides. The replaced adenines with 2-aminopurine were highlighted in red.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shank1a (2AP)</td>
<td>5’ GGGGUUGGGAGGUGUAAGGGGUGGGG 3’</td>
</tr>
<tr>
<td>Shank1b (2AP)</td>
<td>5’ GGGAGGAGAGGUCGGGGUGGGAGUGGGG 3’</td>
</tr>
<tr>
<td>BASP1-5’ UTR (2AP)</td>
<td>5’ GGGCAGGAAGGGAGGGGGAGCGAG 3’</td>
</tr>
</tbody>
</table>

All DNA templates and 2-aminopurine labeled RNAs were re-suspended in sterile deionized water before using. All oligonucleotides were annealed by heating in boiling water at 95 °C, followed by slow cooling at room temperature.

2.3 FMRP RGG box peptide synthesis

The FMRP RGG box peptide was chemically synthesized and purified by the Peptide Synthesis Unit at the University of Pittsburgh, Center for Biotechnology & Bioengineering.
2.4 Expression and purification of recombinant FMRP ISO1 and ISOP

The pET-21a-FMRP plasmid, encoding ISO1 fused with a C-terminal 6x histidine tag, was a gift from Dr. Bernhard Laggerbauer (47). Sara Katrancha, a former undergraduate in the Mihailescu laboratory, designed a phosphorylated mimic of the FMRP ISO1, ISOP, in which serine 500 was replaced by aspartic acid to mimic phosphorylation on serine 500 (15). The pET-21a-FMRP ISOP plasmid was produced by GenScript USA, Inc. To recombinantly express, purify and dialyze FMRP ISOP, we used the protocol previously developed in our laboratory for the expression of FMRP ISO1 (18). Briefly, each plasmid was transformed into the Rosetta 2(DE3) pLysS E. coli cell line, cells were incubated at 37 °C, 250 rpm until an OD$_{600}$ of 0.8 – 1.0, and target protein expression was induced by adding isopropyl β-D-1-thiogalactopyranoside to 1 mM, and incubating the cells at 25 °C, 250 rpm for 12 h. Cells were harvested and lysed, and each FMRP isoform was purified using Ni-NTA Superflow resin (Qiagen) as described in (18). Purified proteins were concentrated using dialysis tubing filled with polyethylene glycol (PEG) 20,000 (48). The two isoforms were dialyzed into a buffer devoid of K$^+$ and Na$^+$, suitable for the analysis of their binding activity to G-quadruplex forming mRNAs. Dialysis occurred in the presence of 5% glycerol and 1 mM EDTA with the gradual removal of imidazole, as described in (18). The concentration of the FMRP isoforms was determined by A$_{280}$ using the same molar extinction coefficient of 46,370 M$^{-1}$cm$^{-1}$ for ISO and ISOP (49, 50). Successful production of each FMRP isoform was analyzed using 10 % tris-glycine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized using Coomassie blue.
2.5 UV spectroscopy thermal denaturation

UV thermal denaturation curves were obtained using a Varian Cary 3E UV-Visible Spectrophotometer with a Peltier temperature control cell holder. Samples were annealed prior to performing the experiments in the presence of various concentrations of KCl in 10 mM cacodylic acid, pH 6.5. Samples and reference cells were covered with 200 μL of mineral oil to prevent evaporation at high temperature. The RNA samples were heated from 25 to 95°C at a rate of 0.2°C/min, and absorbance points were recorded at every 1°C at 295 nm, wavelength identified to be most sensitive to G-quadruplex dissociation (30). To determine if these G-quadruplex forming RNAs adopt an intermolecular or intramolecular conformation, the melting temperature of the G-quadruplex structure was determined at different RNA concentrations.

The hypochromic transition of the G-quadruplex dissociation in Shank1a RNA and Shank1b RNA was identified and fitted with Equation 2.1, which assumes an independent two state model:

\[
A(T) = \frac{A_U + A_F e^{-\Delta H_0 / R T} e^{\Delta S_0 / R}}{e^{-\Delta H_0 / R T} e^{\Delta S_0 / R} + 1}
\]

Equation 2.1

where \(A_U\) and \(A_F\) respectively represent the absorbance of the unfolded and native G-quadruplex structures, and \(R\) is the gas constant.

2.6 Circular dichroism (CD) spectroscopy

The CD spectra were recorded on a Jasco J-810 spectropolarimeter at 25°C. The G-quadruplexes formation in 10 mM cacodylic acid, pH 6.5, was monitored as increasing
amounts of KCl were titrated from a 2 M stock solution to a final concentration of 150 mM. The spectra were measured between 200 and 350 nm and corrected for solvent contributions and dilutions. Each spectrum was scanned seven times with 1 s response time and a 2 nm bandwidth. For the binding studies, increasing amounts of the RGG peptides (0–80 µM) were titrated into a fixed concentration of RNA (10 µM) in 10 mM cacodylic acid, pH 6.5, and 25 mM KCl.

2.7 1H-NMR spectroscopy

The one dimensional 1H NMR spectra of the RNA oligonucleotides were acquired at 25°C on a 500 MHz Bruker AVANCE spectrometer. Water suppression was carried out by using the Watergate pulse sequence (51). Maximum concentrations of RNA samples (~ 200 µM) were prepared in 10 mM cacodylic acid buffer, pH 6.5, in a 90% H2O/10% D2O ratio. G-quadruplex formation was monitored by titrating increasing concentrations of KCl from a 2 M stock to each sample. Annealed samples were prepared by removing the RNA samples from the NMR tube followed by 5 minutes boiling and 10 minutes cooling to 25 °C.

2.8 Native polyacrylamide gel electrophoresis (PAGE) and electromobility gel shift assays (EMSA)

10 µM of RNA samples in the presence of various concentrations of KCl were annealed by boiling for 5 minutes followed by cooling down at room temperature for 10 minutes, prior to their use in native gel electrophoresis. The 20% native gels in 0.5X Tris/Borate/EDTA buffer were run at 4°C, 85 V for 6 h using vertical gel apparatus and visualized by UV shadowing at 254 nm using an Alphalmager (AlphaInnotech, Inc.).
To examine the effect of the FMRP RGG domain binding, a total volume of 15 μL RNA in the presence of desired concentrations of KCl was prepared for each RNA sample, prior to the 5 minutes annealing. Synthetically produced FMRP RGG peptide was added to the RNA in various ratios and incubated for 20 minutes at room temperature. 1 μL of loading buffer (15% glycerol in 10X TBE buffer) was added to each sample after incubation. The samples were run on 20% native polyacrylamide gels (containing 5 mM KCl) in 0.5X Tris/Borate/EDTA buffer in the presence of 5 mM KCl at 4°C, 85 V for 6 hours. Electrophoretic motilities of RNA sample were visualized using UV-shadowing at 254nm using an AlphaImager (AlphaInnotech, Inc.).

2.9 Fluorescence spectroscopy

Steady-state fluorescence spectroscopy measurements of Shank1a RNA binding with the FMRP RGG box were performed on a Horiba Jobin Yvon Fluoromax-3 equipped with a temperature controller set up to 23 °C. All the other fluorescence spectroscopy measurements were performed on a Horiba Jobin Yvon Fluoromax-4 spectrofluorometer at room temperature in the range of 24.5 – 27 °C. The excitation wavelength was set at 310 nm and the emission spectrum was recorded in the range of 330 – 450 nm. The bandpass for excitation and emission monochromators were both set to 5 nm. Increasing concentrations of the FMRP RGG peptide or the FMRP isoforms were titrated in 50 nM increments to a fixed 200 nM RNA. 1 μM of HCV peptides or BSA was added into the RNA sample before titrating RGG peptides and isoforms, respectively. The emission values were corrected and normalized to free RNA fluorescence intensity at 371 nm. Each binding experiment was performed in triplicate.
The binding dissociation constant $K_d$, was determined by fitting the binding curves to the equation:

\[
K_d = \frac{I_F}{I_B} = \frac{1}{1 + \frac{[RNA]}{[P]}}
\]

**Equation 2.2**

where $I_F$ and $I_B$ represents the steady-state fluorescence intensities of the free and bound RNA, $[RNA]_t$ is the total concentration of RNA and $[P]_t$ is the total RGG box peptide or FMRP isoform concentration. The reported $K_d$ value is the average from triplicates with the highest error in these three trails.
CHAPTER 3: CHARACTERIZATION OF THE SHANK1 RNA G-QUADRUPLEX STRUCTURES

3.1 Characterization of the Shank1a RNA

It has been reported that FMRP locally controls the synthesis of proteins in dendrites, such as components of the postsynaptic density (PSD) (34, 35). SH3 and multiple ankyrin repeat domains proteins (Shank), one of the PSD components, regulate the size and shape of dendritic spines and represent master scaffold proteins of the PSD (31). Shank1 mRNA has been identified as an in vivo FMRP target, supported by the

![Figure 3.1](image)

**Figure 3.1** The $^1$H NMR spectra of the Shank1a RNA shows the existence of the G-quadruplex structure reflected by the imino proton resonances from 10 – 12 ppm. There is no Watson-Crick base pair resonances from 12 – 14.5 ppm observed in the spectra. Increasing the KCl concentration doesn’t lead to any conformation change in the Shank1a RNA.
molecular evidence that FMRP represses translation of Shank1 mRNA via its 3’ UTR (37). We hypothesize that the interaction of FMRP with Shank1 mRNA 3’ UTR is dependent upon the recognition of G-quadruplex structure. Two G-quadruplex forming sequences in Shank1 RNA 3’ UTR were identified and named Shank1a RNA and Shank1b RNA in our study. First, we performed the analysis of the Shank1a RNA, one of the selected G-quadruplex forming sequences in Shank1 RNA.

To examine the existence of G-quadruplex structures in the Shank1a mRNA, we first performed 1H NMR spectroscopy experiments, monitoring the imino proton resonance region. Resonances located between 12-14.5 ppm report upon the formation of guanine and uracil imino protons involved in Watson-Crick base-pairs, whereas those from 10 – 12 ppm correspond to the guanine imino protons involved in the formation of G-quadruplex structures (26). Spectra were acquired for the Shank1a RNA sample in the presence of various concentrations of KCl. Figure 3.1, (blue spectrum) indicates that even in the absence of KCl, a G-quadruplex structure is formed in Shank1a RNA, as reflected by the presence of the broad resonance in the region 10-12 ppm. In the presence of 10 mM KCl, a few resonances on the broad envelope centered around 11 ppm become more refined, indicating the structure stabilization (Figure 3.1, red spectrum). No additional changes were observed once the KCl concentration was increased to 25 and 50 mM (Figure 3.1, green and purple spectra). No resonances are observed in the region 12-14.5 ppm, indicating that Shank1a mRNA does not form alternate structures involving Watson-Crick base pairs. Taken together the 1H NMR spectroscopy results indicate that Shank1a RNA forms a G-quadruplex structure even at the 0 mM KCl, structure further stabilized by the presence of K+ ions. The G-quadruplex imino proton resonances
observed are on a broad envelope, indicating a dynamic exchange between conformations.

To examine the conformations of the G-quadruplex formed in Shank1a RNA, we performed native polyacrylamide gel electrophoresis in the presence of increasing KCl concentrations, in the range 0-50 mM (Figure 3.2). Two bands are present in the absence of KCl (Figure 3.2, lane 1), indicating the co-existence of alternate conformations. Upon increasing the KCl concentration the equilibrium between the two conformations shifts, as reflected by the decrease in the intensity of the lower migrating band.

![Figure 3.2](Image)

**Figure 3.2** The native polyacrylamide gel electrophoresis of 15 μM Shank1a RNA at various KCl concentrations: 0 mM (lane 1), 5 mM (lane 2), 10 mM (lane 3), 25 mM (lane 4) and 50 mM (lane 5). Two bands at 0 mM KCl indicates there are alternate conformations coexisting in the Shank1a RNA (arrows).

The QGRS mapper software predicts a single G-quadruplex structure in the Shank1a RNA, which consists of four G₄ groups (GGGG)s (underlined in Table 3.1), a longer loop and two short loops. However, upon the further analysis of this sequence, we noticed that two alternate conformations could form within this sequence. This dynamic conformation change is due to a single guanine shift between the third G₄ group and its adjacent short loop (highlighted in red in Figure 3.3). In conformation I (Table 3.1 I and Figure 3.3 I), the guanine located in position 19 is part of the third G₄ group and the
guanine in position 23 forms a short loop along with the uracil in position 24. In conformation II (Table 3.1 II and Figure 3.3 II), the guanine in position 19 is involved in a long loop and the guanine 23 is now part of the third G₄ group. The native polyacrylamide gel electrophoresis results (Figure 3.2) support the existence of two distinct G-quadruplex conformations. However, we cannot assign the upper or lower band to conformations I or II, respectively.

Table 3.1 The two different conformations of the four-plane Shank1a RNA G-quadruplex structure, due to a guanine shift from the third G-quartet in conformation (I) to the short loop in conformation (II).

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Figure 3.3 The two different conformation predictions of the Shank1a RNA G-quadruplex structure based on Table 4.1. There is dynamic equilibrium between conformation (I) and (II). The guanines highlighted in red shift between these two conformations.
To obtain further information about the fold of the Shank1a RNA G-quadruplex structures we performed CD spectroscopy experiments in the presence of different concentrations of KCl. At 0 mM KCl, a negative band was observed at 240 nm and a positive one at 265 nm, the typical CD signature of a parallel G-quadruplexes (27, 28) (Figure 3.4, yellow spectrum). Once the KCl concentration was increased to 25 mM (blue spectrum), the intensities of both the positive and negative bands increased significantly, indicating that KCl facilitates the stabilization of the G-quadruplex structures in the Shank1a RNA. At higher concentrations 50 mM (red spectrum) and 100 mM (green spectrum), the intensities of these bands increased further, but not as much as from 0 mM to 25 mM KCl. Taken together the $^1$H NMR spectroscopy, CD spectroscopy and native
gel electrophoresis experiments, indicate that Shank1a RNA forms two alternate parallel G-quadruplex structures, one of which is further stabilized by the presence of K$^+$ ions.

To determine if the G-quadruplex structures formed by Shank1a RNA are intermolecular or intramolecular we employed UV spectroscopy thermal denaturation experiments.

For intermolecular G-quadruplexes, the melting temperature, $T_m$, depends on the RNA concentration, $C_T$:

$$\frac{1}{T_m} = \frac{R(n - 1)}{\Delta H_{vH}^0} \ln C_T + \frac{\Delta S_{vH}^0 - (n - 1)R \ln 2 + R \ln n}{\Delta H_{vH}^0}$$

Equation 4.1

where $n$ is the number of strands, $R$ is the gas constant and $\Delta H_{vH}^0$ and $\Delta S_{vH}^0$ are the Van’t Hoff thermodynamic parameters (Equation 4.1). For intramolecular conformations, $n$
equals to 1. The melting temperature $T_m$ is therefore independent of the total RNA concentration $C_T$ (Equation 4.2).

$$n = 1 \text{ and } \frac{1}{T_m} = \frac{\Delta S^0_{\nu H}}{\Delta H^0_{\nu H}}$$

**Equation 4.2**

Several Shank1a RNA concentrations in the range 5-25 μM in 10 mM cacodylic acid, pH 6.5, 2.5 mM KCl were thermally denatured and the absorbance changes were monitored at 295 nm, wavelength shown to be most sensitive to G-quadruplex dissociation (30).

As seen in Figure 3.5, at all RNA concentrations investigated, a single hypochromic transition is present in the UV thermal denaturation profile of Shank1a RNA, typical of G-quadruplex structure unfolding. The melting temperature does not

![Figure 3.6](image)

**Figure 3.6** The melting temperatures of the Shank1a RNA G-quadruplex in the presence of 2.5 mM KCl was plotted versus the RNA concentrations. It shows that the melting temperature is independent on the RNA concentrations.
change significantly upon changing the RNA concentration (Figure 3.5), indicating that the G-quadruplex structure formed by Shank1a RNA is intramolecular.

To further quantitatively measure the melting temperatures of the Shank1a RNA, we identified and fitted the hypochromic transitions at various RNA concentrations into an independent two-state model mentioned in the Materials and Methods section (Equation 2.1). The calculated melting temperatures of each transition at different RNA concentrations were plotted against the RNA concentration (Figure 3.6) to show that the melting temperature of the Shank1a RNA G-quadruplex is independent of the RNA concentration. Its melting temperature in the presence of only 2.5 mM KCl is 73 °C, indicating a highly stable G-quadruplex structure.

Taken together the biophysical and biochemical analysis of Shank1a RNA, we found that it adopts very stable parallel G-quadruplex structures that forms in an intramolecular manner. Additionally, there is equilibrium between alternate conformations existing in this G-quadruplex forming sequence, one of which is further stabilized by the presence of K⁺ ions.

### 3.2 Characterization of the Shank1b RNA

We next performed the analysis of the other predicted G-quadruplex forming sequence, Shank1b RNA, which was also identified in Shank1 3’ UTR RNA. To examine if a G-quadruplex forms also in Shank1b RNA, we monitored the imino proton resonance region by ¹H NMR spectroscopy. Spectra of the Shank1b RNA were recorded in the presence of different concentrations of KCl. The broad resonance located in the 10 -12 ppm region indicates that a G-quadruplex structure is formed in the Shank1b RNA even
in the absence of KCl (Figure 3.7, blue spectrum). In the presence of 10 mM KCl, the more refined resonances on the broad envelope centered around 11.2 ppm suggests an enhanced stability of G-quadruplex structure (Figure 3.7, red spectrum). No additional changes were observed once the KCl concentration was increased to 25 and 50 mM (Figure 4.10, green and purple spectra). No resonances were observed in the region 12 – 14.5 ppm, indicating that Shank1b RNA doesn’t adopt alternate structures involving Watson-Crick base pairs. Taken together, the $^1$H NMR spectroscopy results suggest that Shank1b RNA forms a G-quadruplex structure even in the absence of KCl, its stability being further facilitated by the addition of K$^+$ ions. The broad envelope of the G-
quadruplex imino proton resonances indicates a dynamic exchange between conformations.

To examine the conformation of the Shank1b RNA G-quadruplex we employed native polyacrylamide gel electrophoresis in the presence of increasing KCl concentrations, in the range 0 – 50 mM, (Figure 3.8). Two bands are presents in the absence of KCl, indicating the co-existence of alternate conformations in the Shank1b RNA (Figure 3.8, lane 1). The intensity of the lower band indicates that it corresponds to the dominant G-quadruplex conformation, whereas the diffuse upper band corresponds to an alternate conformation. Upon increasing the KCl concentration the two separated bands migrate as one single broad band which positions in the middle of them (Figure 3.8, lane 2), indicating another conformation is formed in the presence of K⁺ ions. Equilibrium exists among these conformations in the presence of high concentration of KCl, as reflected by the very broad band at 25 and 50 mM KCl (Figure 3.8, lane 4 and 5).

Figure 3.8 The native polyacrylamide gel electrophoresis of 10 μM Shank1b RNA at various KCl concentrations: 0 mM (lane 1), 5 mM (lane 2), 10 mM (lane 3), 25 mM (lane 4) and 50 mM (lane 5). The arrows represents the two G-quadruplex conformations formed in the absence of KCl.
By performing CD spectroscopy experiments in the presence of various concentrations of KCl, we obtained further information about the fold of the Shank1b RNA G-quadruplex. A negative band was observed at 240 nm and a positive one at 265 nm in the absence of KCl, indicating that the Shank1b RNA forms parallel G-quadruplexes (27, 28) (Figure 3.9, yellow spectrum). Once the KCl concentration was further increased, the intensities of these two bands were slightly increased, suggesting that K$^+$ ions stabilize the G-quadruplex structures in the Shank1b RNA. Taken together the $^1$H NMR spectroscopy, CD spectroscopy and native gel electrophoresis experiments, indicate that Shank1b RNA forms alternate parallel G-quadruplexes, their overall stability being further stabilized by the presence of K$^+$ ions.

Next, to determine if the formed G-quadruplex is intermolecular or intramolecular, we acquired the UV spectroscopy thermal denaturation profiles of the
Shank1b RNA. Several Shank1b RNA concentrations in the range 5 – 25 μM were thermally denatured in the presence of 10 mM cacodylic acid, pH 6.5, and 10 mM KCl and the absorbance was monitored at 295 nm. We observed only a single hypochromic transition at all RNA concentrations investigated (Figure 3.10), typical for G-quadruplex structure unfolding. The melting temperature did not change significantly upon varying the RNA concentration (Figure 3.10), indicating that the Shank1b RNA G-quadruplex structure is intramolecular.

![Figure 3.10](image)

**Figure 3.10** The UV thermal denaturation profiles of the Shank1b RNA in the presence of 10 mM cacodylic acid and 10 mM KCl at various RNA concentrations. It shows no hypochromic transitions shift throughout the various RNA concentrations indicating the stability of the Shank1b RNA G-quadruplex is independent on RNA concentration.

To quantitatively obtain the melting temperature of the Shank1b RNA, we identified and fitted the hypochromic transitions at investigated RNA concentrations into the two-state model with Equation 2.1 (Materials and Method section). In the presence of
10 mM KCl, the Shank1b RNA melting temperature is 76°C (Figure 3.11) versus $T_m$ (73°C) of the Shank1a RNA at only 2.5 mM KCl, indicating that Shank1a RNA forms a more stable intramolecular G-quadruplex structure than Shank1b RNA.
4.1 Characterization of the interactions between Shank1a RNA and FMRP

To determine the potential of the Shank1a RNA G-quadruplex to interact with the FMRP RGG box, we employed electromobility gel shift assays. Shank1a RNA samples were mixed with the FMRP RGG peptide at different ratios and to observe the effect of KCl on their interactions, similar samples were prepared in the presence of 25 mM and 50 mM KCl. At both of KCl concentrations the free Shank1a RNA migrates as two separated bands (Figure 4.1 lanes 1 and 4), corresponding to the two G-quadruplex conformations discussed in Chapter 3 (Figure 3.3). At a 1:1 ratio Shank1a RNA to FMRP RGG box the intensity of the upper band corresponding to the dominant G-quadruplex conformation was significantly reduced while the intensity of the lower band

![Figure 4.1 EMSA of 15 μM Shank1a RNA in the presence of 25 mM KCl (lane 1, 2 and 3) and 50 mM KCl (lane 4, 5 and 6) binding to the FMRP RGG box at different RNA: RGG ratios: 1:0 (lane 1 and lane 4), 1:1 (lane 2 and lane 5) and 1:2 (lane 3 and lane 6). It shows both conformation of the Shank1a RNA G-quadruplex bind to the FMRP RGG box. The upper band corresponding to the dominant G-quadruplex conformation has higher binding affinity with the RGG peptides (arrow).](image-url)
corresponding to the alternative G-quadruplex conformation remains the same (Figure 4.1 lanes 2 and 5). The Shank1a RNA-FMRP RGG complex is not observed as a sharp retarded band in the gel due to the fact that the FMRP RGG box contains multiple positive charged arginine residues, affecting the overall charge of the complex. Thus, the binding event is monitored through the disappearance of the band corresponding to the free Shank1a RNA. At a 1:2 ratio Shank1a RNA to FMRP RGG box, both the upper and lower bands corresponding to the free Shank1a RNA vanished (Figure 4.1 lanes 3 and 6), with the concomitant appearance of a diffuse retarded band corresponding to the formation of the Shank1a RNA-FMRP RGG complex. These results suggest that although the FMRP RGG box binds with higher affinity to the predominant Shank1a G-quadruplex conformation (upper band in lanes 1 and 4), both Shank1a RNA G-quadruplex conformations can interact with the FMRP RGG box.

Figure 4.2 The CD spectra of 10 μM Shank1a RNA at various RNA: RGG ratios prepared in 10 mM cacodylic acid with 25 mM KCl (A) and 150 mM KCl (B). It shows that the FMRP RGG box induces the unwinding of the Shank1a RNA G-quadruplex at high RNA: RGG ratios.

To investigate the effect of the FMRP RGG box on inducing G-quadruplex unwinding we employed CD spectroscopy. Increasing amounts of the FMRP RGG box
were titrated into a fixed concentration of Shank1a RNA in the presence of either 25 mM KCl or 150 mM KCl. At both 25 mM and 150 mM KCl, the free Shank1a RNA showed the unique parallel G-quadruplex signature (Figures 4.2 A and B grey spectrum). As increasing FMRP RGG ratios of 1:1, 1:2 and 1:4 (Figures 4.2 A and B green, orange and red spectra), were added into the sample the intensity of the positive band at 265 nm started to decrease. At high FMRP RGG ratios of 1:6 and 1:8 (Figure 4.2 A and B purple and blue spectra), the intensity of this band, as well as that of the negative band at 240 nm are significantly decreased, indicating the unwinding of the Shank1a RNA G-quadruplex structure by the RGG peptide. As expected, since the G-quadruplex structure is more stable in the presence of higher salt concentration the intensity of the band at 260 nm in the presence of 6 or 8 fold excess of FMRP RGG box, is higher in 150 mM KCl, versus 25 mM KCl.

To obtain quantitative information about the interactions between the FMRP RGG box and the Shank1a G-quadruplex we employed fluorescence spectroscopy. In these experiments we introduced a fluorophore into the Shank1a RNA sequence by replacing the adenine located at position 18 by its fluorescent analog 2-aminopurine (2AP) (Figure 4.3). The steady-state fluorescence of 2AP is very sensitive to its surrounding environment, especially to stacking interaction. At its excited state, the fluorescent properties of the 2AP are strongly dependent on the electron transfer quenching process from guanine (52). As part of the loop in the formed G-quadruplex, the inserted 2AP at position 18 is predicted to have direct contact with the binding protein. Its fluorescence change therefore reports the interaction between the Shank1a RNA and the FMRP RGG box. The changes of the steady-state fluorescence signal of the 2AP labeled Shank1a
RNA upon titrating the FMRP RGG peptides were monitored at its emission wavelength 371 nm. We observed a decrease in the fluorescence intensity after titrating the FMRP RGG box into the fluorescent Shank1a RNA, and the binding curve (Figure 4.4) was fitted with Equation 2.2 (Materials and Methods section) to obtain the dissociation constant of the Shank1 RNA: FMRP RGG box. The binding experiments were performed in triplicate, each binding curve being fitted independently, and the dissociation constant value of $68 \pm 9 \text{nM}$ was calculated as the average of the Kd values obtained from each experiment. It is interesting to note that even though it adopts a very stable G-quadruplex, the FMRP RGG box doesn’t bind tighter to the Shank1a RNA as compared with other RNAs investigated in Mihailescu laboratory. The difference is approximately two orders

**Figure 4.3** A fluorescently labeled Shank1a RNA was created by replacing the adenine at position 18 using its fluorescent analog 2-aminopurine (2AP).

**Figure 4.4** The FMRP RGG box was titrated into 200 nM of the fluorescently labeled Shank1a RNA in the presence of 1 μM HCV peptides. The steady-state fluorescence data was fitted into the predicted model (Equation 2.2). The reported Kd value represents the average of three Kd values with the highest error in the triplicates.
of magnitude: Kd of 68 nM for the Shank1a RNA versus 0.7 nM for one of the G-quadruplex forming RNAs human semaphorin RNA (19). One of the major causes could be the difference in the G-quadruplex surrounding loops, as Shank1a contains an extended loop of eight nucleotides, whereas in the case of the other RNAs, the loops contain between one to three nt.

Once we confirmed the FMRP RGG box binding to the Shank1a G-quadruplex structure, we investigated the interactions of this RNA with the full-length FMRP, as the FMRP RGG box might behave differently in the context of the full-length protein. It has been shown that FMRP regulates the transport and translation of multiple messenger RNA targets involved in neuronal development (53-55). The mechanism of how FMRP exerts its translation regulator function is still under investigation, however, previous findings have shown that unphosphorylated FMRP is associated with actively translating polyribosomes and phosphorylated FMRP associates with stalled ribosomes, suggesting that phosphorylation might play a role in the FMRP translation regulation function (16).

To mimic the phosphorylated form of full-length FMRP we have used the S500D mutant (15) in which serine 500 was mutated to aspartic acid (named FMRP ISOP in this study), as it has been shown that the phosphorylation at serine 500 is the main phosphorylation site of FMRP (15). In this study, we aim to investigate if the FMRP phosphorylation results in any potential binding affinity difference to the G-quadruplex forming Shank1a RNA.

We expressed the recombinant FMRP ISO1 (longest FMRP isoform) and ISOP in E. coli cells and purified them as described in the Materials and Methods section. The purity of FRMP ISO1 and ISOP was verified by performing SDS PAGE (Figure 4.5 A
and B). The selected eluate fractions were combined, concentrated and dialyzed extensively against a buffer of 5% glycerol and 1 mM EDTA with the gradual removal of imidazole. The final yield of FMRP ISO1 was 6.6 μM (sample prepared by the graduate student Snezana Stefanovic in Mihailescu laboratory), whereas for FMRP ISOP was 6.2 μM.

To quantitatively measure the binding affinity of the Shank1a RNA G-quadruplex with the FMRP isoforms, we recorded the steady-state fluorescence emission of the 2AP labeled Shank1a RNA at 371 nm while titrating the respective FMRP protein isoform into the RNA samples. The experiments were performed in triplicate and the binding curves were fitted with Equation 2.2 to determine the dissociation constant of the

Figure 4.5 The selected eluate fractions (lane 1 to lane 7) of the FMRP ISO1 (A) and ISOP (B) were run through the sodium dodecyl sulfate polyacrylamide gel with a protein ladder. It shows the purity of the expressed protein ISO1 and ISOP.
Shank1a RNA: FMRP ISO1 complex, $K_d = 198 \pm 59\, \text{nM}$, and of the Shank1a RNA: FMRP ISOP complex, $K_d = 281 \pm 64\, \text{nM}$, respectively (Figures 4.6). These values are higher than the $K_d = 68 \pm 9\, \text{nM}$ obtained for the Shank1a RNA: FMRP RGG box, likely due to a more complex structure of the full-length protein and therefore the reduced accessibility of the RGG box. The reduced binding affinity from the FMRP RGG box to the full-length FMRP isoforms was also observed for the other FMRP G-quadruplex forming RNA targets, such as Semaphorin 3F mRNA ($K_d = (0.7 \pm 0.3)\, \text{nM}$ for binding to the FMRP RGG box versus $(104 \pm 11)\, \text{nM}$ for the FMRP ISO1) (19, 41).

However, the $K_d$ values of the complexes formed by FMRP ISO1 and ISOP with Shank1a RNA are within error of each other, indicating that the posttranslational modification of phosphorylation does not modulate the translation regulator function of FMRP through different binding affinity to its G-quadruplex forming targets. The trend was also observed on a FMRP binding sequence (FBS), located in FMRP’s own mRNA,
binding to ISO1 and ISOP. Specifically, the FMRP ISO1 binding to FBS mRNA is not affected by the mutation of S500D (ISOP) that mimics the post-translational modification of phosphorylation (Kd = (120 ± 17) nM of FBS mRNA binding to ISO1 versus Kd = (130 ± 15) nM of ISOP) (56).

4.2 Characterization of the interactions between Shank1b RNA and FMRP

Using EMSA we analyzed the interactions between the Shank1b RNA G-quadruplex and the FMRP RGG box. To observe the effect of KCl on their interaction, the Shank1b RNA was incubated with the FMRP RGG box at different ratios in the presence of 10 mM and 25 mM KCl. The free RNA bands (Figure 4.7 lanes 1 and 5) correspond to the G-quadruplex formed in the Shank1b RNA. At 1:1 ratio Shank1b RNA to FMRP RGG box, the intensity of free RNA bands begins to decrease (Figure 4.7 lanes 2 and 6). We observed a Shank1b RNA-FMRP RGG complex at 1:2 ratio Shank1b RNA

![Figure 4.7 EMSA of 15 μM Shank1b RNA in the presence of 10 mM KCl (lane 1 to lane 4) and 25 mM KCl (lane 5 to lane 8) binding to the FMRP RGG box at different RNA: RGG ratios: 1:0 (lane 1 and lane 5), 1:1 (lane 2 and lane 6), 1:2 (lane 3 and lane 7) and 1:4 (lane 4 and lane 8). The arrow indicates the band representing the complex formed by the Shank1b RNA and the FMRP RGG box.](image-url)
to FMRP RGG (Figure 4.7 lanes 3 and 7) even though the corresponding bands are not sharp retarded bands due to the positive charged arginine residues in the FMRP RGG box. We therefore mainly monitor the binding event by the disappearance of the band corresponding to the free Shank1b RNA, which was further reduced at 1:2 ratio Shank1b RNA to FMRP RGG (Figure 4.7 lanes 3 and 7). At the highest FMRP RGG box concentration, 1:4 ratio Shank1b to FMRP RGG (Figure 4.7 lanes 4 and 8), the free RNA bands have further disappeared while the concomitant diffuse retarded bands corresponding to the Shank1b RNA-FMRP RGG complex show the highest intensity. The EMSA results indicate that the Shank1b RNA G-quadruplex interacts with the FMRP RGG box. Its binding affinity to the FMRP RGG box, however, is not as high as that of the Shank1a RNA G-quadruplex accordingly to these results *per se*, since the free Shank1a RNA band disappeared completely at a FMRP RGG box at 1:2 ratio RNA: RGG (compare Figure 4.1 lanes 3 and 6 with Figure 4.7 lanes 3 and 7).

**Figure 4.8** The CD spectra of 10 μM Shank1b RNA at various RNA: RGG ratios prepared in 10 mM cacodylic acid with 25 mM KCl. It shows that the FMRP RGG box induces the unwinding of the Shank1b RNA G-quadruplex at high RNA: RGG ratios.
Next, we performed CD spectroscopy to investigate the effect of the FMRP RGG box on inducing the unwinding of the Shank1b RNA G-quadruplex. At 0 mM KCl, the free Shank1b RNA showed the parallel G-quadruplex CD signature (Figure 4.8 grey spectrum). The addition of FMRP RGG box at ratios of 1:1, 1:2 and 1:4 RNA: RGG (Figure 4.8 green, orange and red spectra) started to reduce the intensity of the positive band at 265 nm. At high FMRP RGG ratios of 1:6 and 1:8 (Figure 4.8 purple and blue spectra), the intensity of negative band at 240 nm along with the positive one was significantly reduced, suggesting that the FMRP RGG box induces the unwinding of the Shank1b RNA G-quadruplex.

Using fluorescence spectroscopy, we aimed to obtain quantitative information about the interactions between Shank1b G-quadruplex and the FMRP RGG box. A fluorescently labeled Shank1b RNA was constructed by replacing one of the adenines at

![Figure 4.9](image)

**Figure 4.9** Two fluorescently labeled Shank1b RNAs were created by replacing the adenine at position 5 and position 24 respectively with its fluorescent analog 2-amino purine (2AP).
position 5 with its fluorescent analog 2-aminopurine (Figure 4.9). Being part of the longer loop surrounding the G-quadruplex, this inserted fluorophore was predicted to report the binding activity by directly contacting the FMRP RGG box as what observed in 2AP labeled Shank1a RNA binding with the RGG box. However, upon monitoring its emission wavelength at 371 nm after titrating the FMRP RGG peptides into this fluorescent Shank1b RNA, we didn’t observe any significant change of the steady-state fluorescence signal (data not shown). From our prior experience, selecting the position of the adenine to be replaced by 2AP labeled one has been a challenge when it comes to a sequence containing multiple adenines in its G-quadruplex structure, due to the difficulty to accurately predict which adenine is directly involved in the binding event. In the case of Shank1b RNA, if the longer loop in which the replaced adenine 5 is located is not the contact face with the RGG box, the introduced fluorophore will not report the binding activity accordingly. Based on the binding event observed from EMSA and CD spectroscopy, we are convinced that Shank1b RNA binds to the FMRP RGG. Accordingly, we next constructed another 2AP labeled Shank1b RNA by replacing the adenine 24 located in the short loop (Figure 4.9), which is predicted to be the alternative contact face to FMRP RGG box. The investigation of its binding affinity with FMRP is still in progress.

We started with our hypothesis that the interaction of Shank1 mRNA with FMRP occurs in a G-quadruplex dependent manner. Two G-rich sequences were identified and predicted to form G-quadruplex in Shank1 mRNA. The results have shown that both G-quadruplex forming sequences, Shank1a mRNA and Shank1b mRNA, form very stable parallel G-quadruplex structures intramolecularly, whereas Shank1a mRNA tends to
adopt a more stable structure at the same condition. In terms of interacting with FMRP, Shank1a mRNA G-quadruplex binds to the FMRP RGG box and the two FMRP isoforms, ISO1 and ISOP. Interestingly, we found that Shank1a mRNA has similar binding affinity to ISO1 and its phosphorylated mimic ISOP. It fails to support the assumption that phosphorylated FMRP represses the mRNA translation by weakening the binding affinity to its mRNA targets. The other selected G-quadruplex forming sequence Shank1b mRNA also shows the binding affinity to the FMRP RGG box, though it’s slightly weaker than Shank1a mRNA reflected by EMSA. Taken together, the results have shown that multiple G-quadruplex RNA motifs exist in the 3’ UTR of human Shank1 mRNA, which are required to facilitate its interaction with FMRP. This is consistent with the previous finding that FMRP represses Shank1 expression via its 3’ UTR (37). In summary, the interaction of FMRP with Shank1 mRNA 3’ UTR is dependent upon the recognition of G-quadruplex structure.
CHAPTER 5: CHARACTERIZATION OF THE BASP1 RNA G-QUADRUPLEX
STRUCTURE AND THEIR INTERACTION WITH FMRP

5.1 Characterization of the BASP1-5’ UTR RNA

BASP1 mRNA encodes for the brain abundant, membrane attached signal protein 1 (BASP1). It was identified as a potential FMRP mRNA target and predicted to form an intramolecular G-quadruplex recognized by FMRP RGG box (17). Two sequences having high potential to form a G-quadruplex structure in human BASP1 RNA were selected based on the prediction by the QGRS mapper, and named in this study BASP1-

![Figure 5.1](image)

**Figure 5.1** The $^1$H NMR spectra of the BASP1-5’UTR RNA shows the existence of the G-quadruplex structure reflected by the imino proton resonances from 10 – 12 ppm along with the duplex structure demonstrated by the Watson-Crick base pair resonances from 12 – 14 ppm.
5’ UTR and BASP1-Coding RNA, respectively. First, we performed the analysis of the BASP1-5’ UTR RNA, one of the selected G-quadruplex forming sequence in BASP1 RNA. To examine the existence of the G-quadruplex structure in the BASP1-5’ UTR RNA, we employed ¹H NMR spectroscopy, monitoring the imino proton resonance region. We observed imino proton resonances in the region of 10-12 ppm, indicating the G-quadruplex formation in this sequence, even in the absence of KCl (Figure 5.1, blue spectrum). Resonances are also present in the region 12 - 14 ppm, originating from guanine and uracil imino protons engaged in Watson-Crick base pairs, indicating the formation of a duplex structure in the BASP1-5’ UTR RNA. The intensity of the Waston-Crick imino proton resonances was reduced as the KCl concentration increased, but these resonances were present even after the sample was annealed in the presence of 150 mM KCl. Taken together, the ¹H NMR experiments demonstrate that a G-quadruplex and duplex structure coexist in the BASP-5’UTR even at high KCl concentrations.

Next, to obtain information about the fold of the G-quadruplex structure formed by the BASP-5’UTR we performed CD spectroscopy in the presence of various concentrations of KCl. A typical CD spectrum of a B-form DNA duplex structure has a positive band on an envelope from 260 – 280 nm and a negative one at 245 nm (57), whereas a positive band at 265 nm and a negative one at 240 nm is the typical signature of a parallel G-quadruplex (27, 28). At 0 mM KCl (Figure 5.2 orange spectrum), we observed a position band at 268 nm and a negative one on a broad envelop from 235 – 245 nm, indicating a hybrid between duplex and a parallel G-quadruplex structure. Once the KCl concentration was increased to 10 mM (Figure 5.2 green spectrum), the intensities of both the positive and negative bands increased significantly along with a
shift of, the positive one from 268 nm to 265 nm and the negative one to 240 nm. It suggests that KCl facilitates the stabilization of the G-quadruplex structure in the BASP1-5’UTR RNA as it co-exists with duplex. At higher concentrations 25, 50 and 100 mM (Figure 5.2 blue, red and purple spectra), the intensities of both bands further increased, but not as much as from 0 mM to 25 mM KCl. Taken together the ¹H NMR spectroscopy and CD spectroscopy, indicate that BASP1-5’UTR adopts a parallel G-quadruplex and a duplex structure, both of them co-existing at higher concentration of KCl while the G-quadruplex is further stabilized by the presence of K⁺ ions.

![Figure 5.2](image.png)

**Figure 5.2** The CD spectra of 10 μM BASP1-5’UTR RNA prepared in 10 mM cacodylic acid at various KCl concentrations shows a parallel G-quadruplex structures stabilized by KCl.

To examine the conformation change of the G-quadruplex formed in the BASP1-5’UTR RNA, we performed UV thermal denaturation experiments. 10 μM of BASP1-5’UTR RNA in 10 mM cacodylic acid, pH 6.5, was thermally denatured at different conditions, as the absorbance was monitored at 295 nm that has been shown to be most
sensitive to G-quadruplex dissociation (30). In the absence of KCl (Figure 5.3 red spectrum), we observed a major transition and a minor one separated at 35 °C. Once the KCl concentration was increased to 10 mM (Figure 5.3 green spectrum), a hypochromic transition corresponding to the G-quadruplex dissociation was observed from 40 – 75 °C, indicating that KCl enhances the stability of G-quadruplex in the BASP1-5’UTR RNA. At 25 mM KCl (Figure 5.3 blue spectrum), this hypochromic transition slightly shifted towards high temperature, suggesting that KCl further facilitates its stabilization. Accordingly, the minor transition from 0 – 40 °C in the presence of KCl was assigned to be the duplex dissociation due to its less stable structure. In the absence of KCl, the major transition from 35 – 50 °C corresponds to the G-quadruplex dissociation without effect of K⁺ ions. Clearly, its stability was reduced in the absence of KCl. Taken together the CD spectroscopy and the UV thermal denaturation, demonstrate that BASP1-5’ UTR forms G-quadruplex and duplex, the latter is further stabilized by the presence of K⁺ ions.

**Figure 5.3** The UV thermal denaturation profiles of 10 μM BASP1-5’UTR RNA prepared in 10 mM cacodylic acid at different conditions. It shows a hypochromic transitions 40 - 75 °C indicating the G-quadruplex dissociation along with the coexistence of duplex dissociation 0 – 10 °C.
5.2 Characterization of the BASP1-Coding RNA

After identifying the existence of G-quadruplex structure in the BASP1-5’UTR RNA, we analyzed the other G-quadruplex forming sequence in BASP1 RNA, BASP1-Coding RNA. We first performed $^1$H-NMR spectroscopy to examine the G-quadruplex existence in the BASP1-Coding RNA, monitoring the imino proton resonance region. Spectra were acquired for the BASP1-Coding RNA sample in the presence of different concentrations of KCl. In the absence of KCl (Figure 5.4 blue spectrum), we observed imino proton resonances from 10 – 12 ppm on a very broad envelope, indicating that the

![Figure 5.4](image)

**Figure 5.4** The $^1$H NMR spectra of the BASP1-Coding RNA shows the existence of the G-quadruplex structure reflected by the imino proton resonances from 10 – 12 ppm along with the duplex structure demonstrated by the Watson-Crick base pair resonances from 12 – 14 ppm. The G-quadruplex formation is stabilized by adding KCl and annealing the RNA.
BASP1-Coding RNA forms a G-quadruplex structure though it’s less stable than the BASP1-5’ UTR G-quadruplex at the same condition (Figure 5.1 blue spectrum). Very refined resonances located between 12 -14 ppm report upon guanine and uracil imino protons involved in Watson-Crick base-pairs, indicating that BASP1-Coding RNA forms a duplex structure as well. The intensity of the imino proton resonances located in 10 – 12 ppm was increased as the KCl concentration increased, while the Watson-Crick imino proton resonances were present throughout the various concentrations of KCl (Figure 5.4, red, green and purple spectra), suggesting that G-quadruplex and duplex co-exists in the BASP1-Coding RNA even at the high concentration of KCl, G-quadruplex is further stabilized by the presence of K+ ions.

5.3 Characterization of the interactions between the BASP1 RNAs and FMRP

After identifying the existence of G-quadruplex in the BASP1-5’ UTR and BASP1-Coding RNA, we characterized their interaction with the FMRP RGG box by employing electromobility gel shift assay and fluorescence spectroscopy. EMSA showed no binding affinity between these two G-quadruplex forming RNAs and the FMRP RGG box at various RNA: RGG ratios (data not shown). To confirm the EMSA results, we constructed the fluorescent variants of BASP1-5’UTR and BASP1-Coding RNA by replacing one of the adenines in each RNA by its fluorescent analog 2-aminopurine. We didn’t observe significant fluorescence intensity change by titrating the FMRP RGG peptides into the 2AP labeled RNAs (data not shown), confirming that BASP1-5’ UTR and BASP1-Coding RNA G-quadruplexes do not interact with the FMRP RGG box. Since the binding domain RGG box shows no binding affinity with these two G-quadruplex forming RNAs, we didn’t inquire their binding affinity with the full-length
FMRP isoforms. Taken together EMSA and fluorescence spectroscopy, indicate that even though these selected sequences in the BASP1 RNA adopts G-quadruplex structure, this secondary RNA structure *per se* is not sufficient to facilitate the interaction of the BASP1 RNA with the FMRP. The interaction of BASP1 mRNA with FMRP may be mainly determined by other structural elements.
CHAPTER 6: CONCLUSIONS

6.1 Characterization of G-quadruplex formation within G-rich sequences in Shank1 mRNA

We started with the hypothesis that the interaction of Shank1 mRNA with FMRP is dependent on a G-quadruplex structure. Therefore, we first identified two G-rich sequences within the Shank1 mRNA and named Shank1a and Shank1b RNA in this study. The results show the existence of G-quadruplex structure in Shank1a and Shank1b RNA even in the absence of KCl. There are alternate G-quadruplex conformations existing in both of Shank1a and Shank1b RNA, one of which is further stabilized by the presence of K+ ions. Both G-rich sequences forms a very stable parallel and intramolecular G-quadruplex whereas Shank1a RNA G-quadruplex tends to be more stable at the same condition.

6.2 Characterization of the G-rich sequences of Shank1 mRNA interacting with FMRP

To examine the interaction of Shank1 mRNA with FMRP, we have quantitatively determined the binding affinity of the G-quadruplex-forming sequence Shank1a RNA to the FMRP RGG box and to two full-length FMRP isoforms. The results show a high binding affinity of the Shank1a RNA to the FMRP RGG box and a slightly reduced binding affinity to the FMRP isoforms due to the structural complexity of the full-length protein and therefore the reduced accessibility to the RGG box. The other G-quadruplex forming sequence Shank1b mRNA also binds to the FMRP RGG box though its binding affinity is not as tight as Shank1a mRNA. These results have been consistent with the
previous findings that FMRP interacts with Shank1 mRNA via its 3’ UTR (37). Furthermore, it indicates that the interaction occurs at these G-quadruplex forming sites, of which recognition by FMRP is dependent on the G-quadruplex RNA motif.

Previous findings have shown that unphosphorylated FMRP is associated with actively translating polyribosomes while the phosphorylated form interacts with stalled ribosomes, suggesting a role of phosphorylation in modulating the translation regulation function of FMRP (16). Accordingly, we assumed that the phosphorylated form of FMRP represses the gene translation by negatively influence its binding affinity to mRNA targets. It has been shown that FMRP represses translation of Shank1 mRNA via its 3’ UTR (37). Furthermore, our results show that the G-quadruplex RNA motifs located in the Shank1 mRNA 3’ UTR are required to facilitate its interaction with FMRP. If FMRP phosphorylation regulates the translation of Shank1 mRNA by simply varying the binding affinity to its mRNA target, different binding activity is expected between the phosphorylated FMRP and the dephosphorylated form. Interestingly, the Shank1a RNA has shown no difference in terms of binding to the full-length FMRP isoform ISO1 and its phosphorylated mutant ISOP. It suggests that the posttranslational modification of phosphorylation does not modulate the translation regulator function of FMRP through different binding affinity to its G-quadruplex forming target Shank1a RNA, however, it may be important in other aspects of FMRP regulating the mRNA translation involving other pathways. Likely, one of the involvements is miRNA.

MicroRNAs (miRNAs) are short noncoding RNAs (~22 nucleotide) that control translation of specific target mRNAs by complementing with antisense sequences in the 3’ UTR of these mRNAs to inhibit their translation or promote their decay (58-61).
FMRP is biochemically and genetically linked to the miRNA pathway (10). Specifically, the miRNA interacts with FMRP, functioning as translational repressor. It has been shown that miR-125b and miR-132, as well as several other miRNAs, are associated with FMRP in mouse brain (10). It has been shown that one of the miRNA targets, NMDA receptor subunit NR2A, is negatively regulated through its 3’ UTR by FMRP collaborating with miR-125b in hippocampal neurons (10).

The significance of this study is to serve as the molecular groundwork to investigate the mechanism of how FMRP regulates the gene expression by controlling its mRNAs translation and how the absence of FMRP may disrupt this regulatory process. Particularly, by identifying the G-quadruplex structure in the 3’ UTR of Shank1 mRNA and its interaction with FMRP, we provided a FMRP recognition site in Shank1 mRNA that potentially interact with miRNA. Considering the role of miRNA pathways in FMRP functioning as a translation regulator, we reason that FMRP may alternatively regulate the translation of its target Shank1 mRNA via miRNA involvement. Taken together, it contributes to the investigation on the function of FMRP in fragile X syndrome, and furthermore, the molecular pathogenesis of fragile X syndrome and potential therapeutic targets.

6.3 Characterization of the G-quadruplex formation within G-rich sequences of the BASP1 mRNA

Two G-rich sequences were identified within BASP1 mRNA and named as BASP1-5’ UTR and BASP1-Coding RNA according to the regions they are located in. The results show a G-quadruplex structure exists in both sequences along with a duplex structure while the former is stabilized by the presence of K+ ions. Both of BASP1-5’
UTR and BASP1-Coding RNA intramolecularly form a parallel G-quadruplex structure, which have been hypothesized to interact with FMRP. The EMSA and fluorescence spectroscopy have shown, however, these two G-quadruplex forming sequences lack the binding affinity to the FMRP RGG box. Even though the previous findings have shown that BASP1 mRNA is an in vivo target of FMRP (17), our results fails to support the prediction that G-rich regions in BASP1 mRNA are the recognition sites by FMRP RGG box. It suggests that G-quadruplex RNA motif may be required, but not sufficient to facilitate the interaction between BASP1 mRNA and FMRP per se, which may be determined by other structural elements.

6.4 Future direction

As previously mentioned, it’s important to rationalize the role of posttranslational modification of phosphorylation in the translation regulator function of FMRP. Accordingly, we aim to investigate the binding event between the other G-quadruplex forming sequence Shank1b and two FMRP isoforms. If the observation shows no binding affinity difference of Shank1b mRNA to ISO1 and its phosphorylated form ISOP, it indicates a consensus between these two G-rich regions. This analysis will be helpful to illustrate if FMRP phosphorylation modulates its translation regulator function by altering its binding affinity to its G-quadruplex forming target Shank1 mRNA.

We have identified miRNAs associated with FMRP in mouse brain that are also complementing with G-quadruplex forming sequences in Shank1 mRNA. To analyze if miRNAs play a role in FMRP regulating Shank1 mRNA translation at these G-quadruplex forming site, it will be necessary to investigate the interaction among FMRP, Shank1 mRNA and miRNAs by employing biophysical and biochemical methods. This
provides potential information that are important to answer (i) if FMRP regulating Shank1 mRNA translation is modulated by miRNAs (ii) if FMRP phosphorylation functioning in repressing Shank1 expression is dependent on the involvement of miRNAs and (iii) how does the absence of FMRP disrupt this regulatory process in FXS.
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