Structural and Thermodynamic Study of the Fragile X Mental Retardation Protein Interactions with G Quartet Forming MAP1B RNA

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Structural and Thermodynamic Study of the Fragile X
Mental Retardation Protein Interactions with G Quartet Forming MAP1B RNA

A Thesis Presented to
the Bayer School of Natural and Environmental Sciences
of Duquesne University

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

By
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April 11, 2006
Abstract

Fragile X syndrome, the most common form of inherited mental retardation, is the result of an unstable expansion of the CGG trinucleotide repeat in the 5’ untranslated region of the fragile X mental retardation-1 (FMR1) gene. The abnormal hypermethylation of these CGG repeats causes the transcriptional silencing of the FMR1 gene, and consequently the loss of the fragile X mental retardation protein (FMRP). FMRP is an RNA binding protein that has been shown to use its RGG box domain to bind to G quartet forming RNA. The RNA encoding for the microtubule associated protein 1B (MAP1B), a protein that is involved in neuritic extension and neuronal development, has been identified as a potential FMRP mRNA target. In this study, a thermodynamic and structural analysis of MAP1B RNA and its interactions with the FMRP RGG box was performed. It was determined that MAP1B RNA forms an intramolecular parallel G quartet structure. In the presence of 150 mM KCl, MAP1B RNA appears to fold into two monomeric conformations; however, only one is bound by the FMRP RGG box. A careful study was performed to identify the conditions in which MAP1B RNA folds as a single species and it was determined by NMR spectroscopy and native gel electrophoresis that at low salt concentrations (10 mM KCl) only the MAP1B RNA conformation that is bound by the FMRP RGG box is dominant.

This study also investigated the interactions between MAP1B RNA and the FMRP RGG box. Using fluorescence spectroscopy, it was determined that the FMRP RGG box binds with high affinity to MAP1B RNA. Circular dichroism and UV spectroscopy were employed to determine if the binding of the FMRP RGG peptide changes the structure and the stability of the MAP1B RNA G quartet structure.
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LIST OF ABBREVIATIONS

2-AP: 2-aminopurine

D$_2$O: deuterium oxide

EDTA: ethylenediamine tetraacetic acid

EMSA: electrophoretic mobility shift assays

FMR1: fragile X mental retardation 1 gene

FMRP: fragile X mental retardation protein

ICP27: herpes simplex virus 1 protein

kb: kilobase

K$_d$: equilibrium dissociation constant

KH: K homology

MAP1B: microtubule associated protein 1B

MAP1B-19AP: MAP1B RNA with the 2-AP reporter located at position 19

NES: nuclear export signal

NLS: nuclear localization signal

nt: nucleotide

RNP: ribonucleoprotein

TBE: Tris, Boric acid, EDTA pH 8.3

UTR: untranslated region

ΔG°: standard Gibbs free energy change

ΔH°: standard state enthalpy change

ΔS°: standard state entropy change
I. INTRODUCTION
Fragile X syndrome is the most common form of inherited mental retardation, affecting 1 in 4000 males and 1 in 8000 females (Warren & Jin, 2003). Common symptoms associated with this syndrome are hyperactivity, abnormal facial features, autistic behavior and hyperextensible joints. Typically, boys with fragile X are more severely affected than girls, as most boys have mental retardation whereas only one third to one half of girls show intellectual impairment, the rest having normal IQ’s or showing slight learning disabilities (FRAXA, 2006). The emotional and behavioral problems are seen in both sexes. Men carrying the premutation pass it on to all of their daughters, but none of their sons, whereas a carrier woman has a 50% chance of passing on the premutation to their offspring (FRAXA, 2006). The Fragile X premutation can be passed silently through generations before a child is affected by the syndrome (FRAXA, 2006). Males have only one X chromosome, inherited from the mother, and one Y chromosome inherited from the father. Females inherit two X chromosomes, one from each parent. Fragile X is more predominant and severe in males because their only X chromosome contains the mutated gene. A female is usually not as severely affected because each cell of her body needs to use only one of it’s two X chromosomes and randomly inactivates the other (FRAXA, 2006).

Fragile X syndrome is caused by the loss of the fragile X mental retardation protein (FMRP), a protein required for synaptic development and essential for higher cognitive function (Lu et al. 2004). FMRP and its autosomal paralogs, FXR1P and FXR2P, constitute a small family of RNA-binding proteins that share >60% amino acid identity (Jin et al., 2004). FMRP is a predominantly cytoplasmic RNA binding protein that is expressed in various types of tissues and is very abundant in neurons. FMRP
interacts directly with target mRNAs (proposed targets include MAP1B and Semaphorin 3F RNA) and with polyribosomes (Mazroui et al. 2003). In fragile X syndrome patients FMRP is not produced due to the silencing of the fragile X mental retardation 1 gene (FMR-1). FMR-1 contains a 100 nucleotide fragment within its 3’-terminal part of the coding sequence, which retains the ability to bind FMRP specifically (Schaeffer et al., 2001). FMR-1 has 17 exons that can undergo alternative splicing, resulting potentially in 20 FMRP isoforms (Ashley et al., 1993). The most abundant isoform is a 78 kD species that contains an arginine-glycine-glycine (RGG box) and two K homology (KH) RNA binding domains (Figure I.1) (Chen et al. 2003). The RGG box binding domain is an arginine and glycine rich domain that contains a cluster of the tripeptide repeat Arg-Gly-Gly. The KH binding domain was first biochemically characterized in the major pre-mRNA-binding protein K (heterogeneous nuclear ribonucleoprotein K), is approximately 45 amino acids long and is an evolutionary conserved RNA binding domain (Grishin, 2001). FMRP has a functional nuclear localization signal (NLS) within its N terminus and a nuclear export signal (NES) at the C terminus (Eberhart et al., 1996). Near the promoter of FMR-1, in its 5’-untranslated region (5’UTR) there is an expansion of a CGG repeat in the fragile X patients which leads to the silencing of FMR-1, and thus the lack of FMRP. Among normal individuals this repeat is highly polymorphic in length and content, and contains AGG interruptions, normal repeats range from 7-54 base pairs with about thirty CGG repeats (Warren and Jin, 2003). A normal CGG repeat sequence would be as follows: 5’-(CGG)\textsubscript{9} AGG(CGG)\textsubscript{9} AGG(CGG)\textsubscript{9}-3’ (O’Donnell & Warren, 2002). The length of the CGG tracts determines the repeats stability; with a threshold for expansion of about 34-38 uninterrupted repeats (O’Donnell & Warren, 2002). Unstable
permutations arise from the gradual expansion of the 3’ end or by the loss of one or more AGG interruptions (O’Donnell & Warren, 2002). Individuals carrying the premutation have CGG repeats expanded from 60-200 repeats (Figure I.2) (O’Donnell & Warren, 2002). In most affected individuals the repeats are expanded over 200 (full mutation) and become abnormally hypermethylated, silencing FMR-1 (Warren and Jin, 2003). FMR-1 is 38 kb long and encodes a 4.4 kb transcript consisting of 17 exons (O’Donnell & Warren, 2002). FMR-1 RNA and protein are highly expressed in testes and in the fetal and adult brain, with the majority of signal localized to neurons (O’Donnell & Warren, 2002). Since FMRP has both an NLS and NES it is possible that FMRP shuttles between the cytoplasm and the nucleus, possibly assisting in the transport of RNA from the nucleus to the cytoplasm (Kaytor & Orr, 2001). FMRP associates with polyribosomes that actively translate proteins in the cytoplasm, an association that is RNA-dependent via mRNP particles (Kaytor & Orr, 2001). Nucleolin, a component of mRNPs is associated with FMRP-containing mRNPs, leading to the suggestion that FMRP may target mRNAs to translating ribosomes (Kaytor & Orr, 2001). The analysis of mRNA target sequences in vitro has shown a preference for guanine rich sequences that bind to FMRP in a potassium dependent manner (Darnell et al., 2001; Brown et al., 2001; Schaeffer et al., 2001). These guanine rich sequences have been proposed to fold into quadruplex structures which are formed by stacked G quartets (Figure I.3). G quartets are formed in vitro by both DNA and RNA oligonucleotides, and sequences that could form these structures are found in chromosomal telomeres, gene promoter regions, and recombination sites (Davis, 2004). G quartets differ in their orientation and are always stabilized by cations. They can form from four or two strands (intermolecular) or from a
**Figure I.1:** Fragile X mental retardation gene 1 FMR1 with the functional domains of the protein underlined (Schaeffer et al., 2001).

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**Normal**

(CGG)$_{6-60}$

ATG

- phenotype: normal
- transmission: stable
- methylation: no
- transcription: yes

**Premutation**

(CGG)$_{69-200}$

ATG

- phenotype: largely normal
- transmission: unstable, prone to expansion
- methylation: no
- transcription: yes

**Full mutation**

- phenotype: affected
- transmission: unstable
- methylation: yes
- transcription: no

**Figure I.2:** Schematic representation of the repeat expansion seen in premutation and full mutation patients (O’Donnell & Warren, 2002).
single chain (intramolecular) in a parallel or anti-parallel manner (Figure I.4). Quartets formed from stretches of guanines oriented parallel to each other are more stable than those formed in anti-parallel sequences (Hardin et al., 2001). Since a G quartet has four oxygen atoms clustered in its center, a cation is needed to keep the arrangement favorable (Davis, 2004). Large cations like K$^+$ and Na$^+$ stabilize the structure, while small cations such as Li$^+$ do not promote the formation of the structure (Williamson et al., 1989). It is hypothesized in Hardin et al., 2001 that potassium is favored over lithium or sodium because of the greater energetic cost of sodium and lithium dehydration. The quadruplex interior is electron rich, yet the carbonyl oxygen-cation core is embedded within a relatively hydrophobic medium, formed by the bases of the sandwiching quartets (Hardin et al., 2001). Divalent cations such as magnesium and strontium can also be used to stabilize G quartet structures. Divalent cations must be used in lower concentrations than monovalent cations because they have a much higher affinity for nucleic acid binding; lower divalent cation concentrations stabilize quadruplexes whereas higher concentrations destabilize the structures (Hardin et al., 2001). It is not fully understood why this occurs, but according to Hardin et al., 2001, this is due possibly to the specific binding of the divalent cations to the hydrogen-bond acceptor sites of guanines.

It has been proposed that MAP1B RNA, which has been shown to interact with FMRP directly \textit{in vivo}, contains G quartet structural elements in its 5’UTR (Lu et al., 2004). Figure I.5 shows the proposed G quartet structure of the MAP1B RNA determined in this study. FMRP regulation of MAP1B mRNA translation may be important in controlling neuronal network formation; however, no model regarding how FMRP may regulate MAP1B production during normal neuronal development has been
Figure I.3: Structure of a G quartet.

Figure I.4: Quadruplex structures (A) parallel quadruplex; (B), (C) anti-parallel quadruplex; (D) intermolecular anti-parallel quadruplex (Zhang et al., 2000).
Neuronal process extension is dependent on the reorganization of the cytoskeleton, in particular microtubules and microfilaments (Gonzalez-Billault et al., 2002). One of the ways in which microtubules are regulated is by a group of proteins called MAPs or microtubule associated proteins. During brain development MAP1B is the first MAP to be expressed, and it controls neurite extension and growth cone motility via modulating microtubule dynamics (Lu et al., 2004). Growth cones are a flattened area at the end of a growing axon that has filipodia, which act as guides for the outgrowth of embryonic nerve fibers. MAP1B RNA forms cross bridges between microtubules promoting microtubule assembly (Nothias et al., 1996). In vitro studies of MAP1B have indicated that it facilitates microtubule nucleation and elongation of microtubules (Pedrotti & Islam, 1996). Overall, MAP1B plays an important role in neurite growth.
Figure I.5: Proposed structure of MAP1B RNA. Highlighted adenine represents the placement of the 2AP reporter for fluorescence studies.
II. MATERIALS AND METHODS
II.1 Materials

Purification of T7 RNA Polymerase

T7 RNA polymerase is widely used for the *in vitro* synthesis of RNA oligonucleotides. The expression plasmid for the His-tagged T7 RNA polymerase was the pT7-911Q plasmid (Figure II.1), which was expressed in *E.coli* BL21 (DE3) competent cells (both generously given to us by Dr. John Marino, CARB). First, the *E. coli* cells were plated and grown overnight at 37°C on LB plates with 100 µg/mL ampicillin. A single colony from this plate was used to inoculate 5 mL of LB with 100 µg/mL ampicillin, which was grown overnight to saturation at 37°C with shaking at 250 rpm. Next, 4 x 0.5 mL of the overnight culture was used to inoculate 4 x 500 mL of LB medium containing 100 µg/mL ampicillin. These four flasks were grown at 37°C while shaking at 250 rpm until they reached an OD_{600} of 0.4-0.6. The cells were then induced with 100 µM IPTG (isopropyl-β-D-thiogalactopyranoside) and incubated for an additional 4 hours at 37°C. The cells were then pelleted by centrifugation and placed into a freezer set at -20°C for short term storage. Next, the cells were thawed at room temperature and quickly frozen on dry ice, this freeze-thaw cycle being repeated four times. Shocking the cells by freezing and thawing helped weaken the cell membranes before cell lysis. The cells were then resuspended in lysis buffer (50 mM Tris pH 8.0, 0.1 M NaCl, 5.0 mM β-mercaptoethanol, 5% glycerol), lysozyme was added at a final concentration of 2 mg/mL and the suspension was placed into a refrigerator (~ 4°C) and let sit overnight. Lysozyme was used to digest the cellular membranes so the proteins could be extracted. DNase I was added to the cell lysate to cleave the DNA and reduce the viscosity of the solution. The T7 polymerase was purified by affinity
chromatography on Qiagen Ni-NTA agarose resin. The Ni-NTA agarose resin was first equilibrated with the lysis buffer in preparation for protein purification. Each step involved lightly mixing the lysis buffer with the resin in a 50 mL falcon tube, gently rocking for 5 minutes, centrifugation for 10 minutes at 3,500 rpm and 4°C, and decanting off the supernatant. Cell debris and unlysed cells were removed from the cell lysate by centrifugation at 18,000xg for 30 minutes, at 4°C in a 50 mL falcon tube. Next, the cleared lysate was bound to 5 mL of the Ni-NTA-agarose resin by gentle rocking for 30 minutes at 4°C. The T7 RNA polymerase bound to the Ni-NTA-agarose was washed four times with lysis buffer containing 1.0 mM imidazole and four times with buffer containing 10 mM imidazole. T7 RNA polymerase was eluted by carrying out two rounds of washing the resin with lysis buffer containing 100 mM imidazole. When the imidazole concentration is increased the 6xHis-tagged T7 RNA polymerase dissociates from the Ni-NTA resin because it can no longer compete for binding sites on the resin. Glycerol was added to the enzyme at a final concentration of 25% and aliquots were stored at -20 °C. The final yield of the T7 RNA polymerase product was 36.5 mg/L of initial culture.

**Expression and Purification of MAP1B RNA**

A 34 nt fragment located in the 5’-UTR of MAP1B RNA, that has been postulated to be bound by the FMRP RGG box, has been expressed and purified. The sequence of this fragment is:

5' - GGC GCU GGG AGA GGG CGG AGG GGG AGG CGG CGC C – 3’

MAP1B RNA was produced by a T7 RNA polymerase *in vitro* transcription reaction off a synthetic DNA template. The MAP1B DNA template (1 µM) was first annealed with
Figure II.1: T7 RNA Polymerase expression plasmid pT7-911Q.
the CTOP primer (1.1 µM) using a slight excess of primer. The sample was boiled for three minutes then allowed to cool very slowly to allow the formation of the primer-template junction. The final concentrations of the individual NTPs was calculated according to the occurrence of these nucleotides in the sequence of MAP1B RNA under study and is as follows: GTP-8 mM, UTP-2 mM, CTP-4 mM and ATP-2 mM. After the primer-template was cooled, it was added to the 10 mL transcription reaction containing the above NTP concentrations, Tris at pH 8.3 (40 mM), DTT (5 mM), spermidine (1 mM), Triton X 100 (10 mM), MgCl₂ (25 mM) and sterile diH₂O. The reaction was then placed in a 37°C water bath for five minutes, after which the T7 RNA polymerase was added at a final concentration of 0.15 mg/mL. The reaction was placed back into the water bath for approximately eight hours to complete the *in vitro* transcription reaction. After the reaction was complete, EDTA was added at a concentration double to the MgCl₂ concentration to inhibit the reaction. Next, the 50 mL falcon tube containing the reaction was thoroughly vortexed, making the white precipitate (Mg-pyrophosphate) go back into solution. Last, 3 ½ volumes of 200 proof ethanol and sodium acetate (50 µL/mL reaction) were added to the reaction to precipitate the nucleic acids. The reaction was incubated in a -20°C freezer overnight. The next day the nucleic acids were pelleted by centrifugation at 3500 rpm for thirty minutes. The pellet was then lyophilized to remove excess ethanol, dissolved in 1 mL of EDTA (pH 8.0) and resuspended in 10 mL of sterile diH₂O. The resuspended RNA was then placed in an Amicon Ultra centrifuge tube (MW ~ 5000) and concentrated to a volume of approximately 400 µL. The RNA sample was then transferred to a microfuge tube and placed in the refrigerator. Next, a large denaturing gel (20% acrylamide containing 8 M Urea) was run to purify the RNA.
from the DNA template and other byproduct transcripts. The gel was pre-run overnight at 250 V in 1xTBE (0.09 M Tris pH 8.3, 0.1 M Boric acid, and 2 mM EDTA) running buffer. The RNA sample was prepared for electrophoresis by adding 400 µL of loading buffer (20% glycerol, 0.1% bromophenol blue, 2% EDTA & 78% formamide). Prior to its loading on the gel, the sample was annealed by boiling for five minutes and cooling for twenty minutes. The gel was run at 1200 V until the bromophenol dye reached the bottom (~7 hours). The nucleic acid bands were visualized by UV shadowing (254 nm) and the desired band was cut out and the gel slices were stored at -20°C. The purified RNA was eluted using a Schleicher-Schuell Elutrap run in sterile ½ X TBE at 250 V. Fractions were collected every hour and the OD_{260} was measured for each fraction to monitor the elution. Once all of the RNA was eluted out of the gel slices the fractions were combined for concentration and dialysis. After concentration, the sample was dialyzed first with sterile diH_{2}O and then with the desired RNA buffer (10 mM cacodylic acid + 50 mM KCl/LiCl pH 6.0, 10 mM Tris + 10 mM KCl or 10 mM Tris). The final MAP1B RNA concentration was determined using the following equation: \[(\text{absorbance at 260 nm)} \times (\text{dilution factor})] / 329.8 \text{ (extinction coefficient). The extinction coefficient was determined using the Ambion online oligonucleotides MW and extinction coefficient calculator, where the value is determined by entering the 5’ – 3’ sequence of MAP1B RNA.}

**UV-Vis Spectroscopy**

A thermal denaturation experiment determines the stability of the secondary structure of RNA. T_{m} (melting temperature) is defined as the melting temperature or more accurately, the temperature of midtransition. Heating an RNA sample leads to a
change in absorbance properties; reflecting a conformational change of the RNA in solution (Mergny & Lacroix, 2003). All thermal denaturation experiments were performed on a Varian Cary 3E UV-Visible Spectrophotometer equipped with a Peltier cell for temperature control. All samples were annealed by boiling for 5 minutes followed by bench cooling. The thermal denaturation experiments were conducted at 295 nm, a wavelength specific for the dissociation of G quartet structures (Mergny et al., 1998). The UV-Vis absorbance at 295 nm was recorded every 1°C and samples were heated at 0.2°C/min from an initial temperature of 20°C to a final temperature of 95°C. To determine the melting temperature two straight baselines were needed (upper and lower) which correspond to the unfolded and folded forms, respectively (Mergny & Lacroix, 2003). After the baselines were determined a median line was drawn between the middle of the two baselines between the baseline Y-intercept (Mergny & Lacroix, 2003). The cross point between the experimental curve and the median line corresponds to the $T_m$ (Mergny & Lacroix, 2003). The melting temperature of the MAP1B G quartet structure was determined by determining the baselines of the hypochromic transition corresponding to G quartet dissociation (50°- 70°C ) and taking the midpoint. One problem that may occur with the thermal denaturation experiments is that the rapid increase in temperature may lead to experimental curves that do not correspond to the equilibrium curves, leading to an inaccurate $T_m$. To make sure that the $T_m$ is determined at equilibrium, a reversible cycle (heating and cooling) was performed. Recordings were taken every 1°C at a rate of 0.2 °C/min from 95 - 20°C (Data not shown). Theoretically, these two curves should align indicating that the sample is at equilibrium.
Thermodynamic Study of MAP1B RNA Structure

The dependence of the T\textsubscript{m} of a melting transition on the RNA concentration gives information on the molecularity of the transition (intramolecular or intermolecular). If the RNA is intramolecular, then the T\textsubscript{m} will not depend on the RNA concentration. However, if the structure is intermolecular (with n number of strands), 1/T\textsubscript{m} depends linearly on the natural logarithm of the total RNA concentration (C\textsubscript{T}) (Hardin et al., 2001):

\[
\frac{1}{T_m} = \frac{R(n-1)}{\Delta H^0_{vH}} \ln C_T + \frac{\Delta S^0_{vH} - (n-1)R \ln 2 + R \ln n}{\Delta H^0_{vH}}
\]

(1)

where R is the gas constant and \(\Delta H^0_{vH}\) and \(\Delta S^0_{vH}\) are the Van’t Hoff thermodynamic parameters. Experiments were conducted for MAP1B RNA concentrations in the range of 1 µM – 100 µM. As the RNA concentration increased it was necessary to increase the wavelength (295 nm - 310 nm) in order to obtain accurate absorbance readings. Once it was determined that the MAP1B structure is intramolecular, the thermodynamic parameters \(\Delta H^0\), \(\Delta S^0\) and \(\Delta G^0\) for G quartet formation were determined by fitting the G quartet dissociation transition to equation 2 using the Origin 7.5 software.

\[
A(T) = A_U + A_F \frac{e^{-\Delta H^*/RT} e^{\Delta S^*}}{e^{-\Delta H^*/RT} e^{\Delta S^*} + 1}
\]

(2)

where \(A_U\) and \(A_F\) represent the absorbance of the unfolded and the native G quartet RNA, respectively.

FMRP RGG box effect upon the stability of the MAP1B RNA G quartet structure

To determine if the stability of the G quartet structural elements in MAP1B RNA is affected by the binding of the FMRP RGG box, thermal denaturation experiments were
conducted for the RGG peptide-MAP1B RNA complex at an RNA: RGG peptide ratio of 1:2. The RNA sample was annealed and bench cooled then the FMRP RGG box was added and allowed to equilibrate for 30 minutes.

**Fluorescence Spectroscopy**

Fluorescence spectroscopy was used to obtain structural information about the G quartet forming MAP1B RNA and its interactions with the FMRP RGG box. The MAP1B RNA used for the fluorescence spectroscopy studies was labeled with the highly fluorescent purine analog, 2-aminopurine (2-AP) which has been extensively used in structural studies of nucleic acids (Mclaughlin et al, 1998, Law et al. 1996). The RNA was labeled at position 19 which is predicted to be part of the G quartet surrounding loops upon the folding of the RNA molecule (Figure I.5), and this molecule will be called MAP1B-19AP. 2-AP labeled MAP1B RNA was chemically synthesized and purified by Dharmacon Inc. Steady-state fluorescence measurements of the 2-AP labeled MAP1B RNA were conducted in the presence of KCl and LiCl respectively, the latter of which does not promote the formation of G quartets. All fluorescence experiments were performed on a Fluoromax-3 fluorimeter, equipped with temperature control. The excitation wavelength was 310 nm (specific for 2-AP) and the emission spectrum was acquired from 330 - 450 nm. A 10 µM MAP1B RNA stock was annealed by boiling for 5 minutes followed by slow bench cooling for 20 minutes. A 400 nM sample in KCl or LiCl RNA buffer was taken from the stock and placed into a cuvette in a final volume of 150 µL. All fluorescence experiments were conducted at 25°C.

The 2-AP reporter was located at position 19 to assess if the 2-AP reports on the FMRP RGG box binding event. In this case, the binding of the RGG peptide to MAP1B
RNA was tested by monitoring the 2-AP fluorescence changes upon the titration with the RGG peptide in the presence of potassium ions. Similar experiments were performed in the presence of lithium as well, to confirm that binding is G quartet dependent. The FMRP RGG box peptide was titrated into a 400 nM MAP1B-19AP RNA sample in 10 mM cacodylic acid/10 mM KCl at 60 nM peptide increments. After each peptide addition the sample was let to equilibrate for 10 minutes before a scan was recorded. The binding dissociation constant, $K_d$, was determined by fitting the binding curves to equation 3:

$$F = 1 + \left(\frac{I_B}{I_F} - 1\right) \times \frac{(K_d + [P]_t + [RNA]_t) - \sqrt{(K_d + [P]_t + [RNA]_t)^2 - 4 \times [RNA]_t \times [P]_t}}{2 \times [RNA]_t}$$

(3)

Where $I_F$ and $I_B$ represent the steady-state fluorescence intensities of the free and bound MAP1B-19AP, $[RNA]_t$ is the total concentration of MAP1B-19AP, and $[P]_t$ is the total RGG box peptide concentration.

Electrophoretic Mobility Shift Assay

Native gel electrophoresis was employed to analyze if MAP1B RNA folds as a monomeric or dimeric species and to determine if its conformation is dependent upon the $K^+$ ions concentration. Electrophoretic mobility gel shift assays (EMSA) (15% acrylamide, $\frac{1}{2}$ x TBE) were performed to assess the binding of FMRP to MAP1B RNA. All native gels were visualized by UV shadowing at 254 nm and by ethidium bromide staining, using an AlphalImager HP (AlphaInnotech, Inc.). All RNA samples were annealed by boiling for 5 minutes in KCl or LiCl containing RNA buffers and then bench cooled for 20 minutes. Both the running buffer and the gel itself contained 50 mM KCl or LiCl. Samples were run at 60 volts for approximately 3 hours in 1/2x TBE (50 mM KCl/LiCl, 10 mM Tris and Boric acid).
The EMSA experiments were also employed to determine if the FMRP RGG box binds to MAP1B RNA. These experiments complemented the fluorescence spectroscopy measurements of the FMRP RGG box binding to MAP1B RNA. If no binding is detected in the fluorescence studies it could be due to the fact that the 2-AP reporter is not sensitive to the binding event or that the 2-AP insertion altered the structure of the RNA, preventing the FMRP binding. The EMSA results will confirm or rule out one of these two scenarios. The gel shift assays were performed in the presence of either 50 mM or 10 mM KCl or LiCl (gel and running buffer). Samples were annealed then bench cooled for 20 minutes. Next, the FMRP RGG peptide was added in a 1:2 ratio and the samples were equilibrated for 20 minutes. Last, 0.75 µL of loading buffer (no bromophenol blue dye) was added. The final volume of the samples, minus the loading buffer was 15 µL. The gels were run at 60 volts for approximately three hours and were visualized by shadowing (254 nm) and EtBr staining.

Circular Dichroism Spectroscopy

Circular dichroism (CD) spectroscopy was used to obtain additional information about the fold of the G quartet structure of MAP1B RNA. G quartets can form parallel or anti-parallel quadruplexes (Figure I.4). Parallel G quartets exhibit a positive peak at 265 nm and a negative peak at approximately 240 nm in their CD spectrum, whereas anti-parallel G quartets exhibit a positive peak near 295 nm and a negative peak around 260 nm (Hazel et al., 2004) A mixture of parallel and anti-parallel folding may produce peaks at all four wavelengths. All CD experiments were conducted either on a Jasco 710 spectropolarimeter (Dr. Bruce Armitage, CMU) or a Jasco 810 spectropolarimeter (Duquesne University). The samples prepared for the experiments conducted at CMU
contained 10 μM MAP1B RNA in 10 mM cacodylic acid, pH 6.0, and 50 mM KCl buffer in 800 μL final volume. Following annealing and bench cooling, samples were transferred to a 1 cm path length cuvette and equilibrated in the spectropolarimeter at 25°C. Experiments conducted at Duquesne University on the Jasco 810 were 10 μM and 20 μM respectively in RNA concentration, and the samples were in 10 mM KCl and 10 mM Tris buffer in a final volume of 150 μL. These samples were not annealed and were transferred to a 0.1 cm path length cuvette and equilibrated in the spectropolarimeter at 25°C. The scans were performed in the range 380 - 200 nm, with each trace spectra representing an average of a series of six scans with a 1 s response time and a 1 nm bandwidth. A blank spectrum recorded for the buffer was subtracted from the collected data.

The same parameters were used to record the CD spectrum of the FMRP RGG box-MAP1B complex, which was prepared by mixing 10 or 20 μM MAP1B RNA in a 1:2 or a 1:4 ratio with FMRP RGG box, followed by a 20 minute equilibration time. The CD data for the FMRP RGG box-MAP1B complex was corrected by subtraction of the CD spectrum recorded for the free FMRP RGG box.

**NMR Spectroscopy**

All one dimensional $^1$H NMR spectras of MAP1B RNA were recorded on a 500 MHz Varian Unity Plus spectrometer at 29°C. The jump-and–return pulse sequence was used for water suppression (Plateau & Gueron, 1982). The MAP1B RNA concentration for all samples was between 300 - 600 μM at a 90% H$_2$O/10% D$_2$O ratio. The maximum excitation was set at 11 ppm, the recycling delay was 2 s and 256 scans were acquired. Two samples were prepared for the NMR structural studies. The first sample had a
MAP1B concentration of 600 µM in 10 mM cacodylic acid at pH 6.5 and 50 mM KCl. The sample was annealed and bench cooled for 20 minutes. The second sample was not annealed and had a MAP1B RNA concentration of 400 µM in 10 mM Tris and no salt. KCl was titrated into the second sample in 5 mM increments, followed by a 5 minute equilibration time, after which a spectrum at 29°C was acquired.

The sample used to study the binding interactions between MAP1B and FMRP by NMR spectroscopy was 300 µM MAP1B RNA in 10 mM KCl and 10 mM Tris, pH 8.3. RGG box peptide was titrated into the sample at 35 µM increments from a 10 mM stock of FMRP RGG box in 10 mM KCl and 10 mM Tris buffer, pH 8.3. Following each peptide addition, the sample was equilibrated for 10 minutes before a spectrum was acquired.

_Isothermal Titration Calorimetry (ITC)_

All ITC experiments were performed on a Microcal Micro Calorimetry System (MCS). All MAP1B RNA samples were 20 µM RNA in 50 mM KCl and 10 mM cacodylic acid, pH 6.0 in a total volume of 2.5 mL. FMRP RGG box was titrated into the RNA at 2.15 µM per injection (7 µL/injection) from a 0.5 mM or 1.0 mM FMRP RGG stock solution in 50 mM KCl and 10 mM cacodylic acid buffer.
III. RESULTS
III.1 Structural Studies

*MAP1B RNA contains G quartet structural elements*

Fluorescence spectroscopy experiments were carried out for MAP1B-19AP RNA, which was constructed by replacing the adenine at position 19 in MAP1B by the highly fluorescent purine analog 2-AP (Figure I.5). Lithium does not promote the formation of G quartet structures, whereas potassium is involved in the formation of such structures. Thus, if the hypothesis that the MAP1B RNA sequence has the ability to fold into a G quartet structure is correct, the conformation of this RNA should be very different in the presence of potassium versus lithium ions. Therefore, the intensity of the steady-state fluorescence of the 2-AP reporter should be different when MAP1B-19AP is folded in the lithium versus potassium containing buffers. Our results show that indeed the intensity of the 2-AP steady-state fluorescence is lower in the LiCl sample than in the potassium sample (Figure III.1), indicating that there is a change in the MAP1B RNA conformation when it is folded in the presence of potassium versus lithium ions.

Native gel electrophoresis was also used to assess the conformation of MAP1B RNA in lithium versus potassium containing buffers. In the presence of KCl, MAP1B RNA exists in equilibrium between two conformers, whereas in LiCl only a single conformation is present (Figure III.2 A and B, lanes 1). These gels also contained Munc 13 RNA (lanes 2) and sc1 RNA (lanes 3), as controls. Sc1 RNA has been previously characterized in the Mihailescu lab, and it has been shown that in the presence of KCl (Figure III.2 B, lane 3) this molecule exists in equilibrium between a monomeric G quartet (lower band) and a dimeric species (higher band) (Zanotti et al., 2006). Thus, the position of the bands corresponding to the two MAP1B conformations with respect to the
Figure III.1: Steady-state fluorescence change of MAP1B RNA folded in presence of 150 mM KCl (higher intensity curve) and respectively 150 mM LiCl (lower intensity curve).

Figure III.2: Native gel run in the presence of 50 mM LiCl (A) or KCl (B): 80 μM MAP1B short (Lane 1); 40 μM Munc13 (Lane 2); 40 μM Sc-1 WT (Lane 3); in LiCl buffer (A) and KCl buffer (B) respectively. The gels were visualized by UV shadowing at 254 nm.
Sc1 RNA bands, suggest that both these conformations are monomeric.

To obtain more information about the molecularity of these two MAP1B conformers native gel electrophoresis was employed. The MAP1B RNA concentration was varied in each sample, and the KCl concentration in the samples, gel, and running buffer was 50 mM (Figure III.3). In this gel both the lower and upper bands increased with the increase in MAP1B concentration, indicating that both conformations are monomers. In the case of an equilibrium between a monomeric and dimeric structure, the upper band (dimer) should increase in intensity whereas the lower band (monomeric) should decrease in intensity, as the RNA concentration is increased.

To obtain higher resolution information about the structures formed by MAP1B in the presence of KCl, we have employed NMR spectroscopy. The one dimensional (1D) $^1$H NMR spectrum of MAP1B was analyzed, focusing on the imino proton resonance region (10 - 14.5 ppm), which is very informative about the presence of G quartet structures. Typically, the imino protons of Us and Gs involved in Watson-Crick base pairs resonate in the region 12 - 14.5 ppm, whereas imino protons of Gs involved in the Hoogsteen base pairs of the G quartets resonate in the region 10 - 12 ppm. A sample containing 400 $\mu$M MAP1B RNA was prepared in 10 mM Tris buffer and KCl was titrated into it at 5 mM KCl increments. The initial sample that did not contain K$^+$ did not show any peaks in the 10 - 12 ppm range, indicating that G quartets were not present (Figure III.4, pink spectrum). The spectrum did contain resonances up field of 12 ppm, indicating that Watson-Crick base pairs were present, likely from the stem structure of MAP1B. As KCl was titrated into the sample, spectra were recorded for every 5 mM addition of salt at 29°C. It is clear that upon the addition of the first 5 mM KCl, the
Figure III.3: Native gel with varying MAP1B RNA concentrations:

<table>
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<tr>
<th>Lane #</th>
<th>MAP1B RNA [µM]</th>
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<tr>
<td>1</td>
<td>1.5</td>
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<tr>
<td>2</td>
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<td>3</td>
<td>10</td>
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<td>7</td>
<td>75</td>
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<td>8</td>
<td>100</td>
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</table>
structure of MAP1B RNA changes, as resonances appear now in the 10 - 12 ppm region. These sharp peaks are signatures of a G quartet structure and are maintained in the presence of 10 mM KCl (red spectrum). However, as the KCl concentration was increased (15 - 25 mM), the intensity of these peaks decreased and new resonances started to appear around 10.5 ppm, indicating that MAP1B RNA starts to fold into a second conformation. This led to the conclusion that as long as the KCl concentration is maintained below 10 mM, a single conformation is present in MAP1B. Therefore, all experiments carried out after these results were obtained, were performed in 10 mM KCl.

Native gel electrophoresis was used to follow up the NMR data, in order to determine if the relative concentrations of the two MAP1B RNA conformers observed initially on a native gel in the presence of 50 mM KCl, (figure III.2 B, lane 1) is affected by a change in the KCl concentration. The gel and running buffer contained 50 mM KCl, whereas the samples varied in their KCl concentration from 10 mM to 150 mM. The gel was run at 60 V for three hours at room temperature. The gel confirmed the NMR results, as at low KCl concentrations only a single band is present (figure III.5, lane 1), whereas, as the KCl concentration is increased a new lower band appears on the gel, indicating the appearance of the second conformer (Figure III.5).

We have employed UV spectroscopy to determine the thermal denaturation profile of MAP1B in the presence and absence of KCl. The UV spectroscopy melting curves of 20 µM MAP1B RNA folded in 10 mM Tris at pH 8.3, containing either 10 mM KCl or no salt are quite different (Figure III.6 A & B). Three transitions are present in the UV melting curve of MAP1B RNA folded in the presence of 10 mM KCl (Figure III.6 B). The 50°- 70°C hypochromic transition was assigned to the melting of the G
Figure III.4: NMR spectra of MAP1B RNA at various salt concentrations. (Pink) 0 mM KCl. (Blue) 5 mM KCl. (Red) 10 mM KCl. (Green) 15 mM KCl. (Violet) 20 mM KCl. (Orange) 25 mM KCl.

Figure III.5: Native gel of MAP1B in the presence of increasing concentrations of KCl in samples. Lane 1: 10 mM KCl, lane 2: 25 mM KCl, lane 3: 50 mM KCl, lane 4: 75 mM KCl, lane 5:100 mM KCl and lane 6: 150 mM KCl respectively. Runner buffer and gel contain 50 mM KCl. All samples contain 20 μM MAP1B RNA.
quartet structure. As expected, this transition is absent from the UV melting curve of MAP1B in the absence of K⁺ ions, since no G quartet structure should be present in these conditions (Figure III.6 A). The 75°- 95°C hyperchromic transition was assigned to the melting of the MAP1B stem structure. This transition is shifted at lower temperatures in the UV melting curve of MAP1B recorded in the absence of KCl (Figure III.6 A), since in the absence of a G quartet structure the stem will melt at a lower temperature.

The conclusion of this set of experiments is that in 10 mM KCl MAP1B RNA folds into a single conformation that contains a G quartet structure, and as the KCl concentration is increased, the other conformation appears in MAP1B RNA.

MAP1B RNA forms an intramolecular G quartet structure

To confirm that indeed the single conformation formed by MAP1B RNA in the presence of 10 mM KCl is monomeric, we have measured the melting temperature of its G quartets at different RNA concentrations (10 µM, 15 µM, 20 µM, 50 µM and 80 µM). The melting temperature (Tₘ) was determined by taking the midpoint of the 50°- 70°C hypochromic transition that was assigned to the melting of the G quartet structure. Next, Tₘ was plotted as a function of the RNA concentration (Figure III.7 C) and it was concluded that since the Tₘ is independent of the RNA concentration (Equation 1) the G quartet structure is intramolecular. Once it was established that the structure formed by MAP1B RNA in 10 mM KCl was intramolecular, the hypochromic transition corresponding to the G quartet dissociation was fitted with equation 2, to determine the thermodynamic parameters of G quartet formation. Figure III.7 A and III.7 B, depict the thermal denaturation profile of 50 µM MAP1B RNA in the presence of 10 mM KCl and the fitted transition respectively. The red highlighted region in figure III.7 A represents
Figure III.6: UV melting curves of 20 µM MAP1B RNA in 10 mM Tris buffer, pH 8.3 containing no KCl (A) or 10 mM KCl (B).
the 50°-70°C hypochromic transition that was fitted with equation 2. The thermodynamic parameters extracted from the fitted curves are listed in Table III.1. The enthalpy of G quartet formation determined at all RNA concentrations (-98.37 to -115.70 kcal/mol) suggests the presence of 5 G quartet planes, as the enthalpy of formation of a single G quartet plane for most oligonucleotides (measured in experimental conditions similar to those used in this study) is approximately -20.9 kcal/mol (Jin et al. 1992).

**MAP1B RNA folds parallel G quartet structures**

CD spectroscopy was employed to obtain additional information about the fold of the G quartet structure of MAP1B RNA. The CD spectrum of 20 µM MAP1B in 10 mM Tris pH 8.3 and 10 mM KCl shows a positive peak at 264 nm and a negative peak at 223 nm, both signatures of parallel G quartet folds (Figure III.8). Anti-parallel G quartet folds exhibit a positive signal at around 295 nm and a negative signal at around 260 nm. There is a very small shoulder present around 290 nm in the CD spectrum, which suggests that MAP1B may also form anti-parallel G quartets. Overall, the anti-parallel peak is very miniscule and the predominant peaks suggest that MAP1B mainly forms a parallel type G quartet structure.
Figure III.7: (A) UV thermal denaturation profile of 50 µM MAP1B RNA in 10 mM Tris plus 10 mM KCl. Highlighted region corresponds to the data used in the fitting. (B) Fitted data of 50 µM MAP1B RNA using equation II.1. (C) Van’t Hoff plots of absorbance thermal denaturation results for MAP1B RNA. The $T_m$ was plotted as a function of the RNA concentration.
<table>
<thead>
<tr>
<th></th>
<th>( T_m ) (°C)</th>
<th>( \Delta H^\circ ) (kcal/mol)</th>
<th>( \Delta S^\circ ) (cal/mol*K)</th>
<th>( \Delta G^\circ ) (kcal/mol)</th>
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<tr>
<td>20 ( \mu M ) MAP1B</td>
<td>63.0 ± 0.1</td>
<td>-98.4 ± 0.1</td>
<td>-292.8 ± 0.2</td>
<td>-11.1 ± 0.1</td>
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<tr>
<td>50 ( \mu M ) MAP1B</td>
<td>62.3 ± 0.1</td>
<td>-115.7 ± 0.1</td>
<td>-345.1 ± 0.3</td>
<td>-12.9 ± 0.1</td>
</tr>
<tr>
<td>80 ( \mu M ) MAP1B</td>
<td>61.5 ± 0.1</td>
<td>-109.4 ± 0.1</td>
<td>-327.1 ± 0.2</td>
<td>-11.9 ± 0.1</td>
</tr>
<tr>
<td>50 ( \mu M ) MAP1B + 200 ( \mu M ) FMRP</td>
<td>69.8 ± 0.1</td>
<td>-75.9 ± 0.1</td>
<td>-221.1 ± 0.2</td>
<td>-9.9 ± 0.1</td>
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**Table III.1:** Thermodynamic properties of MAP1B RNA determined from fitted UV-Vis thermal denaturation profiles. (Errors are based from the fitting of the data.)

**Figure III.8:** CD spectra of free 20 \( \mu M \) MAP1B RNA depicting a parallel G quartet formation.
III.2 FMRP RGG box interactions with MAP1B RNA

The FMRP RGG box binds MAP1B and MAP1B-19AP RNA.

The binding of the FMRP RGG box to MAP1B and MAP1B-19AP was studied by electrophoretic mobility shift assay (EMSA) and, in the case of MAP1B-19AP, also by fluorescence spectroscopy. Figure III.9 shows the EMSA pattern of FMRP RGG box binding in 1:1 and 1:2 ratios to MAP1B-19AP (lanes 2 and 3). These experiments were performed in 75 mM KCl, prior to our findings that MAP1B RNA folds as a single species at KCl concentrations lower that 10 mM. Thus, two bands corresponding to the two MAP1B conformers are present (lane 1), and only one of the two conformers (upper band) is bound by the FMRP RGG box (lanes 2 & 3). As a negative control for binding, the Munc13 site 1 RNA was used (Figure III.9 lanes 4 and 5) (Darnell et al., 2001). Figure III.10 shows the binding of the FMRP RGG box to MAP1B RNA in a 1:2 RNA to peptide ratio measured in 25 mM KCl.

Figure III.11 shows the binding curve of FMRP RGG box to MAP1B-19AP, determined by fluorescence spectroscopy at 25°C by titrating increasing amounts of the RGG peptide to a fixed concentration of MAP1B-19AP. The steady-state fluorescence of the 2AP fluorophore in MAP1B-19AP decreases upon the titration of the FMRP RGG peptide, indicating that this reporter is sensitive to the binding event. A binding dissociation constant, $K_d$, of $7.3 \pm 40.6$ nM was determined by fitting the binding curve to equation 3. However, this is only an estimate of the $K_d$ since the error calculated was greater than 100%, due to the steep binding curve. Fluorescence binding experiments were also conducted in 150 mM LiCl as a negative control (data not shown). The binding of the FMRP RGG box to MAP1B RNA was also studied by NMR spectroscopy.
Figure III.9: EMSA of MAP1B-19AP RNA in 75 mM KCl (30 µM) and Munc13 (30 µM) (negative control).

Figure III.10: Gel shift assay that shows MAP1B bound by the FMRP RGG box in 25 mM KCl. Lane 1: 10 µM MAP1B, Lane 2: 10 µM MAP1B + 20 µM FMRP, Lane 3: 20 µM MAP1B, Lane 4: 20 µM + 40 µM FMRP, Lane 5: 100 µM MAP1B, Lane 6: 100 µM + 200 µM FMRP.
The FMRP RGG box was titrated into a 300 µM MAP1B RNA sample in increments of 15 µM. The peptide was added to a final FMRP concentration of 300 µM (1:1 ratio). There was no noticeable change in the NMR spectra when the peptide was added, even when the RNA:peptide ratio was 1:1 (data not shown). However, this experiment needs to be repeated because we believe that the FMRP RGG peptide stock was degraded. When the same stock of FMRP RGG peptide was used for a gel shift assay, no binding occurred to MAP1B RNA (Figure III.12) when it was shown that when using a different stock, the FMRP RGG box does in fact bind to MAP1B RNA (Figure III.12). Therefore, a new peptide stock has been ordered and the NMR experiments that measure the FMRP RGG box binding to MAP1B RNA will be repeated.

Isothermal titration calorimetry (ITC) was also used to attempt to determine a more accurate $K_d$ for the FMRP RGG box – MAP1B RNA complex. The MAP1B RNA concentration was 20 µm and the FMRP RGG box concentration was varied from 0.50 – 1.5 mM. All experiments were conducted in 50 mM KCl, however, none of the results were reproducible (data not shown).

*The FMRP RGG box binding decreases the stability of the G quartet structure of MAP1B RNA*

CD spectroscopy was employed to determine if the FMRP RGG box binding alters the conformation of MAP1B RNA. The CD spectrum of free MAP1B RNA was compared with that of the FMRP RGG box – MAP1B complex (1:2 and 1:4 RNA to RGG ratio). As illustrated in figure III.13, the three CD spectra are very similar, indicating that there are no major structural changes in MAP1B RNA upon binding of the FMRP RGG box.
Figure III.11: Binding curve of FMRP RGG box to MAP1B 19-AP determined by fluorescence spectroscopy. The solid red line represents the best fit using equation 3, from which a $K_d = (7.3 \pm 40.6)$ nM was determined. Result was reproducible.

Figure III.12: EMSA showing that the FMRP RGG box used in the NMR studies is not binding to MAP1B RNA, it was concluded that the FMRP stock was degraded. Lane 1: 20 µM MAP1B, Lane 2: 20 µM + 40 µM FMRP, Lane 3: 20 µM MAP1B, Lane 4: 20 µM MAP1B + 40 µM FXR1 RGG box (free band disappears, but no complex band is seen). Gel was visualized by shadowing.
Next, we investigated by UV Vis spectroscopy if the binding of the FMRP RGG box has any effect upon the stability of MAP1B RNA. In this experiment the sample contained 50 µM MAP1B RNA and 200 µM FMRP RGG box (1:4 RNA:RGG peptide ratio). An increase in the T_m of the G quartet structure of about 8ºC was observed with the addition of the peptide (Figure III.14 A), however, the thermodynamic analysis (Table III.1) shows that the ΔG° is actually becoming more positive indicating that the G quartet structure becomes less stable in the presence of the FMRP RGG box. The melting curve of free MAP1B RNA is steeper than the bound melting curve, which is a more gradual curve. This leads to the conclusion that the binding of FMRP to MAP1B RNA destabilizes the structure of MAP1B RNA.

How specific is the recognition of MAP1B RNA by the FMRP RGG Box?

To investigate if G quartet recognition is a more general feature of the RGG box RNA binding domain we studied the interactions of different RGG box domains (derived from other RNA binding proteins) with G quartet forming RNA sequences. Thus, for this study the RGG boxes selected were FXR1, ICP27 of the herpes simplex virus 1 protein and the arginine-glycine cluster of the FXR2 protein, whose sequences are shown in table III.2. The FXR2 protein does not have an actual RGG box, however, it was included in this study since it is an autosomal paralog of FMRP. ICP27 has been reported to bind with high affinity in vivo to RNA sequences containing multiple G repeats that have the potential to fold into G quartets (Sokolowski et al., 2003). The mechanisms by which ICP27 regulates genes are not known; however, there is evidence that it is an RNA binding protein and that the protein domain responsible for this activity is an RGG box-like short arginine-glycine rich sequence (Mears & Rice, 1996).
Figure III.13: CD spectra of free MAP1B RNA and FMRP RGG box – MAP1B complex. (Blue) 20 μM MAP1B RNA. (Pink) 20 μM MAP1B RNA + 40 μM FMRP. (Green) 20 μM MAP1B RNA + 80 μM FMRP. All samples contain 10 mM KCl and 10 mM Tris.

Figure III.14: (A) Fitted thermal denaturation profile of free 50 μM MAP1B RNA (Black). Fitted thermal denaturation profile of the MAP1B RNA-FMRP complex (Red) (B) Two simulated denaturation profiles in which the structure with the highest $T_m$ (triangles) has a lower stability than the structure with a lower $T_m$ (circles) (Mergny and Lacroix, 2003).
Gel shift assays were used to test if the RGG boxes of FXR1 and ICP27 and the RG cluster of FXR2 bind to MAP1B RNA. As illustrated in Figure III.15, in the presence of the FXR1 RGG box (lanes 3 and 4) and ICP27 (lanes 7 and 8) the band corresponding to free MAP1B RNA decreases in intensity, indicating that there is binding between these RGG boxes and MAP1B RNA. In lane 2 it is clear that there is a shifted complex band for the binding with the FMRP RGG box as well as in lane 8 with the ICP27-MAP1B complex. However, the FXR2 cluster only diminishes the free RNA band slightly (lanes 5 and 6), indicating very weak binding.
Primary sequences of the RGG box peptides used in this study

<table>
<thead>
<tr>
<th>Primary sequences of the RGG box peptides with the RGG boxes highlighted in green.</th>
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<tbody>
<tr>
<td><strong>FMRP RGG box</strong></td>
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<tr>
<td><strong>FXR1 RGG box</strong></td>
</tr>
<tr>
<td><strong>FXR2 RG cluster</strong></td>
</tr>
<tr>
<td><strong>ICP27 RGG box</strong></td>
</tr>
</tbody>
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**Table III.2:** Primary sequences of the RGG box peptides with the RGG boxes highlighted in green.

![Image of the gel and running buffer containing 10 mM KCl and gel was visualized by UV shadowing at 254 nm.](image)

**Figure III.15:** Electrophoretic mobility shift analysis of the FMRP RGG box (lanes 1 and 2), FXR1 RGG box (lanes 3 and 4), FXR2 RG cluster (lanes 5 and 6) and ICP27 RGG box (lanes 7 and 8) binding to MAP1B RNA. The RNA concentration was 20 µM and the RGG box peptides were added in 1:2 ratios. Both the gel and running buffer contained 10 mM KCl and the gel was visualized by UV shadowing at 254 nm.
IV. DISCUSSION
IV.1 Structural Study of MAP1B RNA

MAP1B RNA contains G quartet structural elements

It has been proposed that the structure of MAP1B RNA contains a G quartet structure and that FMRP uses its RGG box domain to bind with high affinity to this structure (Darnell et al., 2001). A preliminary NMR spectroscopy study of a 44-mer MAP1B RNA fragment suggested that indeed this RNA folds into a G quartet structure, however, the 1D $^1$H NMR spectrum showed very broad and overlapped resonances indicating a possible conformational exchange between multiple structures (Ramos et al., 2003). In this study we have analyzed a shorter MAP1B RNA fragment (34-mer) that still maintains the region proposed to fold into a G quartet structure and we have employed biophysical methods such as UV, fluorescence, NMR and CD spectroscopy to characterize this RNA and its interactions with the FMRP RGG box domain.

Our fluorescence spectroscopy results confirmed that MAP1B RNA adopts a different conformation in the presence of KCl and LiCl, respectively. In the fluorescence spectroscopy experiments we have used a MAP1B RNA in which the adenine at position 19 was replaced by 2AP, named MAP1B-19AP. In the postulated G quartet structure of MAP1B RNA (Figure I.5) position 19 is in a loop. Thus, it is expected that the steady-state fluorescence of the 2AP reporter, which is sensitive to its environment, will change upon the formation of the G quartet structure since it is likely that the conformation of the G quartet surrounding loops will be highly distorted. The steady-state fluorescence of the MAP1B-19AP increases approximately 2-fold when this RNA is folded in the presence of KCl versus LiCl (Figure III.1), and we attribute this change to the different environments in which the 2-AP reporter exists: G quartet surrounding loop in the
presence of K$^+$ and single stranded stacked in the presence of Li$^+$, which does not support the formation of G quartets.

UV spectroscopy was also used to characterize MAP1B RNA, namely, to confirm the presence of G quartet elements in its structure and to determine if MAP1B RNA forms an intra- or intermolecular structure. The UV thermal denaturation of MAP1B RNA was measured at 295 nm, the wavelength used extensively to monitor the dissociation G quartet structures (Mergny, 1998). A hypochromic transition is typically observed at this wavelength upon the melting of G quartet structures. Thus, the 50°-70°C hypochromic transition observed in the melting curve of MAP1B folded in the presence of K$^+$ ions was assigned to the melting of the G quartet structure (Figure III.6 B). As expected, this hypochromic transition is not present in the melting curve of MAP1B folded only in the presence of TRIS buffer (no other ions), since the formation of a G quartet structure requires the presence of K$^+$ or Na$^+$ ions (Figure III.6 A). However, the 75°C - 95°C hyperchromic transition, assigned to the melting of the MAP1B stem was present in both melting curves. This transition starts earlier in the absence of the K$^+$ ions, at approximately 61°C as opposed to 75°C in the melting curve with KCl present. Since there is no G quartet structure present in the MAP1B folded in the absence of KCl, the stem begins to melt at a lower temperature. There is also a hyperchromic transition from 20-50°C present in the MAP1B RNA melting curve measured in the presence of potassium. The nature of this transition is not known, however it is potassium dependent (Figure III.6 B and figure III.7 A).

To gain more information about the different conformations of MAP1B RNA we have also used native gel electrophoresis. When MAP1B RNA was folded in the
presence of Li$^+$ ions and run on a gel containing LiCl there was only one species present. However, when this RNA was folded in the presence of and run on a gel in the presence of potassium two species were apparent. This led to the conclusion that the formation of the second species is potassium dependent.

*Isolation of the experimental conditions in which MAP1B RNA folds into a single conformation*

The long term goal of this project is to determine the high resolution structure of this MAP1B RNA fragment, but such structural studies are possible only when the RNA folds as a single species. Our native gel electrophoresis results showed that MAP1B RNA folds into two different conformations in the presence of KCl, complicating the future NMR structural studies. Thus, we have performed a careful study to isolate the *in vitro* experimental conditions that would promote the folding of MAP1B RNA into a single conformation. Through the use of NMR spectroscopy and native gel electrophoresis, it was determined that the optimum conditions for MAP1B to form as a single species are to fold it in the presence of 10 mM KCl.

In the 1D $^1$H NMR spectroscopy study, KCl increments were titrated into a sample of 400 µM MAP1B RNA. The initial RNA sample, with no KCl present, did not show any peaks in the 10 - 12 ppm range, indicating as expected, that G quartets were not present in the absence of K$^+$ ions. With the addition of 5 mM KCl, resonances appeared in the G quartet region, indicating the formation of a G quartet structure. At a 10 mM KCl concentration, the resonances present in the 10 - 12 ppm range remained very sharp, however, as more KCl was titrated in the sample, these resonances became broader and new resonances appeared (Figure III.4). The broadening and decrease in intensity of the
original G quartet resonances and the apparition of new ones indicates that a second MAP1B RNA conformation starts to form. Therefore, we concluded that the best KCl conditions for MAP1B RNA were 10 mM KCl.

Native gel electrophoresis was also used to determine if indeed MAP1B RNA folds as a single species in the presence of 10 mM KCl. Samples containing 20 µM RNA folded in the presence of various concentrations of KCl were run on a gel containing 50 mM KCl (Figure III.5). At 10 mM KCl, a single band is present (Figure III.5, lane 1), however, as the KCl concentration increased, a second lower band representing the second species started to appear and increase in intensity. These results correlate well with the NMR data showing that a KCl concentration over 10 mM promotes the formation of the second MAP1B RNA species.

MAP1B RNA folds into parallel intramolecular G quartets

Once these folding conditions were established, we have employed thermodynamic methods to determine if this MAP1B RNA conformation is intra or intermolecular. The UV thermal denaturation profiles were measured for MAP1B RNA at various MAP1B RNA concentrations. If a molecule forms intramolecular G quartets then the $T_m$ of its G quartets should be independent of the RNA concentration (Equation 1). However, if intermolecular G quartets are formed then their $T_m$ should increase with increasing the RNA concentration (Equation 1). The melting curves of various MAP1B RNA concentrations (10 µM – 80 µM) were collected, and the $T_m$ was measured at for each RNA concentration. All of the melting temperatures were similar (Table III.1), indicating that MAP1B RNA forms intramolecular G quartets.
Once we established that MAP1B forms intramolecular G quartets, we have extracted the thermodynamic parameters of G quartet dissociation by fitting the 50°C - 70°C hypochromic transition with equation 2, that assumes a two-state model. The analysis of the thermodynamic data (Table III.1), suggests that there are five G quartet planes present in the structure of MAP1B RNA. However, the sequence of MAP1B contains stretches of Gs and As and it is possible that planes of G quartets might sandwich A tetrads. Since no thermodynamic information is available in the literature about the stability of these mixed quartet structures, we cannot conclude definitively that five G quartet planes are present in the structure of MAP1B RNA.

CD spectroscopy was employed to determine if MAP1B RNA forms parallel or antiparallel G quartets. The CD spectrum of 20 µM MAP1B RNA folded in the presence of 10 mM KCl depicts a positive peak at 265 nm and a negative peak at approximately 240 nm, indicating that this RNA folds into a parallel G quartet structure.

**IV.2 FMRP RGG box interactions with MAP1B RNA**

*EMSA and fluorescence spectroscopy studies of the FMRP RGG box binding to MAP1B and MAP1B-19AP RNA*

In our initial experiments, we have studied the binding of the FMRP RGG box to MAP1B RNA and MAP1B-19AP RNA by electrophoretic mobility shift assays, at KCl concentrations higher than 10 mM (either 150 mM or 50 mM). The EMSA results showed that the FMRP RGG box binds to only one of the two MAP1B RNA conformations formed at KCl concentrations higher than 10 mM KCl (Figure III.9, lanes 1-3). Subsequently, we have repeated these experiments at 10 mM KCl concentrations,
conditions in which only a single MAP1B structure is present, and we established that the FMRP RGG box binds to this MAP1B conformation (Figure III.15, lane 2).

Next, we used fluorescence spectroscopy to measure the binding of the FMRP RGG box to MAP1B-19AP. The 2AP reporter inserted at position 19 was sensitive to the binding event, its steady-state fluorescence intensity decreasing upon the titration of increasing amounts of the FMRP RGG box. The binding curve was fitted with equation 3 and a $K_d$ of $7.3 \pm 40.6$ nM was determined for the FMRP RGG box-MAP1B-19AP complex at 25°C (Figure III.11). Although, this $K_d$ value is only an estimate (given its very large error), it is one order of magnitude lower that the $K_d = 505$ nM value reported in the literature for the complex between the FMRP RGG box and a 44-mer fragment of MAP1B RNA (that contains the 34-mer fragment used in this study)(Darnell et al., 2001). As mentioned earlier, a $^1$H NMR study indicated that there are multiple species formed by the 44-mer MAP1B RNA fragment, and it is likely that the $K_d$ value of 505 nM represents an average value for the binding of the FMRP to these different conformations. It is also worth mentioning that the these studies were conducted at a 50 mM KCl concentration, higher than the 10 mM KCl concentration we determined is required for the folding of the 34-mer MAP1B RNA fragment in a single species.

*The FMRP RGG box binding decreases the stability of the G quartet structure of MAP1B RNA*

We have determined that the binding of the FMRP RGG box decreases the stability of the MAP1B RNA G quartet structure, as reflected by an increase in the free energy of G quartet formation in the MAP1B RNA-FMRP RGG complex, as compared to that of the free MAP1B (Table III.1). Initially it was believed that the FMRP RGG
box increased the stability of the G quartet structure due to the increase of the G quartet \( T_m \) in the FMRP RGG box-MAP1B RNA complex, however, the thermodynamic analysis showed an increase in the free energy of G quartet formation, in this complex. Figure III.14 B depicts two simulated UV denaturation profiles, one with a \( T_m \) of 60ºC and the second 70ºC. The transition with a \( T_m \) of 60ºC is more abrupt and is completed over a narrower temperature range, making the \( \Delta G^\circ \) of this reaction more temperature dependent (Mergny and Lacroix, 2003). Therefore, the transition with the highest \( T_m \) has a lower stability and less favorable \( \Delta G^\circ \) than the structure with a \( T_m \) of 60ºC (Mergny and Larcoix, 2003). The shapes of these simulated denaturation profiles are very similar to the denaturation profiles of MAP1B RNA and MAP1B RNA-FMRP RGG box complex.

CD spectroscopy was used to determine if the binding of the FMRP RGG box alters the conformation of MAP1B RNA. FMRP RGG peptide was added to the free MAP1B RNA at a 1:2 and a 1:4 RNA: RGG peptide ratio. The spectra of the FMRP RGG box-MAP1B RNA complex are very similar to the spectrum collected for free MAP1B RNA, leading to the conclusion that the binding of the FMRP RGG box does not alter the structure of MAP1B RNA.

*How specific is the recognition of MAP1B RNA by the FMRP RGG Box?*

We have also analyzed the binding of two other RGG box peptides, derived from the FXR1 protein and the herpes simplex virus 1 ICP27 protein, as well as of the RG cluster of FXR2, to MAP1B RNA. Many RNA binding proteins contain RGG boxes (Raman et al., 2001), however, there is no clear definition regarding the required number of RGG repeats and their spacing. Thus, we have used gel shift assays to determine if G
quartet recognition is a general property of the RGG box RNA binding domain and to
determine how unique the recognition is between MAP1B RNA and the FMRP RGG
box. Interestingly, the EMSA results (Figure III.15) indicate that all the RGG boxes,
bind MAP1B RNA, whereas the FXR2 RG cluster does not bind. Therefore, it is likely
that FMRP binds its RNA targets by using additional recognition elements (possibly
involving its KH1 and KH2 RNA binding domains) besides the RGG box G quartet
recognition.
V. Conclusions and Future Research
V.1. Conclusions

This study reports on the structure of MAP1B RNA and its interactions with the FMRP RGG box. At KCl concentrations larger than 10 mM, MAP1B RNA exists in an equilibrium between two conformers; however, only one of the conformers is bound by the FMRP RGG box. To pursue further structural studies of MAP1B RNA, we used various spectroscopic techniques to isolate the experimental conditions in which MAP1B RNA folds into a single conformation. Subsequently, thermodynamic methods were used to determine that MAP1B RNA forms an intramolecular G quartet structure. CD spectroscopy was used to further the structural studies and our results indicate that MAP1B RNA forms parallel G quartets.

Various biochemical and biophysical techniques were used to determine that the FMRP RGG box binds with high affinity ($K_d$ in the nanomolar range) to the unique MAP1B RNA conformer formed in the presence of 10 mM KCl. In addition, by using CD spectroscopy, it was determined that the binding of the FMRP RGG box to this MAP1B RNA does not alter its G quartet structure. Interestingly, the comparison of the thermal denaturation profiles of free MAP1B RNA and MAP1B RNA-FMRP RGG box complex showed that the binding of the peptide actually decreases the stability of the G quartet structure.

This study also addressed the question if G quartet recognition is a general feature of the RGG box domain, by analyzing the binding of three RGG boxes derived from different RNA binding proteins to MAP1B RNA. Our results show that MAP1B RNA is bound by two additional RGG boxes, but it is not bound by the FXR2 RG cluster. The next steps would be to measure the affinity with which these RGG boxes bind to MAP1B RNA.
RNA by using the fluorescence spectroscopy. It would also be interesting to determine if these binding events are specific by measuring the binding of these RGG boxes to MAP1B RNA in the presence of a large excess of another unlabeled RNA like Munc13.

In summary, in this study we have determined a minimum size MAP1B RNA fragment that is still bound with high affinity by the FMRP RGG box, we have confirmed the presence of G quartet elements in its structure and established the in vitro experimental conditions in which this RNA folds as into a single conformation. Moreover, we determined that the FMRP RGG box does not affect the structure of these G quartets, but it affects their stability.

These results now make possible, future high resolution heteronuclear NMR structural studies of MAP1B RNA and its complex with the FMRP RGG box.

V.2 Future Research

The determination of an accurate $K_d$ for the FMRP RGG – MAP1B RNA complex was not achieved in this study, and future research will focus on determining a more accurate $K_d$ value measuring the FMRP RGG box binding curves at different concentrations of MAP1B RNA. In addition, preliminary structural information about the RNA regions contacted by the FMRP RGG box (stem versus G quartet structure) will be obtained by repeating the NMR binding experiments with a new FMRP RGG box peptide stock.

The FMRP RGG box has been previously shown to bind with high affinity to Sc1 RNA (Darnell et al., 2001). Darnell et al., 2001 has also reported that the stem region is necessary for the binding of FMRP to Sc1 RNA. Recently, the Mihailescu lab has determined that G quartet recognition is not sufficient for the FMRP RGG box binding to
Sc1, indicating that additional interactions of the peptide with the stem region and/or junction region between the G quartet and stem are present. The sequence of MAP1B RNA does not contain a junction region between the G quartet and stem like Sc1 RNA. To determine if this region is required for the binding of the FMRP RGG box mutational studies will be carried out. The MAP1B stem will be replaced with the Sc1 stem and junction region. Binding experiments will be carried out to determine if the presence of the junction region and stem from Sc1 RNA increases the binding affinity of the FMRP RGG box to the mutated MAP1B RNA.

The role of protein posttranslational modifications in modulating FMRP - MAP1B RNA interactions will also be investigated. Further studies will be needed to determine if phosphorylation regulates FMRP function but altering its RNA binding properties. Protein arginine methylation involves the methylation of arginine residues located in the RGG box domains by protein arginine methyltransferases. This arginine methylation may also affect the FMRP binding activity to specific RNA targets such as MAP1B RNA.

Currently, we are studying the interactions of only one isoform of FMRP (isoform 7) with MAP1B RNA. The FMR1 gene has 17 exons and can undergo alternative splicing, resulting in 20 different FMRP isoforms. To better understand the protein requirements for specific RNA binding the interactions of MAP1B RNA with other FMRP isoforms will be analyzed.
REFERENCES


