Fragile X mental retardation is a disease caused by the loss of function of a single RNA-binding protein, FMRP. Identifying the RNA targets recognized by FMRP is likely to reveal much about its functions in controlling some aspects of memory and behavior. Recent evidence suggests that one of the predominant RNA motifs recognized by the FMRP protein is an intramolecular G-quartet and that the RGG box of FMRP mediates this interaction. Searching databases of mRNA sequence information, as well as compiled sequences of predicted FMRP targets based on biochemical identification, has revealed that many of these predicted FMRP targets contain intramolecular G-quartets. Interestingly, many of the G-quartet containing RNA targets encode proteins involved in neuronal development and synaptic function. Defects in the metabolism of this set of RNAs, presumably in the translation of their protein products, is likely to underlie the behavioral and cognitive changes seen in the disease.

Key Words: fragile X mental retardation protein (FMRP); RNA binding protein; intramolecular G-quartet; in vitro RNA selection; translational control

In 2003, a workshop for the planning of future research on fragile X mental retardation concluded that the number one priority in the field is the identification of RNA targets to which the FMRP protein binds. This prioritization clearly reflects both the successes and challenges facing work in this area.

The mystery of the fragile X syndrome lies in understanding how loss of function of one protein can lead to the complex behavioral, cognitive, and phenotypic changes characteristic of the disorder. The first clue in this mystery came with the identification of the FMR1 gene in 1991 as the gene responsible for fragile X syndrome (reviewed in [O'Donnell and Warren, 2002]). It subsequently became clear that the disorder was due, in most patients, to expanded CGG triplet repeats in the 5' untranslated region of FMR1 that led to hypermethylation of the gene and consequently gene silencing [O'Donnell and Warren, 2002]. A single patient whose study has been of some importance in this story is a severely affected fragile X individual who harbors a single point mutation within the gene (the I304N mutation) in lieu of a triplet repeat mutation [DeBoulle et al., 1993].

THE FRAGILE X MENTAL RETARDATION PROTEIN, FMRP, RECOGNIZES G-QUARTETS

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As FMRP is found predominantly in the cytoplasm of neurons (and other cells) [Devys et al., 1993], a potential role for FMRP in translational regulation was considered. FMRP was found to localize to the polysome fraction of the cytoplasm [Corbin et al., 1997; Feng et al., 1997a; Feng et al., 1997b], and this localization was associated with functional polysomes, as it was lost in the presence of sodium azide or sodium fluoride, agents that cause ribosomes to run off translating mRNA [Feng et al., 1997b]. Moreover, it was found that FMRP proteins harboring the I304N mutation did not associate with polysomes, establishing a link between the fragile-X mental retardation syndrome and the role of the protein in mRNA translation [Feng et al., 1997b].

The I304N mutation is located within the second FMRP KH domain. Although the function for this amino acid has not been precisely determined, X-ray crystallography of a related KH domain bound to its RNA target, the Nova neuron-specific RNA-binding protein hnRNP-K, and a less well-defined element consisting of repeats of the amino acid element RGG (the RGG-box). Initial work on hnRNP-K had revealed a role for this protein in the regulation of translational control of a specific RNA target, the 15-lipoxygenase mRNA expressed in erythrocytes [Ostareck et al., 1997].

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I304N mutation maps precisely to the core of this RNA-binding pocket, suggesting that the mutation in FMRP may interfere with sequence-specific RNA binding. More recently, the crystal structures of two additional KH domains bound to their cognate RNAs have been solved and the analogous hydrophobic amino acid to I304 in each case also lies at the heart of the RNA-binding platform [Ramos et al., 2003].

These results underscored the importance of identifying the RNA targets to which FMRP binds. In 1993, Steve Warren and colleagues identified the FMR1 mRNA message as an in vitro target of FMRP, and further showed that FMRP only bound some of a random group of mRNAs produced from cDNA clones, suggesting selective RNA binding [Ashley et al., 1993]. A few years later this finding was extended to the 3’UTR of myelin basic protein [Brown et al., 1998]. Bob Denman and colleagues used biotinylated FMRP to capture the RNAs to which it bound, amplify them by different display polymerase chain reaction, and determine their identities. In addition to several unknown genes, prothymosin-a, Tip60a (a tat interactive protein), mitochondrial 26S rRNA, and a piece of the FMR1 mRNA were identified as possibly binding to FMRP [Sung et al., 2000]. The biological relevance of these targets in vivo is unclear. The presumption based on these studies is that FMRP binds to a subset of brain mRNAs and that identifying these RNAs and understanding how FMRP acts on them would provide important clues toward understanding the nature of the disorder.

FMRP RNA TARGETS

Two parallel and synergizing biochemical approaches have been used to identify FMRP RNA targets. In the first, in vitro RNA selection was used to identify the preferred RNA target of the protein [Darnell et al., 2001]. This method makes very few presumptions about what RNA elements, whether they be structural or sequence elements, the protein prefers to bind. The method aims to identify the highest affinity FMRP targets, which arguably might be an important first step in identifying in vivo RNA targets in much the same way that identifying the highest affinity protein–DNA or protein–protein interactions have been important first steps in identifying protein function in other systems.

To undertake in vitro RNA selection with FMRP, a series of iterative experiments were begun in which a pool (library) of long (52 nucleotides) random RNA sequences harboring PCR-amplifiable elements on their ends, were passed over FMRP affinity columns. After extensive washing, RNA bound to protein was specifically eluted with imidazole, releasing His-tagged FMRP from the column. The eluted protein was collected, and the RNA extracted, reverse transcribed, and PCR amplified using the fixed elements present on the ends of each RNA in the pool. Because these elements also contained a T7 RNA polymerase promoter on one end, the pool could then be re-transcribed into a new random library of slightly less complexity (for example, complexity in the original pool of $10^{15}$ different 52-mer RNAs was reduced to $\sim 10^{12}$ different RNAs after this purification). This entire process was then repeated, with re-purification of these $10^{12}$ RNAs on a second column, for a total of nine rounds of RNA selection. At the conclusion of this process, a defined set of RNAs was present, approximately 95% of which bound to FMRP directly and with high affinity [Darnell et al., 2001].

The presumption based on these studies is that FMRP binds to a subset of brain mRNAs, and that identifying these RNAs and understanding how FMRP acts on them would provide important clues toward understanding the nature of the disorder.

A series of mutagenesis and binding experiments suggested several important points about the FMRP RNA targets. First, the domain that had selected the RNA target was not the FMRP KH domains, but the RGG box, suggesting that within the context of the full-length protein, this element may have the highest affinity for RNA. Second, the nature of the RNA was such that it appeared to form a complex tertiary structure termed a G-quartet; in essence, a complex stem-loop element that folds back on itself to form non-canonical (Hoogsteen) base-base interactions. Such complex elements have previously been identified as targets for a number of DNA-binding proteins, and in one case (a bacteriophage protein) as the target for an RNA-binding protein involved in translational regulation [Oliver et al., 2000]. G-quartet elements form around a single small monovalent cation (sodium or potassium), and the requirement for FMRP binding to this element was confirmed by the observation that FMRP bound to its selected RNAs in the presence of potassium ions, but not lithium ions, which are too small to support the G-quartet structure [Darnell et al., 2001]. The net result of these studies was identification of an RNA target with enough complexity that a bioinformatic screen of the genome database for the presence of such structures led to the identification of a limited set of targets, which were validated as discussed below.

Concurrently, Herve Moine and colleagues, in pursuing the finding that FMRP binds to its own mRNA in vitro, delineated the sequences within FMR1 message necessary for FMRP binding [Schaefler et al., 2001]. They found that the FMRP-binding element folded into a purine quartet that was bound by FMRP with very high affinity. In addition, they demonstrated that the interaction could occur in a reticulocyte lysate, because placement of the purine quartet near the 5’ end of a transcript repressed translation of a reporter gene in an FMRP-dependent manner. This intriguing result showing modulation of translation of an mRNA in both a G-quartet and FMRP-dependent manner further substantiates the finding that FMRP recognizes G-quartet motifs in RNA targets and may use this mechanism in vivo to control translation of specific mRNAs [Moine and Mandel, 2001].

A biologically relevant approach to identifying FMRP targets came from a combination of immunoprecipitation and gene array analyses [Brown et al., 2001]. One issue hampering the use of immunoprecipitation in the study of FMRP has been the identification of a number of closely related proteins, FXR-1 and FXR-2, both of which are recognized by most “FMRP” antibodies. Therefore the development of an FMRP-specific antibody was an important first step in these experiments. This antibody was found to be able to coprecipitate protein–RNA complexes from mouse brain, although such a coprecipitation by itself was not specific enough to directly identify FMRP targets. Thus cDNAs were generated from such RNA—protein precipitates and used to
probe Affymetrix gene chips. To improve the signal to noise in these experiments, parallel arrays were probed with samples from control immunoprecipitations, including IPs, from FMRP-null mouse brains. Serial subtraction led to the identification of a small number (several mice brains. Serial subtraction led to the samples from control immunoprecipitations, parallel arrays were probed with probe Affymetrix gene chips. To prove the signal to noise in these experiments fit with a number of studies reviewed elsewhere in this volume, suggesting that FMRP plays an important role in the development and function of the dendrite.

**REFERENCES**


The small number of mRNAs passing through these rigorous selection criteria as candidate FMRP mRNA targets share a remarkably coherent biology. Eleven of the set of 13 target RNAs encode proteins whose function relates to the biology of the synapse. These include receptors and channels (the V1a receptor and potassium channel Kv 3.1), proteins involved in synaptic function (munc 13-2, NAP-22, sec-7 related guanine nucleotide exchange factor, and the rab 6-binding protein), and three proteins involved in neurite extension and development (MAP1B, semaphorin 3F, and ID3) [Brown et al., 2001; Darnell et al., 2001]. While work progresses on the further validation of these targets one in particular deserves further mention. MAP1B plays an important role in the extension of dendrites and axons in the developing neuron [Mandell and Banker, 1996; Takei et al., 2000]. It has recently been shown that the Drosophila homolog of FMRP, dFXR, acts as a translational repressor of futsch, the fly homolog of MAP1B [Zhang et al., 2001]. This is consistent with the finding that MAP1B mRNA is increased in the polysome fractions of fragile X patient cells that lack FMRP [Brown et al., 2001]. Interestingly, transcription of the rat MAP1B message begins at one of three sites [Liu and Fischer, 1996]. The G-quartet identified in MAP1B lies within 60 nucleotides downstream of the most 5′ start site [Darnell et al., 2001]. Data suggesting that any protein is a translational repressor if it binds an mRNA within this distance of the cap site suggests that FMRP binding to this site in vivo would necessarily repress cap-dependent translation by steric hindrance [stripecke et al., 1994].

Recently, two other groups have used additional approaches to identify candidate RNA targets for FMRP. Greenough, Eberwine and colleagues used a technique called APRA (antibody-positioned RNA amplification) to amplify RNAs in close proximity to the epitope recognized by the anti-FMRP antibody 1C3 in neuronal cultures [Miya shiro et al., 2003]. Primary rat hippocampal cells were fixed and permeabilized followed by application of the antibody linked to random primers. Using previously established methods of in situ transcription of cDNA on the cells [Eberwine et al., 2001], a pool of probes were obtained that were used to screen cDNA arrays. The proteins encoded by the RNA cargoes of FMRP identified by this approach fall into many categories, including cell signaling and communication molecules, those involved in cell structure and motility, in the secretory system, and in the regulation of transcription or translation (Table 1 [Miya shiro et al., 2003]). Interestingly, the panel of targets was screened for G-quartet-like elements and a similar motif was found in 24 of 83 searched, although a stem surrounding the G-quartet was not included as part of the query, nor was it stated how many mRNAs in the unselected database are positive with that particular query. It should be noted that in both the Darnell/Warren and Greenough/Eberwine searches for G-quartets, these queries are not likely to be exhaustive. That is, until one can better define exactly which G-quartets FMRP recognizes, these queries are likely to pick up only a subset of the actual G-quartet-binding sites present in mRNAs. Conversely, putative G-quartets picked up by sequence homology, but not tested for FMRP binding in a potassium-dependent manner, may not be true FMRP-binding sites. This was shown by the significant number of putative G-quartets picked up by the RNABob program [Eddy; 2001; Gautheret et al., 1990] cited in Darnell et al. [2001] that did not bind FMRP when tested. It is likely that they do not fold into G-quartets because of competing structures in the RNA or that there is sequence specificity in the loops of the motif.

Bagni et al. have recently shown that FMRP associates with the dendritically localized, nontranslated RNAs BC1 in rodents and BC200 in primates [Zalfa et al., 2003]. BC1 and BC200 may anneal with 3′UTR elements in some dendritically localized messages, including MAP1B; α-CaM kinase II and Arc and provide a mechanism through which FMRP negatively regulates the translation of the messages. This would explain their finding that these mRNAs are increased in their translation status in FMR1 null mice. Although this is an intriguing model, it has also been reported that FMRP does not bind BC200 [Kremerskothen et al., 1998]. Therefore, it is difficult to fully evaluate this model until the interaction between FMRP and BC1 has been independently confirmed.

Taken together, these data provide evidence for how FMRP may function as an RNA-binding protein in health and disease—to regulate the expression of proteins that play critical roles in the regulation of the synapse itself. These observations fit neatly with a number of studies reviewed elsewhere in this volume, suggesting that FMRP plays an important role in the development and function of the dendrite.


