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Breakthroughs and Views

Déjà vu all over again: FMRP binds U-rich target mRNAs

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Abstract

The fragile X mental retardation protein (FMRP) contains three RNA binding domains, two of which the KH2 domain and the C-terminal arginine-glycine-rich (RG-rich) region participate in RNA binding. Because fragile X syndrome is the leading cause of inherited mental retardation, there has been an intensive search for the messenger RNA (mRNA) targets that interact with FMRP in vivo. Initial work led to the conclusion that FMRP binds to a nucleic acid tertiary structure element called a G-quartet. Recent studies have shown that FMRP also binds mRNAs containing U-pentameric sequences. Interestingly, both motifs are mimicked by homoribopolymers (poly (rG) and poly (rU)) that were first used to determine that FMRP functioned as an RNA binding protein. The consequences of these discoveries and future areas of investigation are discussed.

In the not too distant past when researchers spoke about the RNA binding properties of the fragile X mental retardation protein (FMRP), they generally referred to its ability to bind to certain homoribopolymer resins. Indeed, Siomi et al. [1] were among the first to demonstrate that FMRP bound to poly(rG) and to poly(rU), but not to poly(rA) or poly(rC). This is not unusual in the RNA binding protein literature. Model RNAs like homoribopolymers are typically used initially when an RNA binding protein's normal cellular targets are unknown. This is true for the multiple RNA binding domain STAR protein Sam68 [2], the RG-rich domain containing protein ICP27 [3], the mitochondrial Y-box protein RBP16 [4], and the survival of motor neuron protein SMN [5].

The next step along the road to determine the nucleic acid binding properties of an RNA binding protein is to identify putative target messages. This usually involves some type of binding-amplification-selection strategy. Heterogeneous ribonucleoprotein K (hnRNP K) exemplifies this approach. Initially, hnRNP K was shown to be one of the cell's major poly(rC) binding proteins [6]. Subsequently, using selective evolution of ligands by exponential selection (SELEX) Thisted et al. [7] determined a consensus binding motif for hnRNP K which consisted of a C-rich patch [UC_{3-4}(U/A)(A/U)] presented atop a stem-loop. The size of the consensus matched the four to five base patches that are recognized by a single KH domain, while the sequence conformed to sequences found in the only two hnRNP K target mRNAs known [8].

Recently, a series of papers using variations on this strategy have begun to elucidate the messenger RNA (mRNA) targets that interact with FMRP [9–13]. The initial focus of the results of the larger microarray studies [11,12] was the discovery that FMRP, and specifically one of its three RNA binding domains, interacts with purine-rich G-quartet motifs. G-quartets are quadraplex structures in which four-G residues are arranged in a planar conformation that is stabilized by Hoogsteen hydrogen bonds [14]; both one-strand and two-strand G-quartets can form in vitro in conditions that mimic the physiological state of cells [15]. Indeed, Schaeffer et al. demonstrated that a 100 base sequence within the coding sequence of FMR1 mRNA contained a G-quartet that FMRP could bind in vitro. In addition, when this element was tagged to the 5′-end of a luciferase reporter gene, it negatively regulated the reporter mRNA's expression in in vitro translation lysates [16]. Because of this it has been suggested that G-quartet motifs mark messages that are involved in the fragile X

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phenotype [17–19]. Nevertheless, even the most generous estimate shows that G-quartet containing mRNAs comprises less than 50% of all putative FMRP targets [11–13].

Now however, in three new papers, two groups of researchers have discovered that mRNAs containing U-rich motifs bind recombinant FMRP in vitro and associate with FMRP-containing mRNPs in vivo [20–22]. Using a procedure called cDNA-SELEX Chen et al. isolated 114 unique FMRP targets, 57 of which corresponded to known mRNAs. While two of the 57 clones encoding known messages contained a putative G-quartet element, 20 contained U-rich stretches of 5–23 bases of repeating U-pentamers, Table 1. Subsequently, additional experiments were performed that verified the fact that FMRP bound the U-rich messages and required the U-rich element for their interaction. Making use of the fact that two of the U-rich mRNAs (rhoA and clathrin) were expressed in HEK293 cells, they immunoprecipitated FMRP (FMRP-IP) and isolated mRNA from the immunocomplex. They then used real-time RT-PCR to show that these U-rich mRNAs were associated with the FMRP-IP. To determine that the U-rich element was required for this interaction they examined the binding of U-rich and non-U-rich fragments from the same mRNAs using an electrophoretic gel mobility shift assay (EMSA) and found that eliminating the U-rich element abrogated binding.

Affinity chromatography coupled to differential display RT-PCR (DDRT-PCR) provided the first evidence that there were specific brain-derived FMRP target mRNAs [10], and the subsequent use of other high throughput screening (HTS) assays has allowed Sung and coworkers to compile a list of more than 30 FMRP target mRNAs [23,24]. Included within this list are mRNAs encoding transcription factors, translation factors, RNA binding proteins, and receptor mRNAs, Table 1. Again, while potential G-quartets were observed within the data set they were not present in most of the messages. Two additional papers by this group illustrate that G-quartets are not absolutely required for an in vivo interaction between FMRP and its target mRNA [20,21]. Furthermore, in consonance with the studies of Chen et al. U-rich sequences were shown to bind to FMRP within the milieu of cells. Specifically, Sung et al. demonstrated that elongation factor-1A (EF-1A) mRNA, which lacks canonical G-quartet motifs, bound purified recombinant FMRP in vitro and associated with FMRP-containing mRNPs in vivo. Additionally, they showed that in fragile X lymphoblastoid cells lacking FMRP, EF-1A protein was significantly elevated compared to normal controls, while its mRNA remained unchanged, demonstrating that FMRP negatively regulates the translation of EF-1A mRNA. In a separate study using a yeast-3-hybrid screen Dolzhanskaya et al. [21] established that two small expressed sequence tags (ESTs) encoding U-rich mRNA fragments without a G-quartet interacted with human recombinant FMRP expressed in yeast and one of these ESTs was found associated with HeLa cell FMRP-IPs. Interestingly, the two ESTs had significant homology to a U-rich region in the 3′-untranslated region (3′UTR) of FMR1 mRNA that was previously shown to bind to FMRP in vitro [23] and U-rich sequence elements were found in most of target mRNAs isolated by this group.

In one respect these new data are not surprising as they were pre-figured by the interaction of FMRP with poly(rU). What is interesting is the preponderance of U-rich containing targets over G-quartets. The data from these new studies suggest that the majority of the mRNAs that bind to FMRP may contain U-rich motifs. While it must be kept in mind that the bulk of these putative U-rich target mRNAs lack hard biochemical evidence for an in vivo interaction with FMRP or FMRP-containing mRNPs the same is true of the G-quartet containing mRNAs. However, in a recent microarray screening study for putative FMRP target mRNAs Miyashiro et al. [13] found that only 18 of 83 candidates contained a recognizable G-quartet. These data led the authors to conclude that there must be other *cis*-acting motifs that confer binding to FMRP. U-rich elements certainly fit this bill.

The discovery of this second type of FMRP interaction motif while advancing the fragile X field also highlights some of the important questions that remain to be addressed. First, what is the minimal U-rich sequence that will bind to FMRP and is it associated with a higher order structure? As Sung et al. demonstrated serendipitously, not all U-rich element-containing mRNAs bind to FMRP, e.g., βAPP mRNA, Table 1. This suggests that context and motif accessibility play important and as yet undefined roles in determining whether a particular mRNA is an FMRP target, Fig. 1A. Second, some FMRP target mRNAs contain multiple U-rich regions that are separated in linear sequence space (SAP49 mRNA, clathrin mRNA, and NKTR mRNA); other target mRNAs contain both G-quartet elements and U-rich motifs (FMR1 mRNA, NF-κB mRNA, and Tip60a mRNA). How does FMRP recognize each of these message-types? Are they regulated differently? The finding that, like G-quartets [11–13], U-rich sequences are present in the 5′-untranslated regions, the coding sequences and the 3′-untranslated regions of different messages imply differing outcomes may result from FMRP binding to these messages, Fig. 1B. Precedent for this can be found in the differential control of ferritin mRNA and transferrin receptor mRNA by the iron-response element binding protein [25]. Third, can FMRP simultaneously interact with multiple types of elements in a single message? Interestingly, the discovery of both G-quartet and U-rich element in FMR1 mRNA may explain the initial binding stoichiometry of four

Table 1
U-rich mRNA that interact with FMRP

Set[a]	Type	Accession No.	Identity	U-rich[b]	Region[c]	Coordinates[d]	In vitro binding[e]	In vivo binding[e]
1	*RBP*	*X69962*	*FMR1 mRNA*	+	*3′UTR*	*2255–3366*	+	+
1	*RBP*	*XM003154*	*FXR1 mRNA*	+	*CDS*	*283–300*	+	*ND*
					3′UTR	*1880–1920*		
1	RBP	AF106860	G3PDH mRNA	+	5′UTR	115–181	+	+
1	RBP/Translation	M25504	xEF-1A mRNA	+	3′UTR	1591–1624	+	NA
1	RBP/Translation	X61043	rEF-1A mRNA	+	3′UTR	1612–1679	+	+
1	RBP/Translation	X03558	hEF-1A mRNA	+	–	ND	+	+
1	*Transcription*	*U74667*	*Tip60a mRNA*	+	*3′UTR*	*2087–2105*	+	+
1	*Transcription*	*M61909*	*NF-κBp65 mRNA*	+	*3′UTR*	*2281–2300*	+	+
1	Transcription	L35049	Bcl-xL mRNA	+	5′UTR	15–35	ND	ND
1	Transcription	J04103	Ets-2 mRNA	–	NA	NA	ND	ND
1	*Receptor*	*D16250*	*(BMP-R) mRNA*	+	*3′UTR*	*1976–2026*	+	*ND*
1	Receptor	S49542	(5-HT_{2c}-R) mRNA	+	CDS	715–733	+	ND
1	Receptor	Z11597	(5-HT_{1b}-R) mRNA	+	CDS	1573–1603	+	ND
					3′UTR	2223–2248		
1	Receptor	S62907	GABAA-R α2 mRNA	+	CDS	247–271	+	ND
					CDS	1481–1519		
1	Receptor	S62908	GABAA-R α3 mRNA	+	5′UTR	4–35	+	ND
					CDS	431–441		
1	Receptor	M85078	GMCSF-R mRNA	+	3′UTR	1596–1640	+	ND
1	Receptor	M28998	b-FGF-R mRNA	–	NA	NA	ND	ND
1	Receptor	M28233	INFγ-R mRNA	+	3′UTR	1573–1602	ND	ND
					3′UTR	1847–1861		
1	Receptor	L10084	Adrenergic-β1-R mRNA	–	NA	NA	ND	ND
1	Enzyme	NM_006297	XRCC1 mRNA	+	CDS	1721–1770	+[f]	ND
1	Enzyme	U11822	Cdk7/MO15 mRNA	+	CDS	320-358	ND	ND
					CDS	682–708		
1	*Enzyme*	*Z49085*	*Mdk2 mRNA*	+	*3′UTR*	*3551-3647*	*ND*	*ND*
1	Unknown	AF040097	EST	+	NA	1–37	+	+
1	Unknown	AF040098	EST	+	NA	9–60	+	ND
1	Unknown	AF040099	EST	+	NA	145–193	+	+
1	*Misc.*	*M33024*	*Prothymosin-α mRNA*	+	*3′UTR*	*716-749*	+	*ND*
1	Misc.	NM_0000086	Cln3 mRNA	+	CDS	1041–1067	+	ND
1	Unbound	Y00264	βAPP mRNA	+	3′UTR	2701–2802	–	–
					3′UTR	2881–2914		
1	Unbound	NM_00408	Dynamin 1 mRNA	–	NA	NA	ND	–
1	Unbound	NM_052970	Hsp70	–	NA	NA	ND	–
1	Unbound	NM_31144	β-Actin mRNA	–	NA	NA	–	–
1	Unbound	XM003842	G3BP mRNA 1–800	+	3′UTR	1630–1663	–	ND
1	Unbound	K02234	Scrapie mRNA 1–733	+	3′UTR	702–720	–	ND
1	Unbound	M16113	BC-1 mRNA	–	NA	NA	–	ND
2	Channel	NM_004983	K^+ Channel (KCNJ9)	+	3′UTR	1821–1851	ND	ND
					3′UTR	2181–2191		
2	Channel	NM_000720	Ca^{2+} Channel α1	+	CDS	945–961	ND	ND
					CDS	986–1031		
2	Channel	NM_003374	VDAC1	+	3′UTR	967–988	ND	ND

Table 1 (*continued*)

Set[a]	Type	Accession No.	Identity	U-rich[b]	Region[c]	Coordinates[d]	In vitro binding[e]	In vivo binding[e]
					3′UTR	1461–1480		
					3′UTR	1523–1551		
					3′UTR	1681–1740		
2	Signaling	L09159	Rho A	+	3′UTR	1174–1200	+	+
					3′UTR	1279–1316		
					3′UTR	1575–1600		
2	mRNA Processing	NM_005850	SAP49	+	CDS	481–514	ND	ND
					3′UTR	1334–1500		
2	mRNA Processing	NM_002137	HnRNPA2B1	+	3′UTR	1441–1500	ND	ND
					3′UTR	1561–1583		
					3′UTR	1621–1645		
2	Trafficking	NM_004859	Clathrin	+	CDS	2274–2315	ND	+
					3′UTR	5305–1539		
					3′UTR	5423–5464		
2	Trafficking	NM_031483	E3 ubiquitin ligase	+	CDS	1775–1790	ND	ND
					3′UTR	3076–3301		
					3′UTR	4400–4464		
					3′UTR	5403–5445		
2	Misc.	NM_005385	NKTR	+	3′UTR	5871–5937	ND	ND
2	Misc.	NM_017832	FLJ20457	+	3′UTR	981–1011	ND	ND
2	Misc.	BC042625	Luc7 homolog	+	3′UTR	1884–1920	ND	ND
					3′UTR	1954–1982		
					3′UTR	2154–2180		
2	Misc.	XM_167633	DKFZp761F0118	+	CDS	5761–5794	ND	ND
2	Misc.	AB033058	KIAA1232	+	3′UTR	1947–2051	ND	ND
2	Misc.	AF227517	Sprouty-4C	+	3′UTR	847–882	ND	ND
					3′UTR	3001–3061		
					3′UTR	6142–6163		
2	Unknown	AK026293	FLJ22640	+	NA	1–250	ND	ND
						428–601		
						1254–1600		
2	Unknown	AK057112	FLJ32550	+	NA	2461–2507	ND	ND
2	Unknown	AK023131	FLJ13069	+	NA	282–344	ND	ND
						1981–2087		
						3251–3360		
2	Unknown	BC041957	IMAGE:5302100	+	NA	133–174	ND	ND
2	Unknown	BC044624	IMAGE:5288080	+	NA	623–666	ND	ND
						892–971		
						1862–1901		

Italicized mRNAs contain a putative G-quartet.
ND, not determined; NA, not applicable.
[a] Set 1 adapted from Sung et al. [10,23] and Dolzhanskaya et al. [21,24] and recent data, Set 2 adapted from Chen [22].
[b] U-rich sequence comprising >50% U residues and containing U-pentamers.
[c] Region within the mRNA containing the U-rich sequence elements; 5′-untranslated region (5′UTR), coding sequence (CDS), 3′-untranslated region (3′UTR).
[d] Location within the cDNA of the U-rich element(s).
[e] In vitro binding by affinity capture. In vivo binding by isolating the mRNA from FMRP immunoprecipitates.
[f] Also found by Miyashiro et al. [13].

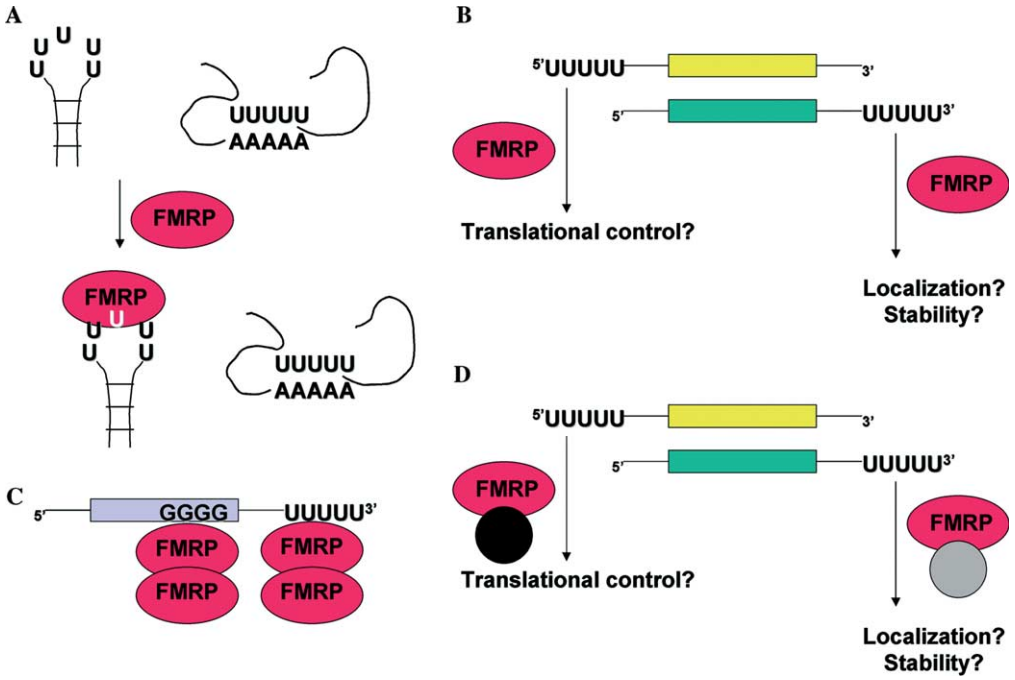

Fig. 1. Possible modes of mRNA binding of FMRP. (A) Accessibility and sequence context may dictate whether FMRP binds to a particular U-pentamer. (B) The location of U-pentamer USER codes within a particular mRNA may affect the outcome of FMRP binding. (C) FMRP may interact with multiple USER codes within a particular mRNA. (D) Other components of the FMRP mRNP may affect the fate of a particular mRNA.

FMRPs/FMR1 mRNA reported by Ashley et al. [26]. Thus, the data presented by these researchers are consistent with a pair of FMRP homodimers (possibly the major form of FMRP in vivo [27]) binding to each of the two sites on FMR1 mRNA, Fig. 1C. Fourth, are mRNAs with different motif compositions parsed into different types of mRNPs? On the basis of yeast-2-hybrid screens and co-immunoprecipitation from cultured cells, FMRP has been shown to associate with more than 14 proteins, and the *Drosophila* ortholog of FMRP, dFXR1, was shown to associate with three more, Table 2. Setting aside known differences in the subcellular localization of some of these proteins (nuclear vs. cytoplasmic), the cytoplasmic protein partners of FMRP, in the absence of mRNA, would form particles that lie far outside the normal size range of mRNPs. This implies that there may be several types of FMRP-containing mRNPs, each with a defined role and whose composition may be regulated: (1) both spatially and temporally within a particular cell, (2) during development, and (3) by second messenger stimuli [28–30]. In addition, tissue- and cell-type-specific differences may also play a role in determining the composition of FMRP-containing mRNPs, Fig. 1D. Indeed, global profiling studies that have focused on identifying mRNA populations in mRNPs have found that there exist multiple populations of mRNPs that contain sets of related mRNAs and that the composition of these mRNPs can vary significantly depending on the state of the cell [31–34]. Based on these data these authors have hypothesized that specific untranslated sequence elements for regulation (USER codes) allow particular messages to segregate to a specific mRNP. Different combinations of USER codes in a single message allow differential regulation of mRNAs depending on various cellular cues [35]. Fifth, the in vitro binding profiles of

Table 2
FMRP-associated proteins

Protein	Accession No.	Molecular weight (kDa)
FXR1P	NP_0055191	70–80
FXR2P	NP_004851	94
hnRNP A1	NP_002127	34–38
Nucleolin	NP_035010	100
Yb1/p50	P27817	50
CYFIP1	NP_055423	145
CYFIP2	NP_598530	146
NuFIP	NP_036477	56
Rac1	NP_776588	23
Purα	AAA60229	42
mStaufen	AAH12959	54
Myosin VA	NP_034994	205
Purβ	AAK72462	39
PABP	NP_006442	53.5
AGO-2	NP_730054	137
VIG	AAN10889	53
P68	P19109	68
Total		1369.5–1383.5

several FMRP isoforms differ significantly from one another [36] and recent work has shown that isoform-specific differences in FMR1 mRNA can be distinguished in different brain regions in mice. Furthermore, alternative splicing appears to be a common mechanism for generating isoforms with particular locations within neurons [37–39]. Do FMRP's various isoforms bind and regulate different sets of messages (soma vs. dendrites), or do they bind a common set of messages but regulate them differently? Sixth, are U-rich target messages involved directly in generating the fragile X phenotype? Based on their data, Chen et al. have hypothesized that a combination of "sub-threshold effects" resulting from multiple changes in gene expression is necessary for eliciting the neuronal alterations involved in fragile X syndrome. Certainly, many of the receptor mRNAs shown in Table 1 have obvious ties to synaptic plasticity, e.g., the L-type voltage sensitive Ca^{2+} channel $\alpha 1$ has a role in memory formation [40]. Additionally, $GABA_A$ receptor reduction has been posited as the mechanism by which fragile X mice, and by implication human patients, are to have increased susceptibility to seizures [41]. Finally, cell-signaling proteins such as sprouty 4c are known to be intimately involved in differentiation by preventing *ras* activation [42,43].

Answering these questions will go a long way in determining the molecular basis of fragile X syndrome.

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