

The Fragile X Syndrome Protein FMRP Associates with *BC1* RNA and Regulates the Translation of Specific mRNAs at Synapses

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Summary

The Fragile X syndrome, which results from the absence of functional FMRP protein, is the most common heritable form of mental retardation. Here, we show that FMRP acts as a translational repressor of specific mRNAs at synapses. Interestingly, FMRP associates not only with these target mRNAs, but also with the dendritic, non-translatable RNA *BC1*. Blocking of *BC1* inhibits the interaction of FMRP with its target mRNAs. Furthermore, *BC1* binds directly to FMRP and can also associate, in the absence of any protein, with the mRNAs regulated by FMRP. This suggests a mechanism where *BC1* could determine the specificity of FMRP function by linking the regulated mRNAs and FMRP. Thus, when FMRP is not present, loss of translational repression of specific mRNAs at synapses could result in synaptic dysfunction phenotype of Fragile X patients.

Introduction

FMRP is an RNA binding protein highly expressed in the brain. Absence or mutation of FMRP leads to the Fragile X syndrome, an X-linked dominant disorder and the most frequent cause of inherited mental retardation (1 in 4000 males and 1 in 6000 females). The syndrome is characterized by mental retardation of variable severity, autistic behavior, macroorchidism in adult males, characteristic facial features, and hyperextensible joints (for recent reviews see Bardoni and Mandel, 2002; O'Donnell and Warren, 2002; Oostra, 2002). The syndrome is mostly associated with an unstable expansion of a CGG repeat located in the 5' UTR of the fragile X mental

retardation gene (*FMR1*), which leads to an abnormal methylation pattern that frequently causes transcriptional silencing of the gene (Verkerk et al., 1991; Oberlè et al., 1991; Sutcliffe et al., 1992). In addition, rare atypical cases of Fragile X syndrome have been reported that are not associated with an amplification of the trinucleotide repeat, but with deletions or single point mutations (Gedeon et al., 1992; Meijer et al., 1994; Hirst et al., 1995; De Boule et al., 1993; Wohrle et al., 1992; Milà et al., 2000).

The FMRP protein contains several RNA binding domains including two KH motifs and one RGG box. As expected from this domain structure, FMRP binds RNA homopolymers and mRNAs in vitro (Ashley et al. 1993; Siomi et al., 1994; Brown et al., 1998; Adinolfi et al., 1999). In particular, FMRP associates with mRNAs and binds to 4% of human fetal brain mRNAs, including the myelin basic protein mRNA (Brown et al., 1998). Further mRNAs that may associate with FMRP and that compose a very heterogeneous family have been recently reported (Sung et al., 2000; Brown et al., 2001). Moreover, specific binding was demonstrated to its own mRNA in a region that may form a particular structure called the purine quartet (Ashley et al., 1993; Schaeffer et al., 2001; Darnell et al., 2001). In mammalian organisms, there are two FMRP homologs, FXR1P and FXR2P (Zhang et al., 1995), which are thought to have distinct but overlapping functions. For example, the pattern of expression in brain and testis as well as the subnuclear distribution mainly overlaps but shows some differences (Tamanini et al., 1997). Several proteins have been shown to interact with FMRP. In the cytoplasm, these include FXR1P and FXR2P and the two proteins CYFIP1 and CYFIP2 (Schenck et al., 2001), while different proteins interact with FMRP in the nucleus (Bardoni et al., 1999; Ceman et al., 1999, 2000). The presence of an NLS and an NES suggests that FMRP may function as a shuttle for mRNA export from nucleus to cytoplasm. Moreover, several lines of evidence suggest that FMRP could modulate stability and/or translation of its target mRNAs in the cell body and also at the synapses. First, FMRP was found to associate with ribosomes in the cell body and in dendrites (Eberhart et al., 1996; Khandjian et al., 1996; Tamanini et al., 1996; Corbin et al., 1997; Feng et al., 1997; Weiler et al., 1997; Greenough et al., 2001). Second, human mRNAs with altered polysomal profiles have been identified by probing microarrays using mRNA isolated from polyribosomes of a human fragile X lymphoblastoid cell line (Brown et al., 2001). Third, two different groups have shown that FMRP functions as a nonspecific repressor of translation in vitro (Li et al., 2001; Lagerbauer et al., 2001) and, more recently, in cotransfection experiments (Mazroui et al., 2002). Finally, it has been shown that the *Drosophila* FMRP regulates *futsch*, a homolog of the mammalian *MAP1B* mRNA, probably at the level of translation (Zhang et al., 2001). However, it is not understood how the translational regulation by FMRP works and whether it exhibits a selectivity for certain mRNAs in vivo.

Here, we show using the *FMR1* knockout (KO) mouse

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model that FMRP is a repressor of translation *in vivo* and regulates translation of specific dendritic mRNAs, including those encoding the cytoskeletal proteins *Arc*/*Arg3.1* and *MAP1B*, and the kinase α -*CaMKII*. FMRP is found in a ribonucleoprotein complex that also contains the small dendritic non-translatable RNA *BC1*. *BC1* binds directly to FMRP and can also associate specifically with mRNAs regulated by FMRP. These results strongly suggest that *BC1* RNA recruits FMRP to the targeted mRNAs, thereby determining the specificity of FMRP action.

Results

FMRP Is a Repressor of Translation at Synapses

FMRP has recently been shown to be a translational repressor in reticulocyte extracts (Laggerbauer et al., 2001; Li et al., 2001). To address the function of FMRP in the neuronal cell, we analyzed the translational efficiency of specific neuronal mRNAs of wild-type as well as *FMR1* knockout mice (Bakker et al., 1994). We selected the mRNAs encoding the following proteins: *MAP1B*, the microtubule associated protein 1B, because its *Drosophila* homolog has recently been suggested to be translationally regulated by FMRP (Zhang et al., 2001); *MAP2*, the microtubule associated protein 2 (Garner et al., 1988); α -*CaMKII*, the α subunit of Ca^{2+} /calmodulin-dependent protein kinase II, whose function is associated to synaptic plasticity (Soderling, 2000) and which is highly expressed at the synapses where its mRNA is translationally regulated (Bagni et al., 2000); *Arc*, the activity-regulated cytoskeleton associated protein, also known as *Arg3.1* (Lyford et al., 1995; Link et al., 1995); the two FMRP homologs, *FXR1P* and *FXR2P*; *GlyR α 1*, the α 1 subunit of the glycine receptor, and *GluR1*, a subunit of the ionotropic glutamate receptor AMPA, because they are thought to play a role in synaptic plasticity (Muller et al., 2002); and, as a control, the small dendritic RNA *BC1* (Tiedge et al., 1991). Since *BC1* does not encode a protein, it should not associate with translating polyribosomes.

A reliable way to assess mRNA translational efficiency is to analyze its partitioning between actively translating polysomes and mRNPs that are not translated (Bagni et al., 2000). To gauge the translational efficiency, the percentage of a given mRNA associated with polysomes (PMP = Percent of Messenger on Polysomes) is quantified. Cytoplasmic extracts, prepared from a whole brain were fractionated through sucrose gradients (see Experimental Procedures). Ten fractions were collected from each gradient while recording the absorbance profile (Figure 1A). Total RNA was extracted from the gradient fractions and then analyzed by quantitative RT-PCR for specific mRNAs. To correct for variations in the efficiency of the RT-PCR reaction, the same amount of a synthetic RNA was added to each sample, amplified, and used for normalization. Exemplarily, the analysis of the α -*CaMKII* mRNA, *BC1*, and the synthetic control RNA is shown for the total brain extracts and the synaptoneurosomes preparation (see below). In each set of panels, the top row shows the distribution of the α -*CaMKII* mRNA, the middle row *BC1*, and the bottom the control RNA.

Quantification demonstrated that some of the dendritic messenger RNAs are translated more efficiently in the *FMR1* KO mice compared to the wild-type mice (Figure 1B). In particular, the PMP for *Arc* increases by 38% in KO mice while that for α -*CaMKII* and *MAP1B* increases by 13% and 17%, respectively. The mRNAs of the two *FMR1* homologs, *FXR1* and *FXR2*, are also translated more efficiently in *FMR1* KO mice compared to the wild-type, presenting a 22% increase in their PMP. Significantly, this is not a general phenomenon as the distribution of mRNAs encoding *GlyR α 1*, *GluR1*, *MAP2*, β -*Actin*, and the non-translatable dendritic *BC1* RNA on the polysome gradient do not change significantly (Figure 1B). Moreover, *Ferritin* mRNA, which is involved in iron metabolism and translationally repressed (Preiss and Hentze, 1999), shows no difference in wild-type mice compared to *FMR1* KO mice.

The data are highly reproducible, and they strongly indicate that FMRP is a repressor of specific mRNAs in the brain. Since FMRP is present at synapses (Weiler et al., 1997), we wanted to verify if FMRP is also a translational repressor at synapses where protein synthesis is known to occur (for reviews see Martin et al., 2000; Steward and Schuman, 2001; Richter and Lorenz, 2002). Therefore, we performed polysome/mRNPs analysis of extracts from synaptoneurosomes as described above for the total brain. These preparations are highly enriched in synaptic termini and virtually devoid of cell body contamination (Bagni et al., 2000).

We found that the translation of some dendritically localized mRNAs is also increased in purified synaptoneurosomes and the increase is, in fact, even higher than in total brain. In particular, the PMPs of *Arc* and α -*CaMKII* mRNAs increase by 34% and 53%, respectively (Figure 1B). The *MAP1B*, β -*Actin*, and *Ferritin* mRNAs were not detectable in the synaptoneurosomes under the same PCR conditions used to amplify the dendritic mRNAs, while the analysis of *GlyR α 1*, *GluR1*, and *MAP2* was not performed on the synaptoneurosomes because they did not show any significant difference in total brain extracts of wild-type and *FMR1* KO mice. In conclusion, several, but not all, mRNAs are translated more efficiently in *FMR1* KO mice, and this effect is significantly stronger in isolated synaptoneurosomes as compared to the total brain extracts.

To determine whether the translational upregulation also leads to higher levels of the proteins in question, we assayed equal amounts of the respective extracts by quantitative immunoblotting. β -*Actin* was used as a control; as expected, its abundance is not affected in the *FMR1* KO (Figure 1C). The level of the other assayed proteins reflects quite well the translational efficiency of their mRNAs. Thus, *Arc*, *FXR2P*, and *MAP1B* are more abundant in total brain extracts from *FMR1* KO versus wild-type mice, and the effect was much more pronounced in the synaptoneurosomes preparation. For α -*CaMKII*, the increase was observed only in the synaptoneurosomes.

Surprisingly, a significant part of the synaptosomal *BC1* RNA cosediments with the polysomes of *FMR1* KO, but not of wild-type mice (Figures 1A and 1B). This shows a clear dysregulation of *BC1* RNA and demonstrates that *BC1* RNA has the ability to associate with large complexes, possibly the translating ribosomes,

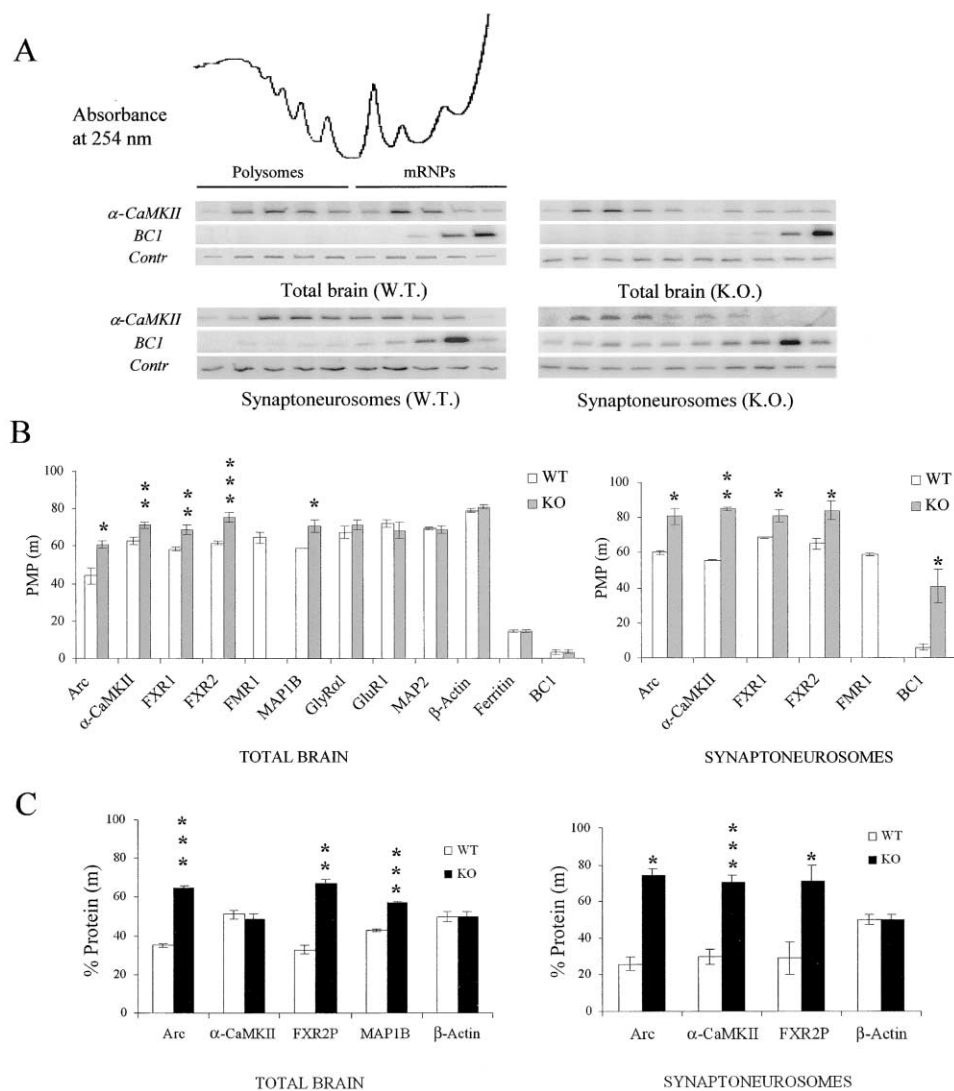


Figure 1. Polysome/mRNPs Distribution of Dendritic and Cell Body mRNAs

Mouse total brain and synaptoneurosomes extracts were fractionated by sucrose gradient centrifugation. Each gradient was collected in ten fractions, and an equal amount of synthetic RNA was added to each fraction and then amplified by radioactive RT-PCR. Radioactive signals in the polysomal and non-polysomal fractions were quantified, corrected versus control RNA, and expressed as PMP (Percentage Messenger on Polysome). (A) Typical polysomal profile from total brain cytoplasmic extracts and example of RT-PCR reaction performed to detect α -CaMKII mRNA and BC1 RNA association with the polysomal gradient. (B) Polysome/mRNPs analysis in total brain extracts and in synaptoneurosomes. Values shown are the mean \pm SEM ($n \geq 3$). (C) Protein level analysis in total brain extracts and synaptoneurosomes. Values shown are the mean \pm SEM. The quantitation was repeated three times. *, $p < 0,05$; **, $p < 0,01$; ***, $p < 0,001$ for KO versus W.T. by Student's t test in all figures.

but this association is inhibited in wild-type mice by the FMRP protein.

FMRP Associates Mainly with Ribonucleoparticles

Previous studies on non-neuronal cell lines have shown FMRP to be present both in fractions containing the polyribosomes and in fractions containing the monomeric 80S ribosome (Eberhart et al., 1996; Feng et al., 1997; Khandjian et al., 1996; Tamanini et al., 1996; Siomi et al., 1996; Corbin et al., 1997; Brown et al., 2001; Mazroui et al., 2002). Considering the possibility of a brain-specific repressor complex, we investigated where

FMRP sediments on sucrose gradients of brain extracts. We fractionated cytoplasmic extracts on a continuous sucrose gradient, as performed to study mRNA translation, and analyzed FMRP distribution by Western blot using polyclonal antibodies against the C terminus of the human FMRP. This region exhibits no homology to the FXR proteins, and the antibodies do not cross-react with the FXR proteins (see Experimental Procedures). Interestingly, FMRP mainly cosediments with the monomeric 80S ribosomes and with mRNPs in the upper fractions of the sucrose gradient (Figure 2). A very similar pattern of FMRP distribution was detected in a gradient derived from synaptoneurosomes (Figure 2). This distribution is

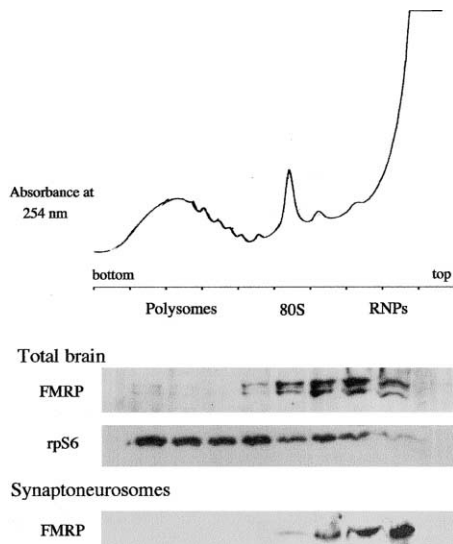


Figure 2. Polysome/mRNPs Distribution of FMRP Protein
 Mouse total brain and synaptoneurosomes extracts were fractionated by sucrose gradient centrifugation. Each gradient was collected in ten fractions. Absorbance profile of the sucrose gradient from total brain showing the polysomal pattern is reported in the upper part of the figure. An aliquot (same volume) of each fraction was analyzed by Western blot using FMRP antibody (rAM1) and, as control, an antibody for the ribosomal protein S6.

consistent with a role of FMRP as repressor of translation. As control for our experiments, we used the ribosomal protein S6 that cosedimented perfectly with the polysomes and, as expected, with the 40S ribosomal subunit. This clearly shows that, in brain, FMRP forms complexes different from those previously reported for non-neuronal cell lines, leading to the initial hypothesis that in the latter cell lines, the repressor complex is absent or less stable.

The Small Dendritic RNA *BC1* Associates with FMRP

The finding that *BC1* significantly changes its partitioning on the polysome gradients in the absence of FMRP prompted us to investigate whether *BC1* is associated with FMRP. For this purpose, we specifically immunoprecipitated the FMRP-RNP particle. Antiserum directed against the FMRP C terminus, but not preimmune serum, efficiently immunoprecipitates FMRP from mouse brain extracts (Figure 3A). Efficient immunoprecipitation requires elevated salt concentrations possibly because the C terminus is masked at physiological salt concentrations; nevertheless, the antibodies precipitate a protein complex that also contains FXR1P, a known FMRP interactor (Figure 3A). To elucidate if the small dendritic RNA *BC1* is part of the FMRP complex, total brain extracts were used to immunoprecipitate FMRP. Analysis of coprecipitating RNA by RT-PCR showed that FMRP can specifically immunoprecipitate *BC1* RNA as well as *Arc* and *MAP1B* mRNAs, whereas the α -*Tubulin* mRNA is not immunoprecipitated (Figure 3B). The binding specificity was demonstrated by using extracts from the *FMR1* KO mice (lanes 6, 8, 10, 12) as well as from preimmune serum (data not shown), which did not im-

muno-precipitate any of the tested RNAs. For these experiments we used stringent salt conditions (250 and 350 mM NaCl). Thus, we conclude that *BC1* RNA, *Arc* and *MAP1B* mRNAs specifically associate with FMRP.

A potential *BC1* analog in primates is called *BC200* RNA (Martignetti and Brosius, 1993; Tiedge et al., 1993). *BC200* RNA, like rodent *BC1* RNA, associates with protein(s) to form an RNP complex (Cheng et al., 1997). Distribution of the human *BC200* reveals a neuron-specific expression and dendritic localization (Tiedge et al., 1993), suggesting a role in dendritic RNA transport and/or translation. This prompted us to investigate whether *BC200* is associated with human FMRP. We specifically immunoprecipitated the FMRP-RNP particle from human neuroblastoma, glioma, and lymphoblast cell line extracts (Figure 3C, left panel), and *BC200* was detected by RT-PCR in extracts from glioma and neuronal cell lines (Figure 3C, right panel, lanes 4 and 5), but not in extracts of lymphoblast cell lines (lane 6).

FMRP Binds Directly to *BC1*

To assess whether FMRP interacts directly with the *BC1* RNA, we performed electrophoretic mobility shift assays (EMSA) using in vitro transcribed, radiolabeled *BC1* RNA and purified, recombinant FMRP protein or brain extracts (Figure 4). Addition of FMRP protein to the RNA leads to formation of an RNP complex that migrates more slowly on the native polyacrylamide gel than the RNA alone (compare lanes 1 and 2). This complex indeed contains the FMRP protein since it is shifted by anti-FMRP antiserum (lane 3). The binding of FMRP to *BC1* RNA is specific and stoichiometric since the complex can be easily competed by a 100-fold excess of unlabeled *BC1* RNA, but not by nonspecific competitor tRNA (lanes 6 and 7). It should be noted that recombinant FMRP is stable only at 1 M salt concentrations. Since the protein preparation makes up 75% of the binding reactions, the binding occurs at 750 mM salt. This high salt stringency underlines the specificity of the interaction. At the same time, only a small part of the *BC1* RNA is bound (25%), despite the 2000-fold excess of the protein. While this may indicate either that the majority of the RNA is folded in an unfavorable structure or that the high concentration may be too far from physiological conditions, we believe that the binary complex has a rather high dissociation constant and needs to be stabilized by additional proteins (see Discussion). This notion is supported by our finding that *BC1* RNA predominantly forms a complex in brain extracts that is bigger than the binary complex (compare lanes 2 and 4). This complex contains FMRP protein, as judged by its shift upon addition of anti-FMRP antibodies.

BC1 Mediates the Interaction between FMRP and the Regulated mRNAs

Since *BC1* RNA associates with FMRP, it might well target, via base-pairing, FMRP to the mRNAs that are to be regulated. To support this hypothesis, we searched for regions of complementarity between *BC1* and the regulated mRNAs. We found that nt. 3-21 and nt. 47-70 of *BC1* RNA are predicted to basepair almost perfectly to *MAP1B* mRNA, while the overlapping region nt. 62-77 is predicted to basepair with α -*CaMKII* mRNA, and the region

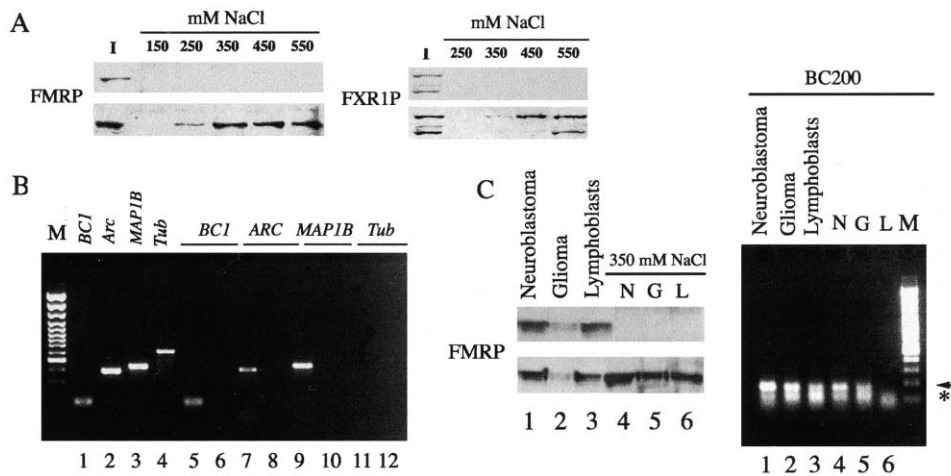


Figure 3. *BC1* RNA, *Arc*, and *MAP1B* mRNA Are Associated with FMRP Complex

(A) Brain lysates were prepared from wild-type mice and immunoprecipitated in presence of different salt conditions with rAM1 antibody and probed with 1C3 anti-FMRP. Preimmune serum was used for IP in the upper panel. Similar immunoprecipitates were probed with antibodies directed against the paralog FXR1P.

(B) Immunoprecipitations were performed from wild-type (lanes 5, 7, 9, 11) and *FMR1* KO mice extracts (lanes 6, 8, 10, 12). RNA was extracted, DNase-treated and RT-PCR was performed using specific oligos for *BC1* RNA, *Arc*, *MAP1B*, and α -*Tubulin* mRNAs. Shown is the product of the PCR reactions. Lanes 1, 2, 3, and 4 contain 1/3 of the input (the mRNAs are equally present in the W.T. as well as in the KO extracts).

(C) Immunoprecipitations were performed from human neuroblastoma, glioma, and lymphoblast cell line extracts with rAM1 antibody and probed with IC3 anti-FMRP (left, upper is preimmune, lanes 1, 2, and 3 contain 1/3 of the input). In a parallel experiment, RNA was extracted, DNase treated, and RT-PCR was performed using specific oligos for *BC200* RNA. The arrow points to the specific *BC200* product while the asterisk points to a nonspecific product (oligo concatamers). M = 100 bp.

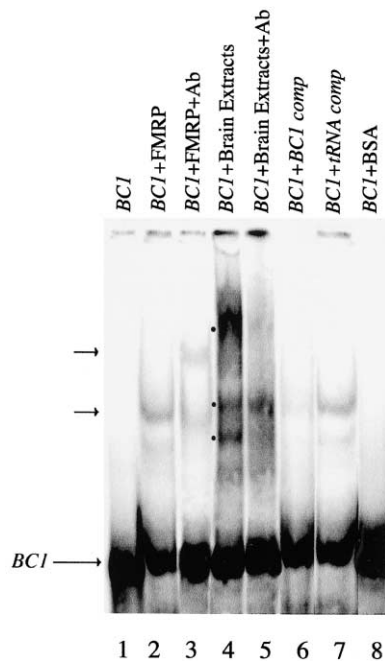


Figure 4. FMRP Binds Directly to *BC1* RNA

EMSA was performed with 32 P-labeled *BC1* RNA and purified FMRP or brain extracts. A retarded band is shown (lane 2) due to FMRP interaction with *BC1*. This band is supershifted after incubation with FMRP (rAM1) Ab (lane 3). Three major complexes (black dots) are formed incubating *BC1* with brain extracts (lane 4), and two of them are destroyed after incubation with rAM1 Ab (lane 5). Competition experiments are reported in lanes 6 and 7 with non-labeled *BC1* and *tRNA*, respectively. A reaction with BSA alone is shown in lane 8.

between nt. 2-20 of *BC1* RNA is predicted to basepair to *Arc* mRNA (Figure 5A). Interestingly, the sequence complementarity is found at the base of the longer stem loop, according to a stable predicted secondary structure of *BC1* RNA (Rozhdetsvensky et al., 2001, Figure 5B). β -*Actin* mRNA, in contrast, is not regulated by FMRP and shows no significant complementarity to *BC1* RNA.

For further support of a direct interaction between *BC1* RNA and the mRNA targets, we designed a 21mer DNA oligonucleotide that anneals to *BC1* RNA in the region of complementarity to *MAP1B* mRNA, which has the longest region of homology among the targeted mRNAs (the region covering 47-70, oligo *BC1*-sl-1). Total brain cytoplasmic extracts were incubated at 37°C with the *BC1* oligo. Then FMRP was precipitated, and the associated RNAs were analyzed by RT-PCR. Initially, two concentrations of oligos corresponding to an estimated 150 and 750 molar excess of *BC1* were used; since both gave the same result, we used the lower concentration in the subsequent experiments.

As shown in Figure 6A, annealing of the specific oligo, as opposed to a control oligo directed against β -*Actin* mRNA, reduced coprecipitation of *BC1* with FMRP. To verify whether this reduction was due to either a destruction of *BC1* by an RNaseH activity in the extract or to an interference of the oligo with FMRP binding to *BC1* RNA, we performed the same experiment using a 2'-O-methylated RNA oligo (O-Me-*BC1* sl-1) that does not induce RNase H degradation of the target mRNA and observed the same reduction in FMRP-*BC1* complex (Figure 6A). Importantly, no *MAP1B* mRNA was detected under saturating PCR conditions when the extract was treated with the specific *BC1* DNA oligo sl-1, whereas treatment with the control oligo caused only a minor

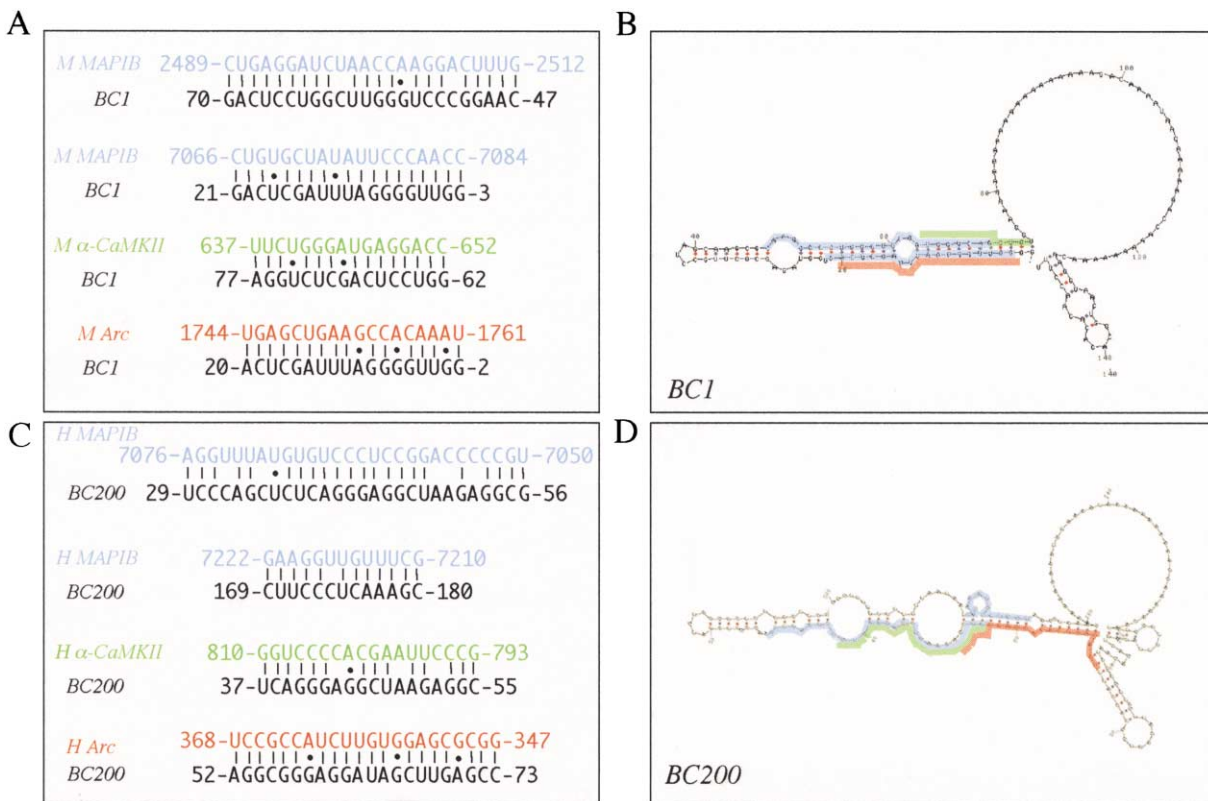


Figure 5. Homology between *BC1* RNA Sequence and the Regulated mRNAs

- (A) Mouse *BC1* exhibits sequence complementarity to *Arc*, α -*CaMKII*, and *MAP1B* mRNAs.
 (B) Secondary structure of *BC1* RNA has been performed using the program FoldRNA. *BC1* sequence is shown in black, while the complementarity of *Arc*, α -*CaMKII*, and *MAP1B* mRNAs with *BC1* RNA are reported in red, green, and blue, respectively.
 (C) Sequence complementarity between human *BC200* and the human *Arc*, α -*CaMKII*, and *MAP1B* mRNAs.
 (D) Secondary structure of *BC200* RNA has been performed using the program FoldRNA.

nonspecific reduction of *MAP1B* mRNA (Figure 6B). Therefore, we conclude that *BC1* is required for the association of *MAP1B* mRNA with FMRP. Moreover, the oligo sl-2 directed against the 3' stem loop of *BC1* does not inhibit the FMRP/*MAP1B* association. These data demonstrate the importance of the *BC1* region that is complementary to the target mRNA.

To assay whether *BC1* RNA and FMRP-targeted mRNAs interact in the absence of proteins, an in vitro annealing reaction was performed with purified total brain RNAs and biotinylated *BC1* RNA. As shown in Figure 6C, *Arc*, α -*CaMKII*, and *MAP1B* mRNAs coprecipitated with biotinylated *BC1* RNA. The specificity of this experiment is shown by the absence of in vitro interaction between *BC1* and the neuronal mRNAs like *Glur1*, *GlyR α 1*, and *MAP2* (neuronal mRNAs that are not regulated at the translational level by FMRP). This finding strongly suggests a base-pairing interaction between *BC1* and the mRNAs.

Discussion

FMRP Represses the Translation of Target mRNAs at Synapses

While it is clear that loss of FMRP protein is associated with the mental retardation of Fragile X (FRAXA) patients,

it is still not understood how the lack of this protein causes such a severe clinical phenotype. The morphological anomaly in the brain of both FRAXA patients and *FMR1* knockout mice appears to be limited to the presence of abnormal dendritic spines, which is reminiscent of a delay in maturation (Hinton et al., 1991; Comery et al., 1997). In the *FMR1* KO mice, longer and denser dendritic spines are observed, consistent with the human phenotype (Nimchinsky et al., 2001). It has been shown that the level of FMRP increases near synapses in response to neurotransmitter activation (Weiler et al., 1997), thus FMRP is thought to have an effect on maturation and/or function of the synapses (Greenough et al., 2001). Since both mRNAs and the protein synthesis machinery are present in dendrites and near postsynaptic sites (Steward and Schuman, 2001), it is now clear that local and regulated synthesis of key synaptic proteins plays an important role in synapsis maturation and neuronal development.

Interestingly, we show that the mRNAs encoding FMRP and the two homologs FXR1P and FXR2P are translated at synapses (Figure 1B). The fact that these proteins are known to interact (Zhang et al., 1995; and Figure 3A) and the fact that all three proteins are synthesized locally at the synapses suggests that these complex(es) can also form in the distal region of the neuron.

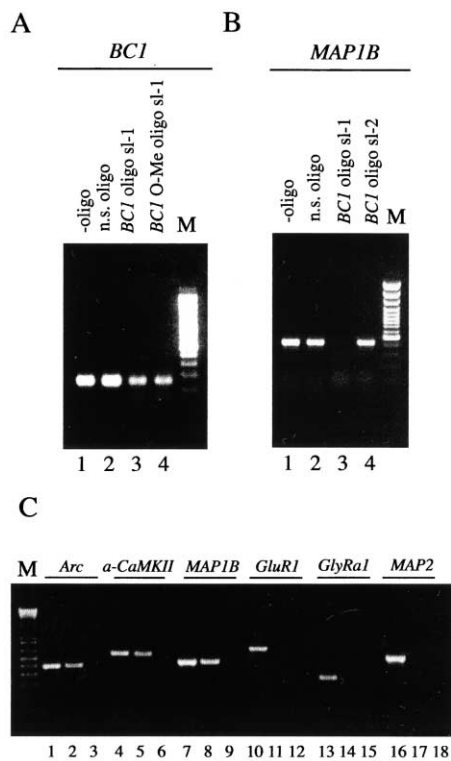


Figure 6. *BC1* RNA Interacts with FMRP-Targeted mRNAs
Total brain cytoplasmic extracts were incubated in the absence of oligonucleotides (lanes 1), in the presence of an oligo complementary to an unrelated RNA (lanes 2), of an oligo complementary to *BC1* RNA in the stem loop 1 (lanes 3), or of a modified RNA oligo O-Me-RNA (lane 4). Then, FMRP was immunoprecipitated and co-precipitating *BC1* RNA (A) or *MAP1B* mRNA (B) was amplified by RT-PCR. Shown is the product of the PCR reaction fractionated on an agarose gel and stained with ethidium bromide. Brain extracts were also incubated in presence of an oligo complementary to *BC1* RNA in the stem loop 2, FMRP was immunoprecipitated, and co-precipitating *MAP1B* mRNA amplified by RT-PCR (Figure 6B, lane 4). M = 100 bp. (C) Biotinylated *BC1* RNA was incubated with isolated total RNA from mouse brain and RNAs annealed to *BC1* RNA were selected on streptavidin beads. RNA was extracted and RT-PCR was performed using specific oligos for *Arc*, α -*CaMKII*, *MAP1B*, *GlyR α 1*, *GluR1*, and *MAP2* mRNAs. Shown is the product of the PCR reactions (lanes 2, 5, 8, 11, 14, 17). Lanes 1, 4, 7, 10, 13, and 16 contain 1/10 of the input. Lanes 3, 6, 9, 12, 15, and 18 show the same experiment but performed in the absence of *BC1* RNA. M = 1 kb plus.

Recent evidence shows that FMRP is a dose-dependent inhibitor of mRNA translation in reticulocyte extracts and *Xenopus* oocytes (Laggerbauer et al., 2001; Li et al., 2001, Schaeffer et al., 2001). In vivo, FMRP could exert a more specific effect. In fact, elegant studies using *Drosophila* genetics showed that dFXR, the homolog of mammalian FMRP, negatively regulates the expression of Futsch, a homolog of the mammalian microtubule-associated protein MAP1B (Zhang et al., 2001). Here, we show that translation of specific mRNAs is up-regulated in *FMR1* knockout mice. Significantly, the translational repression by FMRP is much stronger in isolated synaptoneurosome than in total brain extracts (Figure 1B). Interestingly, the translational repression acts specifically only on a subset of neuronal mRNAs and affects

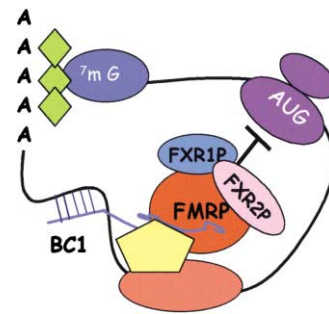


Figure 7. Model of FMRP Function
The model proposes a direct interaction between *BC1* RNA and the targeted regulated mRNAs. The FMRP RNP is thereby brought into vicinity of the initiation codon and blocks the translation. Green rhombi represent Poly(A) binding protein, while the orange and yellow figures represent unknown FMRP protein partners.

mRNAs encoding key regulatory proteins, which could explain how the lack of FMRP could impair the function of synapses.

***BC1* RNA as a Guide to Target FMRP to the mRNAs that Are Regulated**

Several studies have uncovered a variety of mechanisms through which mammalian gene expression can be regulated at the level of translation. In many cases, this happens at the initiation step, where factors can interfere, for example, by phosphorylation or proteolytic cleavage of initiation factors, or by binding to specific sequence elements in the mRNAs (Preiss and Hentze, 1999). Evidence has been reported that FMRP can prevent the formation of the initiation complexes (Laggerbauer et al., 2001).

Here, we present a model in which *BC1* RNA determines the specificity of FMRP repression (Figure 7). *BC1*, a non-translatable RNA, is part of an RNP in the brain (Cheng et al., 1996, 1997). The specific location of *BC1* RNA may indicate a functional role related to translational processes in the somatodendritic compartments of neurons (Tiedge et al., 1991). Part of *BC1* RNA, the ID region, is derived from tRNA^{Ala}, which has been recruited or adapted into a novel function (Brosius and Gould, 1992). Therefore, it is conceivable that *BC1* RNA may interact with ribosomes and may be involved in regulating protein synthesis. Moreover, tissue-specific expression of *BC1*-like RNA is conserved across mammalian species of considerable evolutionary distance, suggesting that the RNA products have an important cellular function.

BC1 RNA cofractionated to a significant degree with the polysome fraction in absence of the FMRP protein. This was observed only in the synaptosomal preparations (Figure 1B), thus linking *BC1* RNA to FMRP regulation at synapses. Further analysis demonstrated that *BC1* RNA coprecipitates with the FMRP protein (Figure 3B) and that it can bind directly to FMRP (Figure 4), establishing a physical link between the two molecules. This association appears to be rather tight, since it was observed at stringent salt concentrations in immunoprecipitation and band shift experiments. Thus, the interesting model arises in which *BC1* RNA binds to FMRP and

also associates with mRNAs, thus bridging FMRP and the mRNAs (Figure 7). In this way, *BC1* might well be responsible for targeting FMRP to the mRNAs that are to be regulated. Since FMRP is thought to inhibit translation at the initiation step, this model predicts that only a minor fraction of *BC1* coassociates with actively translating ribosomes and that this fraction should significantly increase when FMRP and, hence, the block of translation is removed. This is indeed the case. Second, the model predicts that *BC1* RNA would bind to those mRNAs regulated by FMRP. We found significant stretches of complementarity between *BC1* RNA and *MAP1B*, α -*CamKII*, and *Arc* mRNAs, and these regions are located on the longer stem loop of the *BC1* RNA (Figures 5A and 5B). To verify our assumption, we interfered with the putative *BC1*/mRNA interaction using RNA oligos and demonstrated that the interaction of FMRP with its target mRNA is lost. Only *BC1* stem loop 1 is required for *BC1* RNA interaction with the neuronal mRNAs analyzed. In this respect, it is interesting to note that *BC1* stem loop 1 has been shown to be required for *BC1* localization to dendrites (Muslimov et al., 1997). Finally, we show that the association between *BC1* RNA and FMRP-regulated mRNAs can occur in vitro in absence of any protein. The binding is highly specific because it occurs in presence of a large population of mRNAs.

Very recently, it has been shown using the IRES system that *BC1* RNA can repress translation (Wang et al., 2002). This information supports the role of FMRP-*BC1* complex in the repression of synaptic mRNA translation.

A potential analog of *BC1* in primates is called *BC200* RNA. Distribution of the human *BC200* reveals a neuron specific expression and dendritic localization, suggesting a role in dendritic RNA transport and/or translation. Like *BC1* RNA, *BC200* RNA can be divided into three structural domains: a repetitive element, a central region, and a unique region. A possible secondary structure, using computer prediction (Zuker, 1989), revealed high structural homologies to *BC1* RNA (Figure 5D) and, more interestingly, we could detect a strong complementarity between *BC200* and human *Arc*, α -*CaMKII*, and *MAP1B* mRNAs. We have shown here that *BC200* is also able to form a complex with FMRP in neural tumor cells, so it is highly possible that the two *BC* RNAs have the same functional significance.

In conclusion, we suggest that the specificity of FMRP translational repression is defined through base-pairing interactions of the associated *BC1* RNA. In this model, one of the RNA binding domains of FMRP is most likely responsible for binding to *BC1* (Figure 7). Another model has recently been reported in which FMRP binds directly to its target mRNAs. In particular, it has been shown that FMRP binds in vitro to mRNAs that can form G quartet structures (Darnell et al., 2001; Schaeffer et al., 2001). We think that the two binding modes could both occur in the cell, and could possibly be linked to different functions, e.g., translation and transport of the mRNAs. This hypothesis is supported by our band shift experiments using brain extracts, since several complexes are formed on the *BC1* RNA and only one is clearly shifted by FMRP Ab. We find it interesting that the part of *BC1* RNA just before the poly(A) stretch is rather pyrimidine rich and could thus base-pair with a poly(G) stretch like

the one identified as a possible G quartet. Therefore, the putative G quartet structures in the target mRNAs could be bound by *BC1* RNA.

The pathology in the brain of both FRAXA patients and *FMR1* knockout mice appears to be limited to abnormalities in the dendritic spines. Since local protein synthesis is required for synaptic development and function, the repressor role of FMRP likely underlies the behavioral and developmental symptoms of FRAXA patients. The fact that FMRP depletion does not have a lethal effect suggests that FMRP is a regulator only of a subset of mRNAs whose translation occurs in dendrites. We believe that these data offer new and important insights into the molecular mechanisms of the Fragile X syndrome and translational regulation at synapses.

Experimental Procedures

Antisera

Rabbit polyclonal antiserum rAM1 was raised and affinity purified against the hexahistidine-tagged C terminus of FMRP. The antibodies were checked by Western blotting, ELISA, and immunoprecipitation. Monkey COS-7 cells were transfected with full-length *FMR1* (ISO7) or the isoform lacking the C terminus (ISO4) and extracts analyzed with rAM1 and m1C3 antibodies to validate the C terminus specificity.

Cell Culture

Neuroblastoma (SH-SY5Y), glioma (U373MG) and lymphoblast cell lines were grown at a concentration of 10^5 cells/ml. Lymphoblast cells were grown in RPMI 1640 supplemented with glutamax and 10% FCS (both GIBCO-Invitrogen) plus antibiotics. Neuroblastoma and Glioma cell lines were grown in 50% Dulbecco's modified Eagle's medium and 50% F-12HAM.

Protein and RNA Extraction

General procedures for protein and RNA preparation and analysis followed standard laboratory manuals. Proteins from total brain and synaptoneuroosomes were resuspended in Laemli buffer, boiled, separated by polyacrylamide gel electrophoresis, and transferred to Immobilon-P membrane (Millipore), immunostained and visualized using the SuperSignal Chemiluminescent Substrate (Pierce) or, if accurate quantitation was required, ECF Western blotting reagent packs (Amersham Pharmacia Biotech). RNAs were prepared from total brain, synaptoneuroosomes and gradient fractions by Proteinase K treatment, phenol/chloroform extraction, and ethanol precipitation.

Polysomes/mRNPs Distribution of mRNAs or Proteins

Animal care was conducted in conformity with the institutional guidelines that are in compliance with national (DL N116, GU, suppl 40, 18-2-1992) and international laws and policies (European Community Council Directive 86/609, OJa L 358, 1, December 12, 1987; National Institutes of Health Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996). Synaptoneuroosomes and total brain extract preparation, sucrose gradient sedimentation of polysomes, and analysis of the polysomes/mRNPs distribution of mRNAs were carried out as described (Bagni et al., 2000). Total brain or purified synaptosomes were homogenized in lysis buffer (100 mM NaCl, 10 mM $MgCl_2$, 30 mM Tris-HCl [pH 7.5], 1 mM dithiothreitol, 30 U/ml RNasin). After 5 min of incubation on ice, the lysates were centrifuged for 8 min at $12,000 \times g$ at 4°C. The supernatants were sedimented in a 5%–70% (w/v) sucrose gradient by centrifugation for 135 min at 37,000 rpm in a Beckman SW41 rotor. Each gradient was collected in ten fractions. RNA was extracted from gradient fractions and analyzed by RT-PCR. For protein analysis, the supernatant was sedimented in a 15%–50% (w/v) sucrose gradient by centrifugation for 110 min at 37,000 rpm in a Beckman SW41 rotor.

RT-PCR for Polysome/mRNPs Analysis

RNA samples were extracted DNase treated and reverse transcribed into cDNA using 100 U of M-MLV RTase (RNaseH⁻, Invitrogen). For quantitative RT-PCR analysis, an equal amount (10 pg) of an internal control RNA was added to each fraction before RNA extraction. This RNA was obtained by in vitro transcription (Ambion, Austin, TX) of the *Xenopus* ribosomal protein L22 sequence (a.n. X64207) and amplified with oligonucleotides annealing to the vector and the coding region. An aliquot of RT reaction was PCR amplified in a final volume of 50 μ l, using 20 pmol of each primer, 100 μ M of each dNTP and 0.5 U of Taq DNA Polymerase (Amersham Pharmacia Biotech). The amount of template and the number of amplification cycles were preliminarily optimized for each PCR reaction, so as to avoid saturation. For radioactive PCR, dCTP was reduced to 10 μ M and 0.2 μ Ci of α -³²P-dCTP (Amersham Pharmacia Biotech; 3000 Ci/mmol) was added. Products were run on a 5% polyacrylamide gel and quantified by a PhosphorImager.

Mouse Brain Lysate Immunoprecipitation

Whole brain was washed in cold PBS and homogenized by 10 stroke dounce homogenization in 2 ml/brain ice cold lysis buffer (10 mM HEPES [pH7.4]; 200 mM NaCl, 0.5% TritonX-100, 30 mM EDTA) in presence of protease inhibitors (Sigma-Aldrich) and 30 U/ml RNasin. The following steps were performed according to Brown et al., 2001 with slight modifications. Briefly, nuclei and debris were pelleted at 8000 rpm in a Eppendorf centrifuge for 10 min at 4°C, the pellet was washed with 1 ml lysis buffer and pelleted again. Supernatants were pooled, raised to 400 mM NaCl, and clarified at 20,000 rpm in a Beckman SW41 rotor for 30 min. 300 μ g of protein extract were immunoprecipitated for 1 hr at 4°C with 15 μ g affinity-purified polyclonal antibodies rAM1 conjugated to 20 μ l of protein A sepharose (Amersham Pharmacia Biotech). Beads were then washed in lysis buffer containing 150, 250, 350, 450, or 550 mM NaCl. The immunoprecipitate was analyzed by 10% SDS-PAGE and Western blotting.

Preparation of BC1 RNA

Linearized plasmid containing the *BC1* sequence (Cheng et al., 1996) was used as a template to produce either ³²P-labeled *BC1* using in vitro transcription kit (Ambion) and 50 μ Ci of α -³²P-UTP (Amersham Pharmacia Biotech; 3000 Ci/mmol) or biotinylated *BC1* RNA in the presence of biotin-16-uridine-5'-triphosphate (Roche). RNA integrity was examined by agarose gel electrophoresis, and the biotin incorporation was verified by spotting the RNA onto a nitrocellulose membrane and detection with the streptavidin-alkaline phosphatase conjugate.

BC1 Binding to Dynabeads Streptavidin and Duplex Assay

Biotinylated *BC1* RNA (200 ng) was bound to 20 μ l of streptavidin-conjugated magnetic beads (Dynal) for 20 min at RT in annealing buffer (10 mM Tris-HCl [pH 7.5], 2 mM MgCl₂, 400 mM NaCl, 0.2% SDS). Total RNA from mouse brain (500 ng) was incubated with *BC1*-magnetic beads in annealing buffer at 80°C for 5 min and at RT for 14 hr. After three washes with the same buffer, *BC1* RNA and the annealed RNAs were eluted by extraction with phenol/chloroform and ethanol precipitated.

5 μ l of eluted RNA was reverse transcribed and an aliquot (1/4) was PCR-amplified as described above for the immunoprecipitated RNAs. For all the mRNAs, 30 cycles of amplification were performed.

Preparation of Cytoplasmic Extracts and RNA Binding Assay

Cytoplasmic extracts were prepared from brain and cell lines as previously described for immunoprecipitation experiments without clarification. For cell line extracts, (300 μ g total proteins) were used for immunoprecipitation experiments.

RNA binding reaction was conducted as follows: purified FMRP, 0.4 μ g, and ³²P-labeled *BC1*, 0.05 ng=2x10⁴ cpm, were incubated on ice for 30 min in 15 μ l of RNA binding buffer containing 10 mM HEPES (pH 7.9), 3 mM MgCl₂, 10 mM DTT, 100 mM KCl, 750 mM NaCl, 5% glycerol, 7 mM β -Mercaptoethanol, 15 μ g Albumine, 20 μ g Heparin. For competition experiments or supershift assays, unlabeled *BC1* (5 ng) or tRNA (5 ng) or rAM1 Ab were preincubated with the protein for 10 min before addition of RNA probe. For brain extracts, 10 μ g of proteins were incubated in the same buffer con-

taining 150 mM NaCl. Samples were separated on a non-denaturing 5% polyacrylamide gel. Gel was exposed to a PhosphorImager for quantification.

Blocking of BC1 RNA by DNA or 2'O-Me-oligonucleotide Interference

A whole brain was homogenized as described for the immunoprecipitation experiments. 1/100 of a brain was incubated in presence of 300 pmol, estimated to be 150 \times excess compared to the endogenous *BC1* RNA, or 1500 pmol (750 \times) of *BC1* DNA oligonucleotide (*BC1* sl-1) or O-Me-RNA oligo (MWG Biotech AG) at 37°C for 20 min. After the incubation, FMRP immunoprecipitation and RT-PCR was performed as described below.

RT-PCR for Immunoprecipitated RNAs

To coimmunoprecipitate RNA, brain lysates were precleared for 1 hr with 20 μ l protein A sepharose (preblocked with 0.1 μ g/ml each BSA, yeast tRNA, glycogen) and immunoprecipitated as described above. DNase I (50 U RNase-free, Amersham Pharmacia Biotech) was added during washes. The immunoprecipitate was treated with 50 μ g proteinase K (Sigma-Aldrich) for 15 min at 37°C. RNA was phenol/chloroform extracted and ethanol precipitated.

First-strand synthesis was achieved using p(dN)₆ and 100 U of M-MLV RTase (Invitrogen). An aliquot (3 μ l) was used in a PCR reaction with Taq polymerase using gene-specific primers for *BC1*, *Arc*, *MAP1B*, and α -*Tubulin*.

FMRP Recombinant Proteins

The DNA plasmid containing the human FMRP C terminus fragment (nt 1545-1899) was a generous gift of Salvatore Adinolfi (MRC, London). The construct was expressed in *E. coli* strain BL21 (DE3). Human FMRP was produced in baculovirus Sf21 cells using a His-TAT tag in front of the full-length FMRP protein sequence to purify the recombinant protein (S.R. and B.O., unpublished data).

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