Fragile X Mental Retardation Protein (FMRP) Binds Specifically to the Brain Cytoplasmic RNAs BC1/BC200 via a Novel RNA-binding Motif

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Fragile X mental retardation protein (FMRP), the protein responsible for the fragile X syndrome, is an RNA-binding protein involved in localization and translation of neuronal mRNAs. One of the RNAs known to interact with FMRP is the dendritic non-translatable brain cytoplasmic RNA 1 (BC1) RNA that works as an adaptor molecule linking FMRP and some of its regulated mRNAs. Here, we showed that the N terminus of FMRP binds strongly and specifically to BC1 and to its potential human analog BC200. This region does not contain a motif known to specifically recognize RNA and thus constitutes a new RNA-binding motif. We further demonstrated that FMRP recognition involves the 5’ stem loop of BC1 and that this is the region that exhibits complementarity to FMRP target mRNAs, raising the possibility that FMRP plays a direct role in BC1/mRNA annealing.

The fragile X mental retardation protein (FMRP) is3 the protein involved in the fragile X syndrome, the most common cause of inherited mental retardation. FMRP is highly expressed in neurons, where it is involved in mRNP transport and translation, two processes required for synaptic plasticity (1, 2). Thus, FMRP acts as a translational repressor both in vivo and in vitro (3–8), and its effect is more pronounced at the synapses (7). The specific mechanism(s) through which FMRP regulates translation still remains to be understood; in particular, it is not clear whether the regulation occurs at the level of translation initiation (3), during the translation elongation phase (according to a “stalling polynomials” hypothesis) (8, 9), or both, depending on the different stages of development. With respect to mRNP transport, FMRP has both a nuclear localization signal (NLS) and a nuclear export signal (NES) and is capable of shuttling between the nucleus and the cytoplasm (10); it therefore seems likely that FMRP accompanies specific mRNAs from the nucleus to the cytoplasm. Furthermore, granules containing FMRP are transported to locations throughout the dendrite, where translation is regulated by synaptic activation (11), reminiscent of the granules in which mRNPs are thought to be transported. Indeed, mass spectrometric analysis of RNase-sensitive mRNP transport granules also identified, among several proteins involved in transport along the cytoskeleton, FMRP (12).

As a protein involved in mRNP transport and regulation of translation, FMRP is expected to bind selectively to a subset of the mRNAs. Quite a variety of mRNAs have been identified in vitro and in vivo as potential targets of the entire FMRP (13–16), and it is still not clear how the mRNAs are recognized. There are at least three RNA elements that can direct FMRP binding (for a recent review, see Ref. 17). The first is a G-rich RNA structure called the G quartet (14, 18), and the second consists of U-rich stretches (16). Thirdly, we have demonstrated that FMRP binds specifically to the non-coding RNA BC1, which in turn exhibits significant complementarity to and anneals with some mRNAs regulated by FMRP (7). BC1 is a non-translatable RNA, specific of rodents, that acts as an adapter molecule determining the selectivity of FMRP for some of its target mRNAs. Consistent with a role of the BC1-FMRP complex(es) in translational inhibition, BC1 has also been shown to inhibit the in vitro formation of the 48 S preinitiation complex and to bind two key proteins involved in regulation of translation, the poly(A)-binding protein and the translational initiation factor eIF4A (20, 21). Finally, recent findings suggest that FMRP may associate with microRNAs and with components of the RNA-induced silencing complex (22–24). microRNAs are small non-coding RNA molecules (22–24 nt long) that base pair with mRNAs and either direct their degradation or direct their translational regulation (25). In this way, microRNAs may attract FMRP to specific mRNAs, similar to BC1.

FMRP contains three sequence motifs that are characteristic of RNA-binding domains, namely two copies of the KH motif and an RGG box (26). In addition, the N-terminal 217 amino acids can also bind to RNA homopolymers (27, 28). Of these four domains, only the RGG box has been demonstrated to bind RNA with sequence or structure specificity; it recognizes the above mentioned G quartet (14, 18). The presence of multiple RNA-binding domains on the FMRP protein leaves open the interesting possibility that the competing models on the recognition of RNA by FMRP, i.e. via G quartets, via U-rich elements, or via RNA adapters such as BC1, are actually compatible with each other. To investigate this possibility, we set out to map the FMRP domain that is responsible for binding to the BC1 RNA. We showed here that the N-terminal domain of FMRP binds BC1 RNA in a specific manner and that the BC1 region responsible for FMRP binding is found within the stem loop responsible for mRNA target recognition (7). Moreover, we showed that the BC1 analog in primates, called BC200 (29), binds directly and specifically to FMRP via the

3 The abbreviations used are: FMRP, fragile X mental retardation protein; NLS, nuclear localization signal; NES, nuclear export signal; RNP, ribonucleoprotein; mRNP, messenger RNP; NDF, N-terminal domain of FMRP; BC, brain cytoplasmic; EMSA, electrophoretic mobility shift assay; CT, C terminus; NT, N terminus; snRNA, small nuclear RNA; PABP, poly(A)-binding protein; aa, amino acids; nt, nucleotide.

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7 The abbreviations used are: FMRP, fragile X mental retardation protein; NLS, nuclear localization signal; NES, nuclear export signal; RNP, ribonucleoprotein; mRNP, messenger RNP; NDF, N-terminal domain of FMRP; BC, brain cytoplasmic; EMSA, electrophoretic mobility shift assay; CT, C terminus; NT, N terminus; snRNA, small nuclear RNA; PABP, poly(A)-binding protein; aa, amino acids; nt, nucleotide.
same N-terminal domain, strengthening the idea that the two BC RNAs have the same functional significance in neuronal cells. These results demonstrated that the N terminus, which contains two Tudor motifs of unknown function, is capable of sequence-specific RNA binding. We discuss the possibility that the Tudor motif actually indicates the presence of a nucleic acid-binding domain. Furthermore, the N terminus is well separated, in sequence and space, from the RGG box that recognizes the mRNAs via the G quartet, lending support to the idea that FMRP might recognize its target mRNAs in different ways, which either may be linked to the different functions of the protein or may occur simultaneously and cooperatively to strengthen the binding.

**EXPERIMENTAL PROCEDURES**

**FMRP Recombinant Proteins**—The FMRP constructs used for this study are: FMRP N terminus (amino acids 1–217), NDF (amino acids 1–134), NDF/NLS (amino acids 1–180), FMRP-KH1 (amino acids 205–280), FMRP-KH2 (amino acids 281–422), and FMRP C terminus (amino acids 516–632); they were produced as described previously (27). The purity of the recombinant proteins was checked by SDS-PAGE after each step of purification and by mass spectrometry of the final product. To probe the secondary and tertiary structure of the constructs, circular dichroism spectra and nuclear magnetic resonance experiments were performed as described previously (28).

Human FMRP was produced in baculovirus-infected Sf21 cells using a His-TAT-tagged full-length FMR1 clone. The recombinant protein was purified as described previously (30).

**Preparation of BC1 and BC200 DNA Template and RNA Transcripts**—Dral linearized plasmids pBCX607 containing the BC1 sequence (31) (a gift from H. Tiedge) and pPBC200 containing the BC200 sequence (32) (a gift from J. Tiedge) and pPBC200 containing the BC200 sequence (32) (a gift from J. Brosius) were used as a template for the T7 RNA polymerase to produce 32P-labeled RNAs (BC1 or BC200) in the presence of 50 μCi of [α-32P]UTP (Amersham Bicsiences; 3000 Ci/mmol) or non-radioactive RNAs (BC1, BC200 or BC420 fragments) using an in vitro transcription kit (Ambion).

Templates for the containing portions of BC1 RNA (Δ1–Δ5) were generated as described below. The entire 5’ stem loop (Δ1 deletion mutant; nt 1–76) was generated by PCR using the primers 5′-TAA TAC GAC TCA CTA TAG GGG TGG TGG ATT TAG TCT CTC-3′ and 5′-CCA GAG CTG AGG ACC GAA-3′ and the plasmid pBCX607 as template. The partial BC1 RNAs corresponding to 3′ stem loop (Δ2 deletion mutant; nt 127–152) was amplified by PCR using the primers T7 and 5′-AAA GGT TGT GTG TGT TGC-3′, and as template, the oligonucleotide 5′-TAA TAC GAC TCA CTA TAG AAC AAG GTA ACT GGC ACA CAC AAC CTT T-3′. The construct containing the A stretch (Δ3 deletion mutant; nt 61–138) was amplified using the primers 5′-TAA TAC GAC TCA CTA TAG GGT CCT CAG CTC TGG-3′ and 5′-CCA GTT ACC TTG TTT-3′ and the plasmid pBCX607 as template. BC1 5′ end lacking the distal one-third of the stem loop (nt 26–49) (Δ4 construct; nt 1–25/50–76) was generated by PCR using the same primers used for the Δ1 construct, and as template, the oligonucleotide 5′-TAA TAC GAC TCA CTA TAG GGG TGG TGG ATT TAG TCT CTC AGT GGT TCC GGT TCC GGT CCT CAG CTC TGG-3′. The fragment of BC1 5′ end lacking the two-thirds distal (nt 15–60) of the stem loop (Δ5 construct; nt 1–14/61–76) was generated by PCR using the primers T7 and 5′-CCA GAG CTG AGG ACC GAA-3′, and as template, the oligonucleotide 5′-TAA TAC GAC TCA CTA TAG GGG TGG GGG ATT TCG GTC CTC AGC TCT GCT GG-3′. Amplification was performed with Pfu polymerase (Stratagene), and the generated PCR fragments were sequenced before usage. The PCR products were in vitro-transcribed with T7 RNA polymerase as described above.

**Results**

**Band Shift Experiments**—RNA band-shift experiments were performed under variable conditions of stringency and presence of different competitors. As a standard BC-FMRP interaction assay, 32P-labeled BC1 or BC200 RNA (1 × 106 cpm, 0.02 pmol), prepared by transcription in vitro, was incubated with 400 ng of purified FMRP constructs in the following binding buffer: 150 or 300 mM KCl, 5 mM MgCl2, 2 mM dithiothreitol, 5% glycerol, 20 mM HEPES/KOH, pH 7.6, and 500 ng of total yeast tRNA or 20 μg of heparin. Interaction was performed at room temperature (25 °C) or on ice for 20 min. The free RNAs and RNA protein complexes were subsequently separated by electrophoresis on 5 and/or 7% native acrylamide gels in 0.5 × Tris-borate-EDTA buffer at 4 °C and then analyzed by autoradiography. In competition experiments, unlabeled RNAs (tRNA, full-length BC1, or BC1 fragments) in 10-, 50-, or 100-fold excess (0.2, 1, or 2 pmol) were added 20 min before the binding reaction. To map the minimal BC1 region responsible for the binding, BC1 deletion mutants (corresponding to 3′ stem loop (nt 127–152) and the A stretch (nt 61–143) amplified by PCR) were added in 50- or 100-fold excess before the binding reaction.

**Determination of Dissociation Constant (Kd) by Electrophoretic Mobility Shift Assay**—For determination of the apparent binding constant, the same amount of FMRP (1 or BC200) RNA (1 × 106 cpm, 0.02 pmol) was incubated with increasing concentrations of protein. Following electrophoresis, the radioactive gel was dried and analyzed using a PhosphorImager and ImageQuant software (Amersham Bicsiences) to estimate the amount of free RNA and RNA-protein complex for each protein concentration considered. In the assays described here, a great excess of protein over RNA was employed, and the concentration of free protein does not appreciably change upon complex formation ([FMRP]tot). Under this condition, the Kd can therefore be simplified to Kd = ([BC]free/[BC]/bound−1)−1. Moreover, we have characterized the FMRP-BC Interaction

**UV Cross-linking of In Vitro Reconstituted BC1 RNA-FMRP Complex**—A 150 fmol of [32P]Cp-labeled neuronal BC1 RNA (0.01 mCi/pmol), prepared by transcription in vitro, was incubated on ice for 10 min with 1.5, 3.0, 7.5, 15, 30 or 75 pmol of reconstituted fragile X mental retardation protein FMRP N terminus (aa 1–217) in a final volume of 20 μl of buffer A (20 mM Heps/KOH, pH 7.9, 160 mM NaCl, 5 mM MgCl2, 2 mM dithiothreitol). For competition experiments, 150 fmol of the same RNA was incubated on ice for 10 min in the presence of 75 pmol of FMRP and 1.5, 3.0, 7.5, 15, or 30 pmol of non-labeled neuronal BC1 RNA or in vitro-transcribed human U1 snRNA. UV cross-linking of the BC1 RNA-FMRP complex prepared by reconstitution in vitro was performed exactly according to a previously described method (33). 20 μl of SDS-PAGE loading buffer was added to each sample. The samples were analyzed on a 10% SDS-polyacrylamide gel and subsequently visualized by autoradiography.

**Results**

**BC1 RNA Binds FMRP Specifically**—The small dendritic brain cytoplasmic RNA 1 (BC1) is part of the FMRP complex in neurons of rodents and binds directly and specifically to FMRP in vitro (7). To better understand the specificity of the FMRP-BC1 interaction, electrophoretic mobility shift assays (EMSA) were performed using in vitro-transcribed, 32P-labeled BC1 RNA and recombinant human FMRP protein (Fig. 1A). Incubation of FMRP with BC1 leads to the formation of a slower migrating complex (lane 2), which can be competed by a 50-fold excess of unlabeled BC1 RNA (BC1c competitor) but not by a nonspecific competitor like total yeast tRNA tested at the same molar excess (tRNAc competitor) (Fig. 1A, compare lane 3 with lane 4). Moreover, we have
previously shown that this complex can also be super shifted by antibodies specific for FMRP (7).

A similar experiment was performed with bovine serum albumin and two different RNA-binding proteins, the microbial transcription and translation modulator NusG (34) and the splicedosomal 15.5KD/15Smu13p protein (35), respectively. Neither of these proteins formed a complex with BC1 (lanes 5–7), showing that BC1 RNA does not bind to any RNA-binding protein and pointing out the specificity of the FMRP-BC1 interaction.

Titration of FMRP indicates a half-saturation point for BC1 binding of ~200 nM (Fig. 1, B and C). An apparent Kd of 200 nM would be in the range of Kd values observed for other specific RNA-protein interactions. The true Kd is probably lower, and the FMRP-BC1 binding is even stronger; the recombinant full-length FMRP protein tends to aggregate, which is evidenced by the signal seen in the wells of the EMSA gels. Therefore, the available concentration of FMRP in each lane was certainly lower than indicated.

The Entire and Structured N-terminal Domain of FMRP Binds Specifically to BC1 and BC200 RNAs—To map the minimal BC1-binding domain, we tested a series of FMRP deletion constructs for BC1 binding activity. We produced a number of FMRP constructs on the basis of the structural properties of the protein (Fig. 2A); the domain boundaries were designed according to our previous work in which the fold of each construct had been checked by circular dichroism and nuclear magnetic resonance (27). We prepared six constructs spanning the sequences of the isolated FMRP N terminus (NT, aa 1–217), of the two KH motifs (KH1, aa 205–280; KH2, aa 281–422), and of the C terminus (CT, aa 516–632; Fig. 2A). NT is known to comprise three motifs: the well folded N terminal domain of FMRP, NDF, (aa 1–134), the putative nuclear localization signal, NLS, (aa 135–180), and a putative helix-turn-helix motif (aa 181–217), ref28. Of the two KH domains, the first is known to be properly folded, whereas KH2, possibly due to the absence of interactions with regions not directly flanking the motif, is unstructured. CT contains long low complexity stretches and is not folded in a stable three-dimensional structure in the absence of RNA (Fig. 2A).

To map which of these FMRP domains is responsible for binding to BC1 RNA, we performed EMSA experiments, incubating each domain with BC1 RNA (Fig. 2B). Only NT and CT were able to bind to BC1 (Fig. 2B, lanes 3 and 8). Binding of NT to BC1 RNA is specific and stoichiometric since complex formation can be competed by a 50-fold excess of unlabeled BC1 RNA but not by nonspecific competitors (tRNAs) at the same molar excess (Fig. 2C, compare lane 3 with lane 4). Moreover, the addition of lithium, a chaotropic agent that destabilizes nonspecific binding, to the EMSA binding buffer, does not modify the strength of BC1-NT interaction (Fig. 2C, compare lane 2 with 5). In contrast, binding of CT is nonspecific; the complex appears as a smear rather than a defined band on the EMSA gel, and both the tRNA and lithium are able to dissociate BC1 from the FMRP CT (Fig. 2C, compare lanes 6 to 8 and 9). This nonspecific binding could be due to electrostatic interactions with the RGG region, a cluster rich in arginines. The Kapp of the BC1-N terminus complex is ~260 nM (Fig. 2D). This value was, within experimental error, in excellent agreement with that obtained for the full-length protein, indicating that the NT gives the major contribution to the FMRP-BC1 interaction, whereas other regions of FMRP could give additional contributions to stabilize this binding. Two shorter versions of NT, named NDF-(1–134) and NDF/NLS-(1–180) in Fig. 2A, are not able to bind to BC1 RNA. Therefore, the fragment including the amino acids 180–217 and containing the putative helix-turn-helix motif was essential for binding. Attempts to produce this isolated region were, however, impaired by its tendency to aggregate and to go into inclusion bodies. This suggested that the region of FMRP comprising residues 180–217 is unable to fold independently of the flanking regions.

The potential BC1 analog in primates is called BC200 RNA (29). Distribution of the human BC200 RNA reveals neuron-specific expression and dendritic localization comparable with BC1 (36). Recently, we demonstrated that BC200 RNA is able to form a complex with FMRP in human neuroblastoma and glioma cell lines (7), suggesting that these two BC RNAs have the same functional role in FMRP-dependent regulation of translation. Moreover, BC200 RNA has also been shown to bind the entire FMRP in vitro (Ref. 37 and data not shown). To see whether BC200 is recognized in a manner similar to BC1, we also checked the same FMRP domains for BC200/binding (Fig. 3). As for BC1 RNA, only the entire NT of FMRP is able to bind to BC200 RNA (Fig. 3, lane 2), and the apparent Kd is similar (300 nM), within experimental

![FIGURE 1. BC1 RNA binds specifically and with high affinity to FMRP. A, EMSA experiments were performed incubating the 32P-labeled BC1 RNA (0.02 pmol) with (lane 2) or without (lane 1) FMRP (6 pmol). The formation of RNA-protein complexes was monitored by autoradiography. The well-folded N-terminal domain of FMRP, NDF, (aa 1–134), the putative nuclear localization signal, NLS, (aa 135–180), and a putative helix-turn-helix motif (aa 181–217), ref28. Of the two KH domains, the first is known to be properly folded, whereas KH2, possibly due to the absence of interactions with regions not directly flanking the motif, is unstructured. CT contains long low complexity stretches and is not folded in a stable three-dimensional structure in the absence of RNA (Fig. 2A).](http://www.jbc.org/doi/abs/10.1074/jbc.M505512200)
error, to the one observed for the BC1-NT complex (data not shown). Thus, despite the considerable divergence of the two BC RNAs, FMRP bound both through the same domain and with similar affinity.

The N-terminal Domain of FMRP Can Be Cross-linked Specifically to BC1 RNA—Specific UV cross-linking at 254 nm between protein and RNA in native or reconstituted protein-RNA complexes reflected efficient binding between these two components, as it indicates that the protein is in very close vicinity to certain bases of the RNA. We thus investigated whether the NT of FMRP (aa 1–217) can be specifically cross-linked to BC1 RNA.

Increasing amounts of FMRP NT were incubated with radioactively labeled BC1 RNA prepared by transcription in vitro, and the mixture was UV-irradiated at 254 nm. Subsequent analysis of the complexes on a denaturing SDS-polyacrylamide gel revealed three labeled bands that appear upon UV irradiation (Fig. 4A). Two of these bands (marked with an asterisk) also appear with weaker intensity in UV-irradiated naked BC1 RNA samples and are thus not considered to be protein-dependent. The lowest, strongest band (XL), with an apparent molecular mass of ~60 kDa, is obtained only from samples that contain both BC1 RNA and FMRP protein. This particular band is also sensitive toward RNase and proteinase K treatment (data not shown). We therefore concluded that this band represents a cross-link between FMRP and BC1 RNA.

Increasing amounts of FMRP NT were incubated with radioactively labeled BC1 RNA prepared by transcription in vitro, and the mixture was UV-irradiated at 254 nm. Subsequent analysis of the complexes on a denaturing SDS-polyacrylamide gel revealed three labeled bands that appear upon UV irradiation (Fig. 4A). Two of these bands (marked with an asterisk) also appear with weaker intensity in UV-irradiated naked BC1 RNA samples and are thus not considered to be protein-dependent. The lowest, strongest band (XL), with an apparent molecular mass of ~60 kDa, is obtained only from samples that contain both BC1 RNA and FMRP protein. This particular band is also sensitive toward RNase and proteinase K treatment (data not shown). We therefore concluded that this band represents a cross-link between FMRP and BC1 RNA.

Although non-labeled BC1 RNA was able to compete with the binding between NT and labeled BC1 RNA already at the lowest concentrations tested, human U1 snRNA did not show any effect on the binding of FMRP NT to BC1 RNA. These results demonstrated unambiguously the highly specific nature of the protein-RNA interaction between the N terminus of the FMRP protein and the BC1 RNA.
**Characterization of the FMRP-BC Interaction**

FMRP Recognizes the 5' Stem Loop of BC1 RNA—To determine which domain of BC1 RNA was responsible for FMRP N terminus binding, an excess of unlabeled RNA constructs representing parts of the BC1 RNA (Fig. 5A; Δ1–Δ3) was used to compete for the interaction with labeled BC1 RNA (Fig. 5B). These BC1 RNA constructs are most likely structured in vitro, as suggested by the highly negative ΔG value of putative secondary structures (data not shown). Complex formation (Fig. 5B, arrow) was completely inhibited by competition with a 50-fold excess of unlabeled full-length BC1 RNA (Fig. 5B, lane 3) and by competition with a 50–100-fold excess of Δ1 construct corresponding to the 5' stem loop (lanes 5 and 6), whereas only weak reduction was observed by competition with a 100-fold excess of the Δ3 construct (lane 12). No competition occurred with the Δ2 construct corresponding to 3' stem loop. These data indicated that FMRP contacts principally the 5' end of BC1. The Δ3 construct encompassing the A-rich stretch and a few nucleotides of the 3' stem loop gave only a minor contribution, and the 3' hairpin (construct Δ2) does not bind. The same experiment was repeated with the full-length FMRP, and also, in this case, only the Δ1 construct was able to compete with BC1 full-length (data not shown), strengthening the idea that the 5' stem loop of BC1 is recognized by FMRP. To further restrict the BC1 5' stem loop region involved in the binding to FMRP NT, we made smaller constructs (named Δ4 and Δ5 in Fig. 5A) lacking the distal one-third of the 5' stem loop (nt 26–49) or the distal two-thirds of the 5' stem loop (nt 15–60) (see “Experimental Procedures” for details). As shown in Fig. 5C, the progressive deletion of the distal part of 5' stem loop leads to a decrease of the interaction strength with the NT; although the construct Δ4 is bound only weakly, the proximal part of 5' stem loop alone (construct Δ5) does not bind the NT any longer. These data altogether demonstrated that the integrity of 5' stem loop is required for optimal binding between FMRP and BC1.

**DISCUSSION**

FMRP is known to contain several independent RNA-binding motifs; besides the two well characterized KH domains, FMRP has at least two other regions, including the N and C termini, with affinity for RNA (27, 38). Such a multiple RNA-binding platform may determine whether the FMRP-RNP complex participates in nuclear processes, nucleo-cytoplasmic shuttling, dendritic/axonal mRNA transport, or translational control at different stages of development and cell cycle. Here, we have demonstrated that the N terminus of FMRP contains a novel RNA-binding motif that binds specifically to the rodent BC1 and to its primate analog BC200 RNAs. Although binding of this region to RNA homopolymers had been reported before (27), we have shown for the first time that this isolated region is able to recognize the BC1 and BC200 RNAs. The interaction seems highly specific since another non-translatable RNA of a comparable length, U11 RNA, is unable to bind. An unspecific interaction due to double strand recognition could also be ruled out because both U11 and a fragment of BC1 with a double stranded structure do not bind.

We have demonstrated that NT is the region of FMRP necessary and sufficient for BC1/BC200 RNA binding; no other regions of the protein are able to recognize BC1 on their own, whereas the Kd values obtained for the full-length protein and for the NT construct were comparable. Further mapping of the interaction within the FMRP NT showed that the region aa 180–217 is necessary for the binding. The two deletion mutants of NT, NDF and NDF/NLS, are in
fact unable to bind BC1, thus suggesting that the BC1-binding motif comprises the stretch between the NLS and KH1 (Fig. 2A). A structural characterization of NT had suggested that it contains at least two distinct regions (28): the NDF and a potential helix-loop-helix motif present in amino acids 181–214, the region that we now observe to bind BC1. The structure of NDF has recently been solved and contains two repeats of a Tudor domain fold, a motif known to be involved in protein-protein interactions and in recognition of methylated amino acids (40). Interestingly, NDF is highly flexible and presumably needs other regions of FMRP or other partners to be stabilized. We can therefore envisage that the structure containing the helix-loop-helix motif (aa 180–217), which cannot be produced independently in a soluble form, is determined by its interactions with NDF. The RNA-binding site could be highly localized in the region 180–217 or distributed along the whole NT sequence, possibly making use of the several conserved positively charged residues.

NT also encompasses the NLS, suggesting a role of the complex with BC1 in the regulation of the FMRP cycle in and out of the nucleus. Binding of BC1/BC200 RNA to FMRP could in fact mask this signal, thus preventing import of the protein in the nucleus (10, 41–43), whereas the disassembly of the FMRP-BC1 complex would make the protein free to enter the nucleus again. Data reported here suggested again that a protein with different RNA-binding domains may have multiple roles. The fact that BC1 binds to the N-terminal portion of FMRP leaves the other three RNA-binding domains available for further recognition of additional targets. Therefore, FMRP can bind an mRNA simultaneously through BC1 and a G quartet (Fig. 6), and the resulting cooperativity would considerably strengthen the interaction with the mRNA. In support of this notion, strong FMRP targets such as the MAP1B and FMR1 mRNAs (7, 13, 14) contain both a G quartet and a region complementary to BC1 RNA. In addition, other points of contact

\[4\] A. Ramos, D. Hollingworth, S. Adinolfi, M. Castets, G. Kelly, T. A. Frenkel, B. Bardoni, and A Pastore, submitted for publication.

**FIGURE 5.** The 5’ stem loop of BC1 RNA binds to FMRP-NT. A, a scheme of the BC1 deletion mutants used in the EMSA experiments reported below. Δ1, nt 1–76 (corresponding to the entire 5’ stem loop); Δ2, nt 127–152 (corresponding to the entire 3’ stem loop); Δ3, nt 61–138 (containing the A stretch); Δ4, nt 1–25/50–76 (two-thirds of 5’ stem loop; the nucleotides marked in red were inserted to create a UUCG tetraloop); Δ5, nt 1–14/61–76 (one-third of 5’ stem loop). B, 50-fold excess of unlabeled BC1 RNA (lane 3) or 10-, 50-, and 100-fold excesses of each BC1 subregion corresponding to 0.2, 1, and 2 pmol, respectively (lane 4–12), were used as competitors in EMSA experiments. The mobility of labeled full-length BC1 or the BC1-NT complex is shown in lanes 1 and 2, respectively. Only the entire BC1 RNA and the 5’ stem loop are able to successfully compete with labeled BC1 for N terminus binding. The positions of the BC1-NT complex and the free BC1 are indicated in the left by arrows. C, 32P-labeled, full-length BC1 RNA (lane 2) or deletion mutants of the 5’ stem loop (Δ1, Δ4, and Δ5, lanes 4, 6, and 8, respectively) were incubated with the NT, and the complexes were analyzed as in Figs. 2 and 3. The position of the BC1-NT (lane 2), Δ1-NT (lane 4), and Δ4-NT (lane 6) complexes is indicated on the right of each lane by a black dot. Free full-length BC1, Δ1, Δ4, or Δ5 RNAs, are shown in lanes 1, 3, 5, and 7, respectively.
Characterization of the FMRP-BC Interaction

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REFERENCES

1. Steward, O., and Schuman, E. M. (2003) Neuron 40, 347–359
2. Martin, K. C., Barad, M., and Kandel, E. R. (2000) Curr. Opin. Neurobiol. 10, 587–592
3. Laggerbauer, B., Osterreick, D., Keidel, E. M., Osterreick-Lederer, A., and Fischer, U. (2001) Hum. Mol. Genet. 10, 329–338
4. Li, Z., Zhang, Y., Ku, L., Wilkinson, K. D., Warren, S. T., and Feng, Y. (2001) Nucleic Acids Res. 29, 2276–2283
5. Zhang, Y. Q., Bailey, A. M., Matthes, H. J., Renden, R. B., Smith, M. A., Speese, S. D., Rubin, G. M., and Broaddus, K. (2001) Cell 107, 591–610
6. Mazzoni, R., Huot, M. E., Tremblay, S., Fillion, C., Labelle, Y., and Khandjian, E. W. (2002) Hum. Mol. Genet. 11, 3007–3017
7. Zalla, F., Giorgi, M., Primmero, B., Moro, A., Di Penta, A., Reis, S., Oostra, B., and Baggi, C. (2003) Cell 112, 317–327
8. Lu, R., Wang, H., Liang, Z., Ku, L., O’Donnell W., T., Li, W., Warren, S. T., and Feng, Y. (2004). Proc. Natl. Acad. Sci. U. S. A. 101, 15201–15206
9. Stefani, G., Fraser, C. E., Darnell, J. C., and Darnell, R. B. (2004) J. Neurosci. 24, 7727–7726
10. Tamarini, F., Bonettoke, C., Bakker, C. E., van Unen, L., Anar, B., Willemsen, R., Yoshida, M., Galjaard, H., Oostra, B. A., and Hoogeveen, A. T. (1999) Hum. Mol. Genet. 8, 863–869
11. Antar, L. N., Afroz, R., Dictenberg, J. B., Carroll, R. C., and Bassell, G. J. (2004) J. Neurosci. 24, 2648–2655
12. Kanai, Y., Dohmae, N., and Hirokawa, N. (2004) Neuron 43, 513–525
13. Brown, V., Jin, P., Ceman, S., Darnell, J. C., O’Donnell W., T., Tenenbaum, S. A., Jin, X., Feng, Y., Wilkinson, K. D., Keene, J. D., Darnell, R. B., and Warren, S. T. (2001) Cell 107, 477–487
14. Darnell, J. C., Jensen, K. B., Jin, P., Brown, V., Warren, S. T., and Darnell, R. B. (2001) Cell 107, 489–499
15. Miyashiro, K. Y., Beckel-Mitchener, A., Purt, T. P., Becker, K. G., Barret, T., Liu, L., Carbonnet, S., Weiler, I. J., Greenough, W. T., and Eberwine, J. (2003) Neuron 37, 417–431
16. Chen, L., Yun, S. W., Seto, J., Liu, W., and Toth, M. (2003) Neuroscience 120, 1005–1017
17. Bagini, C., and Greenough, W. T. (2005) Nat. Rev. Neurosci. 5, 376–387
18. Schaeffer, C., Bardoni, B., Mandel, J. L., Ehresmann, B., Ehresmann, C., and Moine, H. (2001) EMBO J. 20, 4803–4813
19. Ramos, A., Hollingworth, D., and Pastore, A. (2003) RNA (N. Y.) 9, 1198–1207
20. Wang, H., Iacangioli, A., Popp, S., Musilm, I. A., Imataka, H., Sonenberg, N., Lomakin, I. B., and Tiedge, H. (2002) J. Neurosci. 22, 10322–10324
21. Muddashetty, R., Khanam, T., Kondrashov, A., Bundman, M., Iacangioli, A., Kremerskothen, J., Duning, K., Barnekow, A., Huttenhofer, A., Tiedge, H., and Brosius, J. (2002) J. Mol. Biol. 321, 433–445
22. Caudy, A. A., Myers, M., Hannon, G. J., and Hammond, S. M. (2002) Genes Dev. 16, 2491–2496
23. Ishizuka, A., Siomi, M. C., and Siomi, H. (2002) Genes Dev. 16, 2497–2508
24. Jin, P., Zarnescu, D. C., Ceman, S., Nakamoto, M., Mourey, J., Jongens, T. A., Nelson, D. L., Moses, K., and Warren, S. T. (2004) Nat. Neurosci. 7, 113–117
25. Tomari, Y., and Zamore, P. D. (2005) Genes Dev. 19, 517–529
26. Khandjian, E. W. (1999) Biochem. Biol. Cell. 77, 331–342
27. Adinolfi, S., Bagini, C., Musson, G., Gibson, T., Mazzarella, L., and Pastore, A. (1999) RNA (N. Y.) 5, 1248–1258
28. Adinolfi, S., Ramos, A., Martin, S. R., Dal Piaz, F., Pucci, P., Bardoni, B., Mandel, J. L., and Pastore, A. (2003) Biochimica 42, 10437–10444
29. Martignetti, J. A., and Brosius, J. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 11563–11567
30. Reis, S. A., Willemsen, R., van Unen, L., Hoogeveen, A. T., and Oostra, B. A. (2004) J. Mol. Histol. 35, 389–395
31. Cheng, J. G., Tiedge, H., and Brosius, J. (1996) DNA Cell Biol. 15, 549–559
32. Bovia, F., Wolf, N., Byser, S., and Strub, K. (1997) Nucleic Acids Res. 25, 318–326
33. Urlaub, H., Hartmann, K., and Lührmann, R. (2002) Methods (Orlando) 26, 170–181
34. Steiner, T., Kaiser, J. T., Marinovich, S., Huber, R., and Wahli, M. C. (2002) EMBO J. 21, 4641–4653
35. Nottrott, S., Hartmann, K., Fabrizio, P., Urlaub, H., Vidovic, I., Finer, R., and Lührmann, R. (1999) EMBO J. 18, 6139–6145
36. Tiedge, H., Chen, W., and Brosius, J. (1993) J. Neurosci. 13, 2382–2390
37. Gabus, C., Mazzoni, R., Tremblay, S., Khandjian, E. W., and Darlix, J. L. (2004) Nucleic Acids Res. 32, 2129–2137
38. Siomi, H., Siomi, M. C., Nussbaum, R. L., and Dreyfuss, G. (1993) Cell 74, 291–298
39. Huang, Y. S., Carson, J. H., Barabese, E., and Richter, J. D. (2003) Genes Dev. 17,
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40. Maurer-Stroh, S., Dickens, N. J., Hughes-Davies, L., Kouzarides, T., Eisenhaber, F., and Ponting, C. P. (2003) Trends Biochem. Sci. 28, 69–74
41. Verheij, C., Bakker, C. E., de Graaff, E., Keulemans, J., Willemsen, R., Verkerk, A. J., Galjaard, H., Reuser, A. J., Hoogeveen, A. T., and Oostra, B. A. (1993) Nature 363, 722–724
42. Eberhart, D. E., Malter, H. E., Feng, Y., and Warren, S. T. (1996) Hum. Mol. Genet. 5, 1083–1091
43. Feng, Y., Gutekunst, C. A., Eberhart, D. E., Yi, H., Warren, S. T., and Hersch, S. M. (1997) J. Neurosci. 17, 1539–1547
44. West, N., Roy-Engel, A. M., Imataka, H., Sonenberg, N., and Deininger, P. (2002) J. Mol. Biol. 321, 423–432
45. Darnell, J. C., Fraser, C. E., Mostovetsky, O., Stefani, G., Jones, T. A., Eddy, S. R., and Darnell, R. B. (2005) Genes Dev. 19, 903–918
46. Muslimov, I. A., Santi, E., Homel, P., Perini, S., Higgins, D., and Tiedge, H. (1997) J. Neurosci. 17, 4722–4733
