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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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Francesca Zalfa[‡1], Salvatore Adinolfi[§], Ilaria Napoli[‡], Eva Kühn-Hölsken[¶], Henning Urlaub[¶], Tilmann Achsel[‖], Annalisa Pastore[§], and Claudia Bagni[‡‖2]

From the [‡]Dipartimento di Biologia, Università "Tor Vergata," 00133 Rome, Italy, the [§]Molecular Structure Division, National Institute for Medical Research, London NW7 1AA, United Kingdom, the [‖]Istituto di Neuroscienze Sperimentali, Fondazione Santa Lucia, Istituto di Ricovero e Cura a Carattere Scientifico, 00179 Rome, Italy, and the [¶]Department of Cellular Biochemistry, Mass Spectrometry Group, Max Planck Institute for Biophysical Chemistry, 37077 Göttingen, Germany

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)[3] is 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 vitro* and *in vivo* (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 polysomes" 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, 19), 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

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[2] To whom correspondence should be addressed: Università di Roma "Tor Vergata," Dipartimento di Biologia, Via della Ricerca Scientifica 1, 00133 Rome, Italy. Tel.: 39-06-72594223; Fax: 39-06-2023500; E-mail: claudia.bagni@uniroma2.it.

[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.

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—DraI linearized plasmids pBCX607 containing the BC1 sequence (31) (a gift from H. 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 ^{32}P-labeled RNAs (*BC1* or *BC200*) in the presence of 50 μCi of [α-^{32}P]UTP (Amersham Biosciences; 3000 Ci/mmol) or non-radioactive RNAs (*BC1*, *BC200* or *BC1* 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 TTG GGG ATT TAG 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 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 TAC 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 TTG GGG ATT TAG CTC AGT GGT TTC GGC CCT GGG TTC 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 TTG GGG ATT TCG GTC CTC AGC TCT 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.

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, ^{32}P-labeled *BC1* or *BC200* RNA (1×10^5 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 $MgCl_2$, 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 (K_d) by Electrophoretic Mobility Shift Assay—For determination of the apparent binding constant, the same amount of *BC1* (or *BC200*) RNA (1×10^5 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 Biosciences) to quantify 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 ($[FMRP]_{free} \approx [FMRP]_{tot}$). The equation $K_{dapp} = ([BC]_{free} \cdot [FMRP]_{free})/[FMRP\text{-}BC]$ can therefore be simplified to $K_{dapp} = ([BC]_{free}/[BC]_{bound}) \cdot [FMRP]_{tot}$. Under this condition, the K_{dapp} is the concentration of FMRP at which half-of the RNA is bound ($[BC]_{free} = [BC]_{bound}$).

UV Cross-linking of in Vitro Reconstituted BC1 RNA-FMRP Complex—150 fmol of [^{32}P]pCp-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 recombinant fragile X mental retardation protein FMRP N terminus (aa 1–217) in a final volume of 20 μl of buffer A (20 mM Hepes/KOH, pH 7.9, 160 mM NaCl, 5 mM $MgCl_2$, 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, ^{32}P-labeled *BC1* RNA and recombinant human FMRP protein (Fig. 1*A*). 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. 1*A*, 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 spliceosomal 15.5KD/hSnu13p 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 K_d of 200 nM would be in the range of K_d values observed for other specific RNA-protein interactions. The true K_d 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. 2*A*); 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. 2*A*). 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. 2*A*).

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. 2*B*). Only NT and CT were able to bind to *BC1* (Fig. 2*B*, *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 (tRNA*s*) at the same molar excess (Fig. 2*C*, 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. 2*C*, 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 competitor and lithium are able to dissociate *BC1* from the FMRP CT (Fig. 2*C*, 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 $K_{d\text{app}}$ of the *BC1*-N terminus complex is ~260 nM (Fig. 2*D*). 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. 2*A*, 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.

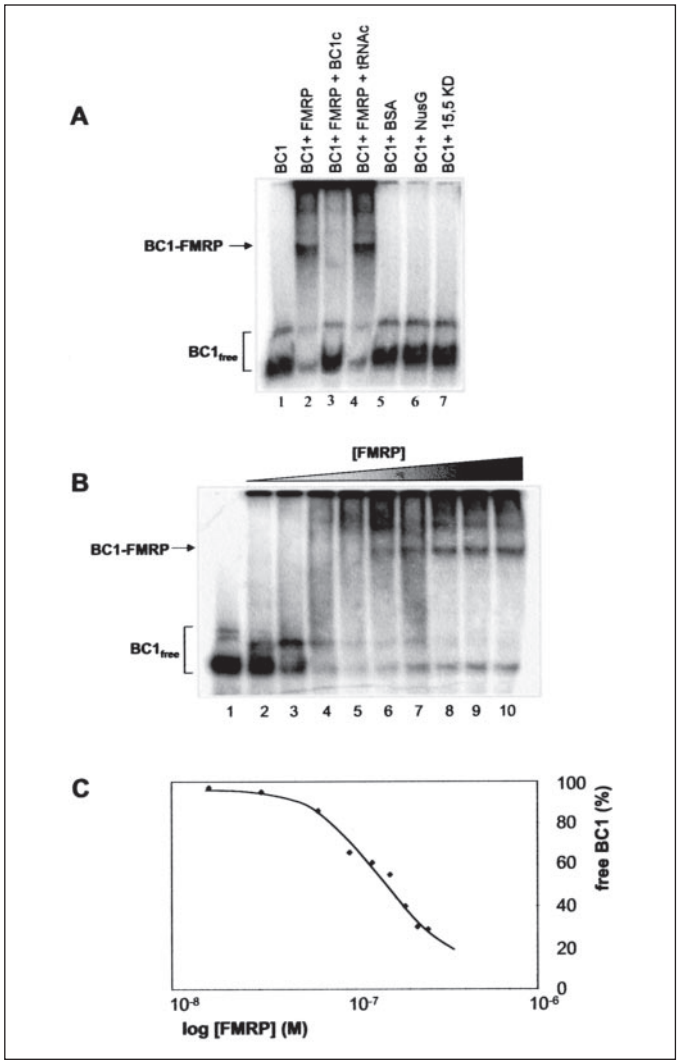

FIGURE 1. ***BC1* RNA binds specifically and with high affinity to FMRP.** *A*, EMSA experiments were performed incubating the ^{32}P-labeled *BC1* RNA (0.02 pmol) with (*lane 2*) or without (*lane 1*) purified FMRP (6 pmol). The formation of RNA-protein complexes was analyzed on native polyacrylamide gels. The position of the band corresponding to the *BC1*-FMRP complex is marked by an *arrow*. *Lanes 3* and *4* represent competition experiments performed with a 50-fold excess of non-labeled *BC1c* (BC1 competitor) and total yeast tRNAc (tRNA competitor). Reactions with bovine serum albumin (*BSA*) or with two RNA-binding proteins, NusG and 15.5KD, are shown in *lanes 5*, *6*, and *7*, respectively. *B*, to determine the *BC1*-FMRP $K_{d\text{app}}$, increasing concentrations of FMRP protein (from *lanes 2* to *10*: 16, 30, 63, 94, 125, 156, 188, 219, and 250 nM) were used. Labeled *BC1* RNA is shown in *lane 1*. The *arrow* points to the band corresponding to the *BC1*-FMRP complex, whereas the *square brackets* mark the free *BC1*. *C*, to estimate the $K_{d\text{app}}$ of *BC1*-FMRP complex, $[BC1]_{\text{free}}$ values were plotted *versus* log [FMRP] as reported under "Experimental Procedures."

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 K_d is similar (300 nM), within experimental

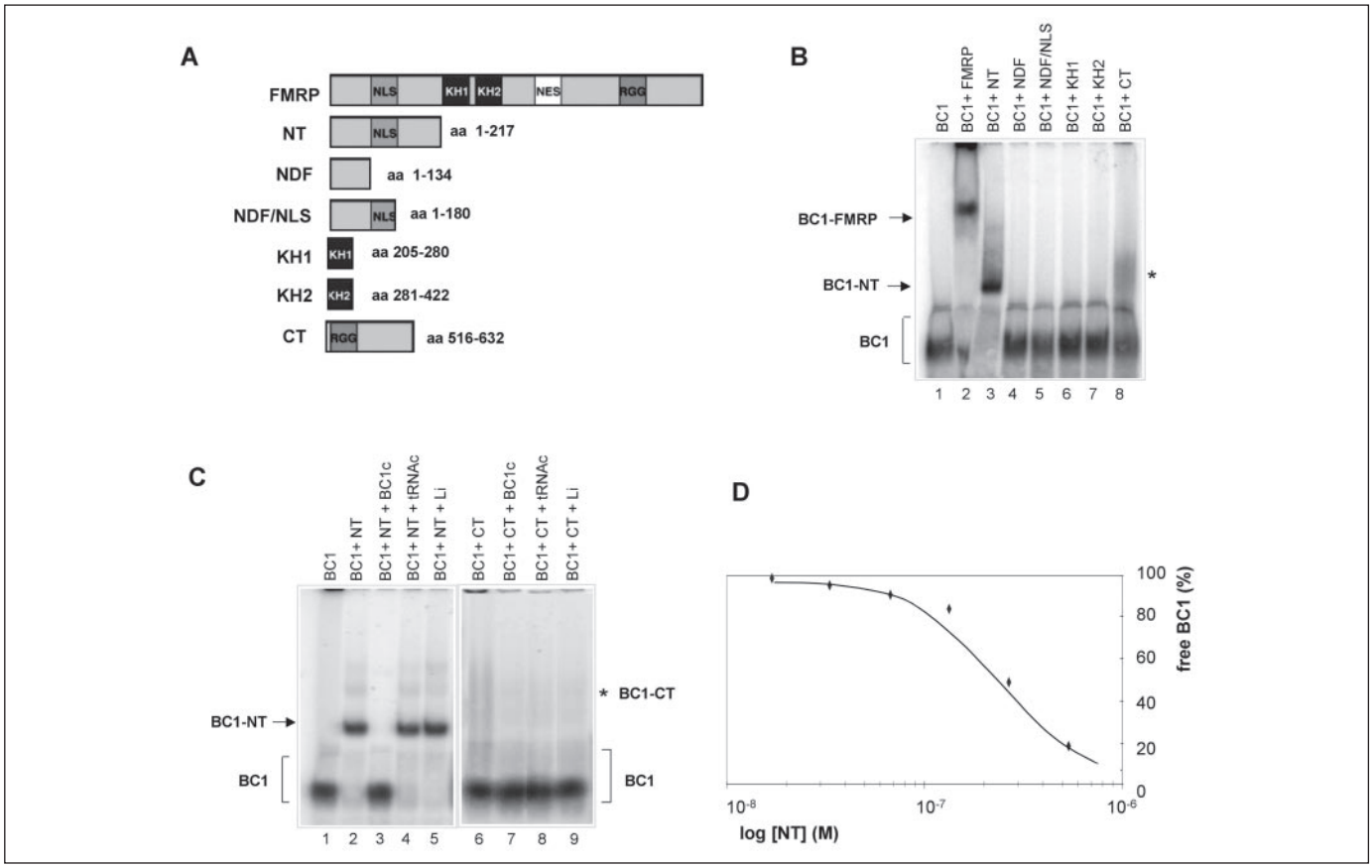

FIGURE 2. **The N-terminal region of FMRP binds specifically to *BC1* RNA.** *A*, a scheme of the FMRP deletion mutants used in EMSA experiments. *B*, ^{32}P-labeled *BC1* RNA (0.02 pmol) was incubated with 6 pmol each of the full-length FMRP (*lane 2*) or the FMRP domains (*lanes 3–8*), and the complexes were analyzed as above. Free *BC1* RNA is shown in *lane 1*. The positions of *BC1*-FMRP and *BC1*-NT complexes are indicated by *arrows*, free *BC1* RNA is marked by a *square bracket*, and the smear corresponding to *BC1*-CT complex is indicated with an *asterisk*. *C*, competition experiments to determine the specificity of *BC1*-NT and *BC1*-CT interactions were performed with a 50-fold excess of non-labeled *BC1* RNA (*BC1c*, *lanes 3* and *7*, respectively) or total yeast tRNA (tRNA competitor, *lanes 4* and *8*, respectively) or in the presence of Li^+ ions (*lanes 5* and *9*, respectively). *BC1* RNA alone is shown in *lane 1*, whereas the complex *BC1*-NT or *BC1*-CT is shown in *lanes 2* and *6*, respectively. *D*, the plot represents the quantification of a titration experiment performed by incubating 0.02 pmol of ^{32}P-labeled *BC1* RNA with increased concentrations of protein (17, 33, 67, 133, 267, and 533 nM). The $K_{d_{app}}$ was determined as reported under "Experimental Procedures."

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. 4*A*). 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.

The specificity of the UV cross-link was verified by competition experiments. For this purpose, FMRP NT-*BC1* complexes were reconstituted and subsequently cross-linked in the presence of non-labeled competitor RNAs, *i.e. BC1* RNA and human *U1* snRNA (Fig. 4*B*).

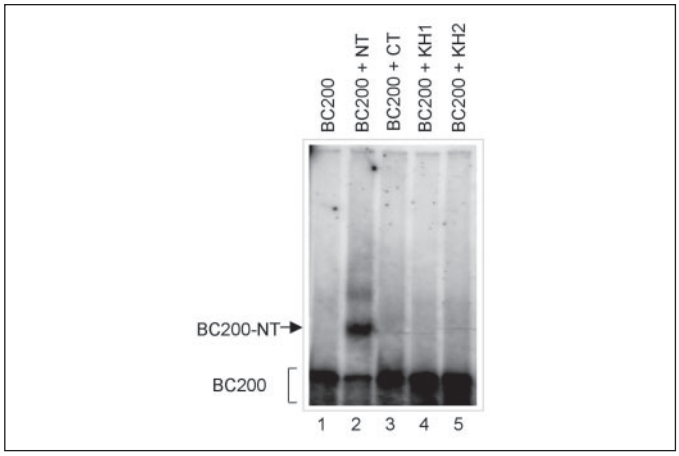

FIGURE 3. ***BC200* RNA binds directly and specifically to N terminus of FMRP.** Band shift experiments were performed incubating the ^{32}P-labeled *BC200* RNA (0.02 pmol) in the presence of four FMRP RNA-binding domains (N terminus, C terminus, KH1, and KH2). A retarded band is shown (*arrow*) only for the *BC200*-N terminus complex (*lane 2*).

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.

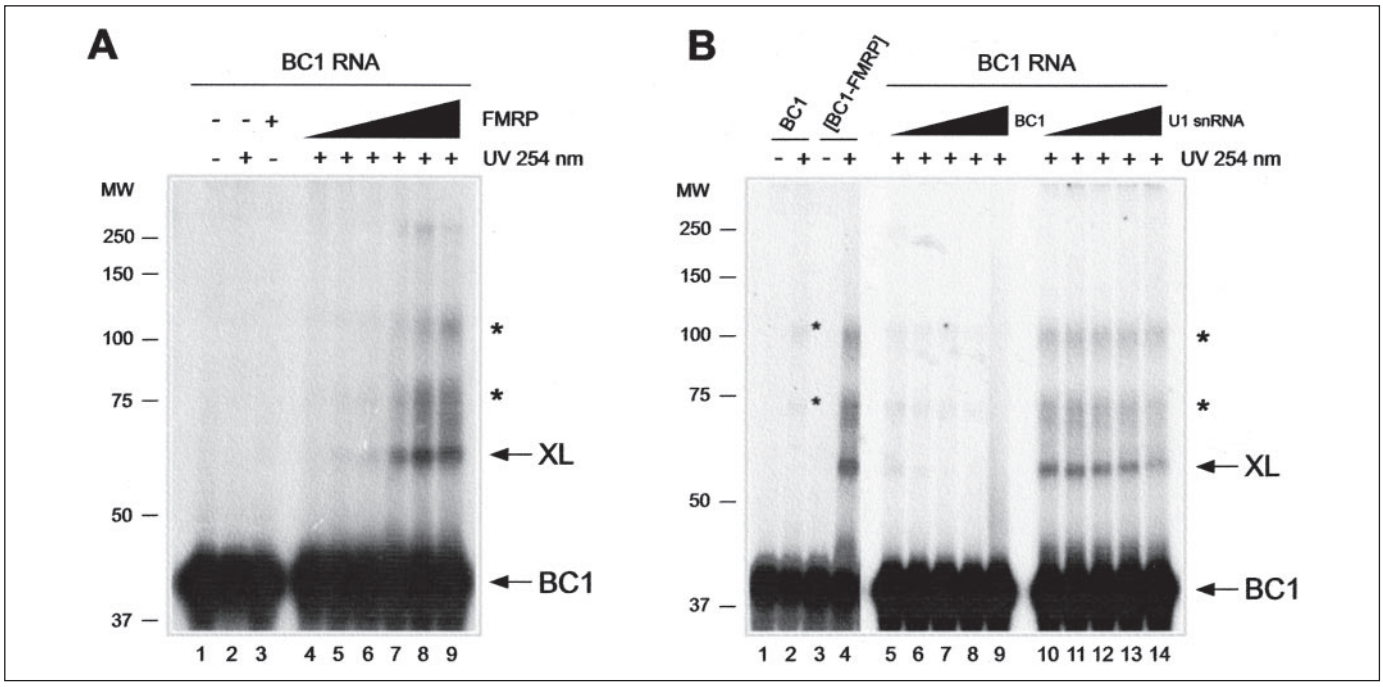

FIGURE 4. **The N-terminal domain can be specifically cross-linked to *BC1* RNA.** *A*, cross-linking of increasing amounts of the N-terminal domain of FMRP to *in vitro*-transcribed *BC1* RNA. *Lane 1*, non-UV-irradiated *BC1* RNA; *lane 2*, UV-irradiated *BC1* RNA; *lane 3*, non-UV-irradiated *in vitro* reconstituted *BC1* RNA-NT complex; *lanes 4–9*, UV-irradiated *in vitro* reconstituted *BC1* RNA-NT complex. For *in vitro* reconstitution of the *BC1* RNA-NT complex, an increasing amount of the N-terminal domain of FMRP (1.5, 3.0, 7.5, 15, 30, and 75 pmol, respectively) was used. Positions of the *BC1* RNA and of FMRP cross-linked to *BC1* RNA (*XL*) are indicated on the *right*. Molecular size markers (indicated by *MW*) are shown on the *left*. Bands marked with an *asterisk* also appear with weaker intensity in UV-irradiated naked *BC1* RNA samples (Fig. 1*B*, *lane 2*) and are thus not considered to be strictly protein-dependent. *B*, UV cross-linking of the N-terminal domain of FMRP to neuronal *BC1* RNA in the presence of non-labeled competitor RNAs (neuronal *BC1* RNA and human *U1* snRNA). For the competition experiment and for the control, *lane 4*, the *BC1*-NT complex was formed by incubating 150 fmol of *BC1* RNA with 75 pmol of FMRP NT. *Lanes 1–3*, control lanes, as described for *panel A*; *lane 4*, UV-irradiated *in vitro* reconstituted *BC1* RNA-NT complex; *lanes 5–9*, UV cross-linking of *in vitro* reconstituted *BC1* RNA-NT complexes formed in the presence of increasing amounts of non-labeled *BC1* RNA (1.5, 3.0, 7.5, 15 and 30 pmol, respectively); *lanes 10–14*, UV cross-linking of *in vitro* reconstituted *BC1* RNA-NT complexes formed in the presence of increasing amounts of non-labeled human U1 snRNA (1.5, 3.0, 7.5, 15, and 30 pmol, respectively). For further details, see the description of *panel A*.

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. 5*A*; Δ1–Δ3) was used to compete for the interaction with labeled *BC1* RNA (Fig. 5*B*). 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. 5*B*, *arrow*) was completely inhibited by competition with a 50-fold excess of unlabeled full-length *BC1* RNA (Fig. 5*B*, *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. 5*A*) 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. 5*C*, 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, *U1* RNA, is unable to bind. An unspecific interaction due to double strand recognition could also be ruled out because both *U1* 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 K_d 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

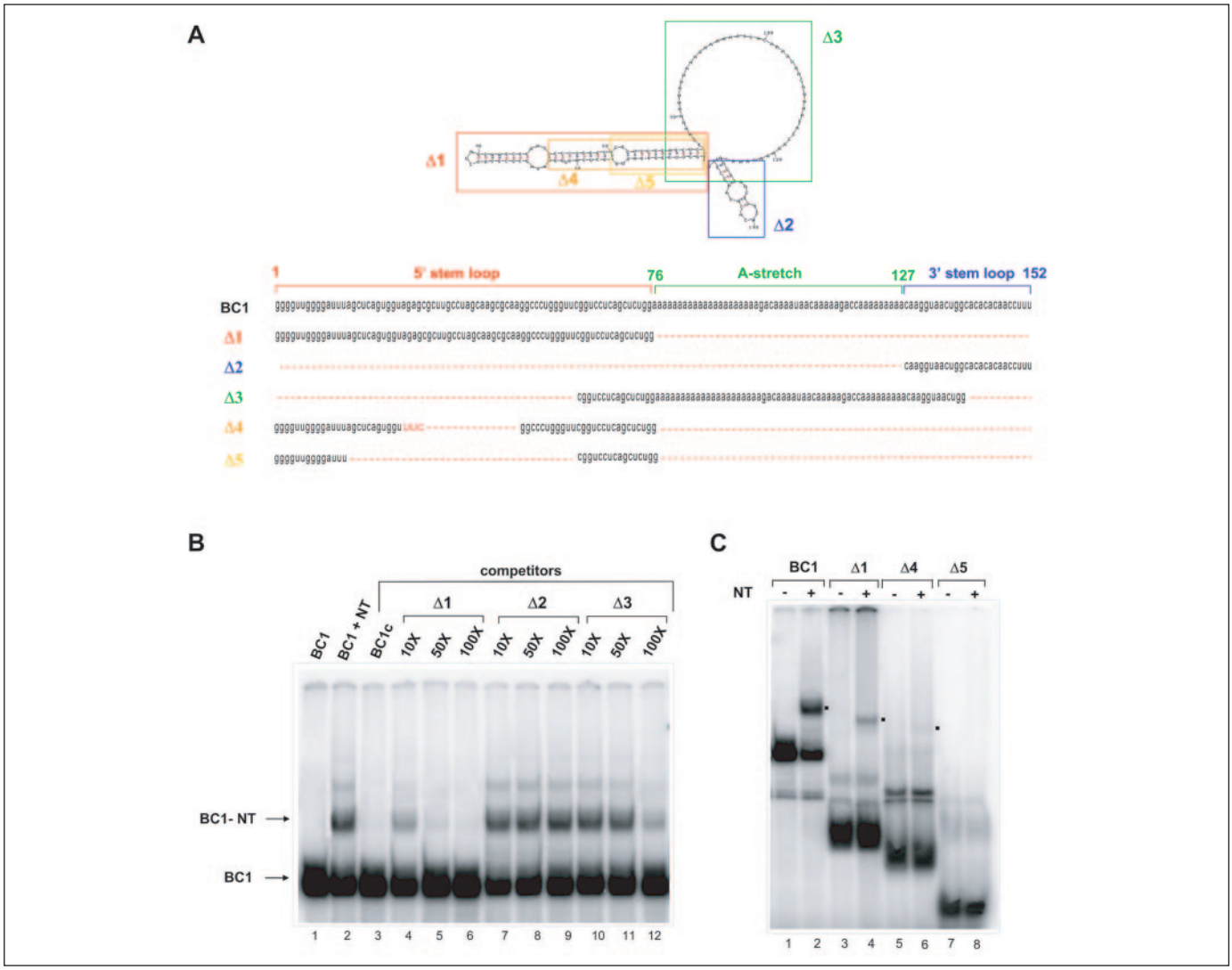

FIGURE 5. **The 5′ stem loop of *BC1* RNA binds to FMRP-NT.** *A*, scheme of the *BC1* deletion mutants used in the EMSA experiments reported below. Δ1, nt 1–76 (corresponding to the entire 5′ stem loop); Δ2, nt 127–152 (corresponding to the entire 3′ stem loop); Δ3, nt 61–138 (containing the A stretch); 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*, ^{32}P-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.

fact unable to bind *BC1*, thus suggesting that the *BC1*-binding motif comprises the stretch between the NLS and KH1 (Fig. 2*A*). 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,[4] 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. Frenkiel, B. Bardoni, and A Pastore, submitted for publication.

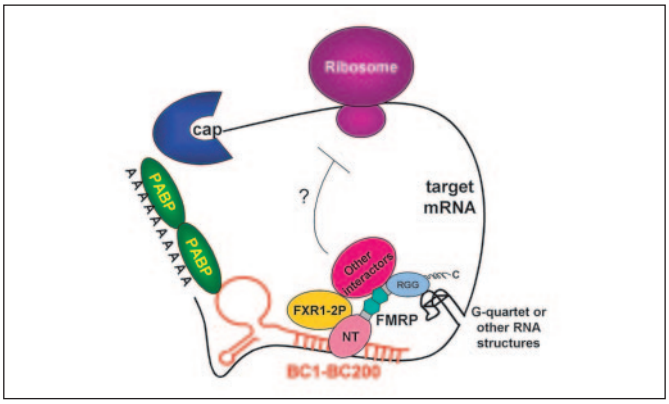

FIGURE 6. **Model representing the mechanism of mRNA recognition and translational repression mediated by the *BC*-FMRP complex.** In the proposed model, the N terminus of FMRP would contact the 5′ region of the longer stem loop of *BC1* RNA stabilizing and/or helping the interaction with the targeted mRNAs that are translationally repressed. The mRNP containing FMRP and *BC1* RNA would be able to contact the PABP on the basis of *in vitro* interaction previously reported between *BC1*, the PABP, and the eIF4A. As suggested for other inhibitor complexes, additional factors could also be involved in modulating the repression. Finally, the indirect (through *BC1* RNA) binding of the N terminus to the mRNAs would still leave the other RNA-binding domain free to strengthen the RNA-protein interaction.

to further strengthen the FMRP-*BC1*-mRNA complex are possible; the poly(A)-binding protein (PABP) associates with *BC1* (44), and those two could interact with the poly(A) tail of the mRNAs (Fig. 6). The two KH domains may recognize further, still uncharacterized, elements on the mRNA. Recently, it has been proposed that the KH2 domain could bind structured double stranded RNAs (45). Such a high synergy not only makes binding stronger, it can also render mRNA recognition more flexible; lack of one binding element can be compensated by a high affinity version of another element.

On the *BC1* side, we mapped the binding to the 5′ stem loop, with a minor contribution from the adjacent A-rich stretch. Binding occurred to the upper part of this stem loop, as deletion of the top third severely reduced binding, whereas deletion of the top two-thirds completely abolished binding. The 5′ stem loop of *BC1* has been implicated in several functions; first, this is the part of *BC1* that exhibits complementarities to mRNAs targeted by FMRP (7). We envisage a role for FMRP in mediating recognition/annealing between *BC1* and the FMRP target mRNAs as presented in Fig. 6. In agreement with this model, it has recently been shown *in vitro* that FMRP has nucleic acid chaperone properties (37), *i.e.* that it is able to catalyze RNA-RNA recognition and annealing. The *BC1*/mRNA duplex that results from the chaperon activity of FMRP is still bound by FMRP (Fig. 6). This hypothesis is supported by the fact that all three components have to be present to get a strong FMRP-mRNA interaction (7). The fact that FMRP recognizes both the *BC1* 5′ stem loop and the mRNA as well as the *BC1*/mRNA duplex can be explained with a separation of the two binding sites on *BC1*; the elements that are complementary to mRNAs are located in the bottom two-thirds of the stem loop (7), whereas FMRP recognizes the upper part (see above). However, the two elements could also overlap; annealing of *BC1* to an mRNA, rather than to itself, may create a structure that looks similar to the *BC1* stem loop.

Moreover, the 5′ *BC1* segment (the first 62 nt) also contains a cis-acting dendritic targeting element that determines the specific transport of this RNA to dendrites (46). Most likely, this function is intimately linked to the mRNA annealing and FMRP binding roles of the same region. We envision a complex in which *BC1* determines the specificity of FMRP action and maintains the regulated mRNAs in an inactive status. This complex will probably also be part of the translationally repressed transport granules recently described along the dendrites (39).

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

Francesca Zalfa, Salvatore Adinolfi, Ilaria Napoli, Eva Kühn-Hölsken, Henning Urlaub, Tilmann Achsel, Annalisa Pastore and Claudia Bagni

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