

# 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)<sup>3</sup> 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 mRNA 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 polyosomes" hypothesis) (8, 9), or both, depending on the different stages of development. With respect to mRNA 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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<sup>3</sup> 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.

## Characterization of the FMRP-BC Interaction

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 <sup>32</sup>P-labeled RNAs (BC1 or BC200) in the presence of 50 μCi of [α-<sup>32</sup>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, <sup>32</sup>P-labeled BC1 or BC200 RNA (1 × 10<sup>5</sup> 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<sub>2</sub>, 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<sub>d</sub>) by Electrophoretic Mobility Shift Assay**—For determination of the apparent binding constant, the same amount of BC1 (or BC200) RNA (1 × 10<sup>5</sup> 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]<sub>free</sub> ≈ [FMRP]<sub>tot</sub>). The equation  $K_{dapp} = ([BC]_{free} \cdot [FMRP]_{free}) / [FMRP-BC]$  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 [<sup>32</sup>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<sub>2</sub>, 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, <sup>32</sup>P-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

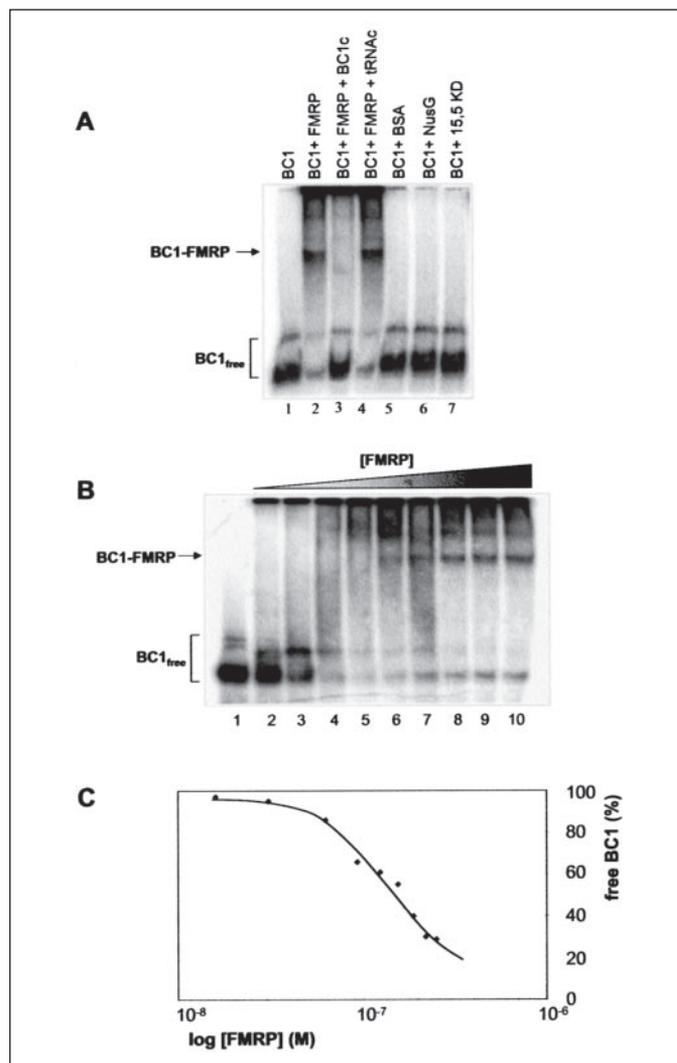
previously shown that this complex can also be super shifted by antibodies specific for FMRP (7).

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 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  $\sim 200$  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. 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).

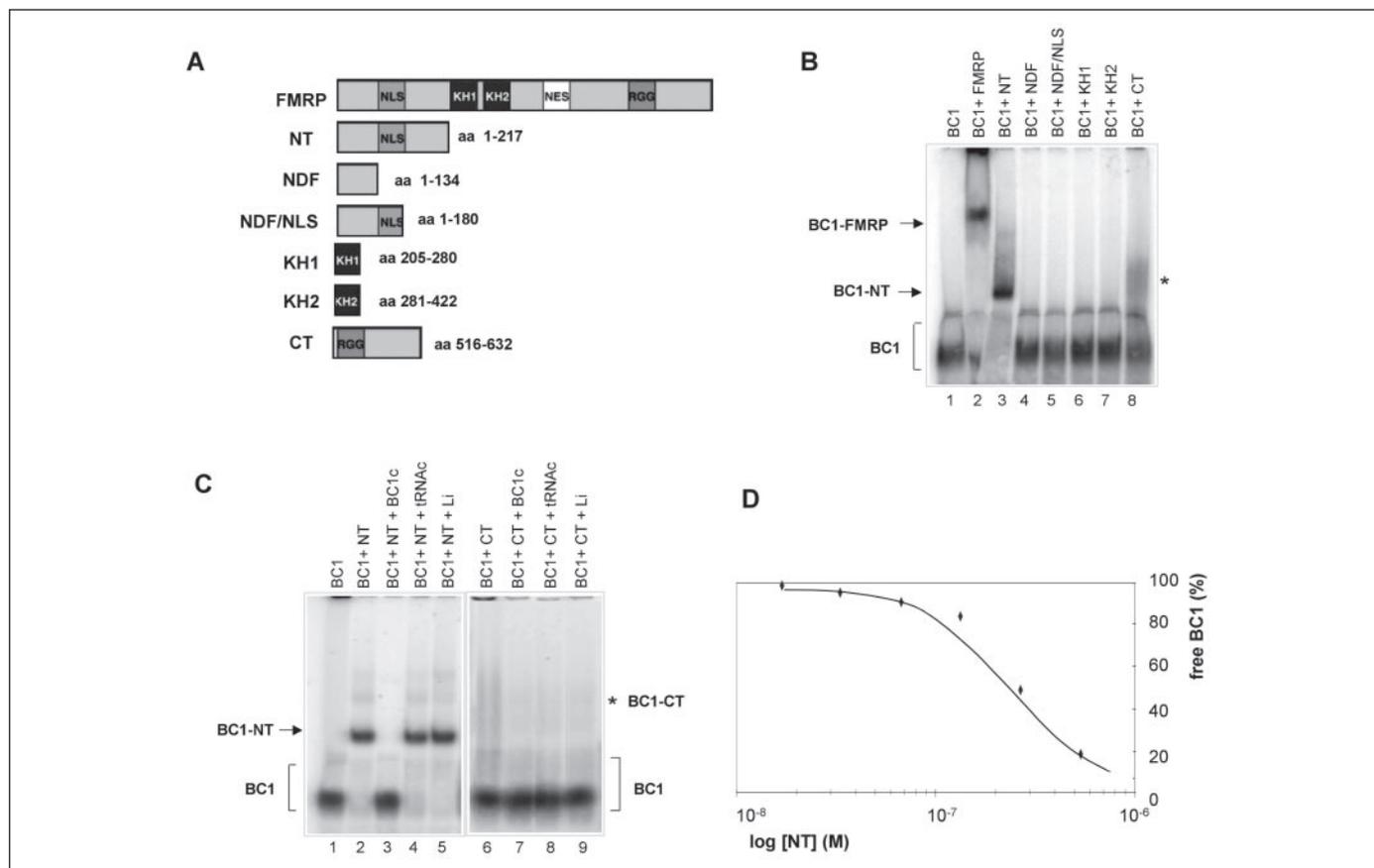
To map which of these FMRP domains is responsible for binding to *BC1* RNA, we performed EMSA experiments, incubating each domain with *BC1* RNA (Fig. 2B). Only NT and CT were able to bind to *BC1* (Fig. 2B, lanes 3 and 8). Binding of NT to *BC1* RNA is specific and stoichiometric since complex formation 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 competitor 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  $K_{dapp}$  of the *BC1*-N terminus complex is  $\sim 260$  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



**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  $K_{dapp}$ , 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  $K_{dapp}$  of *BC1*-FMRP complex,  $[BC1]_{free}$  values were plotted versus log [FMRP] as reported under "Experimental Procedures."

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  $K_d$  is similar (300 nM), within experimental



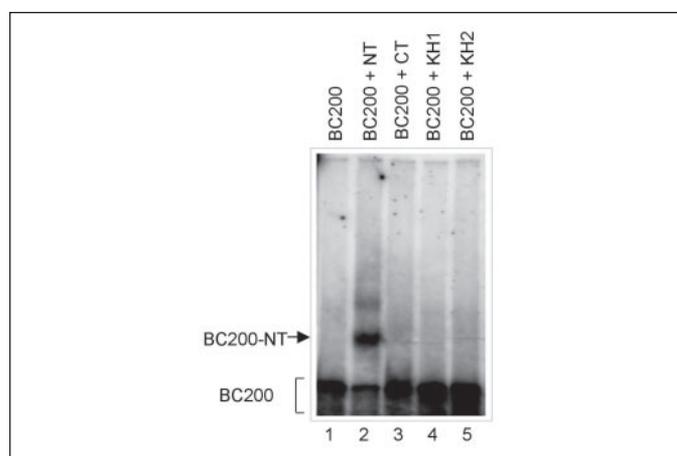
**FIGURE 2. The N-terminal region of FMRP binds specifically to BC1 RNA.** *A*, a scheme of the FMRP deletion mutants used in EMSA experiments. *B*,  $^{32}$ P-labeled BC1 RNA (0.02 pmol) was incubated with 6 pmol each of the full-length FMRP (lane 2) or the FMRP domains (lanes 3–8), and the complexes 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<sup>+</sup> 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  $K_{dapp}$  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.

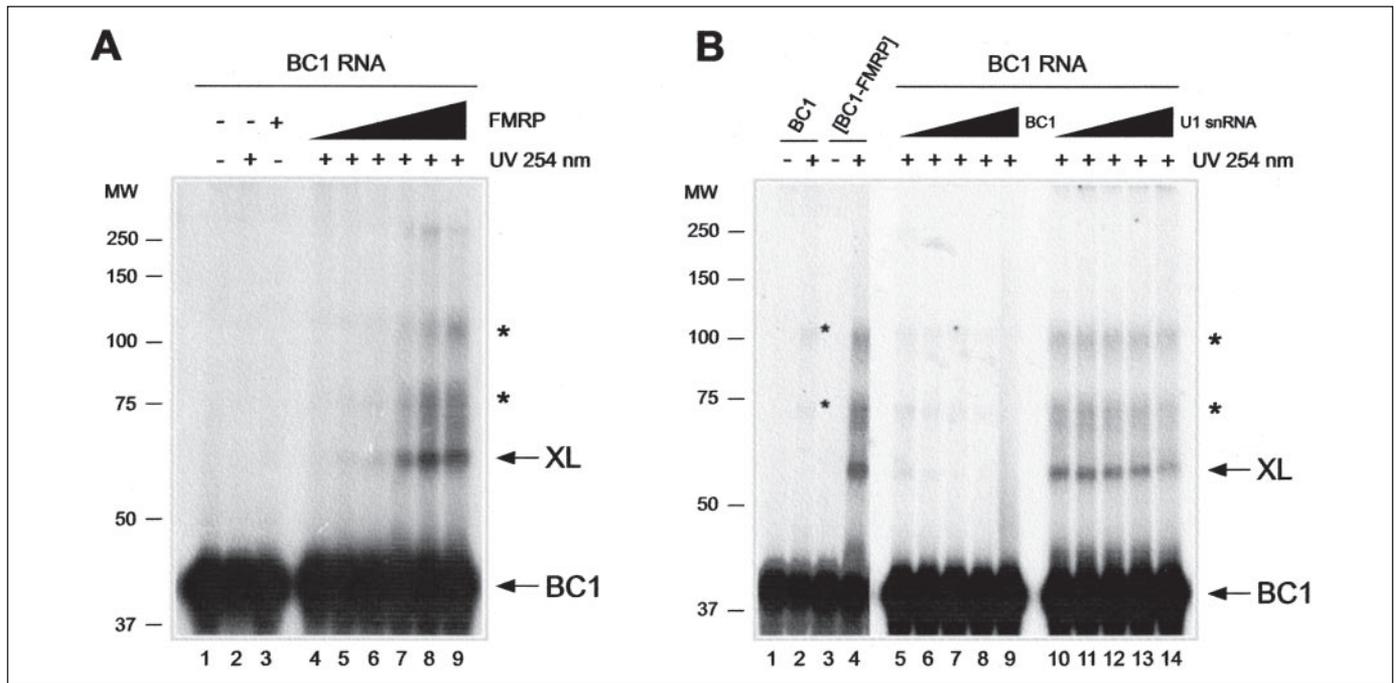
Increasing amounts of FMRP NT were incubated with radioactively labeled BC1 RNA prepared by transcription *in vitro*, and the mixture was UV-irradiated at 254 nm. Subsequent analysis of the complexes on a denaturing SDS-polyacrylamide gel revealed three labeled bands that appear upon UV irradiation (Fig. 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.

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. 4B).



**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*.

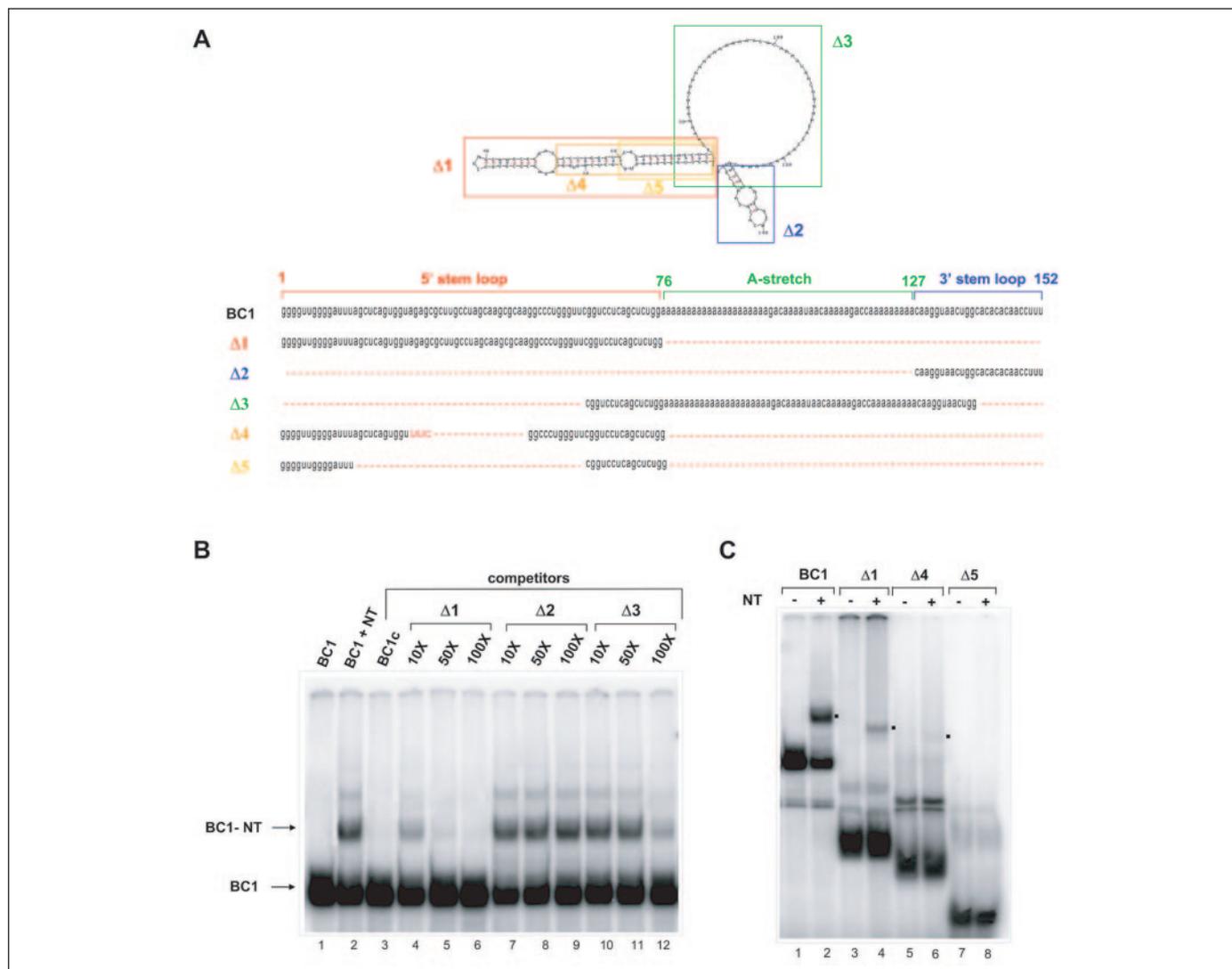
**FMRP Recognizes the 5' Stem Loop of BC1 RNA**—To determine which domain of BC1 RNA was responsible for FMRP N terminus binding, an excess of unlabeled RNA constructs representing parts of the BC1 RNA (Fig. 5*A*;  $\Delta 1$ – $\Delta 3$ ) was used to compete for the interaction with labeled BC1 RNA (Fig. 5*B*). These BC1 RNA constructs are most likely structured *in vitro*, as suggested by the highly negative  $\Delta G$  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  $\Delta 1$  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  $\Delta 3$  construct (lane 12). No competition occurred with the  $\Delta 2$  construct corresponding to 3' stem loop. These data indicated that FMRP contacts principally the 5' end of BC1. The  $\Delta 3$  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  $\Delta 2$ ) does not bind. The same experiment was repeated with the full-length FMRP, and also, in this case, only the  $\Delta 1$  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  $\Delta 4$  and  $\Delta 5$  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  $\Delta 4$  is bound only weakly, the proximal part of 5' stem loop

alone (construct  $\Delta 5$ ) 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



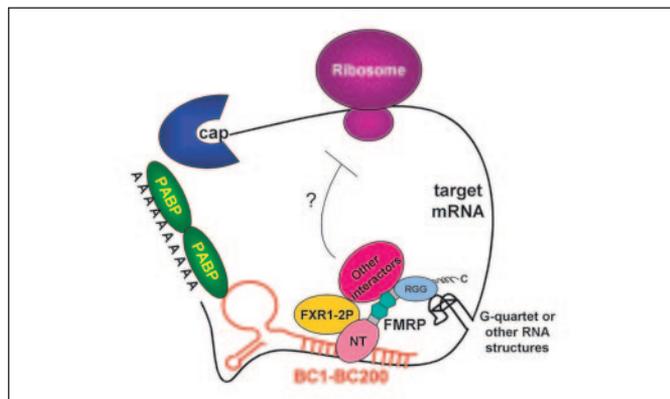
**FIGURE 5. The 5' stem loop of BC1 RNA binds to FMRP-NT.** *A*, scheme of the BC1 deletion mutants used in the EMSA experiments reported below. Δ1, nt 1–76 (corresponding to the entire 5' stem loop); Δ2, nt 127–152 (corresponding to the entire 3' stem loop); Δ3, nt 61–138 (containing the A stretch); 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*, <sup>32</sup>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. 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,<sup>4</sup> 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 FMRI mRNAs (7, 13, 14) contain both a G quartet and a region complementary to BC1 RNA. In addition, other points of contact

<sup>4</sup> 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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## Characterization of the FMRP-BC Interaction

638–653

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