On BC1 RNA and the fragile X mental retardation protein

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The fragile X mental retardation protein (FMRP), the functional absence of which causes fragile X syndrome, is an RNA-binding protein that has been implicated in the regulation of local protein synthesis at the synapse. The mechanism of FMRP's interaction with its target mRNAs, however, has remained controversial. In one model, it has been proposed that BC1 RNA, a small non-protein-coding RNA that localizes to synaptodendritic domains, operates as a requisite adaptor by specifically binding to both FMRP and, via direct base-pairing, to FMRP target mRNAs. Other models posit that FMRP interacts with its target mRNAs directly, i.e., in a BC1-independent manner. Here five laboratories independently set out to test the BC1–FMRP model. We report that specific BC1–FMRP interactions could be documented neither *in vitro* nor *in vivo*. Interactions between BC1 RNA and FMRP target mRNAs were determined to be of a nonspecific nature. Significantly, the association of FMRP with bona fide target mRNAs was independent of the presence of BC1 RNA *in vivo*. The combined experimental evidence is discordant with a proposed scenario in which BC1 RNA acts as a bridge between FMRP and its target mRNAs and rather supports a model in which BC1 RNA and FMRP are translational repressors that operate independently.

fragile X syndrome | non-protein-coding RNAs | translational control

Small non-protein-coding RNAs perform important functions in the regulation of eukaryotic gene expression (1). In the mammalian central nervous system, they have been implicated in promoting organism–environment interactions (2). Small untranslated BC1 RNA is a translational repressor that is thought to participate in the regulation of local protein synthesis at the synapse (2, 3). BC1 RNA represses translation by targeting assembly of 48S initiation complexes (4). Interacting with eukaryotic initiation factor 4A (eIF4A) and poly(A) binding protein (PABP) (4–6), BC1 RNA prevents recruitment of the 43S preinitiation complex to the mRNA. Targets of BC1-mediated repression are those mRNAs that depend on the eIF4 family of factors for efficient initiation (4).

Fragile X syndrome is caused by the functional absence of fragile X mental retardation protein (FMRP) (7, 8). Consensus has developed over recent years that FMRP is, like BC1 RNA, a translational repressor that is active in postsynaptic microdomains (7, 9, 10). However, in contrast to BC1 RNA, FMRP is associated with polysomes (11–15), indicating that BC1 RNA and FMRP operate at different levels in the translation pathway.

In an alternative model, it has been proposed that BC1 RNA and FMRP interact directly with each other (16, 17). In this scenario, BC1 RNA (*i*) physically binds to FMRP, (*ii*) directly interacts, by base-pairing of its 5′ domain, with select mRNAs that are FMRP targets, and (*iii*) acts as a bridge between FMRP and such target mRNAs, thus serving as a requisite adaptor (16, 17).

The above two models cannot be reconciled. We, five independent groups with a longstanding interest in the molecular biology of BC1 RNA and/or FMRP, therefore reexamined the issue of FMRP–BC1–mRNA interactions *in vitro* and *in vivo*. We report that no specific direct interaction can be documented and that BC1 RNA is not required as an adaptor to link previously reported target mRNAs to FMRP.

Results

***In Vitro* Interactions of BC1 RNA with FMRP.** FMRP has been reported to bind to BC1 RNA under conditions of exceptionally high salt concentrations (750 mM NaCl plus 100 mM KCl) (17). We performed EMSAs to reassess binding of BC1 RNA to FMRP as a function of salt concentration *in vitro*. In the presence of 850 mM monovalent cations (buffer Z) (17) we were unable to detect any mobility shift of BC1 RNA over a wide range of FMRP concentrations (Fig. 1*A*). When salt concentrations in buffer Z were reduced to 100 mM KCl (modified buffer Z), a mobility shift of BC1 RNA became apparent at FMRP levels of 100 ng per assay and higher (Fig. 1*A*).

Notably, however, buffer Z does not use competitor RNA to reduce unspecific binding. EMSAs were therefore repeated under conditions of physiological salt concentrations (150 mM KCl, buffer B) (18) in the presence of 100 ng/μl tRNA (Fig. 1*B*). In this case, no mobility shift was observed except at an extreme level of FMRP (800 ng). N8 RNA, an FMR1 mRNA segment that does not specifically bind to FMRP (19), readily displaced BC1 RNA from FMRP in a manner analogous to tRNA (data not shown). In contrast, when we used N19 RNA, an FMR1 mRNA segment that contains a G-quartet-forming structure and is a genuine FMRP target RNA (19), tRNA did not compete because a clear shift was observed in its presence over the entire FMRP concentration range (Fig. 1*B*).

For a quantitative assessment of BC1–FMRP interactions, we used a filter binding RNA competition assay (20) that allows appraisal of nonspecific binding contributions. As shown in Fig. 1*C*, addition of tRNA or BC1 RNA to the reaction had little effect on the binding of N19 RNA to FMRP, demonstrating that BC1 RNA is as ineffective as tRNA at displacing N19 RNA, a bona fide FMRP target RNA. In contrast, both tRNA and N19 RNA displaced BC1 RNA from FMRP, as evidenced by a substantial reduction of binding in the presence of either com-

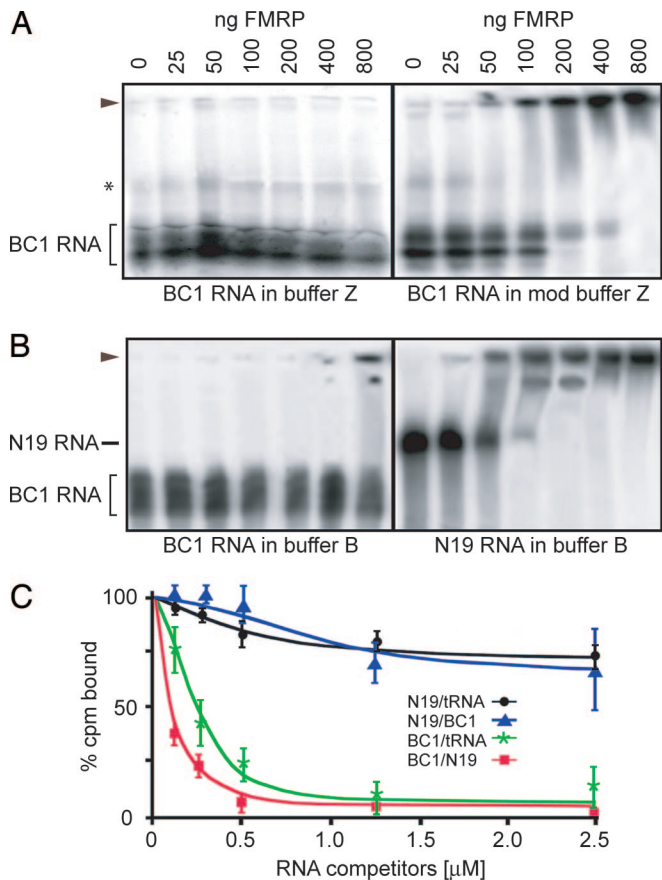www.pnas.org/cgi/doi/10.1073/pnas.0710991105

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Fig. 1. Binding of FMRP to BC1 RNA is nonspecific. (*A* and *B*) EMSA was performed with ^{32}P-labeled BC1 RNA or N19 RNA and increasing amounts of FMRP as indicated. Monovalent cations were used as follows: 750 mM NaCl plus 100 mM KCl (*A Left*, buffer Z), 100 mM KCl (*A Right*, mod buffer Z), or 150 mM KCl (*B*, buffer B). (*A*) In the absence of competitor RNA, binding of BC1 RNA to FMRP was observed under conditions of moderate but not high salt concentrations. (*B*) Binding of BC1 RNA, but of N19 RNA, was abolished in the presence of 100 ng/μl tRNA. Arrowheads indicate FMRP–RNA complexes. In addition, the positions of BC1 RNA and N19 RNA in the absence of FMRP are indicated. The asterisk indicates a minor species of differentially folded BC1 RNA formed under conditions of high salt concentrations. In general, whereas *in vitro* transcribed BC1 RNA resolves as a single band in denaturing gels, several bands reflecting different conformers are visualized in native gels, depending on salt concentrations. (*C*) Competition experiments for FMRP binding to BC1 RNA and N19 RNA were performed by using filter binding assays. Each data point represents the mean of three experimental values. The key lists RNAs in the format labeled/competitor. Error bars represent SEM. The data in this figure are from the E.W.K. laboratory.

Fig. 2. BC1 RNA does not specifically interact with FMRP target mRNAs. Total RNA isolated from WT C57BL/6 mouse brains and from *BC1*$^{-/-}$ KO mouse brains was incubated with biotinylated BC1 RNA or an oligonucleotide complementary to the 3' BC1 RNA region (bio-unique). The experimental protocol of Zalfa *et al.* (17) was followed throughout. RNA was extracted and RT-PCR was performed by using primers specific for the respective RNA species indicated. Lanes were loaded as follows: lane 1, total RNA from KO *BC1*$^{-/-}$ mouse brain was annealed to biotinylated BC1 RNA; lane 2, total RNA from KO *BC1*$^{-/-}$ mouse brain was annealed to biotinylated bio-unique oligonucleotide; lane 3, total RNA from KO *BC1*$^{-/-}$ mouse brain was mock-annealed to streptavidin magnetic beads; lane 4, total RNA from WT mouse brain was annealed to biotinylated BC1 RNA; lane 5, total RNA from WT mouse brain was annealed to biotinylated bio-unique oligonucleotide; lane 6, total RNA from WT mouse brain was mock-annealed to streptavidin magnetic beads; lane 7, total RNA from WT mouse brain was used as template for RT-PCR with specific oligonucleotides as a positive control for each experiment; lane 8, total RNA from KO *BC1*$^{-/-}$ mouse brain was used as template for RT-PCR with specific oligonucleotides as a positive control for each experiment. The data in this figure are from the J.B. laboratory.

petitor RNA (Fig. 1*C*). The data show that whereas N19 RNA, BC1 RNA, and tRNA appear to bind to the same site on FMRP, BC1 RNA but not N19 RNA is competed off this site by tRNA. In view of the these data, it is concluded that *in vitro* BC1–FMRP interactions are nonspecific and are of low affinity. It is possible that many RNAs bind to FMRP in such nonspecific, low-affinity manner *in vitro* (5).

Interactions of BC1 RNA with FMRP Target mRNAs. We next turned to the question of whether BC1 RNA physically interacts with mRNAs that are targets of FMRP regulation. Zalfa and colleagues (16, 17) proposed that the 5' BC1 domain directly interacts, via base-pairing, with such FMRP target mRNAs and that BC1 RNA thus bridges these mRNAs and FMRP. These authors probed BC1–mRNA interactions by annealing biotinylated BC1 RNA with total RNA from mouse brain and by
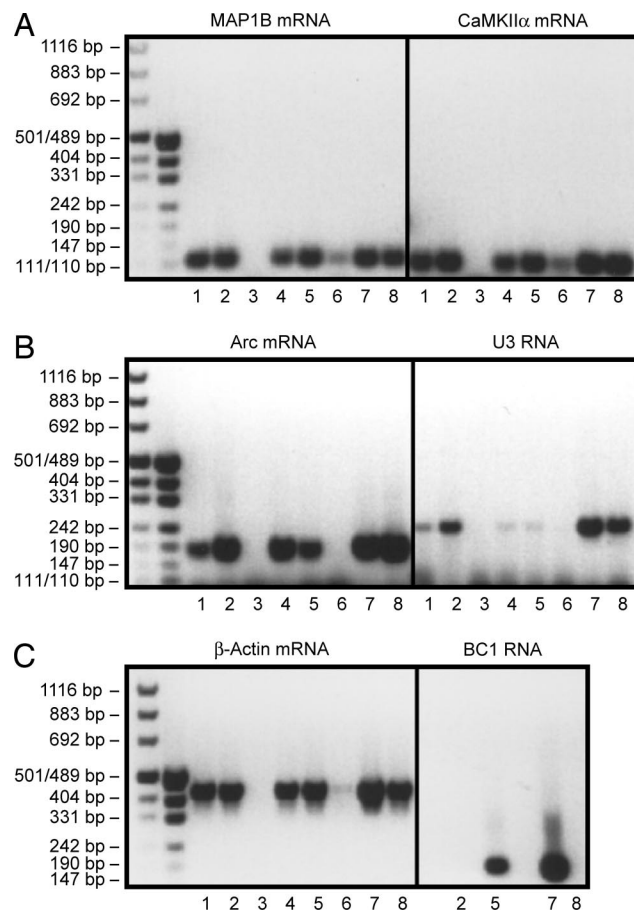
subsequently amplifying RNAs that were recovered from streptavidin beads (17).

To reexamine this issue, we replicated the previously reported experiments under identical conditions and probed for FMRP target and nontarget RNAs (Fig. 2). Two approaches were used in parallel in these experiments. (*i*) Biotinylated BC1 RNA was incubated with total RNA isolated from mouse brain, as described (17). (*ii*) In addition, we used a biotinylated oligodeoxyribonucleotide complementary to the 3' BC1 domain. This probe (called bio-unique) has recently been used for the specific capture, per affinity purification, of BC1 RNA from total mouse brain RNA (21). It will capture endogenous, native BC1 RNA together with any mRNA that may be bound to its 5' domain, an advantage over the first approach, in which any mRNA bound to

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endogenous BC1 RNA has to be competed off and captured by externally added biotinylated BC1 RNA. For further controls, total mouse brain RNA was prepared from $BC1^{-/-}$ [knockout (KO)] animals (22) in addition to WT animals.

As is shown in Fig. 2, we obtained RT-PCR products of microtubule-associated protein 1B (MAP1B), calcium/calmodulin-dependent kinase IIα (CaMKIIα), and Arc (Arg-3.1)mRNAs after capture with biotinylated BC1 RNA from WT and BC1 KO mouse brain total RNA (Fig. 2 *A* and *B*, lanes 1 and 4). Fig. 2*C Right*, lane 8, confirms that BC1 RNA is absent in total RNA from BC1 KO brains. The three FMRP target mRNAs were also captured and amplified from WT mouse brain total RNA using the bio-unique oligonucleotide that is directed against the 3′ BC1 domain (lane 5 in Fig. 2 *A* and *B*). At the same time, however, both approaches also yielded amplification products of RNAs that have not been reported to be FMRP targets, i.e., β-actin mRNA and U3 snoRNA (Fig. 2 *B* and *C*). Both RNAs are ubiquitous RNAs, the latter a small nucleolar RNA that participates in pre-rRNA processing. Neither RNA exhibits any significant sequence complementarity to BC1 RNA. The combined results let us conclude that the BC1 capture approaches used in this and a previous article (17) do not discriminate between FMRP target and nontarget mRNAs.

It appears that this lack of discrimination is the result of nonspecific interactions. Thus, we noted that, under the experimental conditions used and previously reported, MAP1B and CaMKIIα mRNAs also bind, albeit with lower apparent affinity, to the streptavidin matrix (Fig. 2 *A* and *B*, lane 6). Furthermore, when using the bio-unique oligonucleotide directed against the 3′ BC1 domain, RNAs were captured and amplified even from BC1 KO mouse brain total RNA (Fig. 2, lane 2). The results show that the chosen experimental routine is prone to yielding nonspecific amplification products.

The central domain of BC1 RNA contains a single-stranded homopolymeric A segment of 22 nucleotides. We therefore hypothesized that, in capture experiments *in vitro*, BC1 RNA may interact, by base-pairing of at least part of this A22 segment, with RNAs that contain U-rich segments. To test this hypothesis, we annealed total RNA from mouse brain to poly(A) RNA agarose (Fig. 3). Bound RNA was recovered, converted to cDNA, and amplified with gene-specific primers [supporting information (SI) *Materials and Methods*] using previously described conditions (17). From total mouse brain RNA we amplified Fmr1, eukaryotic elongation factor 1A (eEF1A), CaMKIIα, and MAP1B mRNAs (Fig. 3, lane 1). Each of these mRNAs was also present in the poly(A) annealed fraction (Fig. 3, lane 3). These four mRNAs are FMRP targets that contain U-rich segments (23). However, we also recovered β-actin and dynamin A1 mRNAs (Fig. 3), mRNAs that feature U-rich segments but are not known to be FMRP target mRNAs (23). The experimental approach therefore selects U-rich mRNAs regardless of whether they are FMRP targets or not. The strength of the band representing the respective captured mRNA (Fig. 3, lane 3) corresponds to the length of U-element(s) in that mRNA (see SI Table 1). As a further control, we performed the annealing reaction in the presence of excess free poly(A) RNA to verify that amplification did not arise from contaminating unbound RNA (see SI Fig. 6).

Taken together, the above results lead to the conclusion that the chosen RNA capture and amplification procedure (17) is unable to substantiate any specific binding of BC1 RNA to FMRP target mRNAs. Various RNAs are captured and amplified in this procedure because of (*i*) lack of specificity of the procedure *per se* and (*ii*) the selection of U-rich RNAs by an A-rich segment in BC1 RNA.
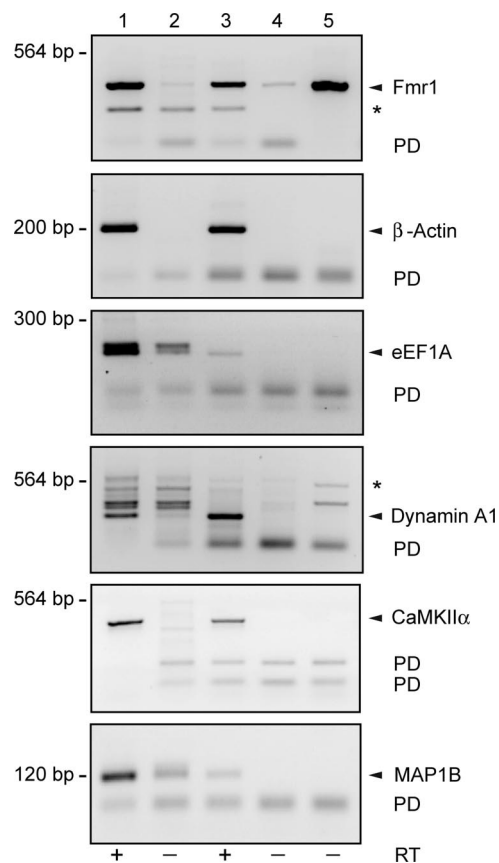

Fig. 3. U-rich FMRP target and nontarget mRNAs bind to poly(A) RNA. Mouse cortex total RNA (1 μg) was annealed to 20 μl of poly(A) RNA agarose resin, and bound RNA was converted to cDNA and amplified (see *Materials and Methods*). Results are shown in lane 3. Lane 4 shows controls performed in the absence of reverse transcriptase. Lanes 1 and 2 show amplifications of cDNA from total RNA (1 μg) after omission of the poly(A) binding step (reverse transcriptase omitted in lane 2). Lane 5 is an amplification of pET21A-FMRP plasmid DNA (10 ng). PD indicates primer–dimer bands, and asterisks indicate nonspecific bands. In the absence of poly(A) annealing (lanes 1 and 2), a minor band was in some cases apparent both in +RT and −RT lanes (e.g., dynamin A1 amplification). Bands in the −RT lane may indicate amplification from carryover of genomic DNA. However, such bands were not observed after poly(A) annealing. The data in this figure are from the R.B.D. laboratory.

On the Interaction of BC1 RNA, FMRP, and FMRP Target mRNAs *in Vivo*. The model proposed by Zalfa and colleagues (16, 17) specifically predicted that BC1 RNA is required as an adaptor to link FMRP with its target mRNAs. If so, FMRP should be unable to interact with such target mRNAs in the absence of BC1 RNA. We tested this hypothesis *in vivo* using $BC1^{-/-}$ animals (22).

We performed immunoprecipitation (IP) with brain extracts prepared from WT and $BC1^{-/-}$ (KO) animals, using FMRP-specific monoclonal antibody 7G1-1 (24). IP RNA was amplified by RT-PCR with mRNA-specific primers. We probed for CaMKIIα, Arc, and MAP1B mRNAs, i.e., those mRNAs that Zalfa *et al.* (17) proposed to be physically linked by BC1 RNA to FMRP. In addition, we probed for Fmr1 mRNA, another FMRP target mRNA, and GAPDH mRNA, a FMRP nontarget control mRNA (24).

As shown in Fig. 4, FMRP target mRNAs, but not nontarget GAPDH mRNA, were amplified from IP RNA. Notably, levels of none of the FMRP target mRNAs that were amplified from IP RNA were found to differ discernibly between preparations from WT and BC1 KO brains (Fig. 4*A*). Quantitative and statistical analysis did not reveal any significant differences, for
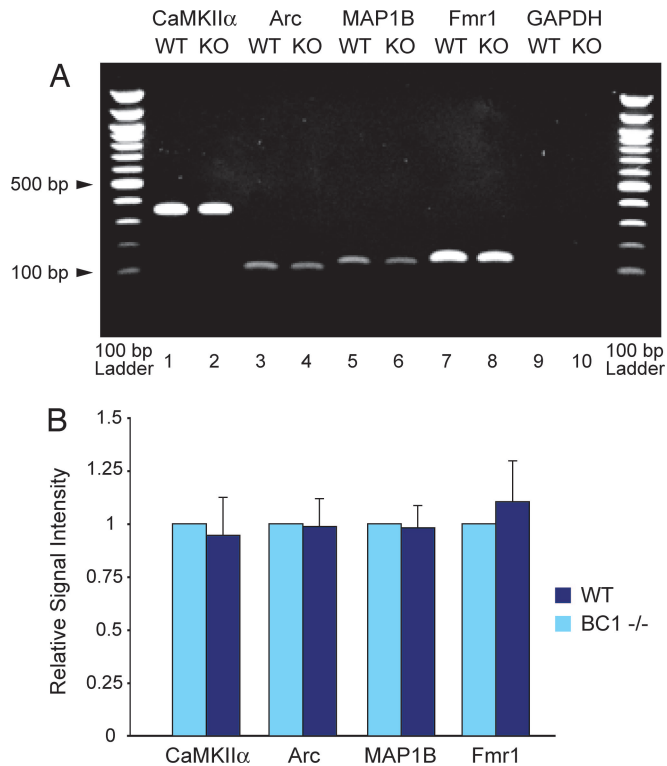
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Fig. 4. FMRP associates with target mRNAs *in vivo* in the absence of BC1 RNA. Using monoclonal anti-FMRP antibody 7G1-1, coimmunoprecipitation was performed in brain extracts from WT and $BC1^{-/-}$ (KO) animals. (*A*) RNA was extracted and assayed for CaMKIIα, Arc, MAP1B, and Fmr1 mRNAs (i.e., FMRP target mRNAs) and for GAPDH mRNA (not an FMRP target). All examined FMRP target mRNAs were indistinguishably identified in WT and KO brains. Expected PCR product sizes were as follows: CaMKIIα, 354 bp; Arc, 86 bp; MAP1B, 119 bp; Fmr1, 134 bp; GAPDH, 233 bp. (*B*) Paired Student's *t* tests revealed no significant difference in FMRP target mRNA levels between WT and KO animals ($P > 0.8$ for each target mRNA; $n \geq 4$ in all cases). For each group, the relative WT level was normalized to 1. The data in this figure are from the H.T. laboratory.

any of the probed FMRP target mRNAs, between immunoprecipitated RNAs derived from WT and BC1 KO brains, respectively (Fig. 4*B*). The data show that FMRP associates with target mRNAs in the absence of BC1 RNA, a result incompatible with a requirement for BC1 RNA to provide a physical link between FMRP and its target mRNAs, as postulated by Zalfa and colleagues (16, 17).

Does BC1 RNA associate with FMRP *in vivo*? We performed IP experiments to address this question. FMRP (F-IP) and PABP (P-IP) were IP-targeted in whole-brain homogenates prepared from $fmr1^{-/-}$ (KO) and WT mice. PABP was chosen as a positive control because it has previously been shown to interact with BC1 RNA (4, 25, 26). F-IP from $fmr1^{-/-}$ (KO F-IP) brains was performed to exclude the possibility of unspecific RNA precipitation. As an additional negative control, we performed IP with irrelevant IgGs (IgG-IP).

Extracted IP RNA was probed for BC1 RNA by RT-PCR and real-time PCR. The RT-PCR data show that BC1 RNA was identified in P-IP RNA but not in F-IP RNA (Fig. 5*A*). In contrast, FMRP target PSD-95 mRNA (27) was readily detectable in both F-IP and P-IP (SI Fig. 7). These results were independently confirmed by real-time PCR performed with RNA from three independent tissue preparations. A high P-IP vs. F-IP ΔCT value of 14.01 ± 0.60 confirms that in WT brains BC1 RNA is detectable in PABP but not in FMRP immunoprecipitates. In addition, ΔCT values for KO F-IP vs. WT F-IP
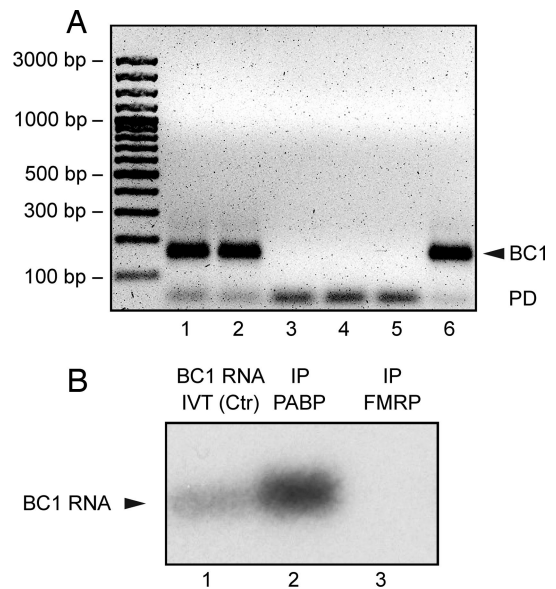

Fig. 5. BC1 RNA does not associate with FMRP *in vivo*. (*A*) RT-PCR was performed with primers specific for BC1 RNA. Template RNA was extracted from input material obtained with WT (lane 1) and *fmr1* KO (lane 2) brains, from irrelevant IgG-IP with WT brains (lane 3), from FMRP-IP (F-IP) with WT (lane 4) and *fmr1* KO (lane 5) brains, and from PABP-IP (P-IP) with WT brains (lane 6). The BC1 PCR product (arrowhead) is detected in both input lanes and in the P-IP lane, but not in F-IP or IgG-IP lanes. PCR products marked by PD are primer dimers. The data in *A* are from the S.K. laboratory. (*B*) Northern hybridization was performed to probe for BC1 RNA in F-IP RNA and P-IP RNA. Whereas a strong signal was observed in the P-IP lane, no signal was detected in the F-IP lane (even after overexposure). *In vitro* transcribed BC1 RNA (10 ng) was used for reference (BC1 Ctr). The data in *B* are from the H.T. laboratory.

of 0.60 ± 0.62 and for WT IgG-IP vs. WT F-IP of 0.46 ± 0.29, i.e., close to 0 in either case, imply that BC1 levels in FMRP immunoprecipitates from WT brains are indistinguishable from background levels. Finally, when detecting PSD-95 mRNA, a ΔCT value for WT F-IP vs. KO F-IP of 3.01 ± 0.83 shows that the antibody efficiently immunoprecipitates PSD-95 mRNA associated with FMRP from WT but not KO brain homogenates. Thus, the lack of BC1 RNA in WT F-IP is not due to a general failure of the antibody to precipitate FMRP–RNA complexes, but to a genuine absence of BC1 RNA from such complexes.

The above experiments were performed with polyclonal anti-FMRP antibody H-120. Analogous work was performed, in separate experiments in a different laboratory, with monoclonal anti-FMRP antibody 7G1-1. Again, an anti-PABP antibody was used for positive control IP. In this case, F-IP RNA and P-IP RNA were probed for BC1 RNA by filter (Northern) hybridization (25). The results (Fig. 5*B*) demonstrate again that BC1 RNA is not detectable in FMRP immunoprecipitates although its presence in PABP immunoprecipitates is clearly evident. The combined data therefore show that BC1 RNA does not associate with FMRP *in vivo*.

Discussion

BC1 RNA and FMRP are translational repressors that have been implicated in the modulation of postsynaptic protein repertoires in neurons (2, 3, 9, 28). BC1 RNA inhibits recruitment of 43S preinitiation complexes to mRNAs; it therefore targets translation particularly of mRNAs with structured 5′ UTRs that require eIF4A-mediated unwinding (4, 5). FMRP targets, on the other hand, include mRNAs with G-quartets (19, 20), kissing complexes (29), and U-rich modules (30), among others. Although the mode of action of FMRP-mediated translational repression

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is currently less well established, it would appear that both repressors may target relatively large subsets of neuronal mRNAs. Therefore, BC1- and FMRP-mediated translational repression may be directed at overlapping sets of target mRNAs.

It is critically important, for this reason, to elucidate the nature of BC1–FMRP interactions, if any, and their relevance for FMRP target recognition. The model proposed by Zalfa and colleagues (16, 17) makes three distinct and directly testable predictions. (*i*) BC1 RNA specifically and directly binds to FMRP. (*ii*) Through its 5′ domain, BC1 RNA specifically and directly binds to various FMRP target mRNAs. (*iii*) BC1 RNA is required as an adaptor, a physical link, between FMRP and a target mRNA. Here we tested these predictions experimentally.

(*i*) No specific binding of BC1 RNA to FMRP was documented either *in vitro* or *in vivo*. Coimmunoprecipitation experiments identified BC1 RNA in PABP IP RNA but failed to do so in FMRP IP RNA. These experiments were performed by two different laboratories—not cognizant of each other's work until after completion—using two different validated anti-FMRP antibodies as well as different precipitation and RNA detection methods. Even when highly sensitive techniques (real-time PCR or RT-PCR with 40 amplification cycles) were used, BC1 RNA was undetectable in FMRP IP RNA. In clear contrast, bona fide FMRP target mRNAs were coimmunoprecipitated by both anti-FMRP antibodies (Fig. 4 and SI Fig. 7).

Low amounts of BC1 RNA were earlier reported in FMRP IP RNA (31); however, slightly higher amounts were detected in the same experiment in MAP2 IP RNA. Because MAP2 is not known as an RNA binding protein, the data suggest that this result occurred from nonspecific precipitation or amplification.

In vitro, BC1 RNA associates with FMRP in a nonspecific manner. Contrary to previous claims (17), no interaction is observed under high concentrations of monovalent cations (850 mM). Under physiological salt concentrations, BC1 RNA associates with FMRP, but this association is nonspecific because it is easily abolished by FMRP nontarget RNAs such as tRNA or N8 RNA. Similarly, under lower than physiological salt concentrations (30 mM NaCl) in the absence of competitor RNA (32), BC1–FMRP interactions are indistinguishable from tRNA–FMRP interactions.

In summary, there is no basis to the claim that BC1 RNA binds to FMRP in any specific way, either *in vitro* or *in vivo*.

(*ii*) The 5′ BC1 domain has been suggested to base pair directly with complementary regions in FMRP target mRNAs (16, 17). However, specific interactions between BC1 RNA and such FMRP target mRNAs could not be corroborated in the present work. The 5′ BC1 domain is a stable stem–loop structure (33). The stability of this domain makes it unlikely that single-stranded segments of sufficient length would be available for base-pairing with other RNAs. In contrast, the single-stranded A-rich segment in the central BC1 domain is readily accessible, at least *in vitro* (33).

(*iii*) The mRNAs encoding MAP1B, Arc, and CaMKIIα have been proposed to require BC1 RNA as a guide to target them to FMRP (16, 17). In *BC1*$^{-/-}$ animals, however, we found that FMRP is associated with these target mRNAs in a manner indistinguishable from WT animals. BC1 RNA therefore does not appear to perform a requisite function in guiding these target mRNAs to FMRP.

In the absence of specific interactions between BC1 RNA and either FMRP or FMRP target mRNAs, and of a requirement for BC1 RNA to guide mRNAs to FMRP, we cannot but conclude that the functional roles of BC1 RNA and FMRP as translational repressors are executed independent of each other. Neither does BC1 RNA require FMRP for its repressor function, nor vice versa. It should be emphasized, however, that these statements do not extend to miRNAs. FMRP has been implicated in the miRNA pathway (reviewed in refs. 3 and 34), but these RNAs

appear to operate via modes of action that are functionally distinct from BC1 RNA.

In summary, the available data are most readily reconciled with the notion that, in the local translation pathway, BC1 RNA and FMRP operate independently. Thus, whereas BC1 RNA represses translation initiation at the level of 48S complex formation, FMRP would target a subsequent step downstream from BC1 RNA. We propose that multiple translational repressors may be required at the synapse to ensure that an adequate activation–repression balance is maintained over time (35).

Materials and Methods

Sequences of oligonucleotides used as PCR primers are listed in *SI Materials and Methods*.

EMSA and Filter Binding Assays. His-tagged FMRP was expressed in *Escherichia coli* as described (36). BC1 and N19 RNAs were ^{32}P-labeled at 40,000 cpm per reaction (≈10 ng) and were incubated with increasing amounts of FMRP (0–800 ng per assay) in 20 μl of 10 mM Hepes (pH 7.9), 100 mM KCl, 750 mM NaCl, 3 mM MgCl$_2$, 7 mM 2-mercaptoethanol, 10 mM DTT, 5% glycerol, 1 μg/μl BSA, and 1.33 μg/μl heparin (buffer Z) (17) or in a buffer containing 50 mM Tris·HCl (pH 7.6), 150 mM KCl, 1 mM MgCl$_2$, 1 mM EDTA, 1 mM DTT, 0.2 units/μl RNase inhibitor (Amersham), 100 ng/μl total yeast tRNA, and 100 ng/μl BSA (buffer B) (18). If competitor RNAs were used, they were preincubated with FMRP for 10 min before addition of the labeled RNA.

Filter binding assays were performed as described (18). For EMSA experiments, RNA–protein complexes were resolved on 5% polyacrylamide gels. It should be noted that under the conditions used RNA–FMRP complexes are typically detected near the top of the gel (see also refs. 16 and 32). We presume that such bands represent homomeric FMRP complexes that bind RNA. However, in the interest of experimental conditions being consistent with previous work (16), we refrained from using dissociating agents such as urea.

In Vitro Annealing to Mouse Brain RNA. Biotinylated BC1 RNA was bound to streptavidin-conjugated magnetic beads and incubated with total mouse brain RNA as described (17). In separate experiments, we used 100 pmol of a biotinylated oligonucleotide complementary to 3′ BC1 domain (bio-unique) (21). First-strand cDNA synthesis was performed by using Transcriptor reverse transcriptase (Roche Diagnostics) and random hexamer oligonucleotides, followed by 30 cycles of PCR amplification (17) with RNA-specific oligonucleotide primers.

Hybridization of mRNAs to poly(A) RNA. Total RNA (1 μg) from mouse cortex was incubated with 20 μl of poly(A) RNA agarose resin (Sigma) at 80°C for 5 min in 20 μl of 10 mM Tris·HCl (pH 7.5), 2 mM MgCl$_2$, 400 mM NaCl, and 0.2% SDS (17) and allowed to cool at room temperature for 2 h. Nonbound material was removed with two washes (100 μl each) of the same buffer. Bound RNA was isolated with Tri-Reagent; after precipitation, half was converted to cDNA with the SuperScript III first-strand synthesis system (Invitrogen), and the other half was incubated in the absence of reverse transcriptase. Samples (1/20 aliquots) were amplified by PCR for the indicated mRNAs.

Immunoprecipitation. IP with anti-FMRP was performed (*i*) to probe for FMRP target mRNAs in the presence or absence of BC1 RNA and (*ii*) to probe for an association of BC1 RNA with FMRP. In the first approach we used a modified version of a previously published procedure (37). Dynabeads protein G slurry (Invitrogen) was washed with 100 mM sodium phosphate (pH 8.0) and incubated overnight at 4°C with 2.5 μg/μl monoclonal anti-FMRP 7G1-1 (Developmental Studies Hybridoma Bank) (24). Beads were incubated with brain extracts at 4°C for 2 h and washed extensively, and RNA was extracted by using TRIzol (Invitrogen).

In the second approach (variant S.K. laboratory), aliquots of brain extracts were incubated overnight with 15 μg of polyclonal anti-FMRP H-120 (Santa Cruz Biotechnology), affinity-purified polyclonal anti-PABP (38) (a gift of Evita Mohr, Institute of Anatomy I, Cellular Neurobiology, University Hospital Hamburg-Eppendorf, Hamburg, Germany), or rabbit IgG (Sigma–Aldrich). Samples were incubated for 40 min with 100 μl of preblocked protein A agarose. Beads were precipitated, washed, resuspended, and treated with proteinase K (Roche). RNA was extracted by using the RNeasy Mini Kit (Qiagen). In the second approach (variant H.T. laboratory), monoclonal anti-FMRP 7G1-1 is used. The anti-PABP used was described earlier (4); in addition, a newly generated affinity-purified polyclonal anti-PABP was used with identical results. IP methods used were those described for the first approach.

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The fact that the laboratories contributing to this article used different materials and methods, as exemplified here by IP methods, is a consequence of their not being aware of each other's work. Regardless of the different methods used, however, the results obtained were congruent. Detailed IP methods are provided in *SI Materials and Methods*.

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