

Dendritic BC1 RNA in translational control mechanisms

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Translational control at the synapse is thought to be a key determinant of neuronal plasticity. How is such control implemented? We report that small untranslated BC1 RNA is a specific effector of translational control both *in vitro* and *in vivo*. BC1 RNA, expressed in neurons and germ cells, inhibits a rate-limiting step in the assembly of translation initiation complexes. A translational repression element is contained within the unique 3' domain of BC1 RNA. Interactions of this

domain with eukaryotic initiation factor 4A and poly(A) binding protein mediate repression, indicating that the 3' BC1 domain targets a functional interaction between these factors. In contrast, interactions of BC1 RNA with the fragile X mental retardation protein could not be documented. Thus, BC1 RNA modulates translation-dependent processes in neurons and germ cells by directly interacting with translation initiation factors.

Introduction

Synaptic plasticity, the input-specific change in synaptic strength after physiological stimulation, is thought to underlie higher brain functions such as learning and memory. Long-term modulation of synaptic efficacy depends on the *de novo* synthesis of proteins, and increasing evidence suggests that translational control of gene expression at the synapse is required for such modulation (for reviews see Wells et al., 2000; Job and Eberwine, 2001; Richter and Lorenz, 2002; Steward and Schuman, 2003; Kindler et al., 2005).

Support for this model comes from several lines of investigation. Diverse types of RNA have been localized to dendrites and, in some cases, to postsynaptic dendritic microdomains (for reviews see Job and Eberwine, 2001; Steward and Schuman, 2003). Dendrites contain the requisite machinery to translate mRNAs (Tiedge and Brosius, 1996; Torre and Steward, 1996), and proteins have been shown to be synthesized on site in synaptodendritic domains (Torre and Steward, 1992; Crino and Eberwine, 1996). The synthesis of some proteins in dendrites

appears to be modulated by physiological parameters such as neurotrophic action and trans-synaptic activity (for reviews see Wells et al., 2000; Job and Eberwine, 2001; Richter and Lorenz, 2002; Kindler et al., 2005). These data suggest a local translation feedback scenario in which synaptic stimulation results in a modification of protein repertoires at the synapse, which in turn produces a change in synaptic strength.

At the same time, the data raise fundamental questions. How is local translational control executed? What mechanisms are used to control translational repression and activation of mRNAs? Recent evidence has indicated that small untranslated BC1 RNA interacts with the translational machinery (Mudashetty et al., 2002; Wang et al., 2002; West et al., 2002; Zalfa et al., 2003). Untranslated RNAs (utRNAs), also referred to as nonprotein-coding or nonmessenger RNAs (Brosius and Tiedge, 2004), play diverse and important roles in the control of gene expression at the levels of transcription, RNA processing, and translation (for review see Barciszewski and Erdmann, 2003). BC1 RNA is a small utRNA that is selectively expressed in nerve cells, where it is specifically targeted to synaptodendritic neuronal microdomains (Chicurel et al., 1993; Muslimov et al., 1997). In one scenario, BC1 RNA has been suggested to repress protein synthesis by interacting directly with the translation initiation apparatus (Wang et al., 2002). In a second scenario, it has been proposed that BC1 RNA mediates translational control indirectly by interacting with the fragile X mental retardation protein (FMRP; Zalfa et al., 2003, 2005).

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Abbreviations used in this paper: AGESA, agarose gel electrophoresis assay; ANOVA, analysis of variance; BMPR, bone morphogenic protein receptor; CPE, cytoplasmic polyadenylation element; CSFV, classical swine fever virus; eEF1A, eukaryotic elongation factor 1A; eIF, eukaryotic initiation factor; EMSA, electrophoretic mobility shift assay; FMRP, fragile X mental retardation protein; IRES, internal ribosome entry site; PABP, poly(A) binding protein; RRL, rabbit reticulocyte lysate; RRM, RNA recognition motif; utRNA, untranslated RNA.

FMRP is expressed at insufficient levels or in dysfunctional form in the fragile X syndrome, a common form of mental retardation (for review see O'Donnell and Warren, 2002). In this second scenario, it is suggested that BC1 RNA, through its highly structured 5' domain, interacts with a subset of neuronal mRNAs and thereby targets such mRNAs for FMRP-mediated translational repression (for review see Antar and Bassell, 2003; Zalfa et al., 2003).

Thus, although small untranslated BC1 RNA has been implicated in the translational control of gene expression (Tiedge et al., 1991; Muslimov et al., 1998), the underlying mechanism remains unresolved. In this paper, we present a functional dissection of the BC1-mediated translational repression mechanism in vitro and in vivo. We show that the 3', but not the 5', BC1 domain is repression competent. The 3' BC1 domain prevents productive recruitment of the small ribosomal subunit to the mRNA. It binds directly to eukaryotic initiation factor (eIF) 4A and poly(A) binding protein (PABP), and functional interactions with both of these initiation factors are required for effective repression. The combined results establish BC1 RNA as a directly acting effector of translational control.

Results

BC1 RNA is a specific repressor of translation in *Xenopus laevis* oocytes

To examine whether BC1 RNA modulates translation in living cells, stage VI *X. laevis* oocytes were coinjected with luciferase mRNA and with titrating amounts of BC1 RNA. Luciferase activity was used as an index for relative translational efficiencies. Luciferase mRNA does not contain any known FMRP recognition motifs such as G-quartets or U-rich elements (for review see Denman, 2003).

We determined that in *X. laevis* oocytes, translation of luciferase mRNA was inhibited by BC1 RNA in a concentration-dependent manner. At 20 ng per oocyte (~400 nM), BC1 RNA reduced translation efficiency by >80% (Fig. 1). In contrast to BC1, U6 RNA at the same concentration had no effect on translation (Fig. 1 B). BC1-mediated translational repression in *X. laevis* oocytes was not further increased after 190 min, as compared with 70 min (Fig. 1 A). Control experiments showed that the decrease in translational efficiency was not attributable to degradation of luciferase mRNA: injected ³²P-labeled luciferase mRNA remained stable in the presence of titrating amounts of BC1 RNA under the experimental conditions used (unpublished data). BC1 RNA also repressed translation in a concentration-dependent manner in the rabbit reticulocyte lysate (RRL) system (Fig. 1 C; Wang et al., 2002). The combined data thus show that BC1 RNA is a translational repressor that is effective in the nanomolar concentration range.

A translational repression element is contained in the 3' BC1 domain

Prominent secondary structure elements naturally subdivide BC1 RNA (152 nt) into three major domains (Rozhdestvensky et al., 2001). The 5' BC1 domain forms a stable stem-loop structure of 74 nt. This domain harbors a dendritic targeting

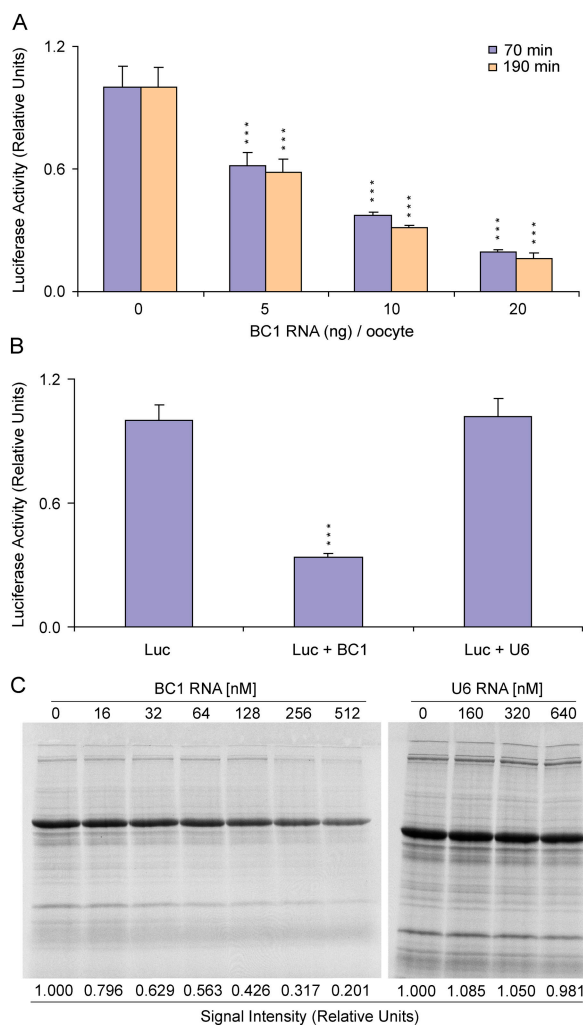


Figure 1. BC1 RNA represses translation in *X. laevis* oocytes. (A) 10 ng of capped and polyadenylated luciferase mRNA was coinjected into stage VI oocytes with increasing amounts of BC1 RNA (10 ng BC1 RNA corresponds to ~200 nM intracellular). The average luminescence signal in the absence of BC1 RNA was assigned a relative luciferase activity value of 1. Quantitative analysis of four experiments revealed a significant reduction of luciferase activity in the presence of BC1 RNA [one-way analysis of variance [ANOVA], $P < 0.001$; Scheffe's multiple comparison post hoc analysis [comparison with 0 nM BC1 RNA control]: ***, $P < 0.001$]. (B) In control experiments, 400 nM BC1 or U6 RNA was coinjected with luciferase mRNA (Luc). Luminescence was measured after 55 min. Quantitative analysis of four experiments revealed a significant decrease of luciferase activity in the presence of BC1 RNA but not in the presence of U6 RNA [one-way ANOVA, $P < 0.001$; Scheffe's multiple comparison post hoc analysis [comparison with Luc control]: ***, $P < 0.001$]. (C) For comparison, translational repression by BC1 RNA is shown in RRL. Incorporation of ³⁵S-methionine into endogenous proteins was examined in untreated RRL and visualized by SDS-PAGE as described previously (Wang et al., 2002). U6 RNA was not repression competent. Quantification is shown for the major bands.

element, a cis-acting element that is responsible for dendritic transport (Muslimov et al., 1997). The 5' domain is separated by one nucleotide from a central domain of 22 uninterrupted A residues, which in turn is followed by a unique 3' BC1 domain (55 nt). This 3' domain consists of a punctuated A-rich subdomain and a 3'-terminal stem-loop structure (Rozhdestvensky et al., 2001).

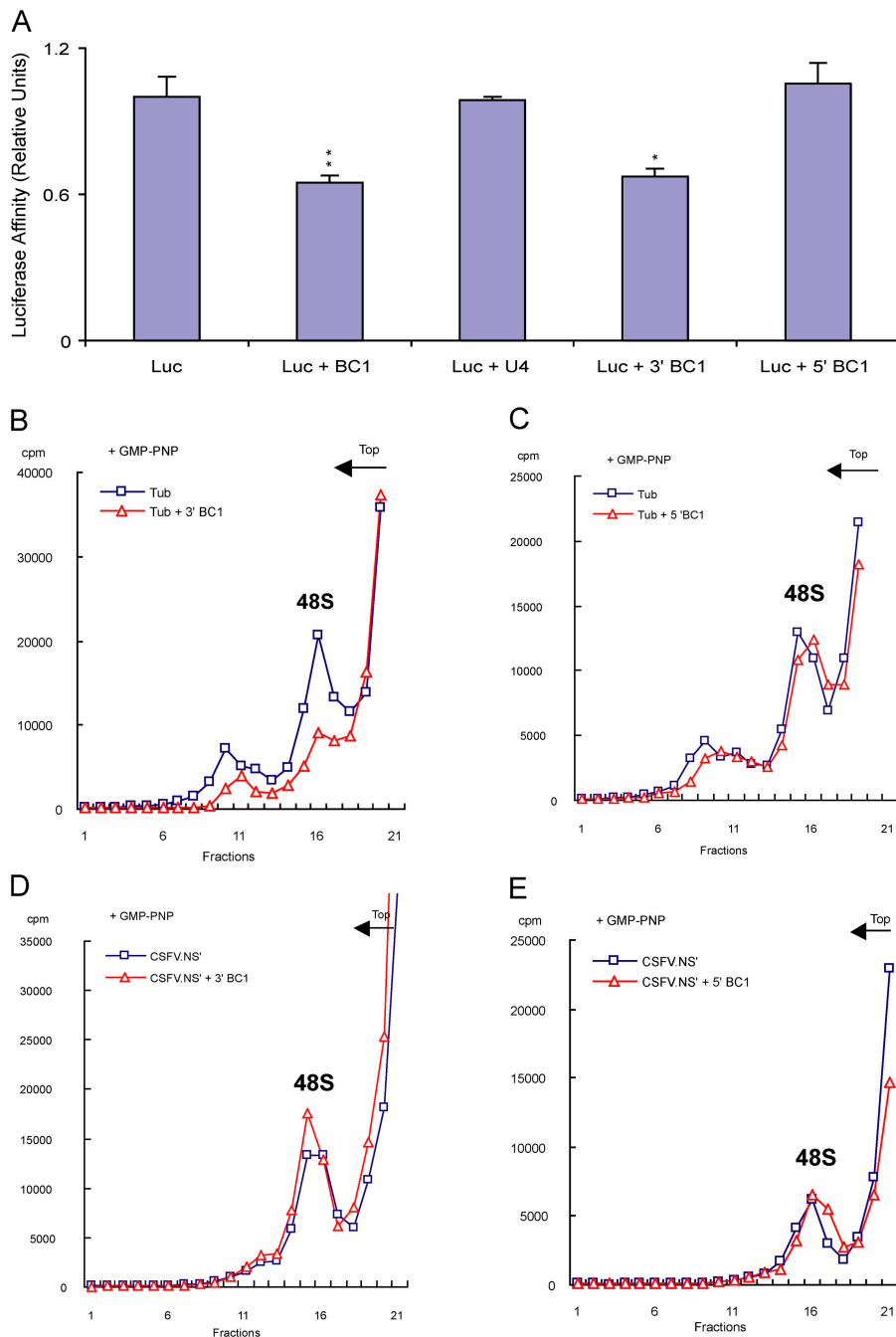


Figure 2. The 3' BC1 domain is repression competent. (A) Luciferase mRNA (Luc) was coinjected with 100 nM of small RNAs into stage VI oocytes and incubated for 55 min. Results from four experiments were quantified by luminescence (one-way ANOVA, $P < 0.001$; Scheffe's multiple comparison post hoc analysis [comparison with Luc control]: **, $P < 0.01$; *, $P < 0.05$). (B and C) 48S complex formation was analyzed in the presence of the 3' (B) or the 5' (C) BC1 domain (500 nM), using capped polyadenylated α -tubulin mRNA as a programming mRNA. (D and E) Analogously, 48S complex formation was probed in the presence of the 3' (D) or the 5' (E) BC1 domain, using uncapped polyadenylated CSFV.NS' mRNA as a programming mRNA.

Which of these BC1 domains is responsible for translational repression? We decided to take advantage of the *X. laevis* oocyte system to dissect BC1 repression competence in vivo, again using luciferase mRNA as a programming mRNA. Full-length BC1 RNA was used as a translational repression standard (Fig. 1 and Fig. 2 A); U4 RNA at the same concentration was used as a negative control (Fig. 2 A). We next tested the 3' BC1 domain and found that it repressed translation as effectively as full-length BC1 RNA (Fig. 2 A). In clear contrast, the 5' BC1 domain failed to repress translation (Fig. 2 A). We conclude that it is the 3', not the 5', BC1 domain that is responsible for BC1-mediated translational repression.

For an in-depth functional dissection of the translational repression mechanism used by the 3' BC1 domain, we next asked which steps in the translation pathway are the targets of such repression. In eukaryotic translation, it is frequently the initiation phase that is subject to regulation (for reviews see Gingras et al., 1999; Dever, 2002). To initiate translation in the cap-dependent mode, the 40S small ribosomal subunit, eIF1, eIF1A, eIF3, and an eIF2-GTP-Met-tRNA_i complex first assemble to form a 43S preinitiation complex (for review see Pestova et al., 2001). This complex is next recruited to the mRNA to form a stable 48S initiation complex after translocation ("scanning") to the AUG start codon. This recruitment step is dependent on the eIF4 group of factors and is coactivated by

PABP. Initiation factors are finally released from the 48S initiation complex, and the 60S ribosomal subunit joins to form the 80S complex (for review see Pestova et al., 2001).

To establish which BC1 domains target which steps in the translation initiation pathway, we used sucrose density gradient centrifugation analysis for an experimental visualization of intermediates along the pathway. The nonhydrolyzable GTP analogue guanylyl imidodiphosphate (GMP-PNP) was used to visualize 48S complex formation in the RRL system (Gray and Hentze, 1994). Using capped α -tubulin mRNA as a programming mRNA, we established that the 3' BC1 domain repressed assembly of 48S initiation complexes (Fig. 2 B). The 3' BC1 domain reduced 48S complex formation substantially, whereas the 5' BC1 domain had no effect (Fig. 2 C). These results demonstrate that the 5' BC1 domain does not play a role in BC1-mediated repression of 48S complex formation. Conversely, the 3' BC1 domain is sufficient to specify such repression, and we therefore conclude that this domain harbors a translational repression element that effectively inhibits 48S complex assembly.

In the cap-dependent initiation pathway, the steps that result in 48S complex formation are mediated by the eIF4 family of initiation factors and include recruitment of the 43S preinitiation complex to the mRNA (i.e., binding to the 5' cap) and subsequent translocation to the initiator AUG codon. Is this eIF4-dependent stage (an important target for regulation in eukaryotic translation) inhibited by the 3' BC1 domain? To address this question, we combined the sucrose density gradient centrifugation assay mentioned in the preceding paragraph with differential internal ribosome entry site (IRES) analysis. Translation of various viral and cellular mRNAs is initiated on IRESs, rather than at the 5' cap as in conventional translation (for reviews see Hellen and Sarnow, 2001; Pestova et al., 2001). On the classical swine fever virus (CSFV) IRES, 48S initiation complexes assemble without the need for any initiation factor of the eIF4 family or PABP, although all other initiation factors (as well as elongation and termination factors) are indispensable and perform the same respective functions as in cap-dependent translation (Pestova et al., 1998). Therefore, translation initiated on the CSFV IRES would be refractory to repression if the inhibition mechanism exclusively targets factors of the eIF4 group. This was indeed observed to be the case with the 3' BC1 domain: if 48S complexes were assembled on an mRNA containing a CSFV IRES, the 3' BC1 domain was no longer an effective repressor and consequently was indistinguishable from the 5' BC1 domain (Fig. 2, D and E). We thus conclude that the 3' BC1 domain represses translation initiation by functionally targeting at least one of the factors of the eIF4 family. Interactions with PABP are also conceivable as the latter is known to coactivate eIF4-dependent initiation (Kahvejian et al., 2005).

The 3' BC1 domain mediates translational repression through functional interactions with eIF4A and PABP

The aforementioned results suggest that the 3' BC1 domain represses translation initiation by targeting eIF4–PABP-mediated recruitment of the small ribosomal subunit to the mRNA.

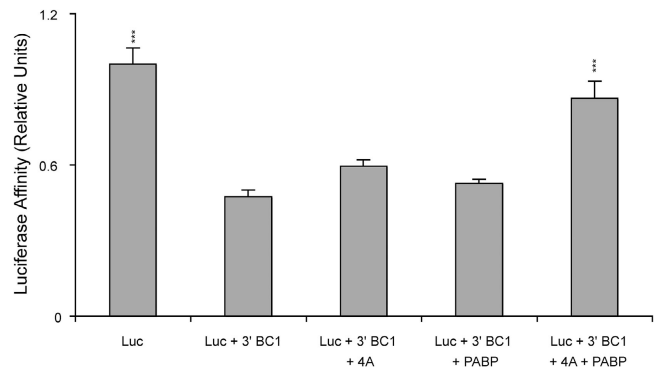


Figure 3. Translation in *X. laevis* oocytes, repressed by the 3' BC1 domain, is restored by joint replenishment with eIF4A and PABP. Luciferase mRNA (Luc) was co-injected into stage VI oocytes with ~400 nM 3' BC1 domain, 100 nM eIF4A, and/or 100 nM PABP. Luminescence was measured after 1 h. Only replenishment with eIF4A and PABP in combination restored 3' BC1-repressed translation to a level that was statistically indistinguishable from unrepressed translation. Quantitative analysis of four experiments is shown (one-way ANOVA, Scheffe's multiple comparison post hoc analysis, ***, $P < 0.001$). The observed differences among 3' BC1, 3' BC1 + eIF4A, and 3' BC1 + PABP were not statistically significant.

Full-length BC1 RNA was previously reported to bind to PABP and eIF4A (Muddashetty et al., 2002; Wang et al., 2002; West et al., 2002). To establish whether interactions of the 3' BC1 domain with PABP and/or eIF4A form the basis for the functional role of BC1 RNA as a repressor of translation, we determined whether translation, repressed by the 3' BC1 domain, could be restored by replenishing with eIF4A or PABP or stoichiometric combinations thereof.

We used the *X. laevis* oocyte system for these experiments. Recombinant eIF4A and PABP were co-injected with the 3' BC1 domain either individually or in combination (Fig. 3). Luciferase activity was used as an index for relative translational efficiency; this efficiency was significantly reduced in the presence of the 3' BC1 domain. Supplementation with eIF4A or PABP alone resulted in a moderate recovery of translation; however, this recovery failed to reach statistical significance (Fig. 3). In contrast, 3' BC1-mediated repression could be overcome by concurrent titration with eIF4A and PABP in stoichiometric ratio (Fig. 3). At 100 nM of both factors, translational efficiency was restored to 87% of nonrepressed levels. (We presume that some of the injected RNA is chaperoned in living cells, i.e., is not functionally available, thus resulting in a lower requirement for rescue proteins relative to repressor.) Higher concentrations of eIF4A and PABP resulted in overtitration, i.e., failure to restore translational efficiency (Kahvejian et al., 2005). eIF4A and PABP are expressed at rather low levels in stage VI *X. laevis* oocytes, unlike ribosomes, eIF4G, ePAB, and other factors that are more abundant (Audet et al., 1987; Zelus et al., 1989; Stambuk and Moon, 1992; Keiper and Rhoads, 1999; Voeltz et al., 2001). Although it remains to be established whether the same two factors are also limiting at the synapse, similarities in translational control mechanisms have been noted between oocytes and neuronal microdomains (Richter, 2000). On the other hand, the target of translational repression does not necessarily have to be limiting as long as

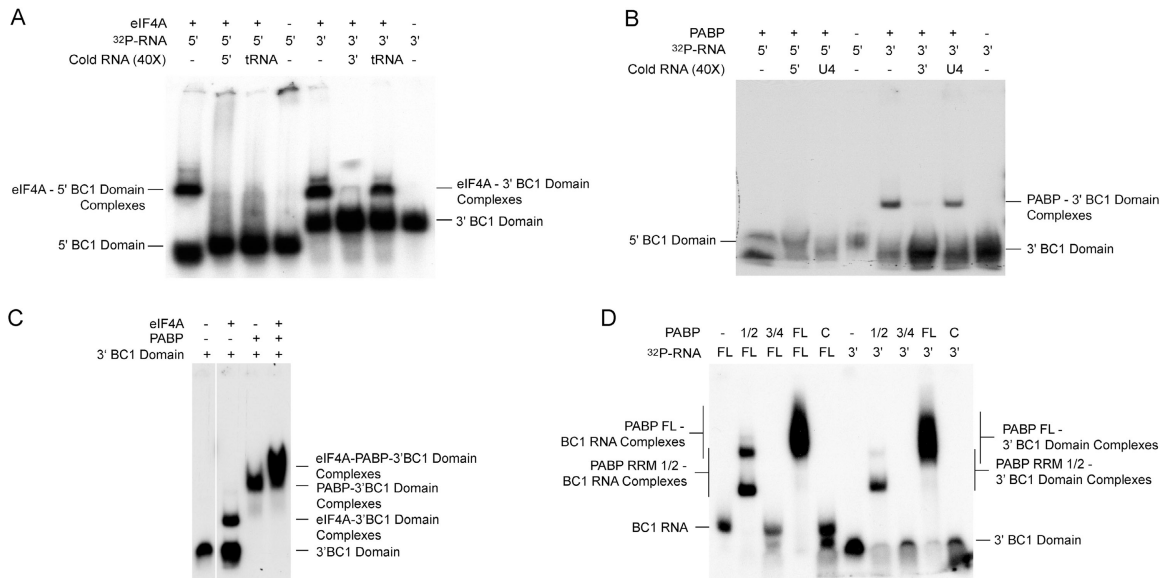


Figure 4. The 3' BC1 domain binds specifically to eIF4A and PABP. EMSA experiments were performed with ³²P-labeled 5' or 3' BC1 domains. (A) BC1 domains were incubated with eIF4A in the absence or presence of unlabeled competitor RNAs. Note that the 5' BC1 domain produced a shift with eIF4A that was abolished by unlabeled tRNAs. (B) BC1 domains were incubated with an NH₂-terminal segment of PABP (aa 1–182) in the absence or presence of unlabeled competitor RNAs. (C) When used in combination, eIF4A and PABP produced a more substantial mobility shift with the 3' BC1 domain than did either factor alone, indicating that both factors can bind simultaneously. (D) A PABP segment containing RRM 1/2 but not a PABP segment containing RRM 3/4 or a PABP segment containing the COOH-terminal domain produced mobility shifts with BC1 RNA (left half of gel) and with the 3' BC1 domain (right half of the gel). Incubation was performed in the presence of 5 mg/ml heparin, followed by RNase T1 digestion (Muddashetty et al., 2002). Omission of heparin/T1 resulted in a weak shift of BC1 RNA by PABP (RRM 3/4), suggesting binding competence of lower affinity and/or specificity of this domain (not depicted).

the local concentration of the repressor is sufficiently high, as is the case with BC1 RNA (Chicurel et al., 1993).

The combined results directly support the notion that the molecular basis for BC1-mediated translational repression is a dual interaction of the 3' BC1 domain with eIF4A and PABP. It is therefore possible that BC1 RNA interacts with both factors concurrently, presumably as they are contained in a complex.

The 3' BC1 domain interacts directly with eIF4A and PABP

In view of these results, we hypothesized that direct interactions of the 3' BC1 domain with eIF4A and/or PABP form the molecular basis for BC1-mediated translational repression. Electrophoretic mobility shift assays (EMSAs) were used to test this hypothesis.

We first examined the 5' BC1 domain. Using recombinant eIF4A with radiolabeled RNA, we observed a distinct shift to lower mobility (Fig. 4 A). However, this shift was abolished not only by preincubation with excess unlabeled 5' BC1 RNA (homologous competition) but also by preincubation with excess unlabeled tRNA (heterologous competition). It is therefore concluded that the binding of the 5' BC1 domain to eIF4A is not specific. Rather, we suggest that it is a reflection of the general ability of eIF4A to interact with RNAs that represent helicase substrates because of their secondary structure content (for review see Gingras et al., 1999).

Unlike the 5', the 3' BC1 domain was found to produce a specific mobility shift with eIF4A (Fig. 4 A). Homologous competition with excess 3' BC1 RNA abolished the mobility

shift; in contrast, heterologous competition with excess tRNA did not (Fig. 4 A). These results indicate that in binding BC1 RNA, eIF4A recognizes attributes that are specific to the 3' BC1 domain, as opposed to double-stranded secondary conformations that are found in a multitude of RNAs. We thus conclude that the 3' BC1 domain interacts directly and specifically with eIF4A and that this interaction prevails over the more general interactions of eIF4A with RNAs featuring secondary structure elements.

Because restoration of BC1-repressed translation requires PABP in addition to eIF4A and because we have shown that the 3' BC1 domain by itself is sufficient to express BC1 repression competence, we sought to determine whether the 3' BC1 domain also bound directly to PABP. Fig. 4 B shows that this is indeed the case. Although PABP did not bind to the 5' BC1 domain, the protein produced a specific mobility shift with the 3' BC1 domain. This shift was abolished by competition with excess unlabeled 3' BC1 RNA but not with excess U4 RNA (Fig. 4 B).

We conclude that the 3' BC1 domain, most likely by virtue of its punctuated A-rich region, displays specific binding activity toward PABP. Is the 3' BC1 domain recognized by eIF4A and PABP at the same time, or is binding to these factors mutually exclusive (e.g., alternating)? EMSA analysis revealed that the simultaneous presence of eIF4A and PABP produced a larger shift (i.e., to lower mobility) than the presence of either factor alone (Fig. 4 C). These data indicate that the 3' BC1 domain can interact with both factors simultaneously.

PABP contains four RNA recognition motif (RRM) domains that mediate RNA binding (for review see Sachs and Varani, 2000). A PABP segment containing the NH₂-terminal domains RRM 1 and 2 has been reported to bind to polyadenylate RNA effectively; however, RRM 3/4 also displays strong RNA binding activity, although presumably mostly to RNA sequences other than poly(A) (Burd et al., 1991; Kuhn and Pieler, 1996; Deo et al., 1999). We therefore asked which PABP domain was responsible for BC1 recognition. EMSA analysis revealed that an NH₂-terminal segment containing RRM 1/2 was as effective as full-length PABP in binding to either full-length BC1 RNA or the 3' BC1 domain (Fig. 4 D). With full-length BC1 RNA but much less so with the 3' BC1 domain, this NH₂-terminal PABP domain apparently formed dimers, probably reflecting an additional binding site provided by the central A₂₂ domain. In contrast to the PABP NH₂ terminus, neither a PABP segment containing RRM 3/4 nor the COOH-terminal PABP domain produced a significant mobility shift with either full-length BC1 RNA or the 3' BC1 domain. We conclude that the NH₂-terminal, RRM 1/2-containing PABP domain is primarily responsible for BC1 binding. It should be noted that it is through the same domain that PABP interacts with eIF4G (Imataka et al., 1998). In summary, our data indicate that the 3' BC1 domain specifically interacts with eIF4A and with the NH₂-terminal domain of PABP. We suggest that these interactions underlie BC1-mediated translational repression.

BC1 RNA fails to bind to FMRP

Our collective data show that BC1 translational repression competence, residing in the 3' domain of the RNA, is mediated through interactions with eIF4A and PABP. Does BC1 RNA in addition bind to FMRP to modulate translation (Zalfa et al., 2003, 2005)? We used several approaches in our efforts to establish whether FMRP is a bona fide BC1 binding protein. All experiments were performed under physiological salt conditions.

To examine BC1–FMRP interactions, we first performed affinity capture assays to probe the capacity of biotinylated BC1 RNA to engage ³⁵S-labeled FMRP. FMRP was generated in a cell-free eukaryotic translation system to permit arginine methylation, which appears to be necessary for proper RNA binding (Denman, 2002). As positive controls, we used biotinylated bone morphogenic protein receptor (BMPR) mRNA and biotinylated eukaryotic elongation factor 1A (eEF1A) mRNA, both of which bind specifically to FMRP (Sung et al., 2003). Bound and unbound fractions were visualized by SDS-PAGE. Fig. 5 A shows that although BMPR and eEF1A mRNAs were able to capture FMRP, BC1 RNA did not react.

To analyze binding of FMRP to BC1 RNA and reference RNAs on a quantitative basis, we next combined solution-binding assays with affinity capture to quantify binding of cell-free-produced FMRP to radiolabeled RNAs. Because it was previously shown that FMRP binds selectively and with high affinity to its own (FMR1) mRNA in vitro (Sung et al., 2000; Schaeffer et al., 2001), we used this mRNA as a standard to normalize all binding results (Miyashiro et al., 2003).

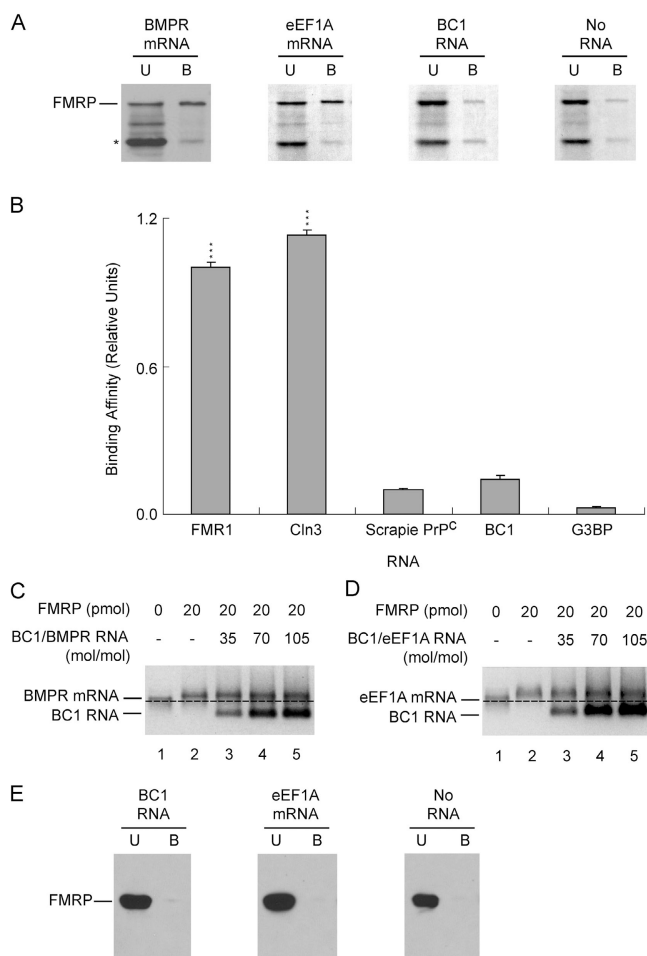


Figure 5. BC1 RNA does not bind specifically to FMRP. (A) Affinity capture reactions were performed between ³⁵S-labeled FMRP and 1 μg of biotinylated BMPR mRNA, 1 μg of biotinylated eEF1A mRNA, and 1 μg of biotinylated BC1 RNA. (Note that on a molar basis, this amount represents ~10 times more BC1 RNA than eEF1A mRNA and 15 times more BC1 RNA than BMPR mRNA.) Full-length FMRP is indicated; asterisk marks incomplete or proteolytic products that were generated in the in vitro translation reaction. Incubation in the absence of RNA was performed as a control for nonspecific binding of FMRP to the resin. (B) A solution RNA binding assay was performed to quantify interactions of FMRP with target RNAs. In these experiments, FMRP was biotinylated and RNAs were ³²P labeled. Binding of FMRP to its own mRNA (FMR1 mRNA) served as a positive control; Scrapie PrP^c mRNA was used as a negative control. Quantitative analysis of three experiments revealed that binding of Scrapie PrP^c mRNA to FMRP was significantly different from binding of FMR1 and Cln3 mRNA but not from binding of BC1 RNA or partial G3BP mRNA (one-way ANOVA, *P* < 0.001; Scheffe's multiple comparison post hoc analysis [comparison with Scrapie PrP^c mRNA, negative control]: ***, *P* < 0.001). (C) AGESA FMRP competition assay between BMPR mRNA and BC1 RNA. Specific FMRP–BMPR mRNA complexes were formed (lane 2, compare with unbound BMPR mRNA in lane 1, indicated by dashed line) and remained resistant to titration with BC1 RNA (lanes 3–5). (D) Analogous AGESA FMRP competition assay between eEF1A mRNA and BC1 RNA. FMRP–eEF1A mRNA complexes were resistant to titration with BC1 RNA. (E) Affinity capture assays with FMRP_{1,280} and biotinylated eEF1A mRNA or biotinylated BC1 RNA. (The amount of BC1 RNA used was again 10 times higher than that of eEF1A mRNA.) FMRP_{1,280} was visualized by Western blotting.

We confirmed that FMRP interacted with FMR1 mRNA (Fig. 5 B) and with Cln3 mRNA, an mRNA that has independently been established to bind to FMRP (for review see Denman, 2003). Scrapie PrP^c mRNA, having previously been

identified as an mRNA that does not bind to FMRP (Sung et al., 2000), was used as a negative control. Quantitative analysis revealed that binding of FMRP to Scrapie PrP^c mRNA was not significantly different from binding to BC1 RNA or to a partial G3BP mRNA, another transcript previously identified as not interacting with FMRP (for review see Denman, 2003). Scrapie PrP^c mRNA, partial G3BP mRNA, and BC1 RNA did not appear to contain any determinants (such as G-quartet or U-rich elements) that are known to be important for interactions with FMRP (for review see Denman, 2003).

We next asked whether FMRP-bound BMRP mRNA or eEF1A mRNA could be displaced by BC1 RNA. An agarose gel electrophoresis assay (AGESA) was previously used to visualize binding of FMRP to target RNAs (Sung et al., 2003). Such a shift was indeed observed with BMRP mRNA; however, titration with BC1 RNA (to >100 times a molar excess over BMRP mRNA) failed to reverse this shift (Fig. 5 C). The result shows that BC1 RNA, even in excess, was unable to displace BMRP mRNA from FMRP. Analogous results were obtained when eEF1A mRNA was used instead of BMRP mRNA (Fig. 5 D).

The NH₂-terminal domain of FMRP (FMRP₁₋₂₁₇) contains a Tudor-like RNA binding motif (Maurer-Stroh et al., 2003). Because BC1 RNA did not compete with two bona fide FMRP-interacting RNAs (which are likely to be recognized by FMRP through central and/or COOH-terminal RNA binding domains; for review see O'Donnell and Warren, 2002; Sung et al., 2003), we wondered if the NH₂-terminal Tudor-like FMRP domain was able to bind BC1 RNA. Having used affinity capture assays to corroborate binding of BMRP and eEF1A mRNA to full-length FMRP, we then used the same assay to probe the binding capacity of FMRP₁₋₂₈₀, as this fragment folds properly and binds homoribopolymers (Adinolfi et al., 1999). As shown in Fig. 5 E, we were unable to document binding of BC1 or eEF1A mRNA to FMRP₁₋₂₈₀. We are left to conclude that the combined data do not provide any evidence for significant direct interactions of FMRP with BC1 RNA.

Discussion

Translational control of gene expression provides a powerful means for the spatiotemporal management of complex protein repertoires in eukaryotic cells. Translational control has been implicated in germ cell differentiation, developmental decisions in early embryos, cellular responses to stress or other environmental factors, and long-term plastic modulation of neuronal synapses (for review see Gray and Wickens, 1998; Sonenberg et al., 2000). The general biological significance of translational control pathways thus invites the question of underlying molecular mechanisms that are used by cells to administer such control.

Molecular mechanism of BC1 control

We illustrate here that in one such mechanism translational control is exercised by a small utRNA. We show that small untranslated BC1 RNA (DeChiara and Brosius, 1987) effectively represses translation in the nanomolar concentration range in

vitro and in vivo. The functional relevance of small utRNAs in the regulation of gene expression is now being increasingly recognized (for review see Barciszewski and Erdmann, 2003). For example, microRNAs base pair with complementary regions of target mRNAs to silence gene expression posttranscriptionally (for review see Barciszewski and Erdmann, 2003). BC1 RNA, in contrast, represses translation by targeting protein factors that are needed for effective initiation. We report that the unique 3' domain of BC1 RNA contains a translational repression element that is responsible for functional repression.

Translational control mechanisms described to date fall into the two categories of mRNA-specific control (e.g., mechanisms that are mediated through microRNAs) and general control (e.g., mechanisms that are mediated through modulation of translation factors). Proteins operate as repressors in the previously described cases of general translational control (Gebauer and Hentze, 2004). In contrast, in the general control mechanism reported here, the repressor is a small utRNA.

Our data show that concerted interactions of the 3' BC1 domain with eIF4A and PABP are responsible for BC1-mediated translational repression. This conclusion is based on results from several experiments, including the finding that translation in *X. laevis* oocytes, under repression by the 3' BC1 domain, can only be restored by concurrent replenishment with eIF4A and PABP in stoichiometric ratio. This result demonstrates that dual interactions with eIF4A and PABP are required for effective BC1-mediated repression. The almost complete restoration of translation after stoichiometric titration with eIF4A and PABP indicates that functional interactions of BC1 RNA with these two factors constitute the major determinants of BC1-mediated translational repression. It appears that any functional interactions of BC1 RNA that fall outside the eIF4A–PABP pathway, if at all relevant, are supplemental in the context of translational repression.

In eukaryotic translation, eIF4A and PABP cooperate in a critical segment of the initiation pathway, the recruitment of the small ribosomal subunit (in form of the 43S preinitiation complex) to the initiator codon (for reviews see Gingras et al., 1999; Pestova et al., 2001). In the cap-dependent mode and IRES-mediated mode of the EMCV subtype (but not in the eIF4-independent CSFV-IRES subtype), this recruitment requires the RNA helicase activity of eIF4A to unwind secondary structure in the 5' untranslated region of the mRNA. PABP stimulates eIF4-dependent initiation by interacting with eIF4A-containing eIF4F (Kahvejian et al., 2005).

What are the molecular interactions that determine the eIF4A–PABP-dependent repression competence of BC1 RNA? Functional dissection revealed that the 3', but not the 5', domain is repression competent in *X. laevis* oocytes. Furthermore, the 3' BC1 domain inhibits 48S complex formation in RRL. In both experimental systems, the 3' BC1 domain acted in a fashion that was indistinguishable from full-length BC1 RNA, whereas the 5' BC1 domain proved ineffective. In contrast, IRES-mediated initiation of the CSFV subtype, which is eIF4 and PABP independent, was refractory to repression by the 3' BC1 domain. We conclude that the 3' domain of BC1 RNA is responsible for translational repression.

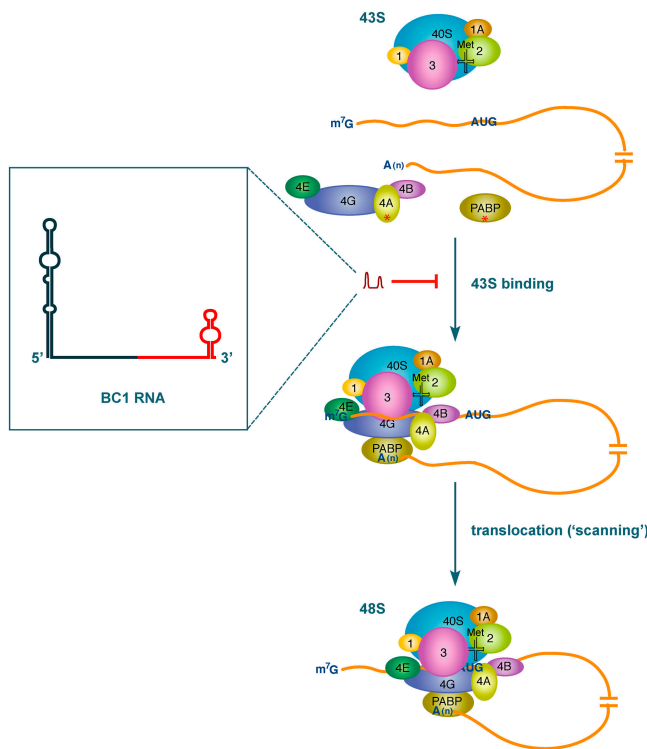


Figure 6. An interaction of the 3' BC1 domain with eIF4A and PABP is responsible for BC1-mediated repression of translation initiation. As a consequence of this interaction, formation of the 48S initiation complex is inhibited. BC1 RNA, shown here targeting recruitment, may in addition interact with one or both factors during translocation. BC1 targets (eIF4A and PABP) are indicated by red asterisks. The 3' BC1 domain is shown in red; the central A₂₂ domain is directly 5' adjacent to the 3' domain. For clarity, some factors that are part of the complex (e.g., eIF5) have been omitted from this sketch. (Reprinted with permission from the *Annual Review of Cell and Developmental Biology*, Vol. 21, 2005, by Annual Reviews, www.annualreviews.org)

Analogously, biochemical evidence indicates that the 3', but not the 5', domain interacts specifically with eIF4A and PABP. Effective binding of PABP to poly(A) requires a homopolymeric stretch of at least 11 A residues (Deo et al., 1999), with a spacing between neighboring PABP molecules of ~27 residues (Baer and Kornberg, 1980). It is therefore likely that one molecule of PABP binds to the central A₂₂ domain of BC1 RNA. This possibility notwithstanding, our data show that PABP binds to the 3' BC1 domain, an interaction that is probably mediated by the punctuated A-rich region in the 5' part of the 3' domain. The combined evidence thus suggests a scenario in which translational repression competence resides in the 3' BC1 domain and is mediated by dual interactions with eIF4A and PABP (Fig. 6). Additional contributions may be derived from PABP–A₂₂ interactions.

Exogenous poly(A) has been reported to reduce translational efficiency under certain conditions, presumably by sequestering PABP (Jacobson and Favreau, 1983; Grossi de Sa et al., 1988; Cao and Richter, 2002). However, poly(A)-dependent repression can be rescued with PABP alone in both RRL (Grossi de Sa et al., 1988) and *X. laevis* oocytes (Cao and Richter, 2002). In addition, a mutant BC1 RNA in which central and 3' A residues have been replaced or deleted does not bind

PABP (Muddashetty et al., 2002) but still represses translation by >60% (Kondrashov et al., 2005). The oligo(A) mechanism by itself is therefore unlikely to account for BC1-mediated translational repression.

We conclude that by targeting and/or sequestering eIF4A and PABP, the repression-competent 3' BC1 domain disrupts a functional link between these two factors that is necessary for efficient initiation. This interaction would be dynamic, with the same RNA molecule interacting with numerous protein molecules over time, and reversible, possibly dependent on the phosphorylation status of interacting proteins. We do not want to rule out a more sustained interaction with PABP as endogenous BC1 RNA has been shown to interact with PABP in RNA–protein complexes (Muddashetty et al., 2002; West et al., 2002). Our conclusions are also consonant with previous observations that functional eIF4A–PABP interactions, supported by eIF4G, eIF4B, and/or Paip-1 (PABP-interacting protein 1), effectively promote translation initiation (Craig et al., 1998; Bushell et al., 2001). Our data raise the possibility, to be addressed in future work, that eIF4G, eIF4B, and/or Paip-1 interact with BC1 RNA and functionally contribute to BC1-mediated repression.

Our experimental results are not compatible, however, with the model of BC1 RNA as a mediator of FMRP-dependent translational repression (Zalfa et al., 2003, 2005). Using a variety of experimental approaches, we were unable to detect significant binding of BC1 RNA to FMRP under physiological conditions or to document competition of BC1 RNA with bona fide target RNAs for FMRP binding. The results shown in Fig. 5 indicate that FMRP may bind nonspecifically to various RNAs in vitro, and FMRP binding to BC1 RNA evidently falls into this category. However, such nonspecific binding can be clearly distinguished from specific binding to genuine FMRP targets such as FMR1 mRNA or eEF1A mRNA.

In addition, a specific interaction of FMRP with the 5' BC1 domain would appear unlikely in view of the fact that this domain has been retroposed and is thus contained in the form of repetitive identifier elements in numerous other RNAs (Deininger et al., 1996). Our data also agree with recent work showing that, unlike kissing complex RNA, BC1 RNA is unable to displace FMRP from polyribosomes in brain homogenates (Darnell et al., 2005). Finally, our results clearly differentiate BC1-mediated translational repression, which targets initiation, from FMRP-mediated repression, which has been reported to occur on polyribosomes (Ceman et al., 2003).

Nonetheless, the possibility that BC1 RNA and FMRP interact indirectly (i.e., other than through direct binding) in the implementation of translational repression cannot be formally ruled out. It is noteworthy in this context that BC1-negative animals exhibit decreased exploratory activity and increased anxiety-related behavior (Skryabin et al., 2003; Lewejohann et al., 2004); the converse phenotype has been reported for FMRP-negative animals (for review see O'Donnell and Warren, 2002). Thus, in conclusion, the physiological relationship between FMRP and BC1 RNA remains an open question.

Translational control in germ cells and neurons

It has become apparent in recent years that some of the translational control mechanisms that are used by neurons are also in place in developing germ cells (for review see Wells et al., 2000). In developing oocytes and spermatogenic cells, various mRNAs are kept in a translationally dormant state for extended periods of time, analogous to synaptically localized mRNAs that are presumed to remain translationally silent until activated (i.e., derepressed) by appropriate input (for reviews see Wells et al., 2000; Job and Eberwine, 2001; Richter and Lorenz, 2002). In addition to neurons, BC1 RNA is also expressed in developing germ cells in a manner compatible with a role in translational regulation (Muslimov et al., 2002). Although such a selective expression profile obviously rules out any global or “housekeeping” cellular function, we submit that BC1 RNA operates as a translational repressor in nerve cells and germ cells.

Translational control in these cell types is also provided through the cytoplasmic polyadenylation mechanism. During *X. laevis* oocyte maturation, the poly(A) tails of selected cytoplasmic polyadenylation element (CPE)-containing mRNAs are extended to ~150 nt, resulting in translational stimulation (for reviews see Richter, 2000; Wells et al., 2000). Analogous mechanisms have been shown to operate in neurons in the translational regulation of CPE-containing dendritic and axonal mRNAs (for review see Wells et al., 2000; Brittis et al., 2002). It is thus conceivable that cytoplasmic polyadenylation and BC1-mediated repression pathways are functionally intersecting.

It is nonetheless important to note that both pathways operate at different levels and with different specificities. The former by definition applies only to mRNAs bearing CPEs, whereas the latter controls eIF4-PABP-mediated translation, including cap-dependent translation and IRES-mediated translation using EMCV-like mechanisms. Several dendritic mRNAs have been reported to harbor IRES elements, indicating that they may be locally translated in a 5' end-independent manner (Pinkstaff et al., 2001; Dobson et al., 2005). Internal ribosome entry would provide a means for independent regulation of translation initiation in dendrites, differentiating it from the mostly cap-dependent mode in somatic translation. Although the mode of action of IRES elements in dendritic mRNAs remains to be established (e.g., eIF4 dependent or not), BC1 RNA is well suited to orchestrate such differential translational control.

With RNA having arisen much earlier than DNA, translational control has in all likelihood predated transcriptional control as the predominant mode in the regulation of gene expression. We posit that utRNAs have accompanied and driven the evolution of translational control, and they continue to acquire, as is exemplified by BC1 RNA, novel functional roles in increasingly specialized eukaryotic cell types.

Materials and methods

Plasmids

Plasmid pBCX607 was used to generate full-length BC1 RNA (Cheng et al., 1996; Muslimov et al., 1997). A 75-nt 5' BC1 domain was transcribed from plasmid p5'(75)pA, which was generated by cloning a pBCX607-

derived PCR product into the pSP64 Poly(A) vector (Promega) between the HindIII and SacI sites. It was linearized with SacI and transcribed with SP6 RNA polymerase. A pBCX607-derived 65-nt 5' BC1 segment, previously shown to contain a dendritic targeting element (Muslimov et al., 1997), produced results indistinguishable from the 75-nt 5' segment. A 60-nt 3'-terminal segment of BC1 RNA was transcribed from plasmid pMK1, and U4 and U6 RNAs were transcribed from plasmids pSP6-U4 and -U6, respectively, as described previously (Tiedge et al., 1991; Muslimov et al., 1997). BC1 segments used were thus the 5' BC1 domain, nt 1–75, and the 3' BC1 domain, nt 93–152. The 3' BC1 domain contains 5 nt of the central A₂₂ stretch and 22 A residues in its punctuated A-rich region.

The following RNAs were used as FMRP targets or controls: BMRP mRNA (available from GenBank/EMBL/DBJ under accession no. D16250; Suzuki et al., 1994), eEF1A mRNA (available from GenBank/EMBL/DBJ under accession no. M25504; Sung et al., 2003), FMR1 mRNA (available from GenBank/EMBL/DBJ under accession no. X69962; Sung et al., 2000), Cln3 mRNA (available from GenBank/EMBL/DBJ under accession no. NM_000086; Denman, 2003), Scrapie PrP^{Sc} mRNA (available from GenBank/EMBL/DBJ under accession no. K02234; Sung et al., 2000), BC1 RNA (available from GenBank/EMBL/DBJ under accession no. M16113; DeChiara and Brosius, 1987), and G3BP₁₋₈₆₆ mRNA (available from GenBank/EMBL/DBJ under accession no. NM_198395; Denman, 2003).

Plasmid pT3LUC(pA) (Wakiyama et al., 2000) was used to generate capped and polyadenylated luciferase mRNA. Plasmid pTub-A98/TA2 (provided by J. Brosius [Universität Münster, Münster, Germany] and S. Kindler [Universität Hamburg, Hamburg, Germany]) was used to produce capped and polyadenylated α -tubulin mRNA (Wang et al., 2002). A rat α -tubulin clone (available from GenBank/EMBL/DBJ under accession no. V01227; Lemischka et al., 1981) containing a G-quartet at nt 1303–1324 was used to generate pTub-A98/TA2. Plasmid pMCG was used to produce polyadenylated EMCV-IRES/GFP mRNA and plasmid pCSFV(1–442).NS'(A) to produce polyadenylated CSFV-IRES/NS' mRNA (Wang et al., 2002).

FMRP and FMRP₁₋₂₈₀ were produced from plasmids pFMR1-CDS (pFMRP-22) and pFMRP-KH₁, using a TNT wheat germ or RRL-coupled transcription-translation system (Promega) as described previously (Sung et al., 2000; Sung and Denman, 2001). In some cases, the proteins were labeled with ³⁵S-methionine or biotin. Recombinant FMRP was expressed in *Escherichia coli* BL21 from pET21A-FMRP and purified on Ni²⁺-NTA resin (Sung et al., 2003). Recombinant eIF4A was expressed from plasmid pET(His₆-eIF4A) in *E. coli* BL21(DE3) (Pestova et al., 1996). Recombinant PABP and PABP segments were generated as described previously (Imataka et al., 1998; Khaleghpour et al., 2001). A COOH-terminal segment (aa 462–633) of PABP was generated from plasmid pGEX.PABP (aa 462–633). Analogously, an NH₂-terminal segment (aa 1–182) of PABP containing RRM 1/2 was generated from plasmid pGEX.PABP (aa 1–182), and a central segment (aa 191–368) containing RRM 3/4 was generated from plasmid pGEX6p2. PABP segments were expressed as GST-fusion proteins and were purified on glutathione-Sepharose beads (GE Healthcare). PABP plasmids were provided by H. Imataka and N. Sonenberg (McGill University, Montreal, Canada), and the eIF4A plasmid was provided by T. Pestova (State University of New York, Brooklyn, Brooklyn, NY).

X. laevis oocytes methods

Oocytes were harvested from mature female *X. laevis* as described previously (Williams et al., 1993). After treatment with 2 mg/ml collagenase (type IA; Sigma-Aldrich) to remove the follicle layer, stage VI oocytes were maintained in a saline solution (5 mM HEPES, pH 7.5, 0.1 M NaCl, 2 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 2.5 mM sodium pyruvate, and 50 μ g/ml gentamycin) at 18°C.

For translation assays in oocytes, capped and polyadenylated luciferase mRNA (6.5–10 ng in a total injection volume of 50 nl per cell) was coinjected with varying amounts of BC1 RNA, BC1 domains, or other small RNAs into the cytoplasm of each oocyte from groups of 20 oocytes. After incubation, pools of five cells were homogenized for each sample and luminescence was measured according to the manufacturer's instructions (Roche), using a luminometer (TD-20/20; Turner Designs). For translation rescue analysis in *X. laevis* oocytes, capped and polyadenylated luciferase mRNA was coinjected with 10 ng of the 3' BC1 domain, and recombinant eIF4A and/or recombinant PABP as indicated into the cytoplasm of each oocyte from groups of 20 oocytes.

In vitro translation and analysis of ribosomal complexes

For in vitro translation assays, the RRL system (Ambion) was used as described previously (Wang et al., 2002). Sucrose density gradient centrifur-

gation assays were used to probe 48S ribosomal complex formation according to established protocols (Gray and Hentze, 1994; Pestova et al., 1996). In brief, *in vitro* translation reaction mixtures (RRL) were preincubated with 1.2 mM GMP-PNP. 5' and 3' BC1 domains were used at 600 nM. Polyadenylated α -tubulin mRNA or CSFV-IRES/NS5' mRNA was used as a programming mRNA (32 P-labeled, 50 ng/reaction). Complexes were resolved by centrifugation through a 5–25% sucrose density gradient in SG buffer (20 mM Tris/HCl, pH 7.5, 100 mM KCl, 2 mM DTT, and 2 mM magnesium acetate).

EMSA

EMSA was done as described previously (Wang et al., 2002). In brief, 32 P-labeled BC1 RNA, 5' BC1, or 3' BC1 domains (50,000 cpm per reaction, ~10 ng) were preheated and then incubated with proteins (typically 0.5 μ g) in binding buffer (20 mM Hepes, pH 7.6, 300 mM KCl, 5 mM MgCl₂, 2 mM DTT, and 5% glycerol) for 20 min at room temperature. When indicated, heparin was used at 5 mg/ml, and optional T1 RNase digestion was performed as described previously (Muddashetty et al., 2002). If unlabeled competitor RNAs were used, they were preincubated with proteins for 10 min before labeled RNAs were added to the reaction. RNA–protein complexes were subsequently resolved on 5% polyacrylamide gels (60:1 polyacrylamide/bisacrylamide) and visualized by autoradiography.

Binding of FMRP to target RNAs

Affinity capture assays were performed as described previously (Sung et al., 2003). Western blotting was done with anti-FMRP mAb 2160 (Chemicon; Sung et al., 2003). For quantitative analyses of FMRP–RNA interactions, we used a modified version of a previously described solution binding assay (Boelens et al., 1993). 32 P-labeled target RNAs, produced by *in vitro* transcription, were incubated at 4°C for 1 h with ~2 ng of biotinylated FMRP or an equivalent amount of mock reaction lysate in 100 μ l of buffer containing 20 mM Hepes, pH 7.9, 2 mM MgCl₂, 0.2% Nonidet P-40, 70 mM NH₄Cl, and 50 mg/ml yeast tRNA (Sigma-Aldrich). Bound RNA was then captured with 10 μ l of SoftLink avidin resin (Promega); free RNA was removed by three 30-column volume washes with 20 mM Tris/HCl, pH 7.5, and 150 mM NaCl. In some cases, affinity capture washes were performed with 150 mM KCl, with identical results. Bound RNA was quantified by liquid scintillation counting. The percentage of FMR1 mRNA bound was arbitrarily set to 100%, and binding of other RNAs was normalized to this value.

For AGESA, recombinant FMRP and RNA were combined in a 10- μ l reaction mixture containing 50 mM Tris/HCl, pH 7.0, 2 mM MgCl₂, and 150 mM KCl. Competition experiments were performed by simultaneously adding target and competitor RNAs via a mixture to FMRP in reaction buffer. Incubations were allowed to proceed for 20 min at room temperature. FMRP–RNA complexes were resolved and visualized as described previously (Sung et al., 2003).

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