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Bruno Acts as a Dual Repressor of *oskar* Translation, Promoting mRNA Oligomerization and Formation of Silencing Particles

Marina Chekulaeva,[1] Matthias W. Hentze,[2,*] and Anne Ephrussi[1,*]
[1] Developmental Biology Unit
[2] Gene Expression Unit
European Molecular Biology Laboratory, Meyerhofstrasse 1, 69117 Heidelberg, Germany
*Contact: hentze@embl.de (M.W.H.); ephrussi@embl.de (A.E.)
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SUMMARY

Prior to reaching the posterior pole of the *Drosophila* oocyte, *oskar* mRNA is translationally silenced by Bruno binding to BREs in the 3′ untranslated region. The eIF4E binding protein Cup interacts with Bruno and inhibits *oskar* translation. Validating current models, we directly demonstrate the mechanism proposed for Cup-mediated repression: inhibition of small ribosomal subunit recruitment to *oskar* mRNA. However, 43S complex recruitment remains inhibited in the absence of functional Cup, uncovering a second Bruno-dependent silencing mechanism. This mechanism involves mRNA oligomerization and formation of large (50S–80S) silencing particles that cannot be accessed by ribosomes. Bruno-dependent mRNA oligomerization into silencing particles emerges as a mode of translational control that may be particularly suited to coupling with mRNA transport.

INTRODUCTION

Translational control of mRNA plays a central role in early development because in most species, zygotic transcription does not occur during the first few hours of life (reviewed in Wickens et al., 2000). *oskar* mRNA encodes the posterior determinant of *Drosophila* (Lehmann and Nüsslein-Volhard, 1986), Oskar protein, whose localized accumulation at the posterior pole of the oocyte and embryo is necessary for development of the abdomen and germline. Posterior accumulation of Oskar is achieved during oogenesis, by localization and translation of *oskar* mRNA at the posterior pole of the oocyte (Ephrussi et al., 1991; Kim-Ha et al., 1991). Restriction of Oskar exclusively to the posterior pole is essential, as ectopic Oskar expression causes anterior patterning defects and lethality (Ephrussi and Lehmann, 1992; Smith et al., 1992). Translational repression of *oskar* mRNA prior to localization is an essential mechanism contributing to the restriction of Oskar activity (Kim-Ha et al., 1995; Markussen et al., 1995; Rongo et al., 1995).

Repression of *oskar* mRNA translation prior to localization is mediated by the RNA binding protein Bruno, which binds to the *oskar* 3′UTR via specific sequences, the Bruno response elements (BREs; (Kim-Ha et al., 1995). Mutations in the BREs that specifically reduce Bruno binding cause ectopic production of Oskar throughout the oocyte, indicating that Bruno is involved in translational repression in vivo (Kim-Ha et al., 1995; Webster et al., 1997). Direct evidence that Bruno is a translational repressor of *oskar* mRNA was obtained in vitro, using a *Drosophila* cell-free translation system that faithfully recapitulates *oskar* repression (Lie and Macdonald, 1999; Castagnetti et al., 2000). Bruno-dependent repression is reproduced in *Drosophila* ovary extract, which contains endogenous Bruno (Kim-Ha et al., 1995). Depletion of Bruno from this extract, using either BRE RNA competitor or antibodies directed against Bruno, alleviates translational repression, demonstrating a direct role of Bruno in this process. Furthermore, addition of purified recombinant Bruno protein to *Drosophila* embryo extract, which lacks endogenous Bruno, causes translational repression of BRE-containing mRNAs. Taken together, these experiments demonstrated that Bruno is a bona fide translational repressor whose activity is mediated by binding to the BREs in the 3′UTR of *oskar* mRNA.

Recently, a eukaryotic initiation factor 4E (eIF4E) binding protein, Cup, was shown to be required for *oskar* repression and to interact with Bruno in yeast two-hybrid assays (Wilhelm et al., 2003; Nakamura et al., 2004). eIF4E binds the cap structure at the 5′ end of mRNAs and, through interaction with eIF4G, recruits the 43S preinitiation complex (consisting of the small ribosomal subunit (40S), the initiator

tRNA, GTP, and a group of initiation factors) to the mRNA (reviewed in Preiss and Hentze, 2003). eIF4E binding proteins (4E-BPs) are translational repressors that block the eIF4E-eIF4G interaction and thus inhibit recruitment of the small ribosomal subunit to the mRNA (Gingras et al., 1999). As Cup contains a functional eIF4E binding motif, it was proposed that Cup regulates *oskar* translation through a mechanism characteristic of 4E-BPs. According to this hypothesis, Cup would be recruited by Bruno to *oskar* mRNA and disrupt the interaction of eIF4E with eIF4G, thus preventing binding of the 43S complex to the mRNA. Consistent with this, transgenic flies expressing a mutant Cup protein whose eIF4E binding sequence is disrupted display defects in *oskar* translational regulation: Oskar protein is produced ectopically from unlocalized *oskar* mRNA (Nakamura et al., 2004).

In translation, both recruitment of the 43S preinitiation complex and the downstream initiation steps are often regulated. After binding to an mRNA, the 43S complex moves along the 5′ untranslated region (5′UTR), in a process termed scanning (Kozak, 1978, 2002), until it recognizes the initiation (AUG) codon. The resulting complex is called the 48S initiation complex. Subsequently, the large ribosomal subunit (60S) joins the 48S complex (this process requires GTP hydrolysis), giving rise to an 80S initiation complex competent for elongation.

In this study, we dissect the mechanism of *oskar* translational control, using cell-free translation systems prepared from *Drosophila* ovaries and embryos. Validating the current model, we show that Cup-mediated repression is effected by inhibition of small ribosomal subunit recruitment to *oskar* mRNA. We also uncover that the BREs repress *oskar* translation through a second mechanism that is independent of Cup-eIF4E interaction. This second mode of BRE function involves Bruno-dependent formation of *oskar* mRNA oligomers and assembly of ''silencing particles,'' unusually large (50S–80S) RNP complexes that render *oskar* inaccessible to the translation machinery.

RESULTS

BRE-Mediated Repression Is Recapitulated In Vitro

To investigate the mechanism of *oskar* mRNA regulation, we first used a cell-free translation system prepared from *Drosophila* ovaries (Lie and Macdonald, 1999; Castagnetti et al., 2000). For this analysis, we constructed luc BRE, a reporter mRNA encoding firefly luciferase and, bearing in its 3′UTR, the minimal *oskar* mRNA sequences required for translational repression, the BREs, which bind Bruno repressor present endogenously in the ovary extract (Figure 1A). A second construct, luc BREmut, identical to luc BRE but containing mutated BREs to which Bruno does not bind efficiently (Kim-Ha et al., 1995) was generated as a specificity control. As expected, luc BRE produces considerably less protein than luc BREmut mRNA (Figure 1B). Titration of Bruno from luc BRE by addition of BRE-containing competitor RNA alleviates translational repression: luc BRE is translated more efficiently in the presence of the competitor than in its absence (Figure 1C, red line). This effect is specific, as

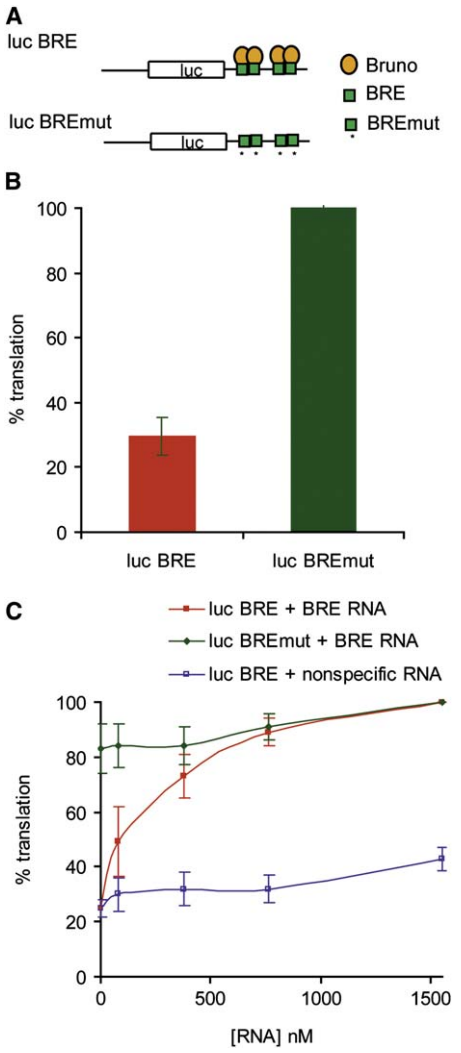

Figure 1. BRE-Mediated Translational Repression

(A) The *oskar* reporters. luc BRE RNA contains a vector-derived 5′UTR (35 nt), the firefly luciferase coding region (1650 nt), and an *oskar*-derived 3′UTR consisting of two copies of the BRE AB region (309 nt). luc BREmut is similar to luc BRE but contains mutated BREs.

(B) BRE-mediated repression in the *Drosophila* ovary cell-free translation system. luc BRE and luc BREmut were translated in *Drosophila* ovary extract, and the amount of product synthesized was estimated by measuring luciferase activity. An equal amount of mRNA encoding Renilla luciferase was added to each reaction, as an internal control. Firefly luciferase activity was normalized to that of the Renilla luciferase. Values are expressed as a percentage of luciferase produced from luc BREmut. Here and in all other experiments, values represent the average of at least four experiments. The error bars show the standard deviation.

(C) Titration of Bruno by BRE RNA competitor causes translational derepression. Increasing amounts of competitor BRE-containing RNA or nonspecific RNA were added to the ovary cell-free translation extract primed with luc BRE or luc BREmut mRNA. Reactions were analyzed as in (B). The output was expressed as a percentage of the most productive luc BRE translation reaction (e.g., in the presence of the optimal amount of competitor BRE-containing RNA). Evaluation of the relative stability of the mRNAs demonstrated that the difference in the amount of protein produced from luc BRE in the presence and in the absence of BRE RNA is not due to decreased stability of the repressed RNA (data not shown).

addition of nonspecific RNA does not increase luc BRE translation (blue line) and competitor BRE RNA does not significantly affect translation from the mutated reporter (green line). Thus, the *oskar* reporter recapitulates BRE-mediated translational repression in ovary extract.

BREs Inhibit mRNA Association with the Small Ribosomal Subunit

To understand the mechanism underlying Bruno-dependent *oskar* mRNA control, we analyzed the translation complexes assembled on the *oskar* reporter by sucrose density gradient centrifugation. To optimize resolution, we created a shorter version of the reporter, replacing the luciferase coding region with a short open reading frame containing the FLAG-tag coding sequence (FLAG BRE). To ascertain that this reporter also recapitulates Bruno-dependent control, we translated FLAG BRE mRNA in ovary extract in the presence of ^{35}S-methionine, with or without added BRE RNA competitor. The FLAG product was immmunoprecipitated and quantified (Figure S1 in the Supplemental Data available with this article online). As with the luc BRE reporter, translation of FLAG BRE increases upon titration of Bruno by the BRE competitor.

To analyze the initiation complexes formed on FLAG BRE mRNA in the ovary extract, we performed translation in the presence of drugs blocking protein synthesis at defined stages. Figure 2A shows the profiles of repressed (+ H_2O; + nonspecific RNA) and derepressed (+ BRE RNA) mRNAs in the presence of the cap analog m^7GpppG, which sequesters eIF4E and thus blocks cap-dependent 43S recruitment. Under such conditions, the mRNA cannot initiate translation and remains as a nonribosomal RNP particle. As revealed by this experiment, RNP particles formed on the repressed mRNA sediment in fractions 5–14 (blue and green lines) and, thus, are significantly heavier than RNPs assembled on derepressed mRNA (red line, fractions 17–19). The same light RNP peak is formed by the FLAG BREmut, which does not bind Bruno repressor efficiently (Kim-Ha et al., 1995; Figure 2D, fractions 17–19).

To investigate the mechanism further and to determine whether Bruno affects association of FLAG BRE mRNA with the small ribosomal subunit, i.e., 48S complex assembly, we performed the analysis in the presence of GMP-PNP, an unhydrolyzable analog of GTP. GMP-PNP allows initiation to proceed to 48S complex formation and blocks translation at this stage, as GTP hydrolysis is required for subsequent joining of the 60S subunit (Trachsel et al., 1977; Lee et al., 2002). In the presence of GMP-PNP, the derepressed reporter forms a 48S peak (Figure 2B, red line, fractions 13–14), in addition to an RNP peak (fractions 17–19). In contrast, the repressed mRNA (blue and green lines) fails to form a 48S peak and remains exclusively in RNP particles (fractions 5–14); its profile is identical to that in the presence of cap analog (Figure 2A). Therefore, we conclude that, in the case of repressed FLAG BRE, 48S complex assembly is inhibited.

The analogous experiment performed in the presence of cycloheximide, a drug that allows translation to proceed to 80S complex assembly and blocks elongation, provides additional evidence for inhibition at the initiation stage (Figure 2C). In the presence of cycloheximide, the derepressed FLAG BRE (red line) completes initiation and forms a 80S peak (fractions 8–9), in contrast, the repressed reporter (blue and green lines) fails to form a 80S peak and remains exclusively in RNP particles (fractions 5–14): its profile is identical to that in the presence of cap analog and GMP-PNP. Thus the BREs mediate inhibition of 48S complex assembly by provoking formation of unusually heavy mRNPs (50S–80S), which we refer to as ''silencing particles.''

To directly assess the role of Bruno in *oskar* mRNA regulation, we repeated this analysis in *Drosophila* embryo extract (Gebauer et al., 1999), which lacks Bruno repressor (Webster et al., 1997; Castagnetti et al., 2000). Bruno protein supplemented to the embryo extract causes formation of heavy RNPs and represses 48S complex assembly, revealing the pivotal role of Bruno in the mechanism of BRE-mediated repression (see Supplemental Data and Figure S2).

BREs Mediate Translational Repression via Two Distinct Mechanisms that Differ in Their Requirement for the Cup-eIF4E Interaction

According to the model proposed by Nakamura et al. (2004), *oskar* mRNA translation is repressed at 43S recruitment, via a trimeric Bruno-Cup-eIF4E interaction. Our data are consistent with this model, and we decided to test it directly by performing in vitro translation in ovary extract prepared from *cup* mutant flies. *cup*$^{\Delta 4212}$ produces a truncated Cup, in which the eIF4E binding motif is disrupted and the Cup-eIF4E interaction abolished (Nakamura et al., 2004). Hence, Bruno-mediated repression should not occur in *cup*$^{\Delta 4212}$ extact.

Surprisingly, when translated in *cup*$^{\Delta 4212}$ extract, luc BRE is derepressed upon addition of BRE competitor RNA (Figure 3A, solid red line). This effect is specific, as nonspecific RNA does not alleviate repression of luc BRE (blue line), and translation of luc BREmut is essentially unaffected by addition of BRE competitor (green line). Thus, the BREs mediate translational repression even in the absence of Cup-eIF4E interaction. However, repression is not as strong in *cup*$^{\Delta 4212}$ extract as in wild-type extract. Indeed, in *cup*$^{\Delta 4212}$ extract, translation of luc BRE increases only about 2-fold upon addition of the competitor BRE RNA (Figure 3A, solid red line), whereas in wild-type extract, translation of luc BRE is stimulated around 4-fold in the presence of BRE competitor (dashed red line). We therefore conclude that the BREs effect repression through at least two distinct mechanisms—one Cup-eIF4E-dependent and one Cup-eIF4E-independent.

48S Complex Formation Is Inhibited even in the Absence of the Cup-eIF4E Interaction

To investigate the Cup-eIF4E independent mechanism of translational repression, we analyzed the initiation complexes formed on FLAG BRE mRNA in *cup*$^{\Delta 4212}$ extract with or without BRE competitor. Sucrose density gradient analysis analogous to that previously described for the wild-type extract revealed that in *cup*$^{\Delta 4212}$ extract, repressed

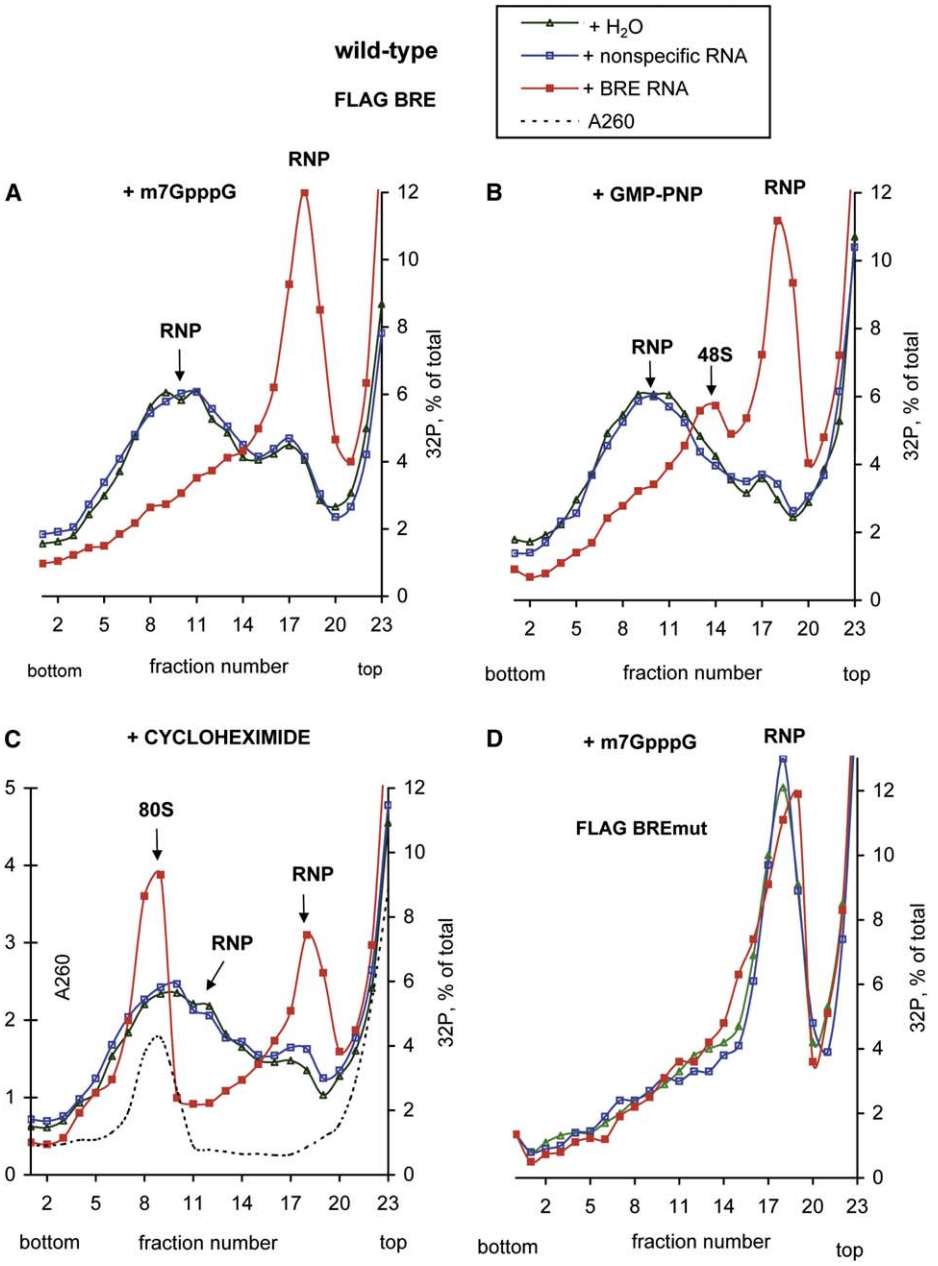

Figure 2. BREs Repress 48S Initiation Complex Formation

(A) BREs mediate involvement of repressed mRNA into unusually heavy RNP complexes. Radioactively labeled FLAG BRE (A–C) or FLAG BREmut (D) mRNA was incubated in the *Drosophila* ovary cell-free translation system in the presence of cap analog m^7GpppG and either competitor BRE RNA (red line), nonspecific RNA (blue line), or H_2O (green line). After incubation, the translation mixture was loaded on a 15%–35% sucrose density gradient, centrifuged, and fractions collected. The radioactivity in each fraction was measured and is represented as a percentage of total recovered counts plotted against the fraction number. The absorbance of each fraction at 260 nm was also measured to reveal the position of monoribosome peak (black dashed line).

(B) BREs repress 48S complex formation. The assay was performed as in (A) but with addition of GMP-PNP.

(C) BREs repress translation at initiation. The assay was performed as in (A) but with addition of cycloheximide.

(D) FLAG BREmut mRNA is not involved in silencing particles. Assay performed as in (A) but with FLAG BREmut mRNA.

FLAG BRE is detected in heavy RNPs (Figure 3B, + m^7GpppG, blue and green lines), as it is in the wild-type extract (Figure 2A). The repressed reporter (Figures 3C and 3D, blue and green lines) is less efficient than the derepressed FLAG BRE (red line) in both 48S (Figure 3C, + GMP-PNP) and 80S complex assembly (Figure 3D, + cycloheximide). Thus, stable association of *oskar* mRNA with the small ribosomal subunit is inhibited independently of the Cup-eIF4E

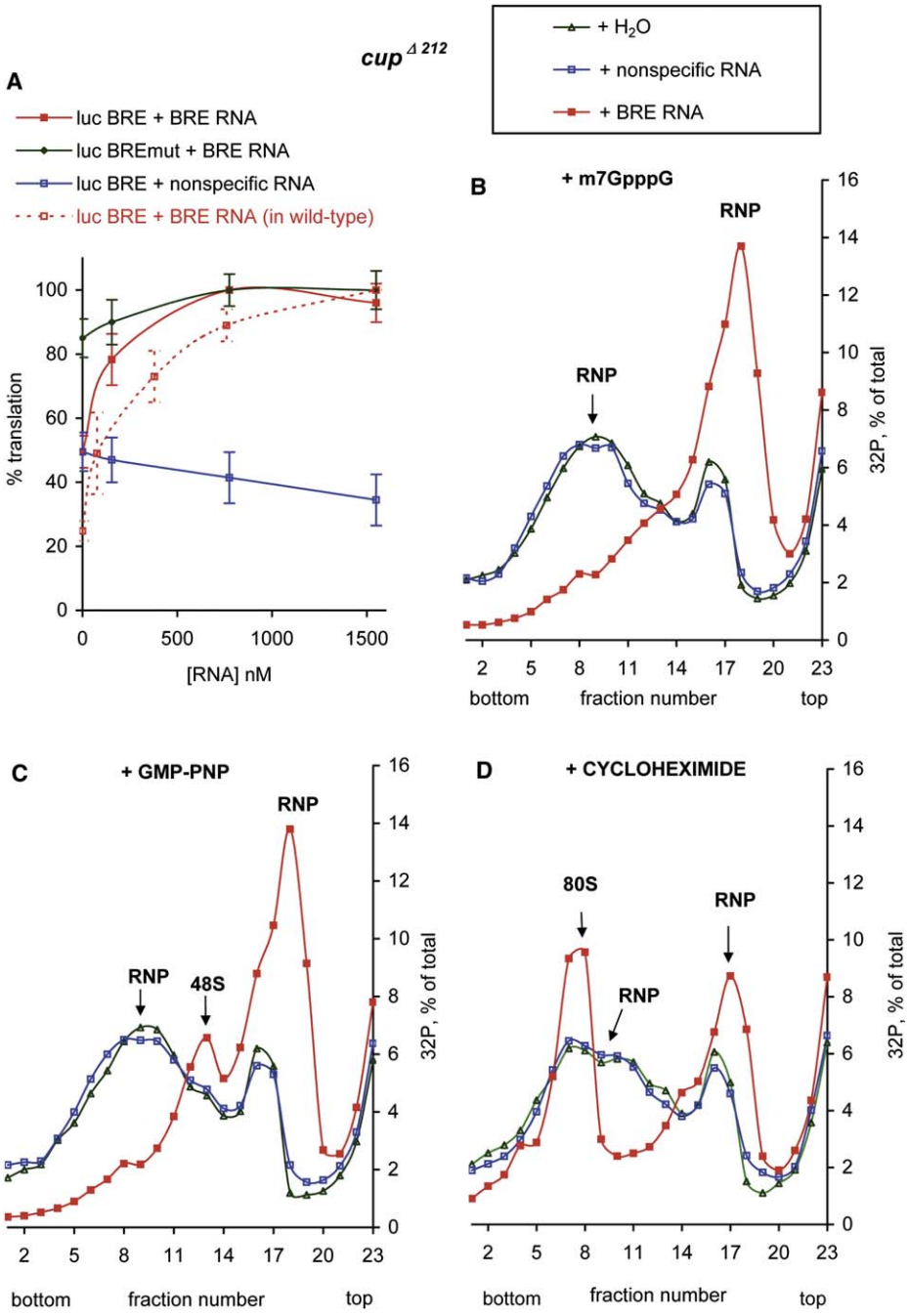

Figure 3. BREs Can Repress Association of *oskar* with the Small Ribosomal Subunit in the Absence of Cup-eIF4E Interaction

(A) Increasing amounts of competitor BRE-containing RNA or nonspecific RNA were added to the *cup*$^{\Delta 212}$ *Drosophila* ovary cell-free translation system primed with luc BRE or luc BREmut mRNA. The assay was performed as in Figure 1C but in *cup*$^{\Delta 212}$ extract instead of wild-type extract.

(B) BREs repress translation at initiation even in the absence of Cup-eIF4E interaction. The assay was performed as in Figure 2A but in *cup*$^{\Delta 212}$ extract instead of wild-type extract.

(C) BREs repress 48S complex formation even in the absence of Cup-eIF4E interaction. The assay was performed as in (A) but with addition of GMP-PNP.

(D) BREs mediate assembly of mRNA into unusually heavy RNP complexes even in the absence of Cup-eIF4E interaction. The assay was performed as in (B) but with addition of cycloheximide.

The error bars show standard deviation.

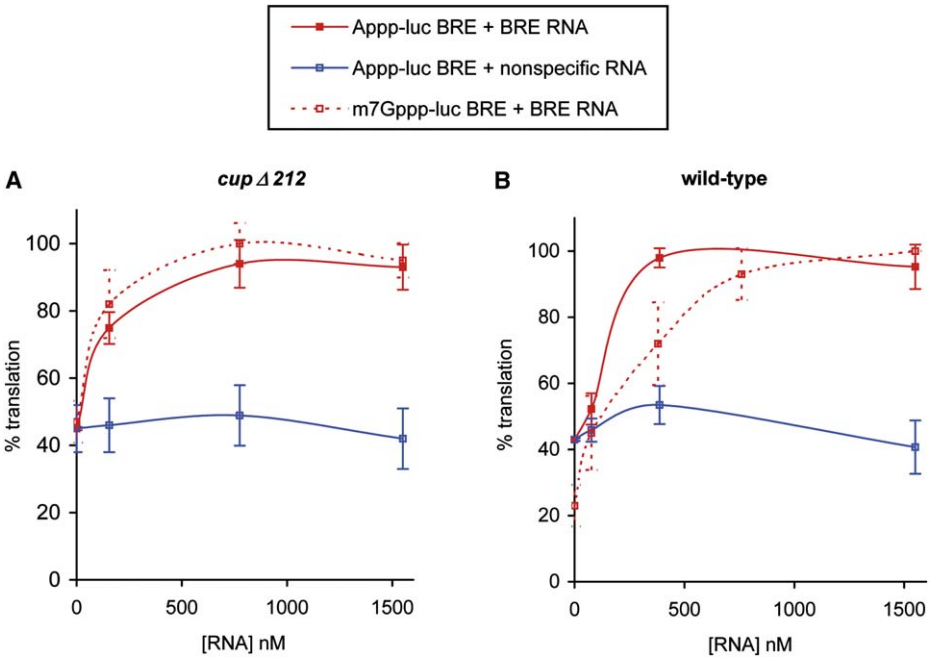

Figure 4. BREs Can Repress Translation Independently of the Cap Structure

(A) luc BRE reporter bearing either an ApppG or a m^7GpppG cap was translated in *cup*$^{\Delta 212}$ *Drosophila* ovary cell-free system in the presence of increasing amounts of the competitor BRE RNA or nonspecific RNA. The reactions were analyzed as in Figure 1C.

(B) The assay was performed as described in (A) but using wild-type extract instead of *cup*$^{\Delta 212}$ extract.

The error bars show standard deviation.

interaction. Likewise, the formation of heavy silencing particles is independent of the Cup-eIF4E interaction.

A Differential Requirement for the m^7GpppG Cap in the Two Mechanisms of BRE-Mediated Translational Repression

To further investigate the Cup-eIF4E-independent repression mechanism, we analyzed its dependence on a m^7GpppG cap. We assessed translational repression of a luc BRE mRNA bearing at its 5′ end an ApppG structure, a cap analog that fails to bind eIF4E. As expected, luc BRE mRNA bearing an ApppG cap (Appp-luc BRE) is translated about 10 times less efficiently than luc BRE bearing the canonical m^7GpppG cap (data not shown). Interestingly, in *cup*$^{\Delta 212}$ ovary extract, Appp-luc BRE and m^7Gppp-luc BRE are derepressed by addition of competitor BRE RNA, with almost identical response curves (Figure 4A, compare solid and dashed red lines). We therefore conclude that this Cup-eIF4E-independent repression mechanism also functions independently of the physiological cap structure. This Cup-eIF4E-independent mechanism can explain the observation of Lie and Macdonald (1999) that BRE-mediated repression occurs even in the presence of saturating amounts of m^7GpppG sequestering eIF4E.

Importantly, in wild-type extract, in addition to effecting repression via a Cup-eIF4E- and cap-independent mechanism, BREs also exert their repressive effect via a second mechanism that requires interaction of Cup and eIF4E (see Figure 4). To test the requirement of a cap for the Cup-eIF4E-dependent mechanism, we compared translational repression of m^7Gppp-luc BRE and Appp-luc BRE RNAs in wild-type ovary extract. Interestingly, the degree of translational derepression achieved by addition of BRE competitor is significantly greater for m^7Gppp-luc BRE mRNA than for Appp-luc BRE mRNA in wild-type extract (Figure 4B, compare dashed with solid red line). As predicted, the second, Cup-eIF4E-dependent repression mechanism requires a m^7GpppG cap. Taken together, our results show there are at least two separate mechanisms underlying BRE-dependent translational repression: one is cap- and Cup-eIF4E-dependent and the other is cap- and Cup-eIF4E-independent.

Bruno-Dependent mRNA Oligomerization into Silencing Particles

The unusually large size of the *oskar* silencing particles led us to hypothesize that such particles might be formed by the association of several mRNA molecules. To test this possibility, we tagged the FLAG BRE reporter with the boxB RNA sequence tag, which binds λN peptide, and primed ovarian in vitro translation reactions with a mixture of radiolabeled FLAG BRE and FLAG BRE boxB-tagged mRNAs. We then isolated the complexes formed on FLAG BRE-boxB mRNA by binding to a λN-GST fusion protein (GRNA chromatography, Czaplinski et al., 2005). RNAs contained in the complexes were separated on a denaturing polyacrylamide gel

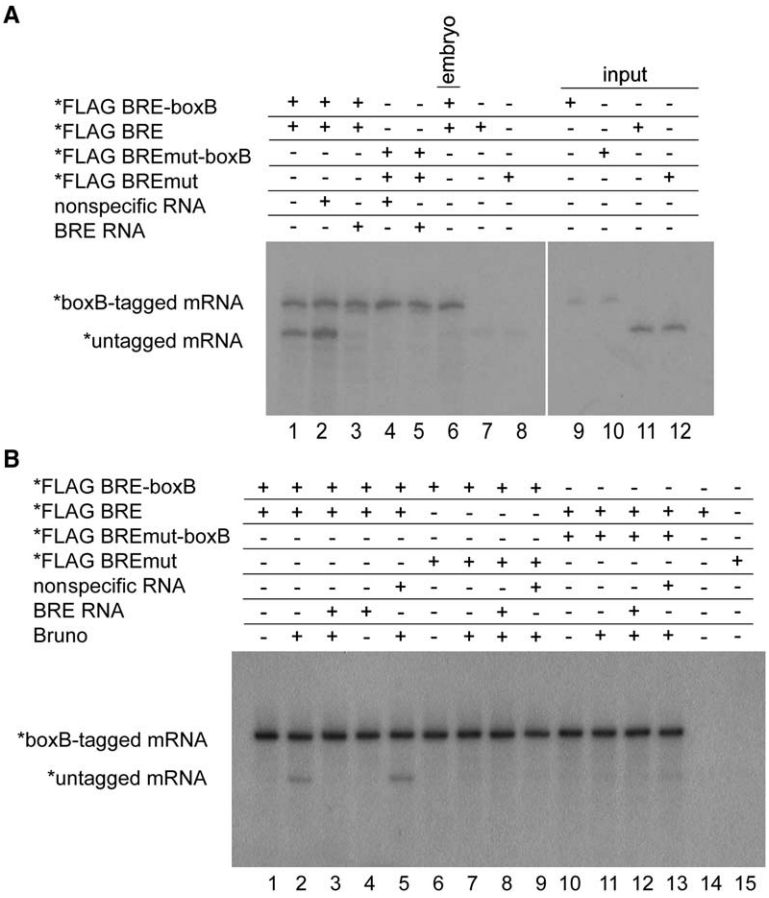

Figure 5. Repressed Complexes Contain RNA Multimers

(A) A mixture of radiolabeled FLAG BRE and FLAG BRE-boxB mRNAs was translated in *Drosophila* ovary extract in the presence of m^7GpppG cap with and without competitor BRE RNA. The complexes formed on FLAG BRE-boxB mRNA were isolated by GRNA chromatography (Czaplinski et al., 2005), and RNAs contained in the complexes were separated on a denaturing 4% polyacrylamide gel and analyzed by autoradiography. RNA recovered from the translation reaction in the absence of BRE RNA competitor (lane 1), in the presence of nonspecific RNA (lane 2); in the presence of BRE RNA (lane 3). Lanes 4 and 5: RNA recovered from the reactions primed with FLAG BREmut-boxB and FLAG BREmut, in the presence of nonspecific RNA (lane 4) or BRE RNA (lane 5). Lane 6: RNA recovered from the *Drosophila* embryo in vitro translation reaction primed with FLAG BRE and FLAG BRE-boxB. Nonspecific (boxB-independent) binding was estimated by GRNA chromatography of reactions primed exclusively with FLAG BRE (lane 7) or FLAG BREmut (lane 8). The amount of loaded material was normalized to the amount of recovered FLAG BRE-boxB (lanes 1–3 and 6) or FLAG BREmut-boxB (lanes 4 and 5). Lanes 7 and 8 contain all recovered RNA. Lanes 9–12 show RNA input: 1/5 of the total FLAG BRE-boxB and FLAG BREmut-boxB added to the reactions (lanes 9 and 10, respectively); 1/30 of the total FLAG BRE and FLAG BREmut in the translation mixture (lanes 11 and 12, respectively). Here and in all subsequent figures radiolabeled RNAs are indicated with an asterisk.

(B) A mixture of radiolabeled FLAG BRE and FLAG BRE-boxB mRNAs was incubated with or without recombinant Bruno. Complexes formed on FLAG BRE-boxB mRNA were isolated and analyzed as in (A). RNA recovered in the absence of Bruno (lane 1); upon Bruno addition (lane 2); after titration of Bruno with BRE RNA (lane 3); in the presence of BRE RNA but in the absence of Bruno (lane 4); in the presence of Bruno and nonspecific RNA (a fragment of FLAG BRE RNA upstream of the BRE sequences; lane 5). Lanes 6–9: RNA recovered from reactions primed with FLAG BRE-boxB and FLAG BREmut, without Bruno (lane 6), in the presence of Bruno (lane 7), in the presence of both Bruno and BRE RNA (lane 8), in the presence of Bruno and nonspecific RNA (lane 9). Lanes 10–13 represent analogous experiments with FLAG BREmut-boxB and FLAG BRE RNAs. Estimations of nonspecific binding (lanes 14 and 15) and normalizations are as in (A).

and analyzed by autoradiography (Figure 5A). This analysis shows that complexes formed on translationally repressed FLAG BRE-boxB mRNA also contain the untagged FLAG BRE mRNA (lanes 1 and 2). Hence, *oskar* silencing particles are indeed formed of mRNA oligomers. Consistent with our observation that heavy silencing particles do not form on FLAG BRE mRNA that is translationally derepressed by addition of the competitor BRE RNA (Figure 2C, + BRE RNA), no oligomerization is observed in the case of derepressed FLAG mRNA (Figure 5A, lane 3). Oligomerization of translationally repressed FLAG BRE mRNAs is specifically mediated by the BREs, as equivalent mRNAs in which the BREs were mutated (FLAG BREmut and FLAG BREmut-boxB) fail to oligomerize (lanes 4 and 5). No oligomerization of FLAG BRE and FLAG BRE-boxB is observed when the mRNAs are translated in the embryo extract (lane 6), which lacks Bruno protein. The fact that FLAG BRE mRNA oligomerization is BRE-dependent in the ovary extract and was not observed in embryo extract suggests that, as is the case for translational repression, FLAG BRE mRNA oligomerization in silencing particles might be mediated by Bruno protein.

To determine if Bruno might be directly responsible for the observed oligomerization of BRE-containing mRNA in silencing particles, we tested whether purified recombinant Bruno protein causes BRE-containing RNAs to oligomerize (Figure 5B). A mixture of radiolabeled FLAG BRE and FLAG BRE-boxB mRNAs was incubated in the absence or in the presence of recombinant Bruno. Complexes formed on FLAG BRE-boxB mRNA were then isolated by GRNA chromatography and RNAs contained in the complexes analyzed by PAGE and autoradiography. While in the absence of Bruno, no untagged FLAG BRE is recovered (lane 1); upon Bruno addition, FLAG BRE is present in complexes formed

on FLAG BRE-boxB (lane 2). No mRNA oligomerization is observed when Bruno is titrated by BRE RNA competitor (lane 3). Furthermore, addition of nonspecific competitor RNA does not prevent mRNA oligomerization in the presence of Bruno (lane 5). The lower recovery of FLAG BRE RNA oligomers in the presence of recombinant Bruno alone (Figure 5B, lanes 2 and 5) than in crude *Drosophila* ovary extract (Figure 5A, lanes 1–3) supports the notion that additional factors present in the ovary extract might be required for a high efficiency of mRNA oligomerization. Consistent with this, the silencing particles formed in embryo extract upon addition of recombinant Bruno are not as large as those formed in ovary extract (Figures 2A, 3B, and S2C).

To simultaneously confirm both the Bruno dependence and the RNA specificity of FLAG-BRE oligomerization, we tested the ability of Bruno to oligomerize FLAG BREmut and FLAG BREmut-boxB, RNAs containing mutated BREs to which Bruno does not bind efficiently (Kim-Ha et al., 1995). Consistent with our previous results, minimal complex formation is observed when one of the interacting RNAs, either FLAG BRE or FLAG BRE-boxB, is substituted with the mutated version, FLAG BREmut or FLAG BREmut-boxB (Figure 5B, lanes 6–13). Hence, the oligomerizing effect of Bruno on BRE-containing RNA is both direct and specific.

To confirm that mRNA oligomerization occurs specifically in the silencing particles, we fractionated translation mixtures by centrifugation in sucrose density gradients, isolated silencing particles by GRNA chromatography (see Figure 2A, fraction 9–11), and analyzed their RNA content as described above. Indeed, oligomerization is highly efficient in the silencing particles (Figure 6A, lane 4), contrary to the low efficiency of oligomerization (Figure 6A, lane 5) in the light RNP peak (see Figure 2A, fractions 17–19). No significant oligomerization is observed when Bruno is depleted by addition of BRE RNA or when mutated reporters (FLAG BREmut and FLAG BREmut-boxB) are used (lanes 6–9). Interestingly, the trace amounts of oligomerization of depressed mRNA that can be detected upon long exposure are restricted to fractions 9–11 (Figure 6A, lanes 6 and 8, and data not shown), suggesting a causal link between mRNA oligomerization and the large size of RNP particles formed by the oligomerized mRNAs.

Silencing Particles Contain Bruno, Cup, and Me31B

To characterize the protein content of the silencing particles, we primed the ovary cell-free system with FLAG BRE-boxB mRNA, in the presence or absence of excess BRE RNA, fractionated the mixtures on sucrose density gradients, and recovered RNA and associated proteins by GRNA chromatography for further analysis. Western blotting revealed that Bruno protein is selectively associated with the repressed mRNA and is present both in silencing particles and in the lighter RNP peak (Figure 6B, lanes 1 and 2). Therefore, the lighter RNP peak (fractions 17–19) might represent an intermediate complex in silencing particle assembly, in which mRNA is bound to Bruno but not yet oligomerized.

Cup is detected only in the heavy but not in the lighter RNP peak of the repressed mRNA (Figure 6B, lane 1), suggesting that silencing particles may also play a role in Cup-dependent repression. The fact that Bruno does not recruit Cup in the lighter RNP peak suggests that effectors may exist that regulate this interaction and cause RNP transition to silencing particles.

Me31B, which has been implicated in translational regulation of *oskar* mRNA during early oogenesis (Nakamura et al., 2001) and is a homolog of the *S. cerevisiae* P body component and translational repressor Dhh1p (Coller and Parker, 2005), is also associated exclusively with the repressed mRNA and is detected in both the light and heavy RNP peaks (Figure 6B, lanes 1 and 2). Taken together, these data show that all three *oskar* translational repressors, Bruno, Cup, and Me31B are specifically associated with the repressed mRNA.

DISCUSSION

Tight restriction of Oskar protein to the posterior pole of the *Drosophila* oocyte is crucial for development of the future embryo and is largely achieved by posterior localization of *oskar* mRNA and its translational inhibition prior to localization. Our molecular analysis of *oskar* mRNA translational repression and of the relative roles of Bruno and Cup in this process has demonstrated the existence of two distinct modes of repression by Bruno and their mechanistic basis. We have demonstrated directly the mechanism hypothesized for Bruno/Cup function, whereby cap-dependent 43S complex recruitment is inhibited (Wilhelm et al., 2003; Nakamura et al., 2004). We have also discovered that Bruno exerts its function through a second mechanism that does not require functional Cup and its interaction with eIF4E. This mode of repression involves Bruno-dependent *oskar* mRNA oligomerization and assembly into silencing particles, unusually large RNPs in which *oskar* remains inaccessible to the translation machinery.

Bruno Is a Dual Regulator of *oskar* Translation

Our analysis of ribosomal complexes assembled on *oskar* reporter mRNA in vitro revealed that 48S initiation complex formation is inhibited both in the presence and in the absence of Cup-eIF4E interaction. This result is compatible with either of two possible mechanisms: (1) inhibition of small ribosomal subunit recruitment and (2) blocking of the following step—scanning of the 5′UTR by the small ribosomal subunit. Indeed, such scanning complexes in which the 43S subunit moves along the mRNA searching for the initiation codon are not stable and can easily dissociate during centrifugation in the sucrose density gradient (Pestova et al., 1998; Beckmann et al., 2005). Therefore, as with a failure in recruitment of the small ribosomal subunit, interfering with scanning would also result in a reduction of the 48S peak.

The first of the two *oskar* repression mechanisms requires the interaction of Cup and eIF4E. This Cup-dependent repression process also requires a m^7GpppN cap on the mRNA (Figure 4). As binding of the small ribosomal subunit represents the cap-dependent step in translation initiation, our results provide a direct demonstration of the

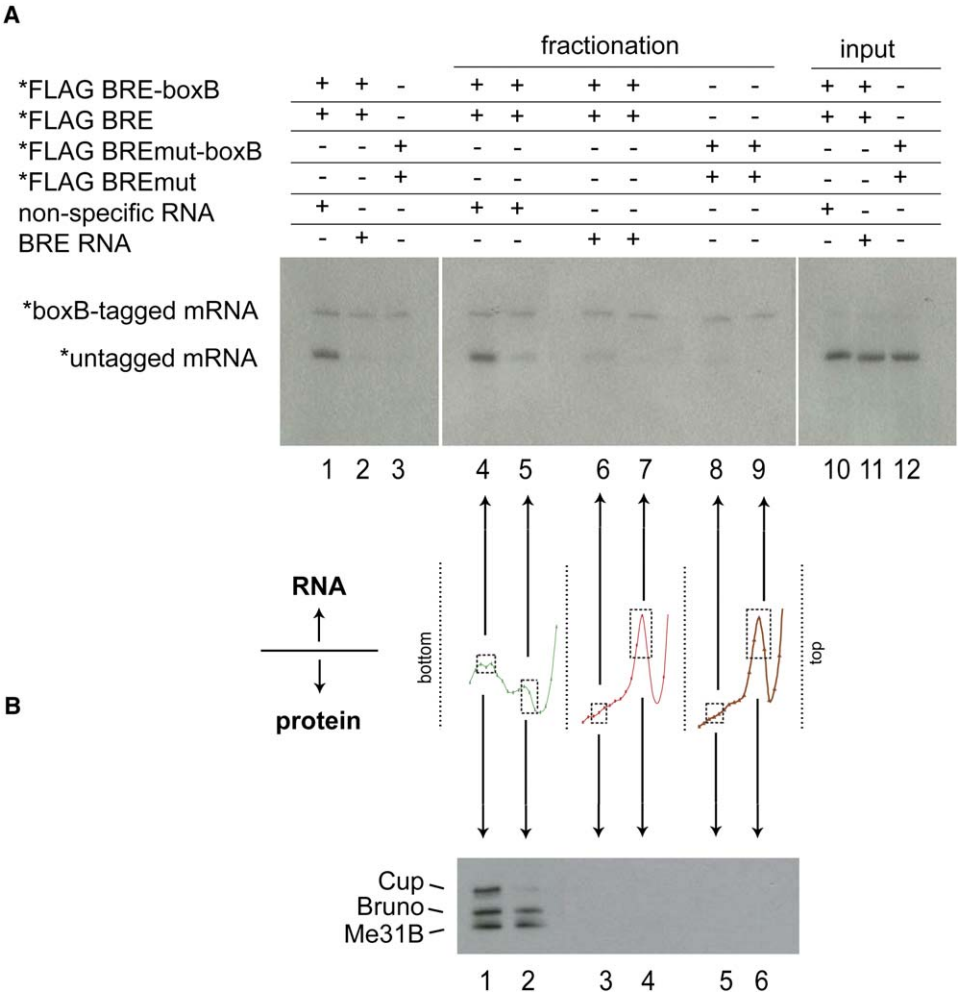

Figure 6. *oskar* Silencing Particles Are Composed of mRNA Oligomers and Contain Bruno, Cup, and Me31B

(A) mRNA oligomerization is specific to silencing particles. Lanes 1–3: RNAs and experimental procedure as in Figure 5A, lanes 2–4. Lanes 4–9: reactions shown in lanes 1–3 were fractionated by centrifugation in sucrose density gradients (as in Figure 2), and complexes assembled on boxB-tagged RNA were isolated from fractions 9–11 and fractions 17–19 by GRNA chromatography. RNA content was analyzed as in Figure 5A. Lanes 4 and 5 contain RNAs from a reaction assembled in the presence of nonspecific RNA and recovered from fractions 9–11 (lane 4) and 17–19 (lane 5). Lanes 6 and 7: RNAs from a reaction assembled in the presence of BRE RNA and recovered from fractions 9–11 (lane 6) and 17–19 (lane 7). Lanes 8 and 9: analogous fractionation of reactions containing FLAG BREmut and FLAG BREmut-boxB RNAs. Lanes 10–12: 1/3 of total RNA present in the translation reactions shown in lanes 1–3, prior to GRNA chromatography. All lanes show same gel, with the pairs of lanes 4 and 5, 6 and 7, and 8 and 9 flipped to show heavy and light fractions on left and right, respectively. Lanes 1–3 and 10–12 are shorter exposures of the same gel.

(B) Protein composition of the silencing particles. Radiolabeled FLAG BRE-boxB or FLAG BREmut-boxB mRNA was translated in *Drosophila* ovary extract in the presence of cycloheximide, with or without competitor BRE RNA. The reactions were fractionated on sucrose density gradients as in (A), and complexes were isolated by GRNA chromatography and processed for Western blot analysis of Bruno, Cup, and Me31B. Lanes 1–6 correspond to lanes 4–9 in (A), with the exception that no untagged mRNA was included in the reactions. The amount of loaded material was normalized to the amount of recovered FLAG BRE-boxB or FLAG BREmut-boxB mRNA. As in (A), the pairs of lanes 1 and 2, 3 and 4, and 5 and 6 were flipped to show heavy and light fractions on left and right, respectively.

hypothesized mechanism for Cup regulation of *oskar* mRNA (Nakamura et al., 2004): a block of cap-dependent 43S recruitment mediated by a functional interaction between Cup-eIF4E and Bruno. Interestingly, we observed that Cup recruits eIF4E to the mRNA in a cap-independent manner suggesting an unexpected role for Cup, over and beyond its role in translational repression (Figure S3). Recruitment of eIF4E to *oskar* mRNA complexes by Cup might ensure co-localization and local enrichment of this otherwise limiting translation factor at the posterior pole, where *oskar* mRNA is translationally activated.

The second mechanism of *oskar* regulation revealed by our analysis also involves Bruno but requires neither Cup-eIF4E interaction nor a m^7GpppN cap. It is therefore unlikely that this mechanism directly interferes with cap-dependent recruitment of the 43S complex.

Cup-eIF4E-Independent Translational Control Involves Formation of Heavy RNPs and mRNA Oligomerization

Our analysis shows that repressed *oskar* reporter mRNA forms unusually heavy complexes sedimenting between the 48S and 80S peaks (Figures 2A–2C and 3B–3D, green and blue lines). Importantly, these complexes form in the absence of the Cup-eIF4E interaction and of ribosomal subunit binding, as revealed by their persistence upon addition of cap analog. We therefore propose that *oskar* mRNA is sequestered in such large RNP complexes and hence inaccessible to the 43S preinitiation complex. Consistent with such a sequestration hypothesis, the repressed mRNA is selectively protected from the degradation machinery (see Supplemental Data and Figure S4). Interestingly, a model of "masked" (translationally inactive, stable) mRNAs was put forward 40 years ago (Spirin, 1966). Masking factors were proposed to bind to mRNA and promote aggregation into higher-order condensed particles, protected from any processive events, including translation, degradation and polyadenylation/deadenylation (Spirin, 1994).

Our experiments reveal that assembly of *oskar* mRNA into RNP complexes as large as monoribosomes can occur without any involvement of the RNA with the ribosomal subunits. These findings shed an unexpected light onto the published literature, where complexes of 80S and larger can be intuitively taken as an indication of ribosomal association and translation elongation. Based on the cosedimentation of *oskar* mRNA with polysomes and experiments involving the polysome-disrupting agent puromycin, Braat et al. (2004) concluded that in the ovary, repressed *oskar* mRNA is associated with translating ribosomes. Our data challenge this conclusion, because we show directly that heavy RNPs (up to 80S in vitro) can form on *oskar* reporter mRNA without ribosomal subunit binding. We note that Braat et al. (2004) employed experimental conditions in which more than one variable was simultaneously changed. Specifically, the Mg^{2+} concentration, which can affect both polysome and RNP stability, differed by an order of magnitude between the puromycin-treated samples (2.5 mM Mg^{2+}) and the cycloheximide control (25 mM Mg^{2+}). We have repeated this experiment, altering only one variable (puromycin). When the Mg^{2+} concentration is kept constant, puromycin does not affect the heavy RNPs that were previously interpreted as being "polysomal" (M.C., unpublished data). We suggest that *oskar* mRNA is engaged in puromycin-insensitive, heavy silencing particles that are sequestered from ribosomal engagement and that cosediment with polysomes.

Remarkably, *oskar* silencing particles comprise not single mRNA molecules but mRNA oligomers, whose formation is dependent on the specific association of Bruno with the BREs (Figures 5 and 6). The fact that the same components, Bruno and BREs, are responsible for both translational repression and mRNA oligomerization into silencing particles suggests a causal relationship between oligomerization and translational silencing.

The interesting finding that Cup is present in the heavy but not in the light RNP peak highlights the role of silencing particles in *oskar* repression. The sucrose gradient analysis of repressed complexes in $cup^{\Delta 212}$ extract (Figure 3) demonstrates that Cup-4E interaction is not required for silencing particle formation. However, the fact that Cup is exclusively associated with the silencing particles but not with the light RNP peak of repressed mRNA (Figure 6B) suggests that particle formation may contribute not only to Cup-independent repression but also to Cup-dependent repression.

mRNA Oligomerization as Mechanism Coupling Translational Control with mRNA Localization

Consistent with our in vitro demonstration of *oskar* mRNA multimerization in silencing particles, it was recently demonstrated that *oskar* mRNA molecules can self-associate through the 3′UTR for localization to the posterior pole of the oocyte (Hachet and Ephrussi, 2004). As *oskar* mRNA is translationally repressed prior to posterior localization, it is tempting to speculate that the large silencing complexes containing *oskar* mRNA multimers we have identified are related to *oskar* mRNA localization complexes. It should be noted, however, that at present, there is no evidence for a role of the translational repressor Bruno in *oskar* mRNA localization. It is also possible that direct intermolecular RNA-RNA interactions might contribute to *oskar* oligomerization, as in the case of *bicoid* mRNA (Ferrandon et al., 1997; Wagner et al., 2001).

Our work suggests that silencing particles in *Drosophila* ovary extracts form by Bruno-mediated mRNA oligomerization from lower complexity precursors (Figure 2A, fractions 17–19). Recent reports have described the presence in yeast and in mammalian cells of large particles, P bodies, from which silenced mRNAs may either return to the translating pool or be targeted for degradation (Coller and Parker, 2005; Brengues et al., 2005). There, also, the idea has emerged of RNP particles that may aggregate from precursors into higher-order structures. In this regard, it is notable that both Cup and Me31B are present in silencing particles, as it has recently been shown that the mammalian eIF4E binding protein, 4E-T, and Dhh1p, the *S. cerevisiae* homolog of Me31B, are P body components (Ferraiuolo et al., 2005; Teixeira et al., 2005). While the factors that promote P body aggregation in mammals and yeast are currently unknown, we have identified Bruno as a critical factor for silencing particle formation. Interestingly, our analysis shows that while Bruno is associated with the repressed mRNA both in silencing particles and lighter RNPs, Cup associates only with the mRNA in silencing particles (Figure 6B). The fact that Bruno does not recruit Cup in the light RNP peak suggests that effectors may exist that regulate this interaction and cause RNP transition to silencing particles by addition/modification of factors and/or conformational change. It will be interesting to further explore the relationship between silencing particles and P bodies.

The exciting finding that *oskar* silencing particles comprise not single mRNA molecules, but mRNA multimers, suggests a mode of mRNA translational control that seems particularly suited to coupling of translational repression with mRNA transport within the cell. Such a repression mechanism

would also allow coordinate repression of multiple *oskar* mRNAs, as well as coordinate derepression of the mRNAs within the silencing mRNP, upon its localization at the oocyte posterior pole. The particles could in principle contain other RNAs regulated and assembled into RNPs by common components. It will be interesting to determine if *gurken* mRNA, which is translationally repressed by Bruno (but not Cup) and colocalizes with *oskar* mRNA during the early stages of oogenesis, is coassembled with *oskar* mRNA in silencing particles.

EXPERIMENTAL PROCEDURES

Plasmids

luc BRE, luc BREmut, FLAG BRE, FLAG BREmut, FLAG BRE-boxB, and FLAG BREmut-boxB were created in several steps. A BglII-BclI fragment containing the AB BRE region (EcoRI-DraI fragment) of the *oskar* 3′UTR was generated by PCR and cloned into pCRII-TOPO. This plasmid was digested with BglII and BclI, and one or two copies of the BRE region were cloned into the BamHI site of pGEM4, yielding pGem-BRE and pGem-BRE2X. An XmaI-PstI BRE-containing fragment was excised from pGem-BRE2X and cloned into the XmaI-PstI sites of a pBluescript plasmid, adjacent to a PstI-HindIII fragment bearing a 150A sequence, generating pBluescript-BRE-150A. The BRE-150A sequence of this plasmid was the source of 3′UTR for the final luc BRE and FLAG BRE reporters. To generate the 3′UTR for the FLAG BRE-boxB construct, we cloned a fragment containing three copies of the boxB sequence (Baron-Benhamou et al., 2004) between XbaI and PstI sites of pGem-BRE2X, yielding pGem-BRE-boxB. Further cloning steps for FLAG BRE-boxB were the same as for the FLAG BRE construct. To create a mutated version of the 3′UTR, we followed the same cloning strategy but used ABmut (Kim-Ha et al., 1995) as the PCR template, generating pBluescript-BREmut-150A.

To generate pSP72-luc, we cloned the luciferase coding sequence between the XhoI and BamHI sites of pSp72. We then inserted the XmaI-KpnI BRE-150A-containing fragment of pBluescript-BRE-150A into the XmaI-KpnI site of pSP72-luc. Finally, the XhoI-HindIII luc-AB-150A-containing fragment of the resulting plasmid was subcloned into pBluescript to create luc BRE. The same strategy was used to generate luc BREmut using pBluescript-BREmut-150A as a 3′UTR source. To generate pSP72-FLAG, most of the luciferase coding sequence was released by digestion with XhoI and EcoNI and was replaced by an XhoI-EcoNI fragment containing the FLAG coding sequence. The fragment bearing the FLAG-tag sequence was generated by PCR using WTs as a template (Gebauer et al., 2003). Further steps in generation of FLAG BRE, including ligation of the 3′UTR and subcloning into pBluescript, were the same as for the luc BRE construct.

pETM82-Bruno, a plasmid encoding recombinant Bruno, fused to the leaderless sequence of DsbC (a subunit of protein disulfide isomerase/disulfide oxidoreductase) and a His-tag, was generated by cloning the Bruno coding sequence between the NcoI and KpnI sites of the pETM82 vector (G. Stier, EMBL).

In Vitro Transcription and Translation

All mRNAs, luc BRE, luc BREmut, FLAG BRE, FLAG BREmut, FLAG BRE-boxB, and FLAG BREmut-boxB, were generated using a T3 Maxiscript in vitro transcription kit (Ambion 1316). The plasmids were linearized using HindIII. Either m^7GpppG or ApppG was added to the reaction (3.5 mM final). To prepare radiolabeled FLAG BRE and FLAG BRE-B mRNAs for sucrose density gradient analysis, we supplemented the in vitro transcription reaction with 3.75 μM ^{32}P-UTP (~800 Ci/mmol, 20 mCi/ml, Amersham, PB20383-1MCI). For GRNA chromatography experiments, in which both FLAG BRE and FLAG BRE-boxB mRNAs were used to prime cell-free translation, FLAG BRE mRNA was radiolabeled by addition of 11.25 μM ^{32}P-UTP (~800 Ci/mmol, 20mCi/ml, Amersham PB20383-1MCI) to the in vitro transcription reaction.

Competitor BRE-containing RNA (BRE RNA) was produced by transcription of pGem-BRE after linearization by XbaI, using an SP6 Megascript in vitro transcription kit (Ambion 1330). Nonspecific RNA of a size similar to BRE RNA was generated by in vitro transcription (T3 Megascript, Ambion 1338) of FLAG BRE linearized using BamHI. All RNAs were purified using an RNeasy Mini kit (Qiagen 74104).

Preparation of *Drosophila* ovary and embryo extracts and in vitro translation assays were performed as previously described (Gebauer et al., 1999; Castagnetti et al., 2000). Translation reactions contained 1.5 nM exogenous mRNA, except in the case of CAT mRNA, which was used at a concentration of 0.3 nM. Unless otherwise stated, BRE RNA and nonspecific RNA were used at a concentration of 800 nM, and Bruno at a concentration of 150 nM. Recombinant Bruno was produced in *E. coli* by A. De Marco in the EMBL Protein Purification Facility (details available upon request).

Sucrose Density Gradient Analysis

For analysis of the translation complexes assembled on radiolabeled FLAG BRE RNA, cell-free translation was performed in a volume of 15 μl and incubated at 25ºC for 30 min. Reactions contained cycloheximide (2 mM) and where indicated, GMP-PNP (2 mM) or m^7GpppG (1 mM). $Mg(OAc)_2$ (2 mM) was supplemented to reactions containing GMP-PNP. Following incubation, translation mixtures were cooled on ice, clarified by centrifugation at 10,000 × g for 15 min at 4ºC and loaded on a 15%–35% sucrose gradient (24 mM HEPES [pH 7.4], 3 mM $Mg(OAc)_2$, 100 mM KOAc, 2 mM DTT). Centrifugation was performed at 45,000 rpm 4ºC in a SW-60 rotor; the run time was 3 hr, 15 min for ovary translation system and 15 min longer for embryo translation system. Fractions of the gradient were collected manually from the top, and radioactivity in the fractions was estimated by scintillation counting.

mRNA Oligomerization Assay

Oligomerization of BRE-containing mRNAs was evaluated in two different assays: (1) in the *Drosophila* ovary cell-free translation system; (2) using purified recombinant Bruno. The cell-free translation system from *Drosophila* ovaries (Castagnetti et al., 2000) was assembled in a volume of 30 μl in the presence of 1 mM m^7GpppG and primed with the radiolabeled mRNAs: 1.2 nM FLAG BRE (or FLAG BREmut) and 0.3 nM FLAG BRE-boxB (or FLAG BREmut-boxB). Where indicated, BRE RNA or nonspecific RNA were added at a final concentration of 800 nM and preincubated in ovary extract for 10 min at 4ºC. The assembled in vitro translation mixture was incubated at 25ºC for 30 min, then subjected to GRNA chromatography. In Figure 6A, 100 μl of the mixtures were fractionated on sucrose density gradients and selected fractions were subjected to GRNA chromatography.

The assay involving recombinant Bruno was performed in a 30 μl volume containing 25 mM Hepes-KOH (pH 7.4), 1.5 mM $MgCl_2$, 150 mM NaCl, 0.3 μg/μl tRNA (Sigma R-5636), and 0.2 U/μl Ribonuclease Inhibitor (Promega 2515). The reaction was primed with the radiolabeled mRNAs: 1.2 nM FLAG BRE (or FLAG BREmut) and 0.3 nM FLAG BRE-boxB (or FLAG BREmut-boxB) and incubated for 30 min at 25ºC prior to GRNA chromatography. Where indicated, 150 nM recombinant Bruno was added to the reaction; competitor RNAs (BRE RNA and nonspecific RNA) were used at a concentration of 1.5 μM.

GRNA Chromatography

Complexes formed on FLAG BRE-B mRNA were purified according to a protocol developed by Czaplinski et al. (2005), with modifications. The protocol is based on the protein-RNA tethering approach using bacteriophage λ antiterminator protein (λN) and its specific RNA binding site, boxB (Baron-Benhamou et al., 2004). Per 100 μg of GST-λN fusion peptide (Czaplinski et al., 2005), 40 μl of a 50% slurry of Glutathione-Sepharose 4B (Amersham, 17075601) in binding buffer (BB: 20 mM TRIS-HCl [pH 7.5], 200 mM NaCl, 1.5 mM $MgCl_2$, 9% Glycerol, 0.05% NP-40, 12 μg/ml heparin) were incubated on an orbital rocker for 1 hr

at 4ºC. To remove the unbound GST-λN, Glutathione-Sepharose beads were washed twice in 1 ml of BB and incubated in a reaction mixture primed with the boxB-containing mRNA diluted with BB 1:10 or in the sucrose gradient fractions diluted with BB 1:1. For analysis of RNA content in total extract or in sucrose gradient fractions, we used 30 or 100 μl of translation mix, respectively. For Western blot analysis of total extract or sucrose gradient fractions, we used 200 μl or 1ml of translation mix, respectively. Sixty micrograms of GST-λN protein were used per one hundred microliters of translation mixture. After 1 hr of incubation at 4ºC on an orbital rocker, Glutathione-Sepharose beads were washed three times in 1 ml of BB. For RNA isolation, samples were treated with 20 μg of proteinase K in 100 μl 1% SDS 10mM EDTA for 30 min at 30ºC, with shaking. After a short spin, the supernatant was collected and extracted with Trizol LS Reagent (Invitrogen 10296028). The isolated RNAs were separated on a 4% polyacrylamide gel containing 7 M Urea and analyzed by autoradiography. For Western blotting, the RNP complexes bound to Glutathione-Sepharose were treated with 0.1 μg RNase A in 40 μl BB for 30 min at 30ºC, with shaking. Proteins in the supernatant were separated on a 10% Laemmli gel and the Western blot probed using anti-Bruno, anti-Cup, anti-Me31B, and anti-eIF4E antibody.

Supplemental Data

Supplemental data include four figures, Supplemental Results and Discussion, and Supplemental References and can be found with this article online at http://www.cell.com/cgi/content/full/124/3/521/DC1/.

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