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BREs Mediate Both Repression and Activation of *oskar* mRNA Translation and Act In *trans*

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SUMMARY

Asymmetric positioning of proteins within cells is crucial for cell polarization and function. Deployment of Oskar protein at the posterior pole of the *Drosophila* oocyte relies on localization of the *oskar* mRNA, repression of its translation prior to localization, and finally activation of translation. Translational repression is mediated by BREs, regulatory elements positioned in two clusters near both ends of the *oskar* mRNA 3′ UTR. Here we show that some BREs are bifunctional: both clusters of BREs contribute to translational repression, and the 3′ cluster has an additional role in release from BRE-dependent repression. Remarkably, both BRE functions can be provided in *trans* by an *oskar* mRNA with wild-type BREs that is itself unable to encode Oskar protein. Regulation in *trans* is likely enabled by assembly of *oskar* transcripts in cytoplasmic RNPs. Concentration of transcripts in such RNPs is common, and *trans* regulation of mRNAs may therefore be widespread.

INTRODUCTION

Formation of the body plan of the *Drosophila* embryo relies on the action of several localized determinants (reviewed in Lipshitz and Smibert, 2000; Palacios and St. Johnston, 2001). One of these, the Oskar (Osk) protein, is localized to the posterior pole of the oocyte and initiates formation of the pole plasm, which is responsible for posterior body patterning and germ cell formation. In the absence of Osk, both processes fail (Lehmann and Nüsslein-Volhard, 1986). Conversely, overexpression of Osk posteriorizes the embryo: a low level causes anterior patterning defects, while higher levels lead to replacement of all head and thoracic segments with a mirror image duplication of posterior abdominal segments, the bicaudal phenotype (Smith et al., 1992). Similarly, specific misexpression of Osk at the anterior efficiently produces bicaudal embryos (Ephrussi and Lehmann, 1992). Thus, proper deployment of Osk is a critical step in embryonic pattern formation.

Several mechanisms are used to ensure that Osk appears at the appropriate level and only at the correct position at the posterior pole of the oocyte. Two such mechanisms act on *osk* mRNA. The first is mRNA localization (Kim-Ha et al., 1991; Ephrussi et al., 1991). At the early stages of oogenesis, *osk* mRNA is efficiently transported from the nurse cells to the oocyte. During stage 9 *osk* mRNA is localized to the posterior pole. The second mechanism is translational control. Translation of *osk* mRNA is repressed prior to its localization and then activated when posterior localization is achieved (Kim-Ha et al., 1995; Rongo et al., 1995; Markussen et al., 1995).

Translational repression of *osk* mRNA is mediated by sequences in the *osk* mRNA 3′ UTR called BREs (Bruno response elements), which are bound by the Bruno (Bru) protein. The BREs are clustered in two regions, called AB and C. The AB region is near the *osk* open reading frame, while the C region is close to the polyadenylation site. Mutation of the BREs greatly reduces Bru-binding in vitro and leads to precocious Osk protein expression in vivo, implicating Bru as a translational repressor (Kim-Ha et al., 1995; Webster et al., 1997). Repression must be released upon localization of *osk* mRNA. How this occurs is unknown, although two activating elements have been identified. One lies within the coding region near the 5′ end of the *osk* mRNA (Gunkel et al., 1998). The second element is the IBE (D*imp* *b*inding *e*lement), a short sequence present in multiple copies throughout the *osk* mRNA 3′ UTR (Munro et al., 2006).

Here we show that BREs have two roles, not one. Either of the two clusters of BREs mediates translational repression of unlocalized mRNA. The C region cluster of BREs has an additional role in translational activation. Remarkably, both repressive and activating BRE functions can be provided in *trans* by *osk* transcripts with wild-type BREs. We propose that *osk* RNP particles enable crossregulation between transcripts.
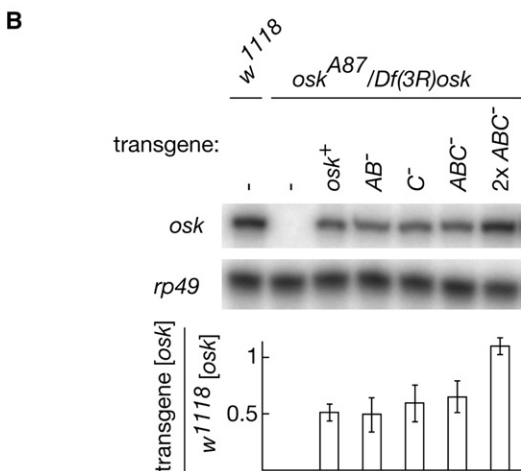
RESULTS

BREs Have Both Positive and Negative Roles in Control of Osk Activity

Analysis of BREs provided evidence of translational regulation of *osk* mRNA. Mutation of BREs in both AB and C regions of an *osk* transgene (*osk ABC⁻*) resulted in precocious expression of Osk protein and disruption of embryonic body patterning (Kim-Ha et al., 1995). We expected that the two clusters of BREs make additive or redundant contributions to repression. This is indeed the case, but selective mutation of subsets of the BREs reveals an unexpected role in activation. Transgenes were constructed in which only AB or C region BREs were mutated. All transgenes,

A

transgene	Cuticular phenotype (%)						
	0-1 abd. segments	2-7 abd. segments	wt	head defects	loss of head and thoracic segments	bicaudal	n
osk+	-	-	100	-	-	-	344
osk AB⁻	-	-	96	4	-	-	236
osk C⁻	83	14	3	-	-	-	444
osk ABC⁻	-	1	4	24	49	21	340
osk IBE⁻	100	-	-	-	-	-	643

B

Figure 1. Body Patterning Activity of *osk* Transgenes

(A) Transgenes were tested in the osk^A87^/Df(3R)osk background, and progeny embryos were examined for cuticular phenotypes. Examples of the classes of phenotypes are shown in Figure S1. See also Figure S2 for data on additional transgenes.

(B) Transcript levels for *osk* transgenes. All transgenes were tested in the osk^A87^/Df(3R)osk (RNA null) background. Top: RNase protection assays of *osk* and *rp49* mRNAs from ovaries of the indicated genotypes. Bottom: Levels of transgene mRNAs relative to the level of endogenous *osk* mRNA in wild-type (w^1118^) flies. RNA levels were quantified by phosphorimaging, and *osk* levels were normalized using the *rp49* signal. Three or more assays were used to generate the average levels (and standard deviations). Flies had one copy of the transgene-bearing chromosome.

Based on the embryonic patterning phenotypes of the mutant *osk* transgenes, we hypothesize (1) that Bru/BRE-dependent translational repression of unlocalized *osk* mRNA relies on contributions from both AB and C region BREs, and (2) that the positive role of C region BREs is in activation of *osk* mRNA translation. This activation is required when all or a subset of BREs are intact and conferring repression.

including the original *osk ABC⁻*, were introduced into the osk^A87^/Df(3R)osk genetic background, in which endogenous *osk* mRNA is absent (Jenny et al., 2006), and tested for their ability to support embryonic body patterning (Figure 1A).

The wild-type *osk* transgene (*osk+*) provides full Osk activity, and only wild-type embryos are found (Figure 1A; see Figure S1 available online). Mutation of just the AB region BREs (*osk AB⁻*) does not substantially alter the patterning activity of the *osk* transgene, as almost all of the embryos have wild-type segmentation (Figure 1A). However, a very small fraction of embryos have anterior patterning defects, indicating of a low level of excess Osk and an extremely mild disruption of translational repression.

In contrast, mutation of just the C region BREs (*osk C⁻*) dramatically reduces patterning activity of the transgene (Figure 1A), with no corresponding reduction in mRNA level (Figure 1B). Thus, mutation of the C region BREs reveals a previously undetected positive role for BREs in control of Osk activity, which we show below to be translational activation.

We also tested the transgene in which BREs from both AB and C regions were mutated. This transgene produces excess Osk activity, consistent with the established role for the BREs in translational repression (Figure 1A). This phenotype is not due to elevated mRNA levels (Figure 1B). When taken together with the properties of the *osk AB⁻* and *osk C⁻* mutants, the phenotype of the *osk ABC⁻* mutant has two implications. First, the AB and C region BREs make redundant contributions to repression. Second, the requirement for the C region BREs in positive control of *osk* expression is reduced or eliminated when repression of *osk* is defective.

Both AB and C Region BREs Contribute to Translational Repression

To test the prediction that the two sets of BREs act redundantly in translational repression, Osk protein accumulation was monitored in stage 8 oocytes (all assays were performed in the RNA null osk^A87^/Df(3R)osk background). At this and earlier stages of oogenesis, *osk* mRNA is highly concentrated in the oocyte. However, translation is repressed in wild-type oocytes (Figure 2A). Each of the transgenes with at least a subset of the BREs intact (*osk+*, *osk AB⁻*, and *osk C⁻*) also fails to direct accumulation of any detectable Osk protein in stage 8 oocytes (Figure 2B and data not shown). By contrast, the *osk ABC⁻* transgene does produce readily detectable Osk (Figure 2C). The results from this direct test of Osk protein accumulation are fully consistent with the assays of Osk body patterning activity: only the *osk ABC⁻* transgene produces high levels of ectopic Osk activity, while the other transgenes produce little or none (Figure 1A). Therefore, BREs in either the AB or C region are sufficient to confer translational repression.

A *GFP* reporter mRNA was used to confirm that both AB and C regions mediate translational repression. The *UAS-GFP* reporter transgene includes UAS transcriptional control elements, the *GFP* coding region, and the polyadenylation signal and flanking sequences from the *fs(1)K10* 3′ UTR (but not the portion of the 3′ UTR that directs transport to the oocyte). In combination with the *maternal alpha tubulin GAL4* driver (*matGAL4*), the *GFP* mRNA is expressed in the nurse cells of the ovary and produces a high
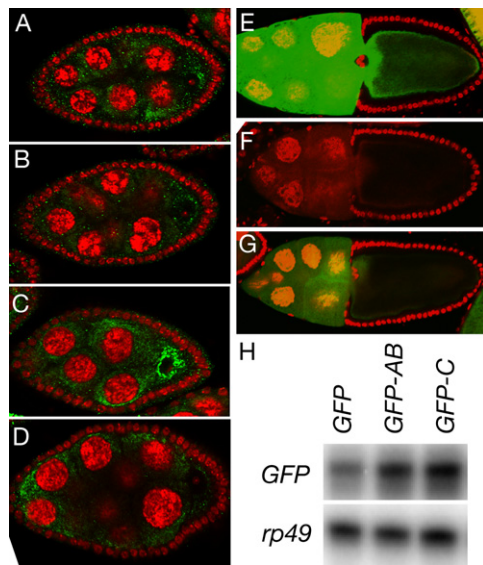
Figure 2. Redundant Contributions of BREs to Translational Repression

(A–D) show stage 8 egg chambers with Osk in green and nuclei in red. (A) is wild-type and (B) and (C) are *osk*^{A87}/*Df(3R)osk* with the *osk AB*$^-$ (B) or *osk ABC*$^-$ (C) transgenes. Results similar to that shown in (B) were obtained for the wild-type and *osk C*$^-$ transgenes. (D) is *osk*^{54}/*Df(3R)osk* with transgene *osk ABC*$^-$. All egg chambers show a low level of green background signal in both germline and somatic cells. The only consistent difference among the different genotypes was the higher level of Osk in the oocytes of *osk*^{A87}/*Df(3R)osk* ovaries with the *osk ABC*$^-$ transgene present, detected at roughly the level shown in 69% (n = 18) of the egg chambers expressing *osk ABC*$^-$, with many of the remaining egg chambers showing a lower level. None of the other genotypes ever showed Osk above background.

(E–G) show stage 10A egg chambers expressing a GFP transgene (green, nuclei in red). All samples were fixed in parallel and imaged together with the same laser power and confocal settings. A, *UAS-GFP*; B, *UAS-GFP-AB*; C, *UAS-GFP-C* (AB and C are the eponymous regions of the *osk* 3′ UTR).

(H) RNase protection assays of mRNA levels of the transgenes.

level of GFP (Figure 2E). The reporter was modified by addition of the *osk* AB or C regions. The AB region confers very strong repression of translation: GFP fluorescence in the ovary is dramatically reduced (Figure 2F) despite somewhat higher mRNA levels than for the control reporter (Figure 2H). The C region also confers repression (Figure 2G), although it is less effective than the AB region. Thus both AB and C regions mediate repression.

C Region BREs Mediate Activation of Translation

The extremely low level of Osk protein patterning activity from the *osk* transcripts with mutated C region BREs suggests that the normal activation of Osk protein expression at the posterior pole of the oocyte does not occur. In wild-type ovaries Osk protein initially appears at the posterior of the oocyte beginning at stage 9, with increasing levels by stage 10. This time course of Osk appearance is reproduced by the wild-type *osk* transgene (Figure 3A and data not shown). Similarly, the *osk AB*$^-$ transcripts direct localization-dependent expression of Osk at the posterior pole of the oocyte (Figure 3A). However, the *osk C*$^-$ transcripts are impaired in Osk synthesis (Figure 3A). Only

a tiny fraction of oocytes display the strong posterior Osk accumulation of wild-type, while the vast majority have low or undetectable levels of Osk. Thus, the C region BREs are required for normal Osk expression at the posterior pole of the oocyte.

The reduction or absence of posterior Osk protein from mutated C region BREs is not due to a reduction in mRNA level (Figure 1B) and must arise from either of two defects: a failure of posterior *osk* mRNA localization, or a failure of translational activation. To distinguish between these options, the distribution of transgene mRNAs was monitored by in situ hybridization. Localization of *osk C*$^-$ transcripts was, as for wild-type, robust (Figure 3B). Thus, the very substantial defects in posterior Osk expression are not due to mRNA localization defects; mutation of the C region BREs interferes with activation of *osk* mRNA translation.

Disruption of translational activation by mutation of the BREs has been interpreted to be due to loss of the Bru-binding sites. However, the mutations could have fortuitously created a novel binding site, to which an unknown ovarian factor binds and inhibits translation. A very strong argument against the latter interpretation comes from functional analysis of additional Bru-binding sites recently identified by in vitro selection experiments (unpublished data). Several of the new sites are found in the *osk* mRNA, and all are clustered with the BREs in the AB and C regions (Figure S2). Mutation of the type II Bru-binding sites in the C region interferes with activation of translation, just as for mutation of the C region BREs, although to a somewhat lesser extent (Figure S2 and Table S1). Because the mutated BREs and type II sites are highly dissimilar (Supplemental Information), the possibility that both types of mutations fortuitously caused the same type of artifactual translation defect seems implausible.
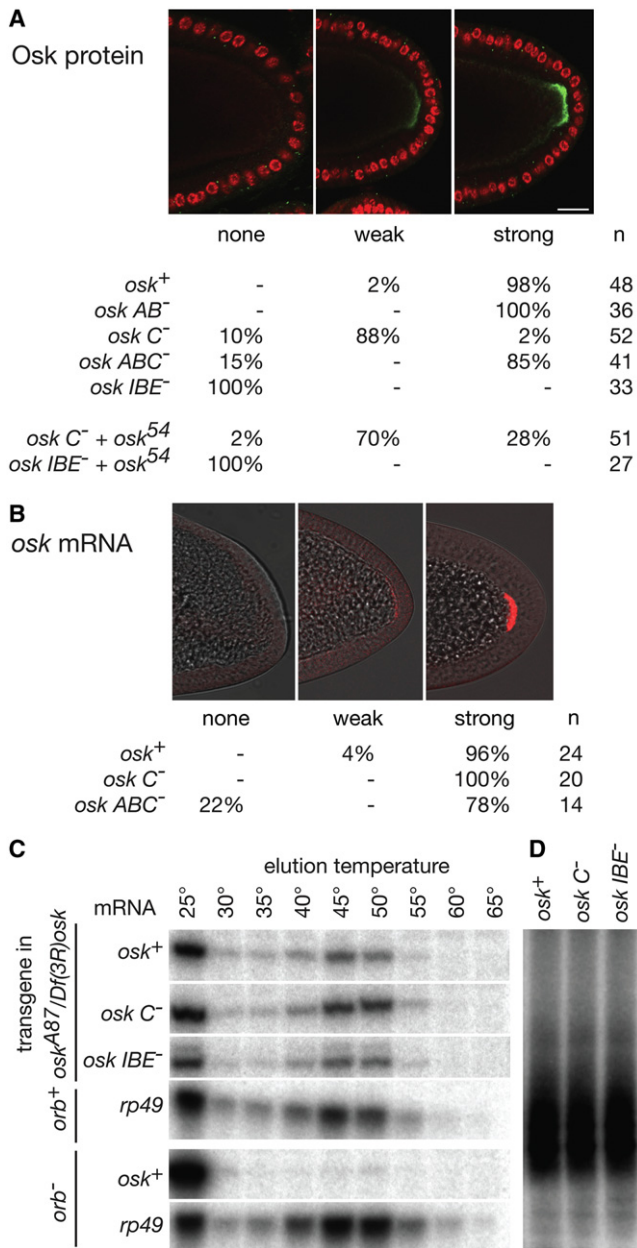
Translational Activation by C Region BREs Is Not Required When Repression Is Defective

Mutation of the C BREs alone leaves repression of the unlocalized mRNA intact (Figure 2) but significantly disrupts activation, with greatly reduced posterior Osk (Figure 3A). When both AB and C BREs are mutated and repression is disrupted, then posterior accumulation of Osk protein is restored (Figure 3A). These experiments illustrate why the activating role of the C region BREs was not detected in the initial analysis of BRE function, which relied on the *osk ABC*$^-$ transgene with both AB and C region BREs mutated (Kim-Ha et al., 1995).

Translational Activation by the C Region BREs Does Not Affect Poly(A) Tail Length

Cytoplasmic polyadenylation serves as one form of translational activation of *osk* mRNA (Chang et al., 1999; Castagnetti and Ephrussi, 2003). Repression by BREs does not involve alteration of the poly(A) tail (Lie and Macdonald, 1999; Castagnetti and Ephrussi, 2003), making it highly unlikely that activation by the BREs could involve cytoplasmic polyadenylation. However, the mechanism of activation by the IBEs is unknown. We evaluated relative poly(A) tail lengths of transgene mRNAs using a thermal elution assay (Simon et al., 1996) in which mRNAs bound via their poly(A) tails to poly(U) agarose are eluted stepwise at increasing temperatures. Transcripts with short tails elute at lower temperatures than those with longer tails. Notably, the elution profiles

A

Osk protein

	none	weak	strong	n
osk^+	-	2%	98%	48
$osk\ AB^-$	-	-	100%	36
$osk\ C^-$	10%	88%	2%	52
$osk\ ABC^-$	15%	-	85%	41
$osk\ IBE^-$	100%	-	-	33
$osk\ C^- + osk^{54}$	2%	70%	28%	51
$osk\ IBE^- + osk^{54}$	100%	-	-	27

B

osk mRNA

	none	weak	strong	n
osk^+	-	4%	96%	24
$osk\ C^-$	-	-	100%	20
$osk\ ABC^-$	22%	-	78%	14

C | **D**

Figure 3. Mutation of C Region BREs Interferes with Translational Activation, but Not Cytoplasmic Polyadenylation

(A) Osk protein in oocytes. Shown at top are posterior portions of stage 10A egg chambers, with examples of the different levels of Osk detected by immunofluorescence. Osk is green and nuclei are red. Transgenes were in the $osk^{A87}/Df(3R)osk$ background, except for those at the bottom in which osk^{54} was present (as indicated). The $osk\ IBE^-$ transgene has mutations of the first three IBEs (Munro et al., 2006). The $osk\ ABC^-$ transgene only poorly rescues the oogenesis progression defects of the $osk^{A87}/Df(3R)osk$ mutant, and even those egg chambers that develop to later stages often display morphological abnormalities (data not shown). These defects are largely suppressed by an additional copy of the transgene, suggesting that any reduction in posterior Osk is a secondary consequence of the poor rescue of progression through oogenesis (the osk RNA null phenotype). See also Table S1 for data on additional transgenes.

(B) osk mRNA in oocytes. Shown at top are posterior portions of stage 10A egg chambers, with examples of the different degrees of posterior osk mRNA localization detected by fluorescent in situ hybridization. osk mRNA is red. All transgenes were in the $osk^{A87}/Df(3R)osk$ background. The absence of localization for a minor fraction of the $osk\ ABC^-$ egg chambers is likely due to the incomplete rescue of the oogenesis progression defects by this transgene, as explained above.

(C) Thermal elution assay. Ovarian RNAs were purified, bound to poly U agarose, and eluted at the temperatures indicated. Each fraction was tested by RNase protection assay for the RNA indicated. As indicated, the osk transgenes were tested in the $osk^{A87}/Df(3R)osk$ background. The eluted fractions of RNA from orb mutant ovaries (orb^{MEL}/orb^{DEC}) were tested for both osk and rp49 to confirm that polyadenylated mRNAs were indeed bound to the poly U agarose.

(D) Ovarian RNAs from $osk^{A87}/Df(3R)osk$ females expressing the indicated transgenes were subjected to the PAT assay (Salles et al., 1994). The distribution of the signal in each lane reflects the range of poly(A) tail length.

were indistinguishable for wild-type osk transcripts and osk transcripts with translational activation defects due to mutation of BREs (osk C⁻) or IBEs (osk IBE⁻) (Figure 3C). In contrast, the elution profiles for wild-type osk mRNA from orb⁺ or orb⁻ mutant ovaries were dramatically different (Figure 3C), consistent with previous work (Chang et al., 1999; Castagnetti and Ephrussi, 2003). A PCR-based assay of poly(A) tail length also showed no substantial differences in the poly(A) tails of the osk⁺, osk C⁻ or osk IBE⁻ transcripts (Figure 3D).

trans Regulation of osk mRNA Translation

The above experiments with osk transgenes were all performed in the osk RNA null background, where the only osk mRNA present is that from the transgene. To ask if the misregulation of osk mRNA with mutant BREs can be influenced by osk mRNA with wild-type BREs, these transgenes were also tested in the presence of the osk^{54} mRNA (the osk^{54} allele is protein null and provides no Osk protein activity, but retains the BREs and has wild-type levels of the mutant RNA; some of these experiments were repeated with osk^{84}, which also retains the BREs and lacks both Osk protein activity and detectable Osk, with essentially identical results). Remarkably, the patterning defects caused by disruption of BRE-dependent repression or activation of osk mRNA translation are dramatically suppressed when osk^{54} mRNA is present.
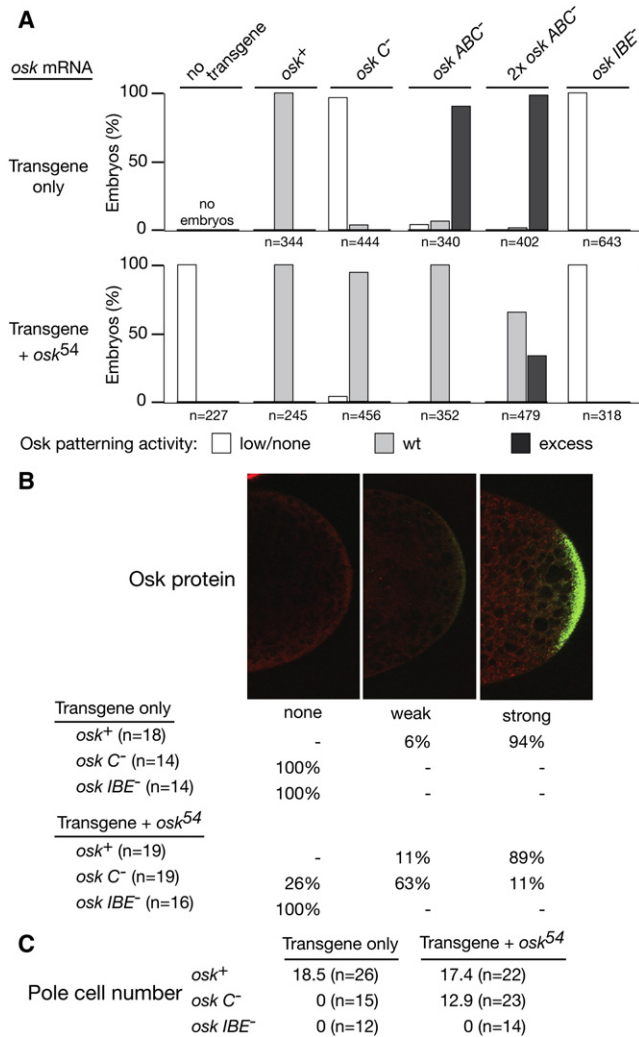
The $osk\ ABC^-$ transgene is defective in translational repression and produces substantial ectopic Osk protein activity in the osk RNA null background. When osk^{54} mRNA is present, the ectopic Osk is largely eliminated (Figures 2D and 4A). We also tested a transgenic line with higher levels of the $osk\ ABC^-$ mRNA (2× ABC⁻) (Figure 1B) and more severe patterning defects (Figure 4A and legend). The extreme patterning phenotype of this transgenic line is also suppressed when the osk^{54} mRNA is present, although suppression is incomplete (Figure 4A).

We next asked if the defect in translational activation of the osk C⁻ transgene could also be suppressed by the presence of osk mRNA with wild-type BREs. Strikingly, this transgene produces little or no Osk patterning activity in the absence of endogenous osk mRNA, yet it supports wild-type body patterning in combination with the osk^{54} mRNA (Figure 4A; see also Figure S3). Although rescue of body patterning is dramatic, restoration of Osk protein expression in stage 10 oocytes is comparatively modest (Figure 3A). A likely explanation comes

Figure 4. Suppression of Regulatory Defects by BRE⁺ mRNA

(A) Body patterning activities of *osk* transgenes in the absence of endogenous *osk* mRNA (upper part) or in the presence of the BRE⁺ *osk⁵⁴* mRNA (lower part). The transgenes are indicated at top. For each transgene the percentage of progeny embryos with different levels of Osk activity is indicated in the graph below (shading key at bottom). Levels of Osk activity: low/none, missing or absent abdominal denticle belts; wild-type, wild-type cuticles; excess, loss of anterior structures or bicaudal phenotypes. 2× *osk ABC⁻* is a transgenic line that expresses twice the level of the line used in all other experiments. Embryos in the "excess" category for the 2× line typically have more extreme phenotypes than for the 1× line (e.g., 56% bicaudal for 2× versus 13% bicaudal for 1×). The *osk IBE⁻* transgene has the 5′ subset of the IBEs mutated. A similar transgene with all IBEs mutated (Munro et al., 2006) yielded identical results (data not shown). See also Figure S3 for data on additional transgenes.
(B) Osk protein in embryos from mothers expressing *osk* transgenes, with or without the BRE⁺ *osk⁵⁴* mRNA. Shown at top are posterior portions of early stage embryos, with examples of the different levels of Osk (green).
(C) Pole cell numbers for embryos from mothers expressing the transgene indicated at top, with or without *osk⁵⁴* mRNA as indicated. See also Table S3 for data on additional transgenes.

from a consideration of the time course of Osk expression. Osk is first detected at the oocyte posterior pole at stage 9, with more present at stage 10. However, the bulk of Osk accumulation occurs later in oogenesis (Snee et al., 2007). Therefore, the

contribution of the C region BREs to activation of *osk* mRNA translation, while detectable at stage 10, may be more significant later. If so, then it may be this later phase in Osk expression that is most effectively restored by coexpression of the *osk C⁻* transgene with the *osk⁵⁴* mRNA. Notably, embryos from mothers expressing only the *osk C⁻* transcripts have no detectable Osk, while coexpression with the *osk⁵⁴* mRNA partially restores embryonic Osk (Figure 4B; see also Table S3). Thus, the later phase of Osk expression is most severely affected by disruption of BRE-dependent translational activation, and is most substantially restored by coexpression with an *osk* mRNA with wild-type BREs.

As a separate assay for rescue of posterior Osk accumulation, we also determined the number of pole cells formed in embryos from mothers expressing the *osk C⁻* transgene, with or without the presence of the *osk⁵⁴* mRNA. Embryos from mothers expressing only the *osk C⁻* transgene had no pole cells, and coexpression with the *osk⁵⁴* mRNA partially restored pole cell formation (Figure 4C).

Rescue of *osk* mRNA regulatory defects in *trans* is selective. There is no rescue at all for the *osk IBE⁻* transgene: Osk protein accumulation (Figures 3A and 4B), Osk patterning activity (Figure 4A), and pole cell formation (Figure 4C) all remain defective when the *osk⁵⁴* mRNA is present.

DISCUSSION

Three types of regulatory elements have been implicated in activation of *osk* mRNA translation: a 5′ activating element, the IBEs, and now the subset of BREs in the *osk* 3′ UTR C region. The BREs present an unusual case, being involved in both repression and activation. In principle, a repressive element could be thought to play a passive role in the activation that relieves repression: the element would need to be unoccupied or unproductively bound for activation to occur. For the BREs the role is active, not passive. In the context of the *osk C⁻* transgene, repression occurs because the AB region BREs are intact. However, despite proper localization of the *osk C⁻* mRNA to the posterior pole of the oocyte, the normal activation of translation to allow Osk protein expression at that site is defective and Osk protein levels are reduced. Thus, the C region BREs are required to release the mRNA from repression conferred by the AB region BREs. In the context of wild-type *osk* mRNA, both AB and C region BREs contribute to repression, and so the C region BREs must switch roles, first repressing and later activating. The activating function of C region BREs could be due to position in the mRNA. For example, activation might only occur when BREs are close to the poly(A) tail. Given the absence of a change in poly(A) tail length when activation is defective, any effect on the poly(A) tail itself would have to be more nuanced under this scenario. Activation could involve cooperation between the BRE-binding factor and another activating factor that binds only in the C region. At present, no protein is known to have that property.

The best candidate for the factor that binds to the BREs to mediate activation is Bru, since the BREs were identified and defined by their ability to bind Bru. Moreover, mutation of the type II Bru-binding sites in the C region also disrupts activation. Therefore, if the activator is not Bru, it must be a protein

or proteins with the ability to bind to the two different types of sites. Mutants lacking Bru function arrest oogenesis at a very early stage (Schupbach and Wieschaus, 1991; Webster et al., 1997), obscuring any potential role in activation of *osk* mRNA translation.

Certain defects in translational regulation of *osk* mRNA can, remarkably, be suppressed by the presence of an *osk* mRNA with wild-type regulatory elements. This phenomenon is reminiscent of transvection, in which regulatory elements controlling transcription of one allele of a gene can influence transcription of the second allele on the homologous chromosome (Lewis, 1954; Duncan, 2002). We suggest a similar model for translational regulation in *trans*, in which regulation imposed on one molecule via direct binding of regulatory factors is then conferred on another molecule via association of the mRNAs. Evidence for a physical association between *osk* transcripts has come from the demonstration that reporter mRNAs containing the *osk* 3′ UTR (which is necessary but not sufficient for localization) localize to the posterior pole of the oocyte only if endogenous *osk* mRNA is also present (Hachet and Ephrussi, 2004). This "piggybacking" of the reporter mRNA relies on the PTB protein. PTB binds to multiple sites in the *osk* mRNA, forming a large aggregate in vitro. Thus, it appears that PTB links multiple *osk* transcripts to form large RNP particles in vivo (Besse et al., 2009). Piggybacking for mRNA localization provides an example of a *trans* effect in posttranscriptional regulation. For piggybacking, all that is necessary is the physical linkage: directed movement of one *osk* mRNA molecule to its destination at the posterior pole of the oocyte would confer the same movement on any other molecule in the same RNP particle.

Would physical linkage alone be sufficient to confer all of the different forms of translational regulation on all *osk* mRNAs in the same RNP particle? At least one type of regulation—activation by the IBEs—is not conferred in *trans*, providing an example where physical linkage is not sufficient. However, under the current models for Bru/BRE-dependent repression, physical linkage could be sufficient for *trans* regulation by BREs. One model for repression involves the formation of silencing particles which in some manner limit accessibility to ribosomes (Chekulaeva et al., 2006). Presumably, any mRNA recruited to the particles would also be protected from ribosomes. A second model for repression involves recruitment of Cup to the *osk* mRNA by Bru. Cup binds to, and inactivates, eIF4E, thus interfering with initiation of translation. If the inactivated molecule of eIF4E is bound to the *osk* mRNA cap, then translation initiation is blocked (Nakamura et al., 2004). A weak point of this model has been the necessity that, for repression to be specific, both RNA contacts of the Bru/Cup/eIF4E complex would have to be with the same mRNA molecule: eIF4E would have to bind the cap of a particular *osk* transcript, and Bru would have to bind the BREs of the same transcript. What would prevent the Cup newly recruited by Bru to *osk* mRNA from inactivating the eIF4E bound to the cap of a different mRNA? In the context of an RNP containing predominantly *osk* mRNAs, inactivation of eIF4E by Cup would interfere with translation of any member of the local population of transcripts, even if the Bru/Cup/eIF4E ternary complex bridges two mRNAs. By this scenario, *trans* regulation would be an inherent feature of the mechanism. The specificity of such *trans* regulation would be limited by the degree to which the local population of

transcripts is homogeneous, and crossregulation between different species of mRNAs would be possible. Recent characterization of sponge bodies has shown that *osk* mRNA is compartmentalized in the oocyte, with large reticulated sponge bodies having *osk* distributed in discrete domains (Snee and Macdonald, 2009). Compartmentalization of *osk* mRNA could impose selectivity on *trans* regulation, preventing features of *osk* regulation from being conferred promiscuously on other mRNAs. Assembly of mRNAs in large RNP particles is common, and elucidation of the rules dictating which types of translational regulation can and cannot be exerted in *trans* should have broad relevance.

EXPERIMENTAL PROCEDURES

Flies and Transgenes

w^{1118} flies were used as the wild-type. Mutant flies are described at FlyBase (http://flybase.bio.indiana.edu/). *Df(3R)osk* was constructed by FRT-mediated recombination using Exelixis transposon insertions *P*(XP)*d09940* and *PBac*f02664. The resulting deletion of 23,158 nt removes four genes: *osk*, *CG11963*, *CG11964*, and *CG11966*.

Flies bearing *osk* transgenes with mutation of IBEs (Munro et al., 2006) were from Bruce Schnapp. The *osk*+ and *osk ABC*− transgenes were described previously (Kim-Ha et al., 1995) and carry the mutations described there (and in Supplemental Information). Novel *osk* transgenes are described in detail in the Supplemental Information.

UAS-GFP has mGFP6 (Haseloff, 1999) inserted into the Asp718 site of pUASp. The AB and C regions of the *osk* 3′ UTR (nt 2669–2795 and 3397−3555, respectively, of the *osk* genomic sequence [GenBank Accession M63492]) were inserted as BamHI-BglII fragments into the BamHI site from the pUASp vector.

Analysis of Proteins and mRNAs

Immunostaining of ovaries and embryos was as described previously (Kim-Ha et al., 1995), except that secondary antibodies were labeled with Alexafluor 488 (Invitrogen). Osk was detected with rabbit anti-Osk (diluted 1:2,000), and Vas was detected with rat anti-Vas (1:500). Fluorescent in situ hybridization was as described (Snee and Macdonald, 2009). In all cases *osk*+ controls were fixed, stained, and imaged in parallel, to confirm that the staining worked properly and to provide a reference for determination of signal levels. Microscopy of all samples made use of a Leica TCS-SP laser scanning confocal microscope or a Nikon epifluorescence microscope.

For RNase protection assays, ovaries were dissected from females maintained with males on well yeasted vials for 3−4 days after eclosion, and RNA was prepared using Tri Reagent-LS (Molecular Research Center, Inc) following the protocol provided by the vendor. RNase protection assays were performed with the RPA III kit (Ambion, Inc), and the results quantitated using phosphorimaging with a Typhoon Trio Imager (Amersham). Probes for RNase protection were transcribed in vitro using the Maxiscript kit (Ambion).

The thermal elution assay was used largely as described (Simon et al., 1996), with minor modifications described in Supplemental Information.

The LM-PAT assay was performed essentially as described (Salles et al., 1994) using ovarian RNA prepared as described above. The oligo dT anchor primer was GAGCTCATTTGCGGCCGCTTTTTTTTTTTT, and the *osk*-specific primer was GTCTTCTAGATAGCTATCTAC.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, three tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.devcel.2009.12.021.

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