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Translational regulation of *oskar* mRNA occurs independent of the cap and poly(A) tail in *Drosophila* ovarian extracts

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SUMMARY

Translational regulation plays a prominent role in *Drosophila* body patterning. Progress in elucidating the underlying mechanisms has been limited by the lack of a homologous in vitro system that supports regulation. Here we show that extracts prepared from *Drosophila* tissues are competent for translation. Ovarian extracts, but not embryonic extracts, support the Bruno response element- and Bruno-dependent repression of *oskar* mRNA translation, which acts in vivo to prevent protein synthesis from transcripts not localized to the posterior pole of the oocyte. Consistent with suggestive evidence from in vivo experiments, regulation in vitro does not involve changes in poly(A) tail length. Moreover, inhibition studies strongly suggest that repression does not interfere with the process of 5′ cap recognition. Translational regulation mediated through the Bruno response elements is thus likely to occur via a novel mechanism.

Key words: Translational regulation, In vitro assay, *oskar* mRNA, Bruno, Polyadenylation

INTRODUCTION

Translational regulation of maternal mRNAs occurs with different degrees of specificity. Global control over all transcripts can be achieved through changes in the activity of the translation machinery. More specific controls rely on cis-acting regulatory elements present within mRNAs, often in their 3′ untranslated regions (3′ UTRs) (reviewed by Richter, 1996; reviewed by Wickens et al., 1996). Message-specific controls that govern the timing of expression commonly involve changes in the length of the poly(A) tail (reviewed by Richter, 1996; reviewed by Wickens et al., 1996). Typically, inactive mRNAs have short poly(A) tails, which are subsequently extended in the cytoplasm coincident with the onset of translation. Translational activation may involve the binding of poly(A) tails by poly(A)-binding protein (PABP), which itself binds to eIF-4G of the cap-binding complex and stimulates translation in a manner not yet fully understood (Tarun and Sachs, 1996; Haghighat and Sonenberg, 1997). In addition, polyadenylation may activate translation by promoting methylation of the 5′ cap (Kuge and Richter, 1995).

Specific forms of translational control also direct spatially restricted patterns of expression. Prominent examples come from *Drosophila* where the Oskar (Osk) and Nanos (Nos) proteins, which act sequentially in patterning the posterior of the embryo (reviewed by Curtis et al., 1995), display spatially restricted synthesis in the oocyte and embryo, respectively (Gavis and Lehmann, 1994; Kim-Ha et al., 1995; Markussen et al., 1995; Rongo et al., 1995). The Osk protein is expressed from mRNA localized to the posterior pole of the oocyte. Translation of *osk* mRNA is repressed prior to its localization in a process dependent on control sequences in the *osk* mRNA 3′ UTR called Bruno response elements (BREs) and the Bruno (Bru) protein which binds to the BREs (Kim-Ha et al., 1995; Webster et al., 1997). Similarly, Nos protein is expressed from mRNA localized at the posterior of the embryo, and translational repression ensures that unlocalized *nos* mRNA is not translated. Repression is mediated by regulatory sequences in the 3′ UTR (Gavis and Lehmann, 1994; Dahanukar and Wharton, 1996; Gavis et al., 1996; Smibert et al., 1996), which are specifically bound by the Smaug (Smg) protein (Smibert et al., 1996).

For both *osk* and *nos* mRNAs, the mechanisms underlying translational control, both repression and subsequent activation, are currently unknown. To facilitate progress in this area, we have developed a novel in vitro system that recapitulates the BRE-dependent translational regulation of *osk* mRNA. Using this system, we have confirmed genetic data defining the function of Bru as a translational repressor. In addition, we have demonstrated that BRE-mediated translational regulation in vitro does not require a poly(A) tail and is not accompanied by a change in poly(A) tail length, results consistent with suggestive data from in vivo experiments. Furthermore, repression is not affected by inhibition of cap-dependent initiation of translation, suggesting that Bruno interferes with a step in translation other than recognition of the cap.

MATERIALS AND METHODS

RNase H experiments

The poly(A) tail lengths of endogenous *osk* transcripts were

determined by RNase H assays (Paek and Axel, 1987) using total RNA prepared from ovaries. For each reaction, 20 μg RNA was mixed with 5 μg oligonucleotide A (3′ CGAACAAACATCGTGTC 5′) or B (3′ CGACGTCTGTGCGGTTGGCC 5′) and 5 μg oligo $(dT)_{12-18}$ (when appropriate) and denatured for 5 minutes at 85°C. Oligonucleotides were hybridized to RNA for 10 minutes at 42°C in RNase H buffer (40 mM Tris-HCl pH 8.0, 4 mM $MgCl_2$, 1 mM DTT, 30 ng/ml BSA) in a total volume of 20 μl. Following hybridization, duplexes were digested with 1 U RNase H (Gibco BRL) for 30 minutes at 37°C. Reactions were terminated by phenol/chloroform extraction and RNA was recovered by ethanol precipitation. Products were resuspended in denaturing gel loading buffer (Ambion), denatured by heating 5 minutes at 95°C and resolved on a 5% denaturing polyacrylamide gel run in 0.5× TBE. RNAs were transferred to GeneScreen membrane (NEN Research products) by electroblotting (Idea Scientific) for 1 hour at 12 V. Membrane was UV cross-linked (Stratagene) and baked under vacuum for 1 hour at 80°C. Standard protocols were used for hybridization to a ^{32}P-labeled DNA probe made from the *osk* C region. RNA fragments were visualized by autoradiography.

Plasmids and reporter RNAs

All plasmids used in these assays were made using the pGEM-4 based plasmid T7lucpA, which includes the *luciferase (luc)* gene under control of the T7 promoter followed by a poly(A) tail 30 residues in length (P. Sarnow, personal communication). All mRNAs were polyadenylated, except where noted otherwise. Messages containing the poly(A) tail were transcribed using T7 RNA polymerase from DNA templates linearized with *Hpa*I, while those without the poly(A) tail were transcribed from templates linearized with *Bam*HI. Constructs with *osk* mRNA 3′ UTR sequences inserted into this plasmid were made by cloning of these sequences into the *Bam*HI site. Transcripts containing the 5′ portion of the 3′ UTR were transcribed from templates linearized with *Sac*II. Transcription reactions were performed as described (Sampson and Saks, 1993), and messages were capped by transcription in the presence of the cap structure analog G(5′)ppp(5′)G (New England Biolabs). Following transcription, template DNA was digested by incubation with 10 U RNase-free DNase I (Boehringer Mannheim) for 15 minutes at 37°C. RNAs were purified through a Sephadex G-50 (Pharmacia) spun column followed by phenol:chloroform extraction, chloroform extraction and ethanol precipitation.

Preparation of in vitro translation extracts

The protocol for extract preparation was adapted from protocols developed for use with yeast (Tarun and Sachs, 1995) and HeLa cells (McBratney and Sarnow, 1996). For ovary extracts, ovaries were hand-dissected from 3- to 4-day-old wild-type females that had been fattened overnight on yeast at 25°C. Ovaries were dissected, placed into hypotonic buffer [10 mM Hepes-KOH pH 7.4, 15 mM KCl, 1.5 mM $Mg(OAc)_2$, 2 mM DTT, 0.5 mM Pefabloc (Boehringer Mannheim)] on ice and washed in approximately 3-5 volumes cold hypotonic buffer 4-5 times with a 3 second centrifugation in between washes to settle ovaries. During the last wash, ovaries were disrupted by pipeting up and down with a 0-200 μl capacity pipet tip. The ovaries were again centrifuged quickly to settle the tissues. All buffer was removed and the ovaries were dounce homogenized. The extract was centrifuged 10 minutes at 15,000 revs/minute at 4°C to pellet debris. The supernatant was transferred to a fresh microfuge tube and centrifuged under the same conditions. The resulting supernatant was collected into a fresh tube.

For embryo extracts, timed collections of embryos were bleached for approximately 3 minutes to dechorionate and then collected by filtration. Embryos were washed 5-6 times with 0.1% Triton X-100 and transferred to hypotonic buffer on ice. Embryos were washed as described for ovaries. All buffer was removed after the final wash and embryos were dounce homogenized. Extract was centrifuged in the manner described above for ovary extract preparation.

Extracts were processed through spun columns prepared with Sephadex G-25 Superfine resin (Pharmacia) preswelled in buffer A (30 mM Hepes-KOH pH 7.4, 100 mM KOAc, 2 mM $Mg(OAc)_2$, 2 mM DTT, 0.5 mM Pefabloc). To prepare the column, resin was centrifuged 3 minutes at 850 revs/minute at 4°C in a Beckman JS-4.2 rotor (approximately 150 ***g***). An equal bed volume of cold buffer A was added and the column centrifuged again. Extract (one-eighth resin volume) was loaded onto the column and centrifuged for 2 minutes at 800 revs/minute (approximately 120 ***g***) at 4°C. The column was transferred to a fresh collection tube, a volume of cold buffer A equal to the extract volume was added and the column was centrifuged for 3 minutes at 150 ***g*** at 4°C. This eluate was collected into a fresh tube for use in in vitro assays. Average protein concentration of these extracts was 30-50 mg/ml.

Endogenous RNAs in the extracts were digested by micrococcal nuclease treatment. Micrococcal nuclease (Pharmacia) was solubilized at a final concentration of 15 U/μl into 20 mM Hepes-KOH plus 50% glycerol and stored at –80°C. 3 μl of 40 mM $CaCl_2$ and 3 μl nuclease were added to 250 μl of extract and incubated 5 minutes at room temperature. 5 μl 0.1 M EGTA was added to terminate the reaction and extract was supplemented with 5 μl 5 mg/ml yeast tRNA. Extract was stored in small aliquots at –80°C.

In vitro translation assay

For in vitro translation assays, a cocktail was made containing the following per reaction: 3.15 μl water, 1.25 μl 6× translation buffer [132 mM Hepes-KOH pH 7.4, 680 mM KOAc, 11.8 mM $Mg(OAc)_2$, 4.5 mM ATP, 0.6 mM GTP, 150 mM creatine phosphate (Boehringer Mannheim), 10.2 mM DTT], 0.3 μl complete amino acid mix (1 mM stock, Promega), 0.1 μl RNase Block (40 U/μl stock, Stratagene), 0.2 μl creatine phosphokinase (10 mg/ml stock, Boehringer Mannheim). For each reaction, 5.0 μl of this cocktail was aliquoted into an individual microfuge tube and mixed with 0.5 μl RNA (25 ng) and 2.0 μl extract. Reactions were incubated at room temperature for 90-120 minutes and assayed for luciferase activity at time points throughout the incubation period. To assay for luciferase activity, 1 μl of the translation reaction was added to 40 μl luciferase assay reagent (Promega) and immediately measured by a 10 second reading in a luminometer (Monolight 2001). The largest degree of repression was observed between 90 and 120 minutes, when the bulk of luciferase activity is produced in the extracts. We use the 90 minute time point when reporting relative translation efficiencies, including experiments in which the time course of the reaction is not presented. This approach is valid, as all differences in translation observed at this (or any other) time point are completely Bru- and BRE-dependent.

For reactions containing free cap analog [m^7G(5′)ppp(5′)G (New England Biolabs)] as a competitor, 1 μl of a 7.5× stock of cap analog was preincubated with extract for 5 minutes at room temperature prior to addition of the reaction cocktail.

Reactions using reticulocyte lysate were completed as per manufacturer's protocol (Promega) and assayed for luciferase activity as described above.

Monitoring of RNA stability and size

RNAs trace-labeled with [α-^{32}P]UTP were transcribed for use in experiments monitoring stability. Large-scale in vitro translation reactions were completed as described above, with aliquots removed at each 30 minute time point into 2× urea/SDS solution (7 M urea, 0.35 M NaCl, 0.01M Tris-HCl pH 7.8, 1% SDS). RNAs were extracted with phenol:chloroform, then chloroform, and ethanol precipitated. RNAs were resuspended in denaturing buffer (Ambion), heated for 10 minutes at 65°C, and resolved on a 1.2% agarose gel containing 7.5% formaldehyde. Following electrophoresis, the gel was dried and exposed using the GS-363 Molecular Imager Phosphorimager and Molecular Analyst software (Bio-Rad).

For experiments in which transcript size was monitored during the in vitro translation reaction, RNAs were labeled and extracted in the same manner as described above. Messages were resolved on a 4% denaturing polyacrylamide gel and detected by autoradiography following exposure to film overnight at –80°C.

Immunodepletion

To immunodeplete Bruno from extracts, 15 μl ovary extract was mixed with 10 μl purified anti-BruB antibodies (Webster et al., 1997) (or the same volume of antibodies purified from normal rat serum (Sigma) as a control), 5 μl buffer A, and 30 μl protein G-agarose beads (Boehringer Mannheim). Following a 3 hour incubation with rotation at 4°C, samples were microcentrifuged for 20 seconds at 4°C to settle beads and the supernatant was collected. The supernatant and the immunoprecipitated material bound to the beads were tested by western analysis for the presence of Bru protein; Bru was not detected in the supernatant but was detected in the immunoprecipitated material (data not shown). 12 μl in vitro translation reactions were set up as follows: 6.5 μl immunodepleted extract, 2 μl 6× translation buffer, 0.48 μl amino acid mix, 0.15 μl RNase Block, 0.32 μl creatine phosphokinase, 1.0 μl RNA (5 ng), 1.55 μl water. For these reactions, the 6× translation buffer was adjusted to give a final concentration of 140 mM KOAc and 2.5 mM $Mg(OAc)_2$ in the in vitro reaction. Reactions were incubated at room temperature and 2 μl assayed at each time point for 30 seconds in the luminometer.

Recombinant proteins and reconstitution experiments

For recombinant protein production, cDNAs were cloned into pBlueBacHis (Invitrogen), a vector that adds an N-terminal 6His tag to the protein. Transfections into Sf9 cells, protein expression and protein purification were completed as per manufacturer's protocol (Xpress System, Invitrogen). Fractions containing protein were identified by western blotting. These fractions were pooled and concentrated 10-fold by dialysis in 25% PEG (M_r 15000-20000) solution prepared in buffer A and subsequent dialysis in buffer A to remove residual PEG. For experiments using immunodepleted extracts (data not shown), reactions were as described in the previous section using 2.0 μl purified protein, 0.55 μl RNA (5 ng) and no additional water. For reconstitution experiments using embryo extract, in vitro translation reactions contained 1.0 μl 0-1 hour embryo extract, 2.0 μl purified protein and 10 ng RNA in a 7.5 μl reaction, with the remaining components added to the concentrations described above for a standard assay (the final concentration of recombinant Bru in the supplemented embryo extract is similar to that found in the ovary extracts, as estimated by western analysis).

As one measure of whether recombinant Bru is properly folded, protein was tested for binding to the *osk* mRNA AB region in a UV cross-linking experiment as described (Lie and Macdonald, 1999). Similar masses of recombinant Bru and Bru protein from ovary extract (as quantitated by Western analysis) were used in cross-linking reactions and similar levels of binding were observed (data not shown).

RESULTS AND DISCUSSION

Numerous examples of translational regulation of maternal mRNAs involve changes in the poly(A) tail (reviewed by Richter, 1996; reviewed by Wickens et al., 1996). Two types of experiments were performed to explore the possibility that *osk* translational regulation occurs by a similar mechanism. First, we used a direct assay to determine the length of the poly(A) tail on *osk* ovarian transcripts (Fig. 1). The poly(A) tail is short, suggesting that polyadenylation is unlikely to function in regulating translation of *osk* mRNA. Second, we determined the distribution of PABP in the *Drosophila* ovary.

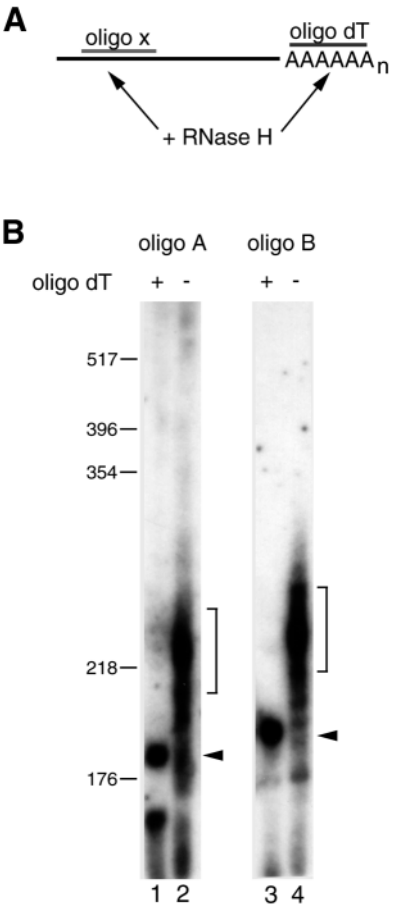


Fig. 1. The poly(A) tail of ovarian *osk* mRNA is short. (A) RNase H experiments, as diagrammed here, were used to determine the length of the poly(A) tail on *osk* transcripts in the ovary. A specific oligonucleotide was hybridized to the *osk* mRNA 3′UTR in the absence or presence of oligo dT. RNase H treatment of these complexes resulted in digestion of DNA:RNA hybrids, and a comparison of the size fragments resulting from RNase H treatment of complexes formed with or without oligo dT indicated the size of the poly(A) tail. (B) RNase H experiments using two different oligonucleotides which hybridize to different parts of the *osk* mRNA 3′ UTR. The RNAs in lanes 1 and 2 were hybridized to oligonucleotide A, while those in lanes 3 and 4 were hybridized to oligonucleotide B. Lanes 1 and 3 included oligo(dT) in the reactions, and lanes 2 and 4 did not. Arrowheads indicate the resulting fragments of *osk* mRNA lacking a poly(A) tail, and bracketed regions indicate poly(A)-containing fragments. Based upon these results, the poly(A) tail of *osk* mRNA in ovaries is approximately 35 residues in length. Sallés et al. (1994) previously reported a slightly longer length for the *osk* mRNA poly(A) tail. This discrepancy may reflect the use of different methods: one direct and the other an indirect method which includes a PCR amplification step.

Although PABP is present at high levels in the germline cells early in oogenesis, it is noticeably depleted from the oocyte at the stage when *osk* mRNA is localized to the posterior pole and translationally activated (Fig. 2). Although these experiments are suggestive, neither observation provides a compelling argument against a role for polyadenylation in translational activation. For example, the poly(A) tail could act in a manner not requiring PABP. Furthermore, the fraction of translationally

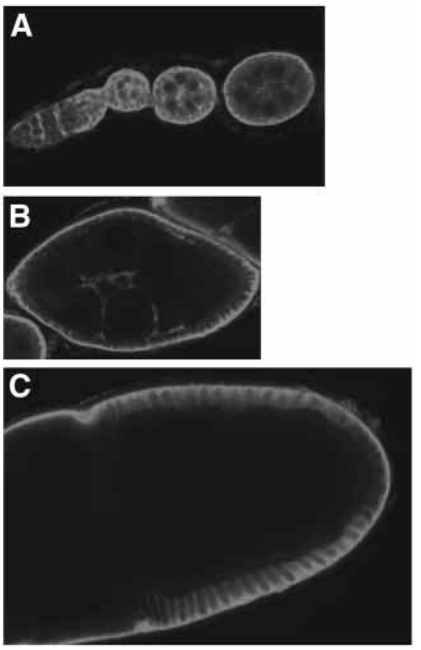
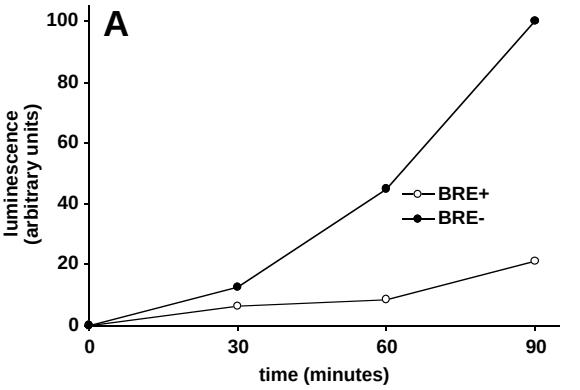


Fig. 2. PABP expression pattern during oogenesis. PABP was visualized using a monoclonal antibody (6E2) (Matunis et al., 1992) and confocal imaging. In this ovariole, developmental stages proceed from left (early) to right (late). Within each egg chamber, anterior is to the left and posterior is to the right. Each egg chamber consists of an anterior cluster of cells, the nurse cells, and a posteriorly positioned cell, the oocyte, surrounded by a layer of somatic follicle cells. (A) PABP is found throughout the egg chamber during the earliest stages of oogenesis. (B,C) At later stages the protein can be detected only in the somatic follicle cells and not in the germline.

active *osk* mRNA may be small, as observed for *nos* mRNA (Bergsten et al., 1999), making it difficult to detect the presence of transcripts with longer poly(A) tails.

To facilitate biochemical analysis of translational control in *Drosophila* and definitively address the role of polyadenylation in regulation of *osk* mRNA translation, we developed in vitro translation systems from *Drosophila* tissues. Although similar systems have been prepared from other sources, they are unlikely to contain the factors necessary for specific translational regulation of *Drosophila* mRNAs. The preparation of the extracts is described in Materials and methods; extensive characterization of the extracts, including demonstration that all assays are performed under conditions in which there is a linear response to changes in RNA concentration and extract dilution, will be described elsewhere (Y. L. and P. M., unpublished data). Translational activity of the extracts was monitored using reporter mRNAs encoding luciferase.

The ability of *Drosophila* extracts to recapitulate regulated translation was tested using the 3′ UTR of the *osk* mRNA, which contains the BRE control elements that mediate Bru-dependent translational repression (Kim-Ha et al., 1995). The *luciferase* (*luc*) reporter mRNA was modified by addition of the following sequences to its 3′ end: either the wild-type *osk* 3′ UTR (BRE+) or a point-mutated version of the *osk* 3′ UTR (BRE−) that is unable to bind Bru protein in vitro and fails to support translational repression in vivo (Kim-Ha et al., 1995). In embryo extracts, as well as reticulocyte lysates, both mRNAs were translated with similar efficiencies (Fig. 3B), revealing no inherent differences in their abilities to be translated. In contrast, translation of the two mRNAs was markedly different in ovary extracts: the *luc BRE+* message was translationally repressed approximately 9-fold, on average, in comparison to the *luc BRE−* message (Fig. 3A). This



B

extract	relative translation BRE-/ BRE+
ovary	9.2 (5.5)
embryo	1.2 (0.1)
reticulocyte lysate	1.1 (0.1)

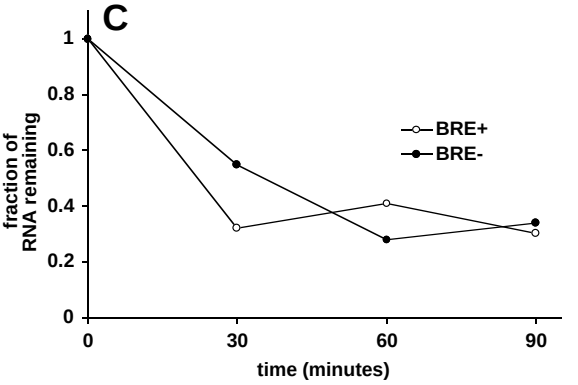


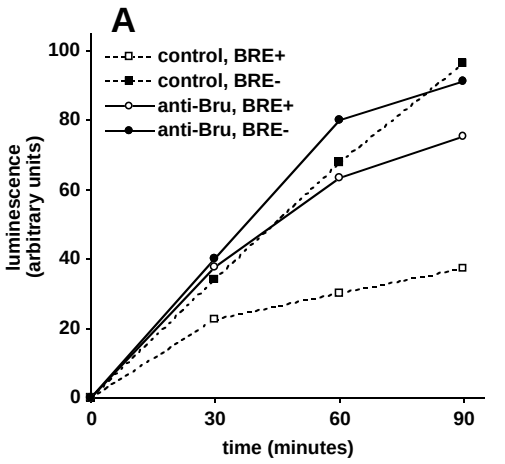
Fig. 3. BRE-mediated translational repression can be reproduced in vitro. (A) Translation of polyadenylated reporter RNAs containing either a BRE+ *osk* 3′ UTR or a BRE− *osk* 3′ UTR was assayed in *Drosophila* ovary extracts. Translation of the BRE− RNA is substantially enhanced relative to the BRE+ RNA. (B) Translational regulation occurs in ovary extracts but not in rabbit reticulocyte lysate or *Drosophila* embryo extracts. Translation of BRE+ and BRE− messages was measured in different extract types. Values shown here (and in Figs 4, 5 below) represent the fold repression as determined by assaying luciferase activity at the 90 minute time point. Calculated standard deviations are indicated in parentheses. Note that the values shown here for ovary extract are an average of the amount of repression observed in 9 independent experiments. (C) Differences in translational activity of reporter RNAs is not a result of altered stability. The stability of radioactively labeled BRE+ and BRE− reporter transcripts was monitored during the in vitro translation reaction. RNA was extracted at 30, 60 and 90 minute time points, resolved on a denaturing agarose gel, and quantitated using a phosphorimager.

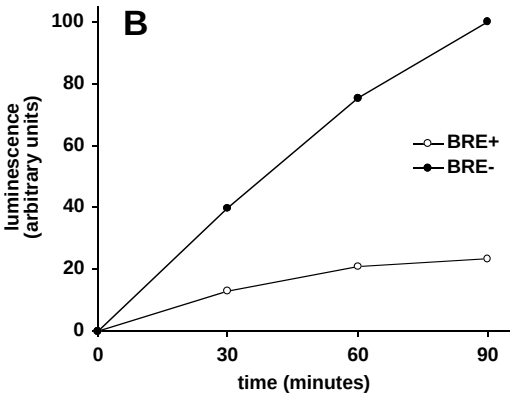
differential translation cannot be attributed to differences in RNA stability. Although both BRE+ and BRE− reporter RNAs were significantly degraded during the first 30 minutes of the translation reaction, the remaining fractions of intact, full-length mRNAs were equally stable for at least the next 60 minutes when the bulk of luciferase activity is synthesized (Fig. 3C). These results indicate that the ovary extract supports specific regulation of translation.

To determine whether the dependence of regulated translation on BREs reflects a requirement for Bru, two related experiments were performed. First, ovarian extracts were immunodepleted using anti-Bru antibodies or antibodies purified from normal rat serum and were then tested for translational activity. Notably, the control antibodies had no effect on the relative levels of BRE+ and BRE− translation, but depletion with anti-Bru antibodies largely eliminated BRE-dependent repression (Fig. 4A). Purified recombinant Bru protein was added to the immunodepleted extracts but was not sufficient to restore translational regulation activity (data not shown, see Materials and methods). This result suggests that, in addition to removing Bru, immunodepletion using anti-Bru antibodies may remove other proteins required for BRE-mediated translational regulation. Indeed, a significant fraction of Bru protein is present in a large macromolecular complex (Y. L. and P. M., unpublished data). In a second type of experiment, purified Bru was added to embryonic extracts, which lack Bru (Webster et al., 1997) and do not support BRE-mediated regulation of translation. Two other proteins, Apontic (Apt) (Lie and Macdonald, 1999) and the N-terminal portion of Smg (NSmg, approximately 635 amino acids) (C. Smibert, personal communication), were expressed and purified in parallel for use as controls in these experiments. Addition of recombinant Bru recapitulated BRE-mediated translational regulation in the embryo extract (Fig. 4B), while addition of either Apt or NSmg had a much more modest effect (Fig. 4C). Both types of experiment reveal that BRE-dependent translational repression in vitro requires Bru.

Although addition of Bru to embryo extract promoted BRE-dependent repression, the relative translation of BRE− versus BRE+ RNAs was less (average 3.2-fold) than that measured in ovary extracts (average 9.2-fold). There are likely explanations for this difference. The recombinant Bru protein may not be fully active, although the protein displays RNA-binding activity indistinguishable from that of ovarian Bru (data not shown, see Materials and methods). Alternatively, additional protein(s) present in ovaries but not in embryos may also contribute to repression and may be required for wild-type levels of activity.

The use of BRE+ and BRE− mRNAs reveals the importance of BREs for translational regulation, but does not address the possible role of other sequences in the *osk* mRNA 3′ UTR. To determine if BREs alone are sufficient, *luc* reporter mRNAs bearing multimerized consensus BREs (8 copies), either wild-type or containing point mutations that abrogate Bru binding (Kim-Ha et al., 1995), were generated and their translation measured in vitro. The BRE+ and BRE− RNAs were translated with only a modest difference in efficiency (Fig. 5A), suggesting that binding of Bru alone is insufficient for complete repression. The 8× BRE was previously found to support translational repression in vivo, but repression was not efficient (Kim-Ha et al., 1995), consistent with the results of





C

added protein	relative translation BRE-/ BRE+
Bruno	3.2 (0.6)
Apontic	1.6 (0.4)
NSmaug	1.5 (0.6)

Fig. 4. Bru is required for translational repression of *osk* mRNA in vitro. (A) In vitro translations using extract immunodepleted for Bru. BRE+ and BRE− reporter RNAs were translated in vitro in ovary extracts immunodepleted using anti-Bru serum or, as a control, antibodies from normal rat serum. The BRE+ RNA is translationally repressed in comparison to the BRE− RNA in the control extract, while extract immunodepleted for Bru no longer supports BRE-mediated translational regulation. Addition of recombinant Bru protein to extracts immunodepleted for Bru did not restore BRE-mediated translational regulation (data not shown, see Materials and methods). (B) BRE-mediated translational regulation can be reconstituted in embryo extracts by addition of purified Bru protein. Recombinant Bru protein was added to embryo extracts, in which translation of reporter transcripts was subsequently assayed. The addition of Bru conferred a substantial level of translational regulation. (C) Relative translation of BRE− RNA in comparison to BRE+ RNA in embryo extracts with the addition of several different recombinant proteins. Although Apt and NSmg cause a small decrease in BRE+ translation, the effect is significantly less than that observed with Bru. The results reported here are an average of the effect observed in a minimum of three independent experiments. Calculated standard deviations are indicated in parentheses.

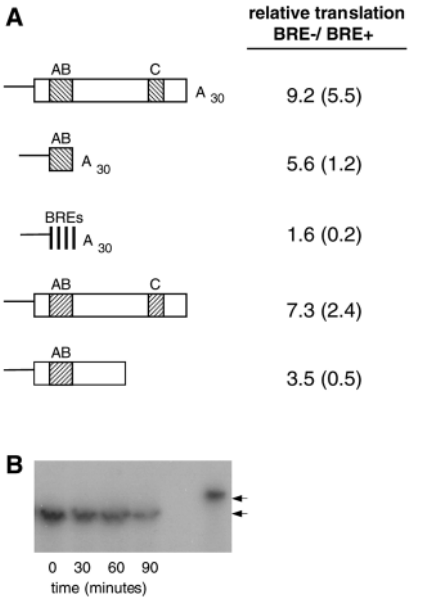


Fig. 5. Sequence dependence of *osk* mRNA translational regulation. (A) Reporter RNAs containing different portions of the *osk* mRNA 3′ UTR were tested to determine if sequences required for translational regulation in vivo are also required in vitro. Multimerized copies of the BREs were insufficient, while the AB region alone was sufficient. To examine the role of the poly(A) tail in translational regulation of these reporter RNAs, extracts were programmed with BRE+ and BRE− transcripts lacking poly(A) tails. The absence of the poly(A) tail had no effect on translational regulation. In addition, reporter RNAs that contained only the 5′ portion of the *osk* mRNA 3′ UTR and lacked polyadenylation signals were also regulated in the same manner. These results are an average of the effect observed in a minimum of 3 independent experiments; standard deviations are indicated in parentheses. Experiments testing the first four RNAs were performed at the same time, while a separate series of experiments comparing the first RNA and the fifth RNA (containing the 5′ portion of the *osk* mRNA 3′ UTR) was done at a later time with different batches of extract. In the later batches of extract, relative translation of the reporter containing the complete 3′ UTR was measured to be 4.3, a value similar to that observed with the poly(A)− RNA containing a truncated 3′ UTR (average 3.5). We attribute this variation in levels of translational repression to differences in batches of extracts. (B) RNAs lacking poly(A) tails are not polyadenylated during the in vitro reaction. Labeled poly(A)- reporter RNAs were monitored throughout the course of the in vitro translation reaction for changes in size that would indicate the addition of a poly(A) tail. No size changes were detected. The lower arrow indicates the reporter RNA, while the upper arrow indicates an RNA (110 nucleotides larger than the reporter transcript) that was used as a size marker.

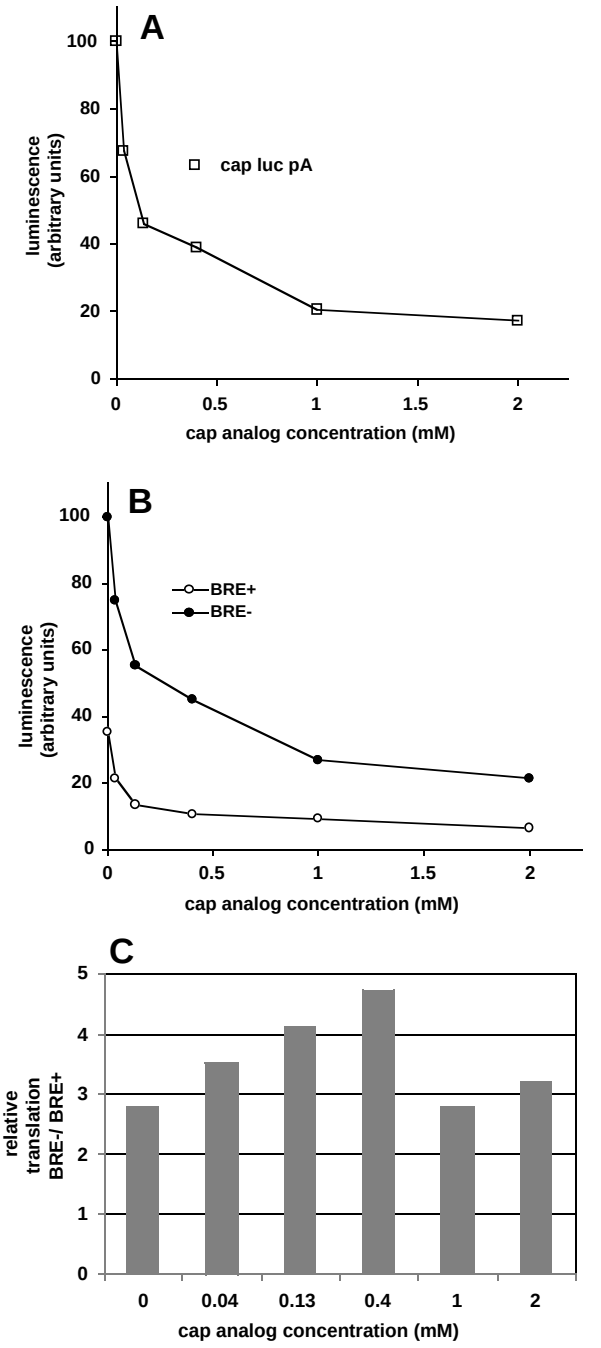


Fig. 6. BRE-mediated translational regulation is independent of the 5′ cap. (A) Cap dependence of translation in *Drosphila* ovary extract. Increasing concentrations of the cap structure analog m^7GpppG were added to in vitro translation reactions using *cap luc pA* RNA. Translation is inhibited by the addition of free cap. (B) In vitro translation reactions with BRE+ and BRE− RNAs in the presence of cap analog. (C) Relative translation of BRE− and BRE+ RNAs in cap competition experiments. The addition of increasing amounts of cap analog does not significantly affect translational regulation.

the more quantitative in vitro assay. In contrast, RNAs containing the full *osk* AB region (a 124 nt region of the *osk* mRNA 3′ UTR containing at least four consensus BREs interspersed among other sequences) were translationally regulated with a much higher efficiency (Fig. 5A). Thus it appears that other cis-acting sequences and presumably other factors contribute to Bru-mediated translational repression (see Lie and Macdonald, 1999; Gunkel et al., 1998), a result consistent with the incomplete repression conferred by addition of Bru to embryonic extracts (see Fig. 4B,C above).

To begin to explore the mechanism of Bru-mediated repression, we used the in vitro system to rigorously test the role of the poly(A) tail, as well as that of the 5′ cap, in translational regulation. A number of maternal mRNAs in *Xenopus* have been shown to be translationally regulated in a manner involving changes in the length of the poly(A) tail (e.g.

cyclin mRNAs and c-*mos* mRNA, Sheets et al., 1994), and RNA control elements and proteins that contribute to this process have been identified (e.g. the cytoplasmic polyadenylation element (CPE) and the CPE-binding protein (CPEB) (McGrew and Richter, 1990; Hake and Richter, 1994)). Similar forms of regulation have been reported for maternal mRNAs from other animals such as *bicoid* and *hunchback* mRNAs in *Drosophila* (Sallés et al., 1994; Wreden et al., 1997) and tPA and c-*mos* mRNAs in mouse (Vassalli et al., 1989; Gebauer et al., 1994), and for mRNAs from somatic tissues such as alpha-CaMKII mRNA (Wu et al., 1998). To specifically study the requirement for the poly(A) tail in *osk* mRNA translational regulation, BRE+ and BRE− reporter RNAs that lacked poly(A) tails were tested in the in vitro system. The translation of these RNAs was regulated in a manner similar to that of transcripts with a poly(A) tail (Fig. 5A). Though the overall level of translation from the poly(A)− messages was slightly reduced (data not shown), BRE-dependent repression remained strong. To address the possibility that reporter mRNAs might be polyadenylated during the course of the reaction, two types of experiments were performed using poly(A)− reporter RNAs bearing only the 5′ portion of the *osk* 3′ UTR, including the AB region. This part of the *osk* 3′ UTR lacks all of the normal polyadenylation elements, including sequences that resemble the binding site for *Xenopus* CPEB, a factor involved in cytoplasmic polyadenylation (McGrew and Richter, 1990; Hake and Richter, 1994). First, translational regulation of these reporter mRNAs was tested and found to be similar to that of RNAs containing the intact 3′ UTR (Fig. 5A). Second, the lengths of reporter mRNAs were monitored over the course of incubation in the in vitro translation extract: no changes in size were observed (Fig. 5B), confirming that polyadenylation did not occur in vitro. We conclude from these data that translational regulation of *osk* mRNA in vitro is independent of the poly(A) tail and that regulation occurs via a novel mechanism.

Another mRNA feature often implicated in control events is the 5′ cap. Cap-dependent translational initiation requires recognition of the cap by the eIF4F complex (or its constituent components), a process that is modulated by several types of regulation (Craig et al., 1998; Imataka et al., 1997; Yamanaka et al., 1997; reviewed by Sonenberg, 1996). A simple test of the importance of the cap in Bru-mediated regulation would be to monitor relative translation efficiencies of BRE+ and BRE− mRNAs lacking cap structures. However, uncapped mRNAs are quite unstable in the extracts (Y. L. and P. M., unpublished data). An alternative approach is to inhibit cap-dependent initiation by addition of excess free cap analog (7-methyl-GpppG). In a preliminary experiment, we showed that translation in the extracts can be substantially inhibited by free cap analog (Fig. 6A) and is thus largely cap-dependent. To test for cap-dependence of Bru regulation, we compared translation of BRE+ and BRE− mRNAs in the presence of free cap. If Bru interferes with recognition or use of the cap, the levels of BRE− and BRE+ translation would be expected to equalize under conditions where cap-dependent initiation is inhibited. Notably, the ratio of BRE−/BRE+ translation remained similar under all conditions tested, ranging from 0 to 2 mM cap competitor (Fig. 6B,C). These results very strongly suggest that Bru interferes with a step in translation that is distinct from cap recognition. Additional evidence supporting this conclusion could come from examination of dicistronic mRNAs, in which translation of one encoded protein is initiated in a cap-independent pathway through use of an internal ribosome entry site (IRES) (reviewed by Jackson, 1996). However, such an experiment must await the identification of an IRES that is active in *Drosophila* ovaries.

Prior analysis of *osk* mRNA translational regulation provided strong but indirect evidence that Bru acts as a repressor. Molecular genetic data revealed the essential role for BREs (Kim-Ha et al., 1995), while genetic evidence demonstrated that Bru acts in controlling the level of *osk* activity (Webster et al., 1997). However, the complex phenotype of mutants defective in Bru prevented a direct demonstration that the absence of Bru led to a derepression of *osk* translation (Webster et al., 1997). The results of our in vitro studies now provide compelling evidence that Bru is in fact required for translational repression mediated through the BREs. How the binding of Bru to the 3′ UTR of *osk* mRNA leads to translational repression remains uncertain, although the availability of the in vitro system defined here is likely to prove useful in addressing that question. Indeed, a definitive demonstration that the 5′ cap and changes in poly(A) tail length are not involved in regulation was only made possible through use of this system.

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