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; Haghhighat 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’ CGAACAAACATGCTGTC 5’) or B (3’ CGACGCTGTTGCCGGTGGCC 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 MgCl2, 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 heating 5 minutes at 95°C and resolved on a 5% denaturing polyacrylamide gel run in 0.5 M urea. Oligonucleotides 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 32P-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 T7lacP, 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 HhaI, while those without the poly(A) tail were transcribed from templates linearized with BamHI. Constructs with osk mRNA 3’ UTR sequences inserted into this plasmid were made by cloning of these sequences into the BamHI site. Transcripts containing the 5’ portion of the 3’ UTR were transcribed from templates linearized with SacII. 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) spin 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 in 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 pipetting 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.

### 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 6x 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 μg/ml 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 2011). 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.

### Monitoring of RNA stability and size

RNAs were labeled with [α-32P]UTP and 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 2x 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 Image 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 60× translation
buffer, 0.48 μl amino acid mix, 0.15 μl RNase Block, 0.32 μl creatine
phosphokinase, 1.0 μl RNA (5 ng), and 1.55 μl water. For these reactions,
the 60× 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
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*
ovoxy.

![Image](image_url)

**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
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}

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**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.

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**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 measured 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 8x 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

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.

### Table 4.1

<table>
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<th>relative translation BRE−/BRE+</th>
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<tr>
<td>Bru</td>
<td>3.2 (0.6)</td>
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<tr>
<td>Aptonic</td>
<td>1.6 (0.4)</td>
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<tr>
<td>NSmg</td>
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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 of these reporter RNAs. 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.

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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.

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Fig. 6. BRE-mediated translational regulation is independent of the 5' cap. (A) Cap dependence of translation in Drosophila ovary extract. Increasing concentrations of the cap structure analog m7GpppG 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.
translational regulation of osk mRNA in vitro

Interferes with recognition or use of the cap, the levels of BRE 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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