Poly(A)-independent regulation of maternal *hunchback* translation in the *Drosophila* embryo

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Development of the *Drosophila* abdomen requires repression of maternal *hunchback* (*hb*) mRNA translation in the posterior of the embryo. This regulation involves at least four components: *nanos* response elements within the *hb* 3′ untranslated region and the activities of Pumilio (PUM), Nanos (NOS), and Brain tumor. To study this regulation, we have developed an RNA injection assay that faithfully recapitulates the regulation of the endogenous *hb* message. Previous studies have suggested that NOS and PUM can regulate translation by directing poly(A) removal. We have found that RNAs that lack a poly(A) tail and cannot be polyadenylated and RNAs that contain translational activating sequences in place of the poly(A) tail are still repressed in the posterior. These data demonstrate that the poly(A) tail is not required for regulation and suggest that NOS and PUM can regulate *hb* translation by two mechanisms: removal of the poly(A) tail and a poly(A)-independent pathway that directly affects translation.

In many organisms, early development occurs in the absence of ongoing transcription; instead, mRNAs and proteins required for development are synthesized during oogenesis and deposited in the egg. Appropriate temporal and spatial expression of these maternal factors is critical for proper development. Elements within the 3′ untranslated region (UTR) of mRNAs have been shown to play an important role in the regulation of mRNA localization, turnover, and translation (reviewed in ref. 1). Although many of the trans-acting factors that interact with these elements have been identified, the mechanisms of regulation remain unresolved.

A key step in the regulation of maternal transcripts is the modulation of poly(A) tail length (reviewed in ref. 2). Many translationally quiescent transcripts are deposited into the oocyte with a short poly(A) tail that is then elongated to activate translation (3–5). Conversely, the poly(A) tail may be shortened or removed from an mRNA, resulting in silencing or degradation of the transcript (6–8). Only recently has it become clear how the poly(A) tail influences translation (9). The poly(A) tail recruits poly(A) binding protein (PABP) to the transcript (10). PABP in turn interacts with eukaryotic initiation factor (eIF) 4G to stabilize the cap-binding complex, thereby leading to more efficient recruitment of the 40S ribosomal subunit and initiation of translation (11). Thus 3′ UTR elements may regulate the initiation of mRNA translation by modulating the length of the poly(A) tail and the recruitment of PABP.

Temporal and spatial control of the translation of maternally transcribed genes has been shown to be critical for the normal establishment of the embryonic axes in the early *Drosophila* embryo. Abdomen formation requires several maternal factors that depend on the proper localization and translation of *oskar* mRNA at the posterior pole (reviewed in ref. 12). Translation of OSKAR protein results in formation of the germ plasm and the subsequent recruitment of *nanos* (*nos*) mRNA to the posterior pole (13). Posteriorly localized *nos* mRNA is translated, resulting in a gradient of NOS protein from the posterior pole (14). In early embryonic patterning, the target of NOS activity is the maternal mRNA for the zinc finger transcription factor Hunchback (HB), a negative regulator of the abdominal segmentation genes (15). Thus HB protein must be excluded from the posterior of the embryo for proper abdomen development.

Maternally supplied *hb* mRNA is deposited into the oocyte and distributed throughout the egg (16). Before fertilization, maternal *hb* mRNA is translationally quiescent and has a short poly(A) tail (≈30 nt) (17). Upon fertilization, *hb* mRNA is activated by the addition of a longer poly(A) tail (≈70 nt) (17). In the posterior of the embryo, *hb* translation is repressed by the joint activities of NOS, Pumilio (PUM), and Brain tumor (BRAT) (15, 18–22). Sequences within the *hb* 3′ UTR, termed *nos* response elements or NREs, also are required for repression (23). PUM binds to the NREs and recruits NOS and BRAT to the RNA (22, 24–27). The NRE and its associated factors is herein referred to as the NRE complex. Formation of the NRE complex results in the deadenylation of the maternal *hb* mRNA in the posterior (17, 23). Polysome gradient analysis of maternal *hb* transcripts in embryos that either lack NOS activity or express NOS throughout the embryo suggest that PUM, NOS, and BRAT affect *hb* translation at the initiation step (28). Although the correlation between poly(A) removal and translational silencing is compelling, it is not clear how this complex regulates poly(A) tail length of maternal *hb* mRNA or to what extent the length of the poly(A) tail influences translation of the *hb* message.

In this article we address the mechanism of *hb* translational repression in the posterior of the embryo. Specifically, we have developed a sensitive and quantitative RNA injection assay that has allowed us to study the requirement for various cis elements and trans factors in *hb* regulation. We demonstrate here that injected RNAs are translationally regulated and that this requires factors known to be required for repression of the endogenous maternal *hb* mRNA. We describe experiments demonstrating that whereas the poly(A) tail length of the maternal *hb* mRNA is modulated in the developing embryo, poly(A) shortening is dispensable for translational silencing. We further show that the NRE complex can repress translation of *hb* mRNA in the presence of a translational activating signal—the

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Abbreviations: UTR, untranslated region; PABP, poly(A) binding protein; *hb, hunchback*; *nos, nanos*; *pum, pumilio*; *brat, brain tumor*; F-Luc, firefly luciferase; R-Luc, *Renilla reniformis* luciferase; NRE, *nanos* response element; HFH, *hb* 5′ UTR + F-Luc + *hb* 3′ UTR; A/P, ratio of anterior to posterior luciferase activity; RT, room temperature; WT, wild type; F/R, ratio of F-Luc/R-Luc; HSL, histone *H1* 3′ terminal stem loop; HCH, *hb* 5′ UTR + chloramphenicol acetyltransferase + *hb* 3′ UTR; eIF, eukaryotic initiation factor; m^7GpppG, 7-methyl guanosine.

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Table 1. Examples of raw data and calculations from the RNA injection assay

	Experiment 1				Experiment 2			
	HFH_{WT} A25				HFH_{WT} A25		HFH_{GU} A25	
	A	P	A	P	A	P	A	P
F	74,434	10,464	69,886	12,314	61,099	6,335	68,894	23,055
R	13,694	15,058	12,696	17,485	185,206	169,108	174,926	65,463
F/R	5.4	0.7	5.7	0.7	0.33	0.04	0.40	0.35
A/P	7.7		7.8		8.2		1.1	

In vitro-transcribed HFH RNAs (50 pg/μl) containing WT or mutant (GU) NREs were injected into WT embryos, incubated 60 min, and assayed for luciferase activity. A, anterior; P, posterior.

histone *H1* stem loop. From these data, we propose a model where PUM, NOS, and BRAT have two effects on *hb* translation: poly(A)-independent repression and poly(A) removal, which translationally silences the RNA.

Experimental Methods

RNA Injection Assay. The *hb* 5′ UTR + firefly luciferase + *hb* 3′ UTR (HFH) and *Renilla reniformis* luciferase (R-Luc) reporter plasmids (also known as *Pp. Luc and Rr*-Luc) have been described (29). Their construction is described in the supporting information, which is published on the PNAS web site, www.pnas.org. Embryos were collected for 30 min at room temperature (RT) and transferred to 18° for dechorionation and injection. HFH A25 and R-Luc A25 mRNAs were combined at a final concentration of 50 pg/μl (unless otherwise noted) and injected into either the posterior or anterior pole. After injection, embryos were incubated at RT, harvested into 35 μl of passive lysis buffer (Promega), and spun for 10 min at 4° to remove insoluble material. Twenty five microliters of supernatant was assayed by using the Dual–Luciferase Reporter Assay System as described by the manufacturer (Promega). For each data point, 30 embryos were injected into the anterior or posterior pole. Each experiment contained two or three data points, and each RNA was tested in at least three independent experiments. For experiments using mutants, one set containing wild-type (WT) RNAs and WT embryos was injected in parallel to verify that the embryo collection and/or RNAs were regulated. In preliminary experiments, 20 or 50 embryos were injected with no difference in regulation noted.

The ratio of anterior/posterior luciferase activity (A/P) was calculated as follows. To control for the amount of RNA injected, the luminescence (relative light units, RLU) for the test mRNA (firefly luciferase, F-Luc) was divided by the RLU for the control mRNA (R-Luc) giving the ratio of F-Luc/R-Luc (F/R). The relative translation efficiency of the test mRNA in the anterior versus the posterior of the embryo was determined from the ratio of anterior (F/R) to posterior (F/R). Statistical analysis was performed by using VASSARSTATS (http://faculty.vassar.edu/~lowry/VassarStats.html) and STATIBOT (www.statibot.com).

While these experiments were being performed, the R-Luc and F-Luc reagents were improved by the manufacturer. These changes together with improvements in injection needles and technique make direct comparison of raw luciferase numbers uninformative. However, it should be noted that within experiments, raw numbers and F/R are consistent and the A/P remained consistent across all experiments, demonstrating the importance of using the R-Luc control (Table 1).

A small increase was noted in the translation of HFH_{WT} A25 RNA in the anterior of *nos* (1.7 times higher) and *pum* (1.4×) mutant embryos as compared with WT. We did not observe a difference in translation of messages bearing a mutant NRE (GU) versus those with a WT NRE (1.1×) (Table 1). Transcripts containing the histone *H1* 3′ terminal stem loop (HSL) were translated about half (0.5×) as well as their polyadenylated counterparts. Not surprisingly, transcripts lacking a poly(A) tail that could not have one added were translated much less efficiently than their polyadenylated counterparts (≈12× lower).

Poly(A) tail length was determined by using radiolabeled, 7-methyl guanosine (m^7GpppG)-capped RNAs corresponding to the complete *hb* 3′ UTR essentially as described (17). RNAs were fractionated on a 39-cm (see Figs. 1*A* and 2*B*) or 15-cm (see Fig. 4) denaturing PAGE. See supporting information for details.

Quantitation of Injected RNAs. Reverse transcriptase–PCR of R-Luc in a mix of total embryonic RNA was not possible because of the high AT content of R-Luc and low primer specificity. To quantitate translation and mRNA turnover rates an additional mRNA *hb* 5′ UTR + chloramphenicol acetyltransferase + *hb* 3′ UTR $(HCH)_{GU}$ A25 was used. HFH_{WT} A25/HSL, R-Luc A25, and HCH_{GU} A25 were combined at a concentration of 100 pg/μl each. Fifty embryos were injected and incubated at RT. Before harvesting, a capillary tube was used to remove any cytoplasm or RNA that may have leaked as well as any embryo that did not appear to develop. Half of the embryos were processed for luciferase. Total embryonic RNA (≈50 ng) was combined with primers for chloramphenicol acetyltransferase and F-Luc and 1 μCi ^{32}P-dCTP (3,000 Ci/mmol) and used for 20 and 25 cycles of reverse transcriptase–PCR. Ten microliters of the PCR was analyzed by urea-PAGE, dried, and visualized by PhosphorImaging.

Results

An RNA Injection Assay Faithfully Recapitulates *hb* Translation Regulation. Previously, it has been shown that NOS, PUM, and BRAT proteins regulate maternal *hb* translation in the posterior of the *Drosophila* embryo via the NREs in the *hb* 3′ UTR (23). However, it has been difficult to study the mechanism of regulation in detail because *hb* translational regulation has not been recapitulated faithfully in *in vitro* or tissue culture assays. Recently, a number of *in vitro* translation systems from *Drosophila* embryos have been established to study the regulation of *nos* and *oskar* mRNAs (30–32). Unfortunately, we have not been able to recapitulate *hb* regulation with these and similar systems (data not shown). RNA injection assays are a method of choice for analyzing translational efficiency *in vivo* in a variety of WT and mutant backgrounds by using modified RNAs (33). To apply this system to *Drosophila* embryos and increase the sensitivity of the injection assay, we used F-Luc and R-Luc genes as reporters. In this injection assay, two *in vitro*-transcribed RNAs are coinjected into either the anterior or posterior pole of the preblastoderm embryo. The control mRNA contains the R-Luc coding region with short 5′ and 3′ UTRs that lack regulatory elements. This unregulated mRNA is used to normalize for the amount of RNA injected. The test mRNA (HFH) contains the maternal *hb*

5′ UTR, the *F*-Luc coding region, and the maternal *hb* 3′ UTR. After incubation, F-Luc and R-Luc activities are determined. The relative translational efficiency of each mRNA in the anterior versus the posterior of the embryo (A/P) then is calculated (see *Experimental Methods*). An A/P of 1 means the test mRNA is translated equally well in the anterior and the posterior, whereas a ratio of greater than 1 means the test mRNA is translated more efficiently in the anterior than the posterior. Translation of a test mRNA with an intact *hb* 3′ UTR and a WT NRE (HFH_{WT} A25) is repressed in the posterior versus the anterior of WT embryos (A/P 7.8 ± 2.0), mirroring the regulation of endogenous maternal *hb* mRNA (Table 2).

To determine whether injected RNAs require the same factors for regulation as the endogenous maternal *hb* transcript, we studied the effect of *nos*, *pum*, and NRE mutations on the translation of the HFH mRNAs. HFH transcripts injected into *nos* or *pum* mutant embryos are translated equally well in the anterior versus the posterior (Table 2). This finding is consistent with the requirement for NOS and PUM in regulating *hb* translation *in vivo*. Further, HFH transcripts containing mutant NREs in which the six guanosines have been changed to uracil (GU) show no significant difference in translation between the anterior and posterior of the embryo regardless of the genetic background (Table 2). This NRE mutation disrupts PUM binding to the NRE *in vitro* and eliminates translational repression of maternal *hb* mRNA *in vivo* (25, 34). Thus factors that are required for the regulation of the endogenous maternal *hb* transcript also are required for the regulation of the injected transcripts.

Table 2. Injected transcripts require the same factors for regulation as endogenous maternal *hb* mRNAs

	A/P			
Embryo genotype	HFH_{WT} A25	*n*	HFH_{GU} A25	*n*
WT	7.8 ± 2.0*	21	1.1 ± 0.2	9
$nos^{L7/L7}$	1.3 ± 0.3	7	1.2 ± 0.2	6
$pum^{MSC/ET7}$	1.2 ± 0.4	8	1.3 ± 0.4	7

In vitro-transcribed HFH RNAs (50 pg/μl) containing WT or mutant (GU) NREs were injected into WT, *nos,* or *pum* mutant embryos and incubated 60 min. *n* is the number of independent injections included in the data set.
*This data set is significantly different ($P < 0.001$) from 1, the remaining data sets are not ($P > 0.1$).

Poly(A) Tail and Deadenylation Are Not Required for *hb* Translation Repression. Removal of the poly(A) tail is the prevailing model for translational regulation of many maternal mRNAs (8, 35). Indeed, maternal *hb* mRNA is polyadenylated in the cytoplasm concomitant with its translational activation (17). In the posterior, maternal *hb* is rapidly deadenylated in a NOS, PUM, and NRE-dependent process (17, 23). To determine whether deadenylation could account for the regulation of maternal *hb* translation, we examined the effect of the poly(A) tail on HFH translation in our injection assay. HFH mRNAs that lack a poly(A) tail are polyadenylated upon injection and more efficiently translated in the anterior versus the posterior (A/P 7.4 ± 0.8) (Fig. 1). We prepared HFH reporter mRNAs without a poly(A) tail that carry a point mutation in the polyadenylation signal (AAUAAA to AAUACA) and thus cannot be polyadenylated (36). These HFH mRNAs are still regulated (A/P 3.5 ± 0.7), although polyadenylation is no longer detectable on transcripts bearing this mutation (Fig. 1*A*). To confirm this finding, we injected HFH mRNAs that lack a poly(A) tail and were end-labeled with cordycepin, an ATP analogue that lacks a 3′ hydroxyl group and, as a result, blocks further elongation of poly(A) tails (37). These mRNAs are also differentially regulated in the absence of a poly(A) tail (A/P 3.0 ± 1.3) (Fig. 1*A*). This finding suggests that a poly(A) tail is not absolutely necessary for the regulation of *hb* translation in the embryo and that the process of deadenylation is not required for *hb* repression. HFH mRNAs that are not polyadenlyated are less well regulated than reporters with a poly(A) tail (A/P ≈3 as compared with A/P ≈7). Because RNAs lacking a poly(A) tail are less well translated it is possible that this difference in regulation is caused by lower translational activation of unadenylated RNA in the anterior rather than to less efficient repression of this RNA in the posterior.

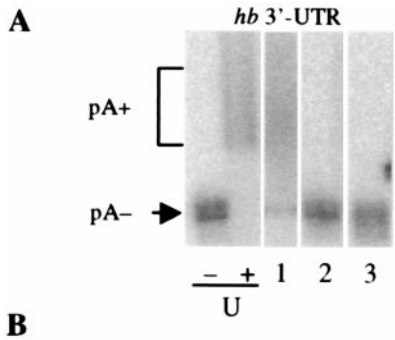
Histone Stem Loop Containing *hb* RNA Is Translationally Repressed. Injected mRNAs lacking a poly(A) tail are poorly translated and thus may not accurately reflect regulation *in vivo*. We therefore developed reporter mRNAs that are efficiently translated independent of a poly(A) tail. To accomplish this we synthesized HFH reporter mRNAs that contained the *Drosophila* HSL in place of the poly(A) tail (HFH HSL). Most histone mRNAs do not contain a poly(A) tail, but rather end with this conserved stem-loop structure. It has been shown that the HSL with the stem loop-binding protein regulates the stability and translational activity of histone mRNAs as the poly(A) tail does for other cellular transcripts (38). To prevent addition of a poly(A) tail to the HSL, the AAUAAA polyadenylation signal was mutated as described. HFH_{WT} HSL mRNAs were translated more efficiently in the anterior than the posterior, demonstrating spatial regulation similar to the endogenous maternal *hb* (A/P 5.4 ± 2.0) (Fig. 2*A*). Further, this regulation depended on an intact NRE and the presence of NOS activity (Fig. 2). There are several possible explanations for this finding: the NRE complex can direct removal of the HSL; HSL-containing messages are destabilized in an NRE-dependent manner; or the NRE complex can directly repress translation.

To determine whether the NRE represses translation of HSL-containing mRNAs by directing removal of the HSL, just as it directs removal of the poly(A) tail, we injected radiolabeled, m^7GpppG-capped *hb* 3′ UTRs containing either a poly(A) tail or the HSL into WT embryos. Consistent with previous findings (17, 23), RNAs containing a poly(A) tail maintain the poly(A) tail in the anterior, but the poly(A) tail is rapidly removed in the posterior (Fig. 2*B Left*). However, when RNAs containing the HSL are injected, the HSL is maintained in the anterior and posterior of the embryo (Fig. 2*B Right*), demonstrating that the NRE complex is not directing removal of HSL. Next, we tested whether the differences in translation of the HSL-containing reporters were caused by differential stability of the mRNAs. In these experiments, we assayed the levels of HSL or poly(A)+ reporter RNAs compared with a poly(A)+ control RNA containing a mutant NRE (see *Experimental Methods*). We found that the relative levels of the reporter mRNAs are not significantly altered in the anterior versus the posterior for either the polyadenylated or the HSL-containing mRNAs (Fig. 2*C*). Together these data demonstrate that translational regulation of *hb* does not require removal of the poly(A) tail. We conclude that NRE-directed repression can be independent of the poly(A) tail.

Deadenylation May Target *hb* mRNA for Silencing. Regulation of poly(A) and HSL containing reporters is quantitatively different, suggesting that there may be differences in some aspects of their regulation. To further compare the translational regulation of polyadenylated and HSL-containing mRNAs, we followed the regulation of HFH reporter mRNAs containing a poly(A) tail versus a HSL at 30, 60, and 90 min after injection (Fig. 3*A*). We found that, whereas absolute production of the reporters rises with time, the A/P ratio of test mRNAs containing a poly(A) tail continues to increase up to the 90-min time point. In contrast, the regulation of the HSL-containing transcripts reaches a plateau

by 60 min. One explanation for this disparity is that the HSL-containing transcripts are translationally active in both the anterior and the posterior and that active repression has to be maintained in the posterior throughout the test period. In contrast, poly(A)-containing mRNAs are translated at a constant rate at the anterior, but are translationally inactivated in the posterior. To evaluate the stability of the injected mRNAs at the later time point, we isolated RNA at various times after injection and determined the levels of the reporter mRNAs. There is not a significant difference in the stability of the mRNAs containing a WT NRE (HFH) versus those containing a mutant NRE (HCH) (Fig. 3). This suggests that poly(A) removal inactivates the *hb* mRNA, targeting it for translational silencing but not for immediate decay.

***hb* Deadenylation Is Cap Independent.** We propose that the NRE has two effects on *hb* regulation: poly(A) removal and poly(A) independent translational repression. One potential mechanism to achieve both of these effects would be through the action of the cap-dependent poly(A) nuclease PARN. Human and *Xenopus* PARN has been shown to compete with eIF4E for binding to the cap, thereby potentially disrupting translational initiation (39). Once bound to the cap, PARN rapidly removes the poly(A) tail (39, 40). The NRE complex could recruit PARN to the RNA or destabilize the cap complex, allowing PARN access. To test this possibility we synthesized RNAs corresponding to the *hb* 3′ UTR that were capped with either m^7GpppG or the cap analog



B

RNA	Ratio A / P	n
HFH WT A0	7.4 ± 0.8 †	12
HFH WT A0 + CD	3.0 ± 1.3 †	8
HFH WT AAUACA A0	3.5 ± 0.7 †	17

Fig. 1. Translational regulation of *hb* does not require a poly(A) tail. (*A*) Radiolabeled, m^7GpppG-capped RNAs (500 pg/μl) corresponding to the *hb* 3′ UTR were injected into the anterior of WT embryos. Uninjected RNA controls (U) show transcript size without (−) and with (+) a poly(A) tail. A poly(A) tail is added to a *hb* 3′ UTR that lacks a poly(A) tail (lane 1). *hb* 3′ UTRs that lack a poly(A) tail and are end-blocked with cordycepin (lane 2) do not have a poly(A) tail added. Mutation of the polyadenylation signal (AAUAAA to AAUACA) also blocks addition of a poly(A) tail to these transcripts (lane 3). The injections shown were performed in the same experiment and analyzed on the same gel; intervening lanes have been removed. (*B*). HFH_{WT} RNAs (50 pg/μl) without (A0) a poly(A) tail were injected and incubated 60 min. *n* is the number of independent injections analyzed. † indicates that the data set is significantly different ($P < 0.0001$) than 1. Comparison of A25 with A0 (AAUACA) RNAs indicates that these are significantly different data sets ($P < 0.0001$). Reporters that lack a poly(A) tail are efficiently translated and differentially regulated in the anterior versus the posterior. mRNAs lacking a poly(A) tail that are also end-blocked with cordycepin are repressed in the posterior of the embryo. HFH_{WT} RNAs bearing a mutant polyadenylation signal (AAUAAA to AAUCAA) were injected at a concentration of 200 pg/μl and incubated for 60 min.

A

RNA	Ratio A/P wild type	n	*nanos* L7/L7	n
HFH WT HSL	5.4 ± 2.0 †	12	1.3 ± 0.3	8
HFH GU HSL	1.4 ± 0.5	7	ND	
HFH WT A25	7.6 ± 1.8 †	4	1.1 ± 0.2	4

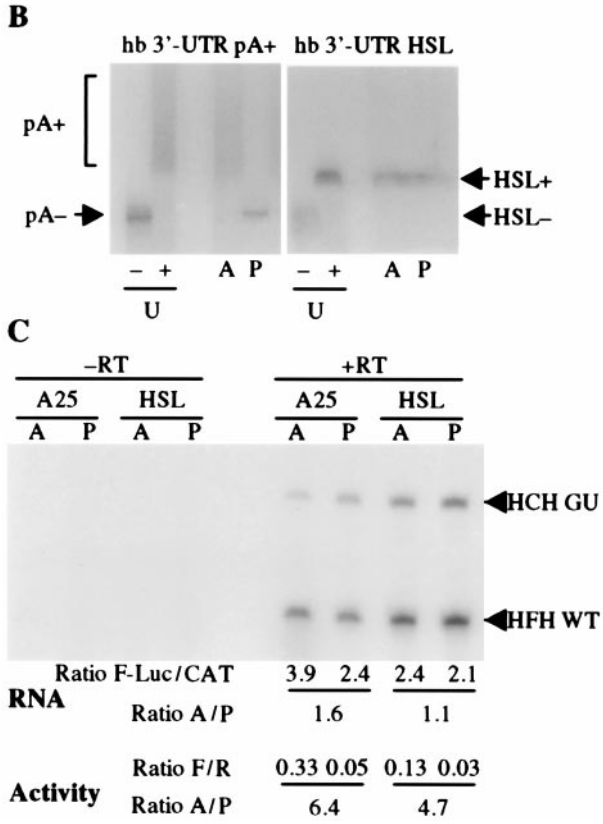


Fig. 2. NRE-dependent translational repression in the presence of the HSL. (*A*) HFH RNAs (50 pg/μl) with WT or mutant (GU) NREs containing the HSL in place of the poly(A) tail were injected into WT or *nos* mutant embryos and incubated 60 min. *n* is the number of independent injections analyzed. † indicates that this data set is significantly different ($P < 0.0001$) from 1, the remaining data sets are not. Parallel injections were performed with HFH_{WT} RNAs (50 pg/μl) containing a poly(A) tail to confirm that poly(A)-containing mRNAs are regulated in a similar manner. Comparison of the aggregate data for HFH_{WT} A25 versus HFH_{WT} HSL indicates that populations are significantly different ($P < 0.04$). (*B*) Radiolabeled, m^7GpppG-capped RNAs (500 pg/μl) corresponding to the *hb* 3′ UTR were injected into the anterior (A) or posterior (P) of embryos. (*Left*) Polyadenylated *hb* 3′ UTR. Uninjected controls (U) showing transcript size without (−) and with (+) a poly(A) tail. The poly(A) tail remains intact when injected into the anterior, but is rapidly removed when injected into the posterior. (*Right*) *hb* 3′ UTR with the HSL. Uninjected controls (U) showing transcript size without (−) and with (+) the HSL. The HSL remains intact in both anterior- and posterior-injected samples. (*C*) Reporter RNAs were injected and incubated for 60 min, the embryos were then divided and either assayed for F-Luc and R-Luc activities or harvested for total RNA. HFH_{WT} A25- or HSL-containing mRNAs were coinjected with HCH_{GU} A25 and R-Luc A25 reporters at a concentration of 50 ng/μl. Equal amounts of total RNA were used for reverse transcriptase–PCR with primers against the chloramphenicol acetyltransferase or F-Luc coding sequences. The numbers shown are for the experiment pictured. Analysis of all of the data demonstrate that no significant differences are found in the stability of poly(A)-containing mRNAs (A/P 1.4 ± 0.3; $n = 5$) or HSL-containing mRNAs (A/P 1.1 ± 0.2; $n = 5$) injected into the anterior versus the posterior of the embryo.

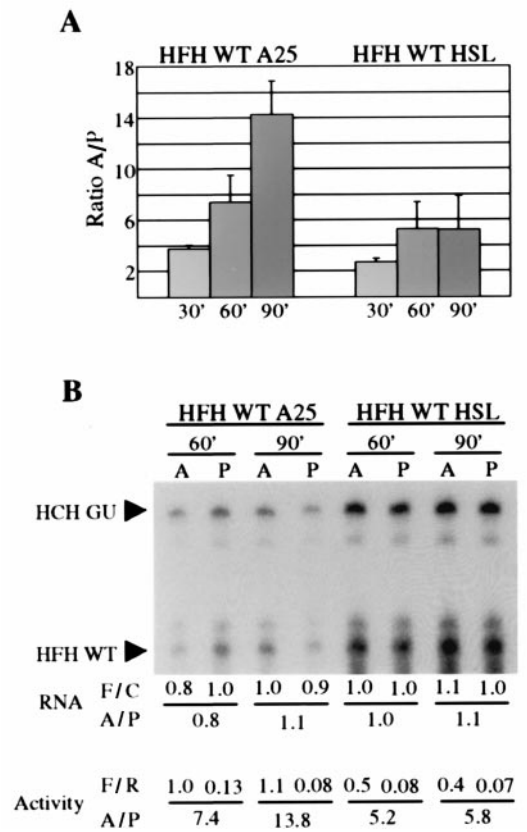


Fig. 3. Poly(A)- and HSL-containing mRNAs are repressed differently. (*A*) HFH RNAs (50 pg/μl) containing either a poly(A) tail (*Left*) or the HSL (*Right*) were incubated 30, 60, or 90 min after injection into WT embryos. The A/P ratio for poly(A)-containing mRNAs continued to increase with longer incubation, consistent with the message being actively translated in the anterior, but silenced or inactivated in the posterior. Comparison of the 60- and 90-min time points for HFH_{WT} A25 demonstrates that they are significantly different ($P < 0.005$). In contrast, the HSL-containing mRNAs reach a maximal A/P difference at 60 min even though luciferase expression levels continue to increase. (*B*) Reporter RNAs were injected and incubated for the designated time and harvested for total RNA. Activity readings were gathered from embryos injected with the same batches of RNA, but they were not processed in parallel. HFH_{WT} A25- or HSL-containing mRNAs were coinjected with HCH_{GU} A25 and R-Luc A25 reporters at a concentration of 50 ng/μl. Embryo equivalent amounts of total RNA were used for reverse transcriptase–PCR with primers against the chloramphenicol acetyltransferase or F-Luc coding sequences. The quantitation shown is for the experiment pictured. Analysis of aggregate data demonstrate no significant differences in the stability of poly(A)-containing mRNAs (60′:1.4 $\pm$ 0.3; 90′: 0.9 $\pm$ 0.2) or HSL-containing mRNAs (60′: 1.1 $\pm$ 0.2; 90′: 1.1 $\pm$ 0.1) injected into the anterior versus the posterior of the embryo throughout the length of the study.

ApppG. It has previously been shown that mRNAs with an ApppG cap are not efficiently deadenylated by PARN (40). Similarly to what is seen with m^7GpppG-capped messages, ApppG-capped messages are deadenylated in the posterior but not the anterior (Fig. 4). Additionally, there is no clear PARN homologue in the *Drosophila* genome. We conclude that PARN or a PARN-related mechanism is not mediating the effects of the NRE on *hb* mRNA.

To determine whether the cap complex is required for regulation, ApppG-capped HFH reporter mRNAs were injected. These mRNAs were very poorly translated (>20 times less well than the m^7GpppG-capped reporters). To obtain luciferase levels that were above background, higher levels of reporter mRNAs (500 pg/μl) were injected. At these RNA levels, m^7GpppG-capped mRNAs were differentially regulated (A/P

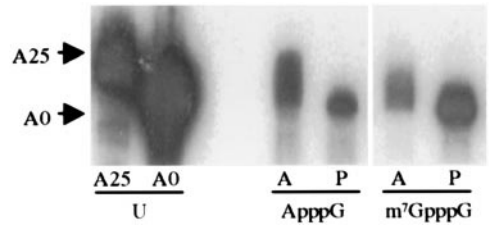


Fig. 4. NRE-directed poly(A) removal is not cap-dependent. Radiolabeled RNAs (500 pg/μl) corresponding to the *hb* 3′ UTR were capped with either ApppG or m^7GpppG and injected into the anterior (A) or posterior (P) of embryos. Uninjected controls (U) show transcript size without (−) and with (+) a poly(A) tail. The poly(A) tail remains intact when injected into the anterior, but is rapidly removed when injected into the posterior regardless of the structure of the cap.

4.9 $\pm$ 1.1, $n = 8$). Regulation of ApppG-capped reporters bearing a WT NRE (2.2 $\pm$ 0.9, $n = 12$) was not significantly different ($P < 0.1$) from that for ApppG-capped reporters bearing a mutant NRE (1.3 $\pm$ 0.5, $n = 4$). These data may indicate a requirement for the cap in NRE-mediated regulations. It is likely, that because of the low translation levels of ApppG-capped mRNAs, the failure to differentially regulate these mRNAs results from a failure to efficiently activate translation in the anterior as opposed to repress translation in the posterior.

Discussion

Repression of maternal *hb* translation in the posterior of the early *Drosophila* embryo is critical for abdomen formation and viability. Previous experiments had shown that NOS and PUM direct deadenylation of NRE containing messages (Fig. 2*B*; refs. 17 and 23). These results suggested that deadenylation may be the principle mechanism of *hb* translational regulation. Using a RNA injection assay we show that the NRE and its associated factors can repress *hb* translation independent of the poly(A) tail and in the presence of a heterologous translation activation signal (the HSL).

At least two models are compatible with this scenario. In the first, the NRE complex may disrupt a single molecule or step in translation that results in both poly(A) removal and translational repression. Because of its central role in linking the 5′ cap complex with the 3′ poly(A) tail, PABP would be a likely target in this model. In this scenario, the NRE complex recruits factors to the *hb* RNA that could modulate PABP activity. *Drosophila* Paip-2, for example, could be such a factor. Recently, human Paip-2 was shown to interact with PABP and disrupt its binding to the poly(A) tail, resulting in an increase in deadenylation and a decrease in translation (41). However, because a poly(A) tail is not absolutely required for *hb* translational repression, it is more likely that the NRE complex would disrupt the cap complex, and that deadenylation is a consequence rather than the cause of cap complex disruption. It has been demonstrated in plant extracts that interaction between eIF4F and eIF4B increases poly(A) binding by stabilizing PABP on the tail (42). Thus it is possible that disruption of the cap complex or its interaction with PABP could result in decreased translation and increased deadenylation.

In an alternative model, the NRE could act by two discrete pathways: one directly interfering with translation and a second partially redundant pathway directing poly(A) removal. Our data as well as the absence of an obvious homologue in the *Drosophila* genome suggest that the cap-dependent nuclease PARN is not the deadenylase. However, the *Drosophila* genome does contain CCR4 and CAF1, which have been shown to be components of the major cytoplasmic deadenylase in yeast, and PAN2 and PAN3, which have a role in both nuclear and cytoplasmic

deadenylation (43, 44). Either or both of these complexes could be recruited to the *hb* mRNA by the NRE complex, thus fulfilling the deadenylation pathway of this model. We show that the NRE complex can directly repress translation of mRNAs containing the HSL. The stem loop is a conserved structure that fulfills many of the functions the poly(A) tail serves on other transcripts and is essential for translation of histone mRNA (38, 45). Recently, stem loop-binding protein has been identified as a sequence-specific factor that interacts with the stem loop and participates in histone mRNA processing and mobilization onto polyribosomes (46). The mechanism of translational activation by the stem loop and its associated factor(s) remains unclear, although it is believed to mediate interaction between the termini, possibly through an interaction with eIFs (45). We speculate that the NRE complex is directly interfering with translation at the same step for mRNAs bearing a histone stem loop or poly(A) tail. Such a common step would likely involve initiation or elongation of the polypeptide rather than PABP. In addition, silencing of polyadenylated RNAs would increase the efficiency of translational repression.

Although the best-studied examples of translational regulation require removal of the poly(A) tail (8), examples of poly(A)-independent regulation have been described (47). For example, human ribonucleoproteins K and E1 repress translation of erythroid 15-lipoxygenase mRNA by inhibiting assembly of the 80S ribosome (48). In *Xenopus*, it has been shown that Maskin interacts with eIF4E, thereby disrupting cap complex assembly (49). Although NOS has two potential eIF4E binding sites, deletion of these sites does not affect NOS regulation of *hb* (data not shown). NOS, ectopically expressed in the fly eye, can repress expression of NRE-containing mRNAs that initiate translation via an internal ribosome entry site, suggesting that NOS, PUM, and BRAT may act downstream of the cap (26). Although we cannot exclude the possibility that these transcripts are degraded in the eye primordia as a result of NRE-mediated removal of the poly(A) tail, it is intriguing to speculate that the NRE complex may act to directly interfere with the function of the general translation machinery on transcripts containing NREs. Interestingly, other maternal RNAs (e.g., *oskar* and *nos*) whose translational repression, like *hb*, is mediated by sequences in the 3′ UTR, do not require the poly(A) tail for their regulation (30, 50, 51). This finding suggests that direct inhibition of the translation machinery may be a common strategy in the *Drosophila* embryo.

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