**Nanos and pumilio establish embryonic polarity in Drosophila by promoting posterior deadenylation of hunchback mRNA**

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**SUMMARY**

Nanos protein promotes abdominal structures in Drosophila embryos by repressing the translation of maternal hunchback mRNA in the posterior. To study the mechanism of nanos-mediated translational repression, we first examined the mechanism by which maternal hunchback mRNA is translationally activated. In the absence of nanos activity, the poly(A) tail of hunchback mRNA is elongated concomitant with its translation, suggesting that cytoplasmic polyadenylation directs activation. However, in the presence of nanos the length of the hunchback mRNA poly(A) tail is reduced. To determine if nanos activity represses translation by altering the polyadenylation state of hunchback mRNA, we injected various in vitro transcribed RNAs into Drosophila embryos and determined changes in polyadenylation. Nanos activity reduced the polyadenylation status of injected hunchback RNAs by accelerating their deadenylation. Pumilio activity, which is necessary to repress the translation of hunchback, is also needed to alter polyadenylation. An examination of translation indicates a strong correlation between poly(A) shortening and suppression of translation. These data indicate that nanos and pumilio determine posterior morphology by promoting the deadenylation of maternal hunchback mRNA, thereby repressing its translation.

Key words: translational repression, polyadenylation, maternal mRNA, Drosophila, hunchback, pumilio, nanos

**INTRODUCTION**

An essential feature of Drosophila embryogenesis is the specific localization of maternal cytoplasmic determinants whose polarized distribution dictates further asymmetry in the developing embryo. The mechanism by which asymmetry is propagated from maternal factors to later embryonic events represents an intriguing aspect of early development.

A classic example of a polarized cytoplasmic determinant is the posterior morphogen nanos (Hülskamp et al., 1989; Lehmann and Nüsslein-Volhard, 1991; Wang and Lehmann, 1991). Nanos (nos) mRNA is localized to the posterior pole of the embryo, and its translational activation during embryogenesis leads to a gradient of nos protein emanating from the site of localization (Gavis and Lehmann, 1992; Wang et al., 1994). It is this asymmetric distribution of nos protein that initiates the morphogenesis of the abdominal posterior structures (Eldon and Pirrotta, 1991; Hülskamp et al., 1990; Kraut and Levine, 1991a,b).

In general terms, the role of nos protein in embryonic patterning has been established. Its target is a maternal mRNA, hunchback (hbmat), which is uniformly distributed in the embryo. hbmat mRNA is not translated in the oocyte, but is activated soon after egg deposition (Tautz and Pfeifle, 1989). However, in the posterior of the embryo, nos represses the translation of hbmat mRNA (Hülskamp et al., 1989; Irish et al., 1989; Struhl, 1989; Tautz, 1988). Thus the gradient of nos protein (highest in the posterior) leads to an opposing gradient of hunchback protein with lowest levels in the posterior. Genetic experiments have established that nos is required during embryogenesis only to suppress hbmat translation: although nos mutant embryos die from lack of posterior structures, embryos doubly-deficient in both nos and hbmat are viable (Hülskamp et al., 1989; Irish et al., 1989; Struhl, 1989). Therefore, the suppression of hbmat mRNA translation in the posterior is required for proper development.

Insight into how nos protein might repress translation came from studies on the BicaudalD (BicD) mutant (Wharton and Struhl, 1989). In embryos from BicD mutant females, nos mRNA along with other posterior factors is ectopically localized to the anterior of the embryo. This mis-localization leads to anterior repression of both hbmat mRNA and the anteriorly localized bicoid (bcd) mRNA. In the case of bcd mRNA, there is a correlation between this translational repression and a decrease in the length of the poly(A) tail (Wharton and Struhl, 1989). The translational activation of bcd mRNA is regulated by cytoplasmic polyadenylation (Lieberfarb et al., 1996; Sallés et al., 1994), a common mechanism for activating maternal mRNAs during oogenesis and embryogenesis (reviewed in Richter, 1996; Wickens et al., 1996). These results suggested that nos activity might repress the translation of bcd and hbmat mRNAs via an alteration of their poly(A) tail length.

To determine how nos activity represses the translation of hbmat mRNA, we first examined the mechanism by which hbmat mRNA...
RNA is translationally activated. In the absence of nos protein, the poly(A) tail of \( h_b^{\text{mat}} \) mRNA is elongated during early embryogenesis; however, in the presence of \( \text{nos} \), the poly(A) tail is shorter, suggesting that polyadenylation regulates \( h_b^{\text{mat}} \) mRNA translation. Injection and recovery in embryos of RNAs derived from the 3' UTR of \( h_b \) mRNA demonstrate that \( \text{nos} \) activity promotes a deadenylated state, and it does so by accelerating the deadenylation of the RNA. This shortening of the poly(A) tail can consequently suppress the translation of maternal mRNAs regulated by polyadenylation.

mRNA deadenylase during development has been associated with the silencing of maternal mRNAs in the mouse oocyte prior to their translational activation (Huaute et al., 1992), and also with default pathways antecedent to mRNA destruction in \( \text{Xenopus} \) embryos (Varnum and Worminton, 1990). The results presented here broaden the scope of this regulatory mechanism by demonstrating its fundamental importance in \( \text{Drosophila} \) pattern formation.

**MATERIALS AND METHODS**

**Fly strains**

The following fly strains were used: Canton-S, \( \text{ry}^{506} \), \( \text{nos}^{\text{BN}} \) (Wang et al., 1994), P[n-b3'UTR; \( \text{ry}^+ \)] (Gavis and Lehmann, 1992), \( \text{Int}(3R)\text{MSC} \) and \( \text{Tp}(3.1)\text{FC8} \) (Barker et al., 1992). \( \text{Pamilio} (\text{pam}) \) embryos were obtained from female flies transheterozygous for \( \text{Int}(3R)\text{MSC} \) and \( \text{Tp}(3.1)\text{FC8} \). In embryos from P[n-b3'UTR; \( \text{ry}^+ \)] females (called \( \text{nos}^{\text{mat}} \) for this paper) nos protein expression is directed to the anterior using the \( \text{bcd} \) mRNA 3'-UTR. These flies are dominant female sterile and are maintained by mating transgenic males with virgin \( \text{ry}^{506} \) females. Canton-S flies were obtained from Peter Gergen, and \( \text{nos}^{\text{BN}} \) and P[n-b3'UTR; \( \text{ry}^+ \)] flies were obtained from Ruth Lehmann. \( \text{Int}(3R)\text{MSC} \) and \( \text{Tp}(3.1)\text{FC8} \) flies were obtained from Ruth Lehmann and Robin Wharton. Canton-S flies are termed wild type for this paper. Egg collection chambers of mutant females contained Canton-S males.

**PCR poly(A) test**

Female fly ovaries and dechorionated embryos were dounce homogenized in approximately 10 volumes of homogenization buffer (8 M urea, 3 M LiCl, 5 mM EDTA, 5 mM DTT). Large particles of debris were removed by centrifugation at 1000 \( g \) for 10 minutes (4°C). The homogenate was incubated overnight on ice and centrifuged for 30 minutes at 17,000 \( g \) (4°C). The pellet was resuspended in 10 mM Tris-HCl pH 7.0, 5 mM EDTA and 0.5% SDS. After resuspension, the RNA was phenol/chloroform (1:1) extracted twice and chloroform extracted once. Next, the RNA sample was precipitated with 0.1 volume of 3 M NaOAc pH 7.5 and 2.5 volumes of ETOH. Approximately 50-200 embryos or 20 female ovaries were prepared at one time. Typically a quarter of the RNA was subjected to PAT reverse transcription at a time (Sallés and Strickland, 1995).

PCR was performed using a reaction mixture spiked with \( ^{32} \text{P} \) dGTP (dATP). The anchor primer used during the reverse transcription step and a primer specific to the 3' end of the \( h_b \) cDNA (5'-AGGCAACTTTCAATCTCTGCTTCTTAT-3') were used in the PCR reaction. The expected size of the PCR product is 403 nt (373 of \( h_b \) and 30 of anchor). The amplified products were separated from the mineral oil overlay by chloroform extraction, phenol/chloroform (1:1) extracted and chloroform extracted. Free label was removed by Sephadex G-50 spin column (Boehringer Mannheim). Equal amounts of radioactivity from each sample were resolved on a 4% acrylamide/8.3 M urea gel (National Diagnostics).

**DNA template for in vitro transcriptions**

Full-length \( bcd \) template pBCD4 was prepared as previously described (Sallés et al., 1994).

The full-length \( h_b \) transcript was derived from the \( \text{J3} \) \( h_b \) cDNA clone (Oro et al., 1988). This clone is a maternal derived cDNA corresponding to an mRNA of approximately 3.3 kb. The \( h_b \) cDNA was excised from the pNB40 plasmid by NotI (NEBL) and HindIII (NEBL) digestion and ligated into the pKS II vector (Stratagene) digested with the same enzymes. The 5'-end of the cDNA containing \( \text{Xenopus} \) globin sequences and 176 nt of the \( h_b \) sequence was removed by digestion with XhoI (NEBL) and PshAI (Panvera) restriction enzymes. The missing 5' \( h_b \) sequence was replaced by PCR using the J3 clone as template and the following primers: (1) 5'-CCAGAACGT-TAGTTGTGGCCCGAAAAACG-3' and (2) 5'-TCCGGGA-CAAAAGTCTTCTTTCGC-3'. Primer (1) contained a HindIII restriction site near the 5'-end off the primer followed by the 5'-most sense sequence of the \( h_b \) mRNA. Primer (2) contained a PshAI restriction site followed by complement sequence 5' to this site within the 5'-UTR of the cDNA. The correct clone was determined by restriction analysis and sequenced to exclude PCR errors.

The pKS-hb clone was used as the template for PCR cloning of the \( h_b \) 3'-UTR. PCR amplification was performed with the following primers: (1) 5'-TATTCGCGCTAATGCTCCCAATCCACCATC-3' and (2) 5'-TATTCGCCGCTATTATACTGATATACTTTAT-3'. Primer (1) contained a \( \text{XhoI} \) site following from sequence from the 5'-most end of the \( h_b \) 3'-UTR. Primer (2) contained a NotI site followed by complement sequence to the 3'-most end of the maternal \( h_b \) transcript. The PCR product was digested with the NotI and \( \text{XhoI} \) (NEBL) restriction enzymes and ligated to a \( \text{XhoI} \) and NotI-digested pKS II vector. Plasmid clones were sequenced to obtain a perfectly copied clone. This plasmid, pHb3'UTR, was linearized with NotI (full length) or DraI (truncated) (NEBL) for use in transcription reactions.

The plasmid containing \( h_b \) 3'-UTR sequences lacking the NRE (pHB3'UTR-NRE) was made by digesting the pHb3'UTR clone with \( \text{XhoI} \) and \( \text{XhoI} \) (NEBL), filling in the overhangs with Klenow (Boehringer Mannheim) and self-ligating the plasmid. This procedure removes the first 146 nucleotides of the 3'-UTR. This plasmid was linearized with NotI for in vitro transcriptions.

**In vitro transcriptions**

For 3'-UTR in vitro transcriptions, approximately 1 \( \mu \)g of linearized template was incubated in a 50 \( \mu \)l reaction containing, 1X epicenter transcription buffer, 10 mM DTT, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 0.012 mM UTP, 0.5 mM CAP analog [m7G(5')ppp(5')G] (Ambion), 100 \( \mu \)Ci [\( ^{32} \text{P} \)UTP (3000 mCi/ mmol), 80 units RNasin (Promega) and 72.5 units T3 RNA polymerase (Epicenter). This reaction was incubated at 37°C for 1.5-2 hours. RNAs were purified by 4% acrylamide/8.3 M urea gel electrophoresis. The RNA was recovered from the gel by incubation of the gel slice containing the appropriate band overnight at 37°C in 0.1% SDS, 1 mM EDTA and 0.5 M ammmonium acetate followed by phenol/chloroform (1:1) extraction (2x), chloroform extraction (1x) and ethanol precipitation. RNAs were resuspended at a specific radioactivity of between 1x10^6 and 1x10^7 cts/minute/\( \mu \)l (approximately 10-100 ng/\( \mu \)l).

Full-length \( bcd \) RNA in vitro transcriptions used for the injection/recovery assay were performed the same as above, except the RNA was purified by two rounds of ethanol precipitation (ammonium acetate (0.5 M) followed by sodium acetate (0.3 M)).

In vitro transcriptions of full-length \( bcd \) RNA used in the translation assay were performed as previously described (Sallés et al., 1994).

**RNA injection and recovery**

Embryo injections were performed by standard procedures (Frohnhofer and Nüsslein-Volhard, 1986). An injection was confirmed by cytoplasmic movement/dilution (approximately 5% egg volume). For this reason, posteriorly injected embryos, in general, contained slightly more radioactivity after recovery than anteriorly injected ones (volume of posterior tip of the embryo is greater than the anterior tip,
and therefore required more injected solution to produce visual detection.

For recovery of injected RNA, 30- to 60-minute-old embryos (prepole cell) were injected at room temperature and allowed to mature for 1 hour. At this point, the embryos were removed from the cover slip and halocarbon oil (series 700; Halocarbon Products Corp.) by a heptane wash. The recovered embryos were washed with 1× PBS/0.1% Triton X-100 and dounce homogenized in a solution of 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.5% SDS, and 10 mM EDTA (Gottlieb, 1992). Carrier tRNA (10 μg) and 1 μl proteinase K (14 mg/ml; Boehringer Mannheim) were added and the mixture was incubated at 65°C for 20-30 minutes. Afterwards, the solution was phenol-chloroform (1:1) extracted and ethanol precipitated in 0.3 mM sodium acetate. Approximately equal amounts of re-isolated RNAs (as determined by radioactivity) were separated by 4% acrylamide/8.3 M urea gel electrophoresis or 0.8% agarose/6% formaldehyde (bcd RNA) and visualized by autoradiography. Approximately equal number of embryos were usually required to produce equal amounts of recovered radioactive. In general, 15-30 embryos were sufficient to generate 1000-4000 cts/minute of RNA, which was loaded in a single lane.

Injections of bcd RNA (200-250 ng/μl) for the assay of translation were performed as above with the injected embryos allowed to develop for 22-26 hours prior to fixation with Hoyer's lactic acid (1:1). Cuticle preparations were baked at 60°C for 2-4 hours prior to visualization using dark-field or phase-contrast microscopy.

**In vitro polyadenylation**

In vitro polyadenylation reactions (20 minutes) were performed as previously described (Sallés et al., 1994).

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**RESULTS**

**hb** mRNA is polyadenylated concomitant with its translation and nanos alters its poly(A) status

Since hb\textsuperscript{mat} is translationally activated during early development, we first examined the mechanism of this activation. To determine if cytoplasmic polyadenylation was involved, we looked at the poly(A) tail length of hb\textsuperscript{mat} mRNA using a reverse transcription-PCR protocol (PCR poly(A) test - PAT; Sallés and Strickland, 1995). The reverse transcription step of the PAT assay generates cDNAs containing a poly(A) tail of approximately the same length as the corresponding mRNA. Amplification of hb\textsuperscript{mat} cDNAs resulted in the appearance of three bands. Each of these bands displayed similar increases in polyadenylation during embryogenesis and, from restriction analysis, all appear to be bona fide cDNAs from hb\textsuperscript{mat} mRNAs with different transcription termination sites (data not shown). Results for the shortest products are described below, although similar conclusions can be drawn from all amplified products.

In the oocyte from wild-type (wt) females, where no hb translation is detected, the mRNA has a poly(A) tail length of approximately 30 nucleotides (Fig. 1, lane 1). However, concomitant with translation of the mRNA at between 0.5 and 1.5 hours after egg deposition (Tautz and Pfeifle, 1989), the poly(A) tail is elongated to approximately 70 nt (Fig. 1, lane 2). The hb\textsuperscript{mat} mRNA polyadenylation profile from oogenesis and early embryogenesis is similar to that of maternal bcd mRNA which is known to be translationally regulated by polyadenylation (data not shown; Lieberfarb et al., 1996; Sallés et al., 1994). Both of these mRNAs are translationally activated at a similar time in development (Driever and Nüsslein-Volhard, 1988; Tautz and Pfeifle, 1989) suggesting that polyadenylation is regulating both maternal bcd and hb mRNA translation.

To determine if nos activity affects polyadenylation, we used the PAT assay to examine the polyadenylation state of hb\textsuperscript{mat} mRNA in embryos lacking nos activity (nos; Wang et al., 1994) and in embryos containing ectopic, anterior nos activity (nos\textsuperscript{ant}; Gavis and Lehmann, 1992). The name of the embryos refers to the genotype of the mother. From the PAT assay, embryos lacking nos protein (nos) have an increased poly(A) tail length, when compared to wt embryos (100 nt in nos embryos versus 70 nt in wt embryos) (Fig. 1, lane 2, 4). However, if nos protein is present throughout the embryo (nos\textsuperscript{ant}), the hb\textsuperscript{mat} mRNA is not further polyadenylated in the embryo when compared to the oocyte (30 nt of adenosine for both) (Fig. 1, lane 5, 6). In fact, it appears as if the mRNA is less adenylated in the nos\textsuperscript{ant} embryo than in the nos\textsuperscript{ant} ovary. In the embryo, the lowest band has greatest relative intensity, whereas in the ovary lane the next highest band (more adenosine residues) has a greater relative intensity.

These changes in polyadenylation status correlate with parallel changes in translation: increased hb\textsuperscript{mat} RNA polyadenylation in the nos embryos correlates with an increase in translation, specifically in the posterior of the embryo (Wharton and Struhl, 1989), and the lack of polyadenylation found in the nos\textsuperscript{ant} embryo coincides with an overall lack of hb\textsuperscript{mat} RNA translation (Gavis and Lehmann, 1992). Thus hb\textsuperscript{mat} RNA is polyadenylated in the embryo, coinciding with its translation, and the presence of nos acts to prevent the acquisition of a long poly(A) tail.
**Nanos activity reduces the polyadenylation of injected hb mRNA via the nanos response element and pumilio**

To investigate how nos activity altered the polyadenylation status of hb mRNA, we injected various exogenous hb RNAs into the embryo and determined their polyadenylation status. Initially, the polyadenylation of full-length hb RNA was analyzed. Injection and later recovery of the full-length hb transcript in the absence of nos activity resulted in a slight increase in size, suggestive of polyadenylation; when this same RNA was injected into the presence of nos activity, there was no change in mobility suggesting a perturbation in polyadenylation (data not shown). However, the size changes relative to the size of the hb RNA (3.3 kb) were not sufficient to accurately determine alterations in poly(A) tail length.

This difficulty in discerning size differences using the full-length hb RNA prompted us to evaluate a smaller substrate. The use of the 3′-UTR allowed us to examine small changes in polyadenylation status that would have otherwise been indeterminable using full-length transcripts. The 3′-UTRs of translationally regulated RNAs usually contain the sequence elements necessary for regulated polyadenylation and have been shown in *Drosophila* and other animal systems to reproduce the polyadenylation and deadenylation characteristics of their respective full-length mRNAs (Sheets et al., 1994; Vassalli et al., 1989; Verrotti et al., 1996).

To determine if the hb 3′-UTR contained sequences sufficient to promote polyadenylation, we injected an RNA corresponding to the entire 3′-UTR of the mRNA into the anterior end of embryos. Injection into the anterior of wt and nos embryos resulted in efficient polyadenylation (Fig. 2A). The maximum length of polyadenylation was approximately 60 nt; subsequent experiments (Fig. 2B, C) showed a maximum poly(A) tail length of the hb 3′-UTR after injection to be 100 nt (comparable to the PAT assay). To confirm that the size alteration of the injected RNA was due to polyadenylation, we performed RNase H/oligo(dT) digestion of RNA injected into the anterior of wt embryos. This treatment, which removes poly(A) tails, resulted in an RNA that co-migrated with the un.injected hb 3′-UTR RNA (Fig. 2B), indicating that the mobility shift of the injected RNA was due to polyadenylation.

In contrast to injections into the anterior, delivery of the hb 3′-UTR RNA into the posterior of wt embryos (nos activity present) resulted in essentially no elongation of the RNA (Fig. 2A). However, injection of the hb 3′-UTR into a posterior lacking nos protein (nos embryo) resulted in efficient polyadenylation of the RNA (Fig. 2A), indicating that nos activity in the posterior results in greatly reduced polyadenylation.

Nos suppression of hb mRNA translation relies on two...
known aspects: a cis-acting sequence in the hb 3′-UTR, and a collaborating protein, pumilio (pum). To further correlate the alteration in polyadenylation with these two aspects of nos function, we first examined the behavior of a hb mRNA lacking the nos response element (NRE) (hb3′UTR-NRE). The NRE is a cis-acting sequence within the 3′-UTR of hb and bcd RNAs that mediates translational repression by nos (Wharton and Struhl, 1991). To determine if this sequence also mediates the ability of nos to alter polyadenylation, we injected a hb 3′-UTR RNA lacking NREs into the posterior of wt and nos embryos. This NRE-minus RNA was no longer responsive to the ability of nos activity to alter polyadenylation, as indicated by efficient polyadenylation after recovery of injected RNA (Fig. 2C). The specific deletion used was the same as previously shown to relieve translational repression of *hb* mat mRNA (Wharton and Struhl, 1991). These results indicate a tight correlation between translational repression and deadenylation, since both processes are disrupted by an identical small deletion in the RNA.

We next determined the influences of the pum gene on the poly(A) status of *hb* RNA. The pum embryo phenotype is very similar to that of *nos* (lacking abdominal structures) and *pum* activity is necessary for proper *nos* function (Lehmann and Nüsslein-Volhard, 1991; Barker et al., 1992). Biochemically, pum protein, not nos, binds to the NREs (Murata and Wharton, 1995). To examine the ability of *pum* activity to alter polyadenylation, we injected the full-length *hb* 3′-UTR into the anterior and posterior of embryos from mothers deficient for the gene. As with *nos*, injection into both the anterior and posterior of *pum* embryos resulted in efficient polyadenylation of the RNA, indicating that the pum protein is needed along with *nos* activity to alter polyadenylation in the posterior (Fig. 2D).

These experiments confirm that the 3′-UTR of *hb* RNA mimics the characteristics of the full-length mRNA regarding polyadenylation and that *nos* activity results in a decrease in poly(A) tail length. Our data implicate the NRE and pum protein in mediating the ability of *nos* activity to alter polyadenylation, further correlating this poly(A) alteration with known alterations in translation (Barker et al., 1992; Wharton and Struhl, 1991).

**Nanos reduces the poly(A) tail length of *hb* RNA by promoting active deadenylation**

The above experiments indicate that the presence of *nos* activity results in *hb* RNA with a diminished poly(A) tail. This observation could stem from complete prevention of polyadenylation by *nos*, or by an alteration of an existing polyadenylation/deadenylation equilibrium to favor deadenylation. The latter mechanism would require active deadenylation to occur in the embryo. In an attempt to discriminate between these two possibilities, we injected an in vitro polyadenylated *hb* 3′-UTR transcript. If there is no active deadenylation in the embryo, the size of the poly(A) tail on this RNA should not be reduced after injection. However, if a polyadenylation/deadenylation equilibrium exists, this transcript will be modified according to the relative rates of addition and removal of adenosines.

The *hb* 3′-UTR RNA was polyadenylated in vitro with approximately 60 nt of adenosine. Injection of this transcript into a region containing *nos* activity (wt posterior) resulted in an apparently complete deadenylation of the transcript. In contrast, the RNA recovered from injections into areas lacking nos protein (wt anterior and nos anterior, posterior) resulted in an increased heterogeneity of poly(A) tail lengths due to partial deadenylation of the RNA (Fig. 3A). The observed reduction in poly(A) tail length of the injected RNAs indicates that, in addition to polyadenylation (Fig. 2A), deadenylation is occurring to these RNAs.

The similarity between the poly(A) status after injection of

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**Fig. 3.** Injection and recovery of various polyadenylated *hb* 3′-UTR RNAs. (A) A polyadenylated *hb* 3′-UTR was injected into the anterior (ANT) and posterior (POST) of wt and *nos* embryos. This RNA was completely deadenylated in the presence of nos protein (wt posterior). ‘NI’ indicates RNA run on the gel without being injected. The ‘−A’ lane indicates transcript size prior to in vitro polyadenylation and ‘+A’ indicates size of RNA after this reaction. The decrease in intensity of bands in wt ANT and nos ANT, POST is due to increased heterogeneity in the size of the RNA and not degradation since approximately equal amounts of radioactivity and equal number of embryos were loaded in each lane. RNAs were recovered 1 hour after injection. (B) Injection of a truncated *hb* 3′-UTR containing a poly(A) tail. This RNA lacks sequences necessary for polyadenylation, thus is only deadenylated. After injection (15 minutes) the RNA was partially deadenylated everywhere. However, the wt posterior lane at this time had increased deadenylation activity due to the presence of *nos* activity. Injected RNAs were recovered at 5 and 15 minutes instead of 1 hour because by 1 hour all RNAs were significantly deadenylated (data not shown). Lane designation is the same as in A with RNA recovered at various times after injection.
the non-polyadenylated and polyadenylated \( hb \) 3'-UTR RNA (compare Figs 2A, 3A) suggests a dynamic equilibrium between polyadenylation and deadenylation. Thus, regardless of the initial length of adenosines before injection, the poly(A) tail of the recovered RNA converges toward a similar length. These results are consistent with \( nos \) either blocking polyadenylation or promoting deadenylation.

To distinguish whether \( nos \) activity blocks polyadenylation or promotes deadenylation, it was necessary to disrupt one of these processes. Since a specific \( cis \)-acting element for polyadenylation in \( Drosophila \) has not been elucidated, we deleted a large region of the \( hb \) 3'-UTR in order to remove sequences necessary for polyadenylation. The DNA template for the 3'-UTR was linearized at a \( DraI \) site producing a truncated RNA representing only the 5'-most 350 nt of the 3'-UTR (\( hb \) 3'-UTR\(^{DraI} \)). This fragment still contained the NREs and therefore would be responsive to the effects of \( nos \) activity. Upon injection into the anterior of a wt embryo, this \( hb \) 3'-UTR\(^{DraI} \) RNA was not polyadenylated, indicating that we had removed essential polyadenylation sequences (data not shown). We then in vitro polyadenylated this RNA (\( hb \) 3'-UTR\(^{DraI}+A \)) and injected it into embryos. If, in the presence of \( nos \) activity, the injected \( hb \) 3'-UTR\(^{DraI}+A \) RNA was deadenylation to a greater extent than in the absence of \( nos \) activity, it would demonstrate that \( nos \) is promoting a deadenylation process. Alternatively, if there is no difference in the length of the poly(A) tail in the presence or absence of \( nos \) activity, this would suggest that \( nos \) was blocking polyadenylation (blocking an already blocked RNA should have no effect). The recovery of this truncated and polyadenylated \( hb \) 3'-UTR 15 minutes after injection resulted in complete deadenylation of the RNA when in the presence of \( nos \) activity (wt posterior; Fig. 3B). The deadenylation was not complete in the absence of \( nos \) activity (wt anterior) indicating that \( nos \) promotes an accelerated deadenylation process.

The polyadenylation of \( bicoid \) mRNA is altered in the presence of \( nos \) activity

The above results show a close correlation between \( hb^{mat} \) polyadenylation and its translation. They also suggest that the lack of polyadenylation seen in the presence of \( nos \) activity results in a lack of translation. To specifically address this issue, we studied the polyadenylation and translation of \( bcd \) mRNA after injection into embryos. \( bcd \) mRNA was chosen as a reporter for translation, and not \( hb \), because we had a convenient morphological assay for \( bcd \) protein production (generation of anterior structures).

As mentioned earlier, studies in which \( nos \) is ectopically expressed in the anterior demonstrated that \( bcd \) and \( hh^{mat} \) RNAs are both translationally repressed in the anterior by the presence of \( nos \) activity and, in the case of \( bcd \) mRNA, this repression was associated with a loss of the poly(A) tail (Gavis and Lehmann, 1992; Wharton and Struhl, 1989). With the results of the PAT on \( nos^{ant} \) embryos correlating a lack of polyadenylation on \( hh^{mat} \) mRNA with its translational repression, it seemed probable that \( nos \) activity was acting on both these RNAs in a similar manner. Therefore, we used the translation of \( bcd \) mRNA as a reporter for the ability of the \( nos \) deadenylation activity to directly affect translation.

To demonstrate that the polyadenylation of \( bcd \) mRNA was altered in the presence of \( nos \), we injected full-length \( bcd \) RNA into the anterior and posterior of wt embryos (Fig. 4). As expected, the RNA was efficiently polyadenylated in the anterior, but not in the posterior. In the anterior, the \( bcd \) transcript obtained a poly(A) tail of approximately 150 nt. In the posterior the RNA was not significantly polyadenylated, with a maximum of 50 nt adenosine added. A poly(A) tail of this short length is unable to efficiently promote the translation of the RNA (Sallès et al., 1994). The ability of \( nos \) protein to alter \( bcd \) mRNA polyadenylation was also observed using the PAT assay and \( bcd \) 3'-UTR injections (data not shown). Thus the \( bcd \) mRNA behaves similarly to \( hb \) mRNA in these injection assays with \( nos \) activity promoting a short poly(A) tail.

\( nos \) activity promotes the deadenylation of maternal mRNA which results in translational repression

The injection of full-length \( bcd \) mRNA into the anterior of embryos from homozygous \( bcd \) females is able to rescue the mutant phenotype of those embryos (Sallès et al., 1994). This ability of injected \( bcd \) RNA to be translated and promote the production of anterior embryonic structures at the site of injection was used here to correlate the ability of \( nos \) activity to alter translation along with polyadenylation. The injection of \( bcd \) mRNA into the posterior end of \( nos \) mutant embryos resulted in the efficient translation of the RNA and the production of head structures at the site of injection (data not shown; indistinguishable from behavior of in vitro polyadenylated \( bcd \) RNA – see later). However, no anterior structures were observed after posterior injections in wt embryos (\( nos \) activity) (data not shown; indistinguishable from behavior of in vitro polyadenylated \( bcd \) RNA – see later). Injection of a chimeric \( bcd \) RNA into wt posteriors generates anterior structures at the site of injection (Driever et al., 1990). This last experiment suggests that the inability of the wild-type \( bcd \) transcript to form anterior structures at the site of injection is due

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**Fig. 4.** Polyadenylation of full-length \( bcd \) RNA is altered in the presence of \( nos \) activity. In vitro transcribed \( bcd \) RNA was injected into the anterior and posterior of wild-type (wt) embryos. In the anterior, the RNA was polyadenylated with approximately 150 nt. This polyadenylation was less in the posterior with \( \approx 50 \) adenosine residues being added to the injected RNA. This short poly(A) tail has been previously shown to not stimulate translation of \( bcd \) mRNA, whereas the same RNA containing a poly(A) tail length of 150 nt is efficiently translated in the embryo (Sallès et al., 1994; Lieberfarb et al., 1996).
to a lack of translation, and not the inability of the wt posterior to produce anterior structures. These results support studies on the BicD mutant in that nos activity can repress the translation of bcd mRNA (Wharton and Struhl, 1989).

As with the hb 3'-UTR, we next polyadenylated the bcd RNA prior to injection and determined the ability of the RNA to be translated in the presence of nos activity. The injected RNA had a poly(A) tail of approximately 150 nt, which is sufficient to promote translation in the embryo (Lieberfarb et al., 1996; Sallès et al., 1994). Even with a long poly(A) tail, the bcd RNA was unable to produce head structures in the presence of nos activity (wt posterior injections) (Fig. 5B; 0/94 embryos had anterior structures at the site of injection). However, this mRNA was translated in the posterior of nos mutant embryos (Fig. 5D,F; 65/74 embryos with anterior structures at the site of injection). This result strongly suggests that the ability of nos protein to deadenylate maternal mRNAs is sufficient to repress their translation.

**DISCUSSION**

Regulated translation of maternal mRNAs is essential for proper development in many diverse species. In this regard, regulated cytoplasmic polyadenylation has been shown to be a major mechanism for this translational activation. In many cases deadenylation accompanies this polyadenylation as a means of temporally controlling poly(A)-driven translation. In this report, we show that deadenylation plays a major role in *Drosophila* posterior pattern formation with *pum* activity and the posterior morphogen, *nos*, initiating abdomen formation through spatial deadenylation and translational repression of maternal *hb* mRNA.

Exactly how nos protein promotes deadenylation or whether it directly deadenylates is not known. Examination of the deadenylation at very short times after injection (5 minutes) suggests a distributive, exonucleolytic deadenylation activity since recovery of the truncated *hb* 3'-UTR resulted in a continuous distribution of deadenylated poly(A) tail lengths (Fig. 3B).

The cis-acting elements responsible for nos responsiveness (NRE) are located at the 5'-end of the *hb* and *bcd* 3'-UTR (Wharton and Struhl, 1991). Deleting these sequences allows for the expression of *bcd* and *hb* in the presence of nos protein (Wharton and Struhl, 1991), and also allows for polyadenylation of the 3'-UTRs of these RNAs (*hb*, Fig. 2C; *bcd*, data not shown), correlating translational repression with repression of polyadenylation. Nos protein does not directly bind to the NRE, but it acts through a ubiquitously expressed maternal protein, pum (Macdonald, 1992; Murata and Wharton, 1995). Whether nos binds directly to pum or through another protein is not known. However, it is apparent that both pum and nos activities are required for both the promotion of deadenylation (Figs 2D, 3B) and translational repression of *hb* mRNA (Barker et al., 1992; Irish et al., 1989; Hülskamp et al., 1989; Struhl, 1989). These data suggest that pum binds to the NRE and initiates the formation of a protein complex, consisting minimally of nos, which promotes the deadenylation and translational repression of the bound RNA (Fig. 6).

Previous reports show that maternal *hb* mRNA is less stable in the presence of nos activity (Tautz and Pfeifle, 1989). A significant amount of degradation of the mRNA is not apparent until after the establishment of a maternal *hb* protein gradient. Therefore, this instability, which is presumably due to nos activity, could not be responsible for the repression of translation. In our study, approximately equal amounts of RNA were obtained per embryo regardless of the injection site, suggesting that stability was not significantly altered in the absence or presence of nos activity.

Nos null mutants are known to have defects in oogenesis.
Fig. 6. Model of maternal hunchback mRNA translational regulation during early embryogenesis. The poly(A) status of maternal hb RNA depends upon an equilibrium between addition and removal of adenosine residues. In the anterior, poly(A) addition results from a greater polyadenylation than deadenylation. In the posterior, poly(A) addition is increased so that the RNA becomes deadenylated. Both nos and pum activity is needed for deadenylation.

(Lehmann and Nüsslein-Volhard, 1991). Studies on the cortex mutation implicate a role for polyadenylation in Drosophila oogenesis (Lieberfarb et al., 1996). These results suggest that nos protein function, possibly its ability to promote deadenylation, may be required during oogenesis to repress the polyadenylation and translation of some other mRNA. However, pum is not necessary for oogenesis (females deleted of the gene are fertile) suggesting that the requirement for nos in oogenesis is not mediated through pum. Translational repression has been shown to be critical in Drosophila oogenesis by studies involving the precocious expression of oskar mRNA, another translationally regulated maternal mRNA (Kim-Ha et al., 1995). Premature translation of oskar mRNA in the oocyte leads to lethality of the embryo.

In Xenopus, polyadenylation has been extensively studied in both oogenesis and embryogenesis. An RNA with homology to nos, Xcat-2, is localized to the vegetal pole of the oocyte (Mosquera et al., 1993). As in Drosophila, the protein product may be generating asymmetry by repressing the expression of a polyadenylated maternal mRNA either in oogenesis or embryogenesis. In C. elegans an asymmetry is established through the translational repression of maternal glp-1 mRNA (Evans et al., 1994). The cis-acting RNA elements responsible for this repression have been defined and are similar to the NREs in hb mRNA. Although the mechanisms of translational activation or translational repression have not been determined for this RNA, the similarities suggest that localized translational repression may be common throughout evolution.

nos and hb mRNA are both translationally activated during development, although by two independent mechanisms. Translational activation of nos mRNA occurs by localization (Gavis and Lehmann, 1994; Gavis et al., 1996), whereas hb expression is likely regulated by polyadenylation. Since the presence of nos protein must precede the polyadenylation and translation of hb mRNA, it is logical that these two maternal mRNAs are activated by different mechanisms. Thus the localization of nos mRNA controls its spatial expression and also its precise temporal translation such that it can repress the translation of maternal hb mRNA resulting in a gradient of hb protein.

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