Development 129, 2699-2710 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
DEV5016

An anterior function for the *Drosophila* posterior determinant Pumilio

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Accepted 1 March 2002

SUMMARY

Bicoid is a key determinant of anterior *Drosophila* development. We demonstrate that the prototypical Puf protein Pumilio temporally regulates *bicoid* (*bcd*) mRNA translation via evolutionarily conserved Nanos response elements (NRE) in its 3′UTR. Disruption of Pumilio-*bcd* mRNA interaction by either Pumilio or *bcd* NRE mutations caused delayed *bcd* mRNA deadenylation and stabilization, resulting in protracted Bicoid protein expression during embryogenesis. Phenotypically, embryos from transgenic mothers that harbor *bcd* NRE mutations exhibited dominant anterior patterning defects and we discovered similar head defects in embryos from *pum*$^{-}$ mothers. Hence, Pumilio is required for normal anterior development. Since *bcd* mRNA resides outside the posterior gradient of the canonical partner of Pumilio, Nanos, our data suggest that Pumilio can recruit different partners to specifically regulate distinct mRNAs.

Key words: Gene regulation, Puf proteins, Translational control, *Drosophila* anterior patterning, RNA-binding proteins, 3′UTR

INTRODUCTION

Post-transcriptional regulation of gene expression plays a fundamental role in embryonic development. This is apparent during the first nuclear divisions when the genome is transcriptionally inactive and gene expression relies on stored maternal mRNAs. Regulation of maternal mRNA localization, translation and stability result in the establishment of embryonic axes and of asymmetric gene expression patterns that are necessary to drive subsequent development. Spatial control produces distinct graded distributions of specific proteins, while temporal control ensures that stored maternal mRNAs are translated at their correct developmental times (Gray and Wickens, 1998; Wickens et al., 2000). Mechanisms also operate generally to degrade maternal mRNAs after translation and locally to avoid expression in inappropriate locales (Cooperstock and Lipshitz, 1997). In most cases, *cis*-acting signals in the 3′ untranslated region (UTR) of the mRNA dictate these post-transcriptional processes (Gray and Wickens, 1998).

In *Drosophila melanogaster*, *bicoid* (*bcd*) is the first maternal gene in a regulatory cascade crucial to anterior patterning: embryos from *bcd*-null mothers fail to develop head and thorax (Frohnhöfer and Nüsslein-Volhard, 1986). *bcd* mRNA, which is synthesized in the nurse cells, is localized to the anterior tip of the oocyte and early embryo (Berleth et al., 1988). At this stage, *bcd* is translationally silent (Sallés et al., 1994) and stable (Surdej and Jacobs-Lorena, 1998). Upon egg activation, the polyA tail of *bcd* is elongated by cytoplasmic polyadenylation and the mRNA becomes translationally active (Sallés et al., 1994). A Bicoid protein gradient emanates from the anterior of the embryo and different concentrations effect distinct developmental fates (Driever and Nüsslein-Volhard, 1988a; Driever and Nüsslein-Volhard, 1988b; Driever et al., 1989; Struhl et al., 1989). Bicoid, a DNA- and RNA-binding homeoprotein (Mayfield, 1996) activates transcription of genes required for anterior development by binding their promoters [e.g. zygotic *hunchback*, hb^{zyg} (Driever et al., 1989; Struhl et al., 1989), and *orthodenticle* (Gao and Finkelstein, 1998)] and translationally inhibits *caudal* mRNA in the anterior by binding its 3′UTR (Chan and Struhl, 1997; Rivera-Pomar et al., 1996). The *bcd* mRNA and protein are degraded around 3 and 4 hours after egg laying (AEL) respectively at 21°C (Driever and Nüsslein-Volhard, 1988a). Hence, Bicoid protein is present for only a discrete time period. Except for two genes (*cor*, *grau*), whose mutations result in disrupted *bcd* polyA addition and a decrease in Bicoid protein (Lieberfarb et al., 1996), specific mechanisms that regulate the translation and stability of *bcd* remain largely unexplored.

Translational regulation is understood in some detail for *Drosophila* embryonic posterior patterning. Here Pumilio and Nanos regulate unlocalized maternal *hunchback* mRNA (hb^{mat}) expression via two copies of a *cis*-acting sequence in the *hb* mRNA 3′UTR: the bipartite Nanos response element (NRE) (Barker et al., 1992; Tautz, 1988; Tautz and Pfeifle, 1989; Wharton and Struhl, 1991). The NRE contains the Pumilio-binding site (Murata and Wharton, 1995; Wharton et al., 1998; Zamore et al., 1997) and Nanos protein associates with an assembled Pumilio-*hb* mRNA complex via protein-protein and protein-RNA interactions (Sonoda and Wharton, 1999). This ternary complex causes deadenylation of *hb* mRNA and translational repression in the posterior of the embryo (Murata and Wharton, 1995; Wharton et al., 1998; Wreden et al., 1997) confining $Hunchback^{mat}$ protein to the anterior half of the embryo.

pumilio (*pum*) and *nanos* (*nos*) were originally characterized genetically: mutations in either of these 'posterior group' genes (Nüsslein-Volhard, 1991; Nüsslein-Volhard et al., 1987) cause abdominal and posterior defects in embryos from homozygous mothers, because of the lack of *hb* repression (Lehmann and Nüsslein-Volhard, 1987; Lehmann and Nüsslein-Volhard, 1991). The Pumilio protein is uniformly distributed in the embryo (Macdonald, 1992), while Nanos protein is distributed in a gradient emanating from its localized mRNA at the posterior pole. This supplies positional information for the translational repression of *hb* (Gavis and Lehmann, 1992; Wang and Lehmann, 1991).

Pumilio is the prototypical member of an RNA-binding protein family evolutionarily conserved from yeast to humans (Wharton et al., 1998; Zamore et al., 1997). Its signature domain is termed a Puf motif after *Drosophila* Pumilio and the *C. elegans* translational regulator FBF (fem-3-binding factor) (Zamore et al., 1997; Zhang et al., 1997). Puf proteins are implicated in post-transcriptional gene expression in *S. cerevisiae*, *C. elegans*, *X. laevis* and *Drosophila* (Nakahata et al., 2001; Olivas and Parker, 2000; Tadauchi et al., 2001; Wharton et al., 1998; Zamore et al., 1997; Zhang et al., 1997). In most characterized situations, these proteins function with Nanos or Nanos-like partners (Parisi and Lin, 2000; Wickens et al., 2000).

Previous work identified an NRE in the *bcd* 3′UTR (Wharton and Struhl, 1991) that subjects *bcd* to concerted Pumilio/Nanos action when Nanos is ectopically expressed in the anterior (Gavis and Lehmann, 1992) or *bcd* mRNA is injected in the posterior of the embryo (Sallés et al., 1994). However, the absence of Nanos protein in the anterior (Gavis and Lehmann, 1992; Wang and Lehmann, 1991) combined with the anterior confinement of *bcd* mRNA led to the conclusion that the *bcd* NRE was not functional in normal development and possibly represented an evolutionarily drifted sequence (Cooperstock and Lipshitz, 1997; Wharton and Struhl, 1991).

We now demonstrate that the evolutionarily conserved *bcd* NRE sequences are indeed operational, temporally regulating *bcd* mRNA expression in a Pumilio-dependent manner. Specifically, in *pum*$^{-}$ embryos relative to wild type, *bcd* mRNA exhibits delayed deadenylation, is stabilized and causes prolonged Bicoid protein expression during embryogenesis. Furthermore, we show that Pumilio-*bcd* mRNA association has developmental relevance by generating and analyzing transgenic embryos carrying *bcd* mRNAs with disrupted NRE sequences. Their molecular phenotype mirrors that of *pum*$^{-}$ embryos with respect to their altered temporal expression of *bcd* mRNA and protein, and their morphological phenotype exhibits head abnormalities consistent with a primary defect in maxillary segment determination. Subsequent analyses of *pum*$^{-}$ embryos reveal similar, previously undetected head defects, uncovering a heretofore unknown role for Pumilio in anterior development. Thus, *bcd* NRE regulation by Pumilio is crucial for normal head development.

MATERIALS AND METHODS

Egg collections, RNA and protein extractions

Fly embryos from well-fed actively laying females were collected on yeasted grape agar plates at 20°C or 25°C, depending on line requirements. Collections and aging times were corrected by 1.7 for slower development at 20°C (Ashburner, 1989). For each experiment, wild-type (Oregon R) and mutant embryos were collected and processed simultaneously and identically. Embryos from wild-type and *pum*^{13}/*pum*^{13} mothers (cold sensitive) were collected at 20°C for 1.5 hours and aged for populations: 0 to 1.5 hours, 20 minutes to 1 hour 50 minutes, 1 hour 10 minutes to 2 hours 40 minutes, 2 hours 5 minutes to 3 hours 35 minutes, 2 hours 54 minutes to 3 hours 24 minutes, 3 hours 45 minutes to 5 hours 15 minutes, 4 hours 46 minutes to 6 hours 16 minutes, and 5 hours 45 minutes to 7 hours 15 minutes. Transgenic embryos (50 minute collections at 20°C) were aged for populations: 0 to 50 minutes, 25 minutes to 1 hour 15 minutes, 50 minutes to 1 hour 40 minutes, 1 hour 40 minutes to 2 hours 30 minutes, 2 hours 30 minutes to 3 hours 20 minutes, 3 hours 20 minutes to 4 hours 10 minutes, 4 hours 10 minutes to 5 hours, 5 hours to 5 hours 50 minutes, 5 hours 50 minutes to 6 hours 40 minutes. *nos*^{BN} and wild-type embryos (53 minute collections at 25°C) were aged for populations: 0 to 53 minutes, 12 minutes to 1 hour 5 minutes, 41 minutes to 1 hour 34 minutes, 1 hour 13 minutes to 2 hours 6 minutes, 1 hour 42 minutes to 2 hours 35 minutes, 2 hours 12 minutes to 3 hours 5 minutes, 2 hours 48 minutes to 3 hours 41 minutes, 3 hours 17 minutes to 4 hours 10 minutes.

Embryos were dechorionated (2.5% bleach for 90 seconds), rinsed (0.7 M NaCl, 0.04% Triton X-100, 0.7 M NaCl) and immediately processed or frozen. Ovaries were manually dissected in PBS. Samples were either dounced in Trizol®, processed (BRL) and quantitated spectrophotometrically (RNA) or homogenized in 15 mM Hepes (pH 7.6), 100 mM KCl, 0.1 mM EDTA, 0.5 mM EGTA, 10% sucrose and protease inhibitors (Roche), cleared by centrifugation, and quantified (proteins).

PAT assay and northern blots

Total RNA (0.5 μg per time point) was mixed with the synthetic internal control RNA and subjected to PAT assay (Sallés and Strickland, 1995) with modifications to be described elsewhere (contact authors for details). Internal control RNA: the oligos 5′CTCGGTACCCATTTGCGCATTCTTTGACCAAGAATCATAGC-TCACATTCTATTTAC3′ (*bcd* 2200-2225 fused to 2306-2328) and 5′CTCGATTCACCCGAGTAGAGTAGTTCT3′ were used to amplify the *bcd* 3′UTR from its cDNA (Berleth et al., 1988). The product, cloned into pBluescript SKII (Stratagene; *Kpn*I-*Eco*RI fragment; pBSΔbcd1), was sequenced. Sense RNA was transcribed in vitro by T7 RNA polymerase with [γ-^{32}P]GTP, gel purified, quantitated (by cpms), in vitro polyadenylated with recombinant bovine PAP, size-selected by polyacrylamide-urea gel (0-50 nucleotide poly A tails) and extracted. Label was eliminated by phosphatase and Sephadex G50 filtration. The internal control RNA amount added to PAT samples was determined empirically to amplify endogenous and synthetic RNAs competitively. For northern blots, 2 μg (4 μg transgenic) total RNA was resolved on modified formaldehyde-agarose gels (Gamberi et al., 1994) transferred to Hybond N^{+} membrane (Amersham) and probed.

Antibodies, western blots and protein assays

Guinea pig α-Bicoid antibodies were raised to the bacterially overexpressed Bicoid C terminus (amino acids 222-438) (Harlow and Lane, 1988). Protein samples resolved via SDS-PAGE were transferred to nitrocellulose (S&S) probed in PBS, 5% NFDM, 0.1% Tween-20 (sera concentration 1:1000/1:3000) and HRP-conjugated secondary antibodies (Cappel, Jackson) and visualized by chemiluminescence (Pierce).

Construction of *bcd* NRE mutants, in vivo assays and fly lines

Drosophila strains: *pum*^{13}, *pum*^{1}, *pum*^{Msc}, *pum*^{FC8} (Barker et al., 1992) *bcd*^{E1} (Lindsley and Zimm, 1992) *nos*^{BN} (Wang et al., 1994).

The NRE1 and NRE2 mutations were introduced between *bcd* 3′UTR *Hpa*I and *Mlu*I sites as annealed oligos in place of the wild-type sequence in the ~6 kb *Eco*RI-*Bam*HI genomic DNA fragment (Seeger and Kaufman, 1990) and cloned in pCasper4.

Multiple independent transgenic fly lines for each construct were obtained by standard means: three wild-type* lines, four each NRE1 and NRE2 constructs wt* (the asterisk indicates transgenics that harbor a wild-type *bcd* transgene and is meant to distinguish these from true wild-type flies). Expression levels of transgenes were similar to endogenous *bcd*. One P-element mobilized NRE1 line exhibited higher expression (approximately two to three times more). Both the wild-type and NRE1 mutant transgenic flies were homozygous for the P elements; all the NRE2 mutant transgenics used were heterozygotes. Two generated lines gave homozygote adults whose embryos died early in embryogenesis, precluding comparison with the other collections. We obtained consistent phenotypic results from pum^{13}/pum^{FC8} and pum^{13}/pum^{Msc} embryos [mouth hook (mh) defect 88% and 93%, head involution defect 81% and 18%, n=171 and 136, respectively]. For cuticles: dechorionated embryos were devitellinized (heptane:methanol) (Su et al., 1998) rehydrated and incubated overnight at 50°C in 9:1 lactic acid:70% ethanol and mounted in Hoyer's medium (light microscopy) or ethanol washed, critical point dried and gold-coated (scanning electron microscopy).

RESULTS

The *bcd* 3′UTR contains evolutionarily conserved NRE sequences

To identify sequences regulating *bcd* mRNA expression, we focused on the perfect bipartite NRE sequence GUUGU-N_5-AUUGUA (A box-N_5-B box) in the 3′UTR of *bcd*, starting 50 nucleotides downstream of the *bcd* translational stop codon (Fig. 1). This *bcd* motif was noticed previously, but its role in normal development was unclear because it resides outside the Nanos embryonic domain (Cooperstock and Lipshitz, 1997; Wharton and Struhl, 1991). The *hb* 3′UTR contains two NRE motifs, while *bcd* has one NRE and an additional B box at position +79 (termed 1 1/2 NREs). By aligning the *bcd* and *hb* 3′UTRs from all available species, we found that the *bcd* motifs are closer to the second *hb* NRE (Fig. 1). Moreover, the 1 1/2 NREs was absolutely conserved in the *bcd* 3′UTR of eight fly species that diverged more than 60 million years, underscoring functional constraint. Thus, we set out to determine the role the NREs play in *bcd* expression and to analyze their contribution to normal embryonic development.

Endogenous *bcd* mRNA deadenylation is delayed in *pum*⁻ mutant embryos

After fertilization, *bcd* polyA tail elongation temporally correlates with a burst of *bcd* translation (Sallés et al., 1994). By analogy to *hb* regulation in the posterior (Murata and Wharton, 1995; Wharton et al., 1998; Wharton and Struhl, 1991; Wreden et al., 1997), we reasoned that the conserved NRE sequences of *bcd* might subject it to Pumilio regulation. Consistently, Pumilio exists throughout the early embryo (Macdonald, 1992) in excess of *hb* mRNA (Zamore et al., 1999); therefore, it is available to interact with additional transcripts.

To determine whether Pumilio affects *bcd* regulation in vivo, we compared *bcd* temporal expression in embryos from wild-type and pum^{13}/pum^{13} homozygous mothers (pum^{-}). As longer polyA tails are often predictive of mRNA translatability, we monitored *bcd* expression by PolyA Tail (PAT) assay, which examines polyA tail size and distribution on a specific transcript species in an RNA population (Sallés and Strickland, 1995) (see Materials and Methods). To compare different samples reliably in a time course and between egg collections,

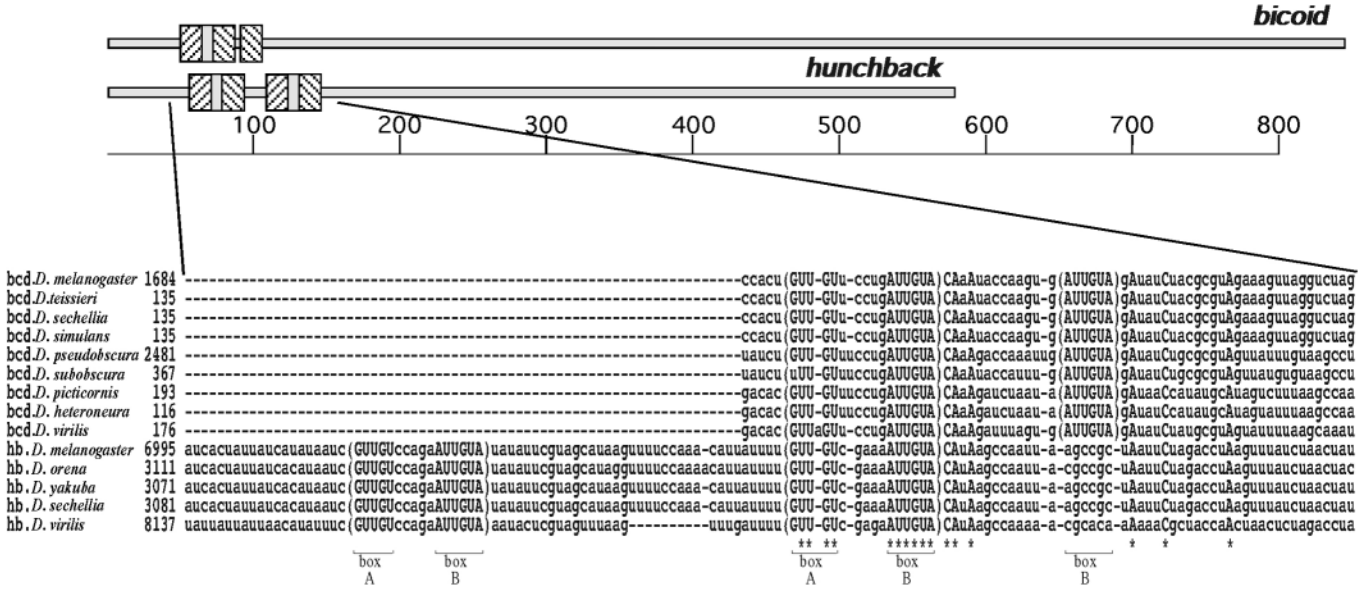

Fig. 1. The *Drosophila bcd* 3′UTR contains conserved NRE sequences. Diagram of *bcd* and *hb* 3′UTRs with size bar (top). NREs with conserved A and B boxes (hatched areas) and spacers (intervening gray areas). The *bcd* 3′UTR contains a bipartite NRE sequence and a downstream B box (1 1/2 NREs). Sequence alignment of the *bcd* and *hb* NRE regions (below) with conserved nucleotides (capitalized) and identical residues (*). Sequence names are the species with a bcd or hb prefix. Sequences in the EMBL/GenBank database: bcd, X14458, M32121, M32124, M32123, X55735, X78058, M32122, M32126, M3125; hb, Y00274, AJ00535, AJ00536, AJ00534, X15359. Numbers are the first nucleotide aligned in their database entry.

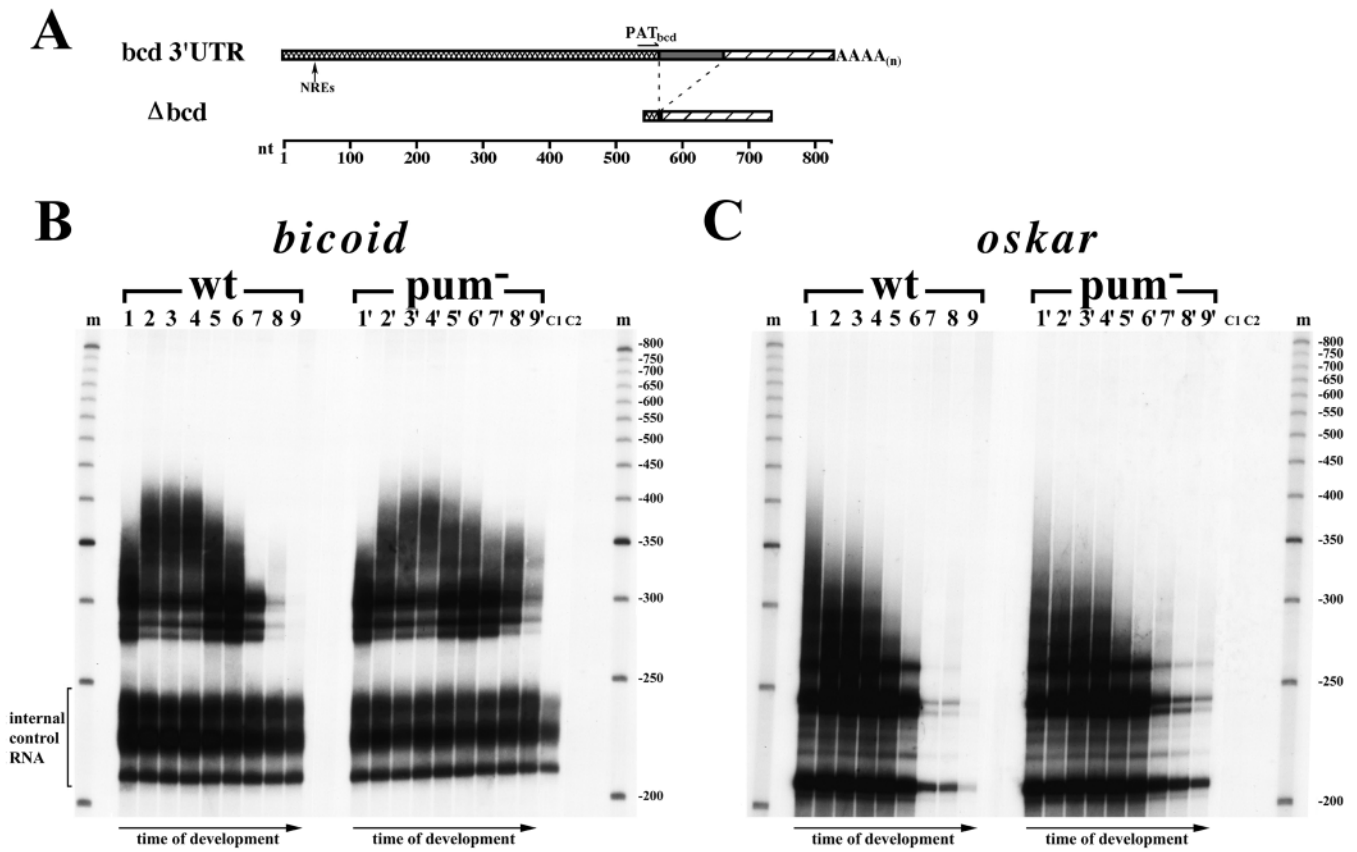

Fig. 2. Endogenous *bcd* mRNA deadenylation is delayed developmentally in *pum*⁻ mutants. (A) The *bcd* 3′UTR and PAT assay internal control (Δbcd, to scale) with 1 1/2 NREs (arrow) and *bcd*-specific oligo priming site (PAT_{bcd}). (B) Internally controlled *bcd* PAT assay profiles from wild type (lanes 1-9) and *pum*⁻ (lanes 1′-9′) samples including ovarian (1,1′) and embryonic (2-9,2′-9′) RNAs (20°C). C1, cDNA synthesis control (only internal control RNA); C2, PCR control (no template). Equal volumes of the final reactions were resolved on acrylamide-urea gel and autoradiographed. 'm', ^{32}P-labeled marker. (C) *osk* PAT assay profile in wild type and *pum*⁻ mutants. The same cDNAs as B were amplified with an *osk*-specific primer (Sallés et al., 1994). *osk* deadenylation does not change noticeably in *pum*⁻ mutants. We observed a slight increase in *osk* stability (8-9 versus 8′-9′). C1, Δbcd cDNA control; C2, *osk* PCR control (no template).

we modified the PAT assay to include a synthetic internal control (Δbcd; Fig. 2A). Δbcd, an in vitro polyadenylated RNA derived from the *bcd* 3′ UTR, was size selected for molecules with 0-50 nucleotide polyA tails. Samples included total RNA extracted from fly embryos collected at timed intervals covering development from egg deposition (0 hours) to 7 hours 15 minutes of embryogenesis and fly ovaries. Collections were at 20°C because the *pum*^{13} mutation is cold sensitive.

A controlled PAT assay (Fig. 2B) from wild type (lanes 1-9) and *pum*⁻ mutants (lanes 1′-9′) revealed *pum*⁻ mutation altered *bcd* polyA tail dynamics. In the ovary, the *bcd* polyA tails are 50-55 nucleotides (lanes 1,1′). Wild-type eggs are activated upon fertilization, and the *bcd* polyA tail is quickly elongated up to 140 nucleotides (lane 1 versus lane 2) (Sallés et al., 1994). Later, *bcd* is deadenylated (lanes 5-7) and eventually degraded (lanes 8-9). In *pum*⁻ embryos examined in parallel, the *bcd* polyA tail is similarly elongated (lanes 2′-4′) but deadenylation is delayed or is less effective (lanes 5-7 versus lanes 5′-7′), resulting in *bcd* mRNA molecules carrying longer polyA tails into later developmental times (lanes 6-9 versus lanes 6′-9′).

bcd cytoplasmic polyadenylation also appears delayed in *pum*⁻ versus wild-type embryos. We observed this reproducibly, in multiple collections using different *pum* genotypes (*pum*^{13}/*pum*^{13}, *pum*^{1}/*pum*^{13}, not shown) indicating this molecular phenotype is *pum* specific. Additionally, we consistently noticed apparent transcript stabilization (lanes 7-9 versus lanes 7′-9′).

We excluded the possibility that delayed *bcd* deadenylation reflected a general defect in maternal mRNA metabolism of *pum*⁻ mutants, rather than a productive Pumilio-*bcd* interaction, by analyzing *oskar* (*osk*) (Ephrussi et al., 1991). This maternal mRNA, which is devoid of an NRE, should be unaffected by the NRE-dependent functions of Pumilio. Indeed, *osk* deadenylation appears unaltered in *pum*⁻ embryos compared with wild type (Fig. 2C) [for wild type, see Sallés et al. (Sallés et al., 1994)]. *osk* mRNA is stabilized at later times (Fig. 2C, lanes 7-9 versus lanes 7′-9′; see Discussion). Other mRNAs likewise exhibited no altered deadenylation in *pum*⁻ embryos (not shown).

Thus, in *pum*⁻ embryos *bcd* mRNA deadenylation is delayed specifically compared with wild-type embryos.

bcd mRNA is stabilized and causes prolonged Bicoid protein expression in *pum*⁻ embryos

The polyadenylation state of an mRNA is positively correlated

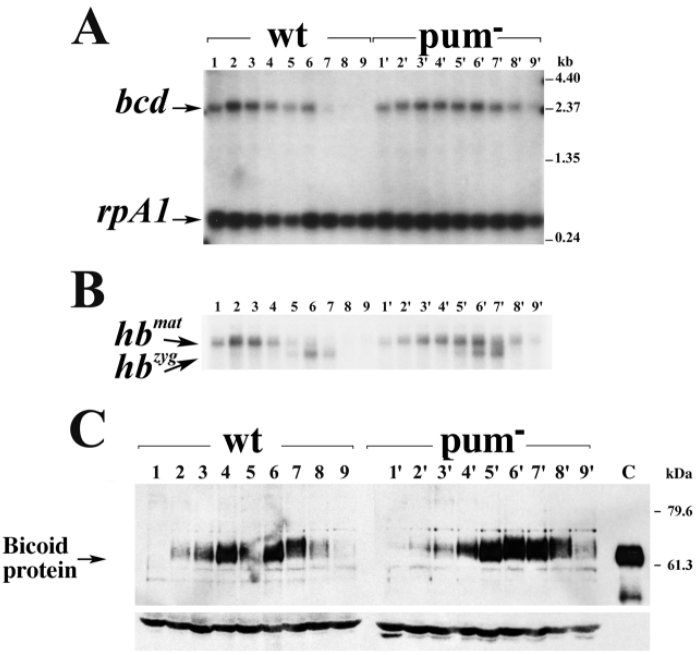

Fig. 3. *bcd* mRNA is stabilized in *pum*⁻ mutants. RNAs used in Fig. 2 analyzed by northern blot. (A) Probed for *bcd* and *rpA1*. *bcd* is stabilized in *pum*⁻ mutants compared with wild type (lane numbering as in Fig. 2). Similar results were obtained for pum^{13}/pum^{13}, pum^{1}/pum^{13} and pum^{Msc}/pum^{FC8} embryos. (B) Reprobing for *hb* shows the embryo populations are developing synchronously and hb^{mat} is also stabilized in *pum*⁻ mutants. (C) Western blot of wild type (lanes 1-9) and *pum*⁻ (1′-9′) embryo proteins probed for Bicoid (time course identical to Fig. 2, and A,B). Lane C, in vitro translated unlabelled Bicoid protein. The lower panel (same gel) shows a secondary antibody crossreacting band that acts as a loading control.

with its stability (Hilleren and Parker, 1999; Richter, 1996) and our PAT (PCR-based) assay suggested *pum* mutation might alter *bcd* stability. Thus, we directly examined the identical RNA samples by northern blot (Fig. 3A). The *bcd* transcript decays at the later developmental times in wild-type embryos (Fig. 3A, lanes 7-9), while it is stabilized in *pum*⁻ embryos for at least three more time points (lanes 7-9 versus lanes 7′-9′). By contrast, the ribosomal protein A1 mRNA (*rpA1*) (Surdej and Jacobs-Lorena, 1998) remains relatively constant. Phosphorimager quantitation on multiple experiments and alleles showed *pum*⁻ embryos contain roughly ten times more *bcd* mRNA (normalized to *rpA1*) than their respective wild-type controls at the last point analyzed.

Consistent with hb^{mat} translational repression by Pumilio (Murata and Wharton, 1995; Wharton et al., 1998; Wreden et al., 1997), our blots showed hb^{mat} mRNA stabilization in *pum*⁻ embryos (Fig. 3B). The hb^{mat} and *bcd* mRNAs are stabilized during identical times, suggesting a similar underlying molecular basis. By contrast, hb^{zyg} temporal expression seems relatively unaffected. The hb^{zyg} level does seem elevated in lane 7′ versus lane 7, consistent with hb^{zyg} mRNA transcription by a distinct Bicoid-dependent promoter activated around nuclear cycle 10 of embryogenesis (Driever and Nüsslein-Volhard, 1989; Struhl et al., 1989). A Pumilio-dependent mechanism analogously regulating hb^{mat} and hb^{zyg} through their identical 3′UTRs is also possible (see Discussion).

The *hb* northern blot (Fig. 3B) reveals zygotic transcription onset is synchronous in wild-type and *pum*⁻ embryos (lanes 5,5′). This eliminates the possibility that delayed deadenylation and stabilization of *bcd* simply reflect a slower development rate of the *pum*⁻ embryos. Both maternal transcripts are eventually degraded about 4 hours AEL (lanes 7,8), coincident with general maternal RNA degradation prior to blastoderm cellularization (Bashirullah et al., 1999).

PolyA tail length positively correlates with mRNA translatability, particularly for maternal mRNAs cytoplasmically polyadenylated after egg activation (Richter, 1996; Richter, 2000). While a long polyA tail allows efficient *bcd* translation in vitro (F. Gebauer and M. Hentze, personal communication), it is presently unclear what polyA tail threshold length affects translation.

Comparative Bicoid protein (Bicoid) expression in wild-type and *pum*⁻ embryos was examined by western blot (Fig. 3C). In wild type, no Bicoid is detectable in ovaries where *bcd* has short polyA tails (lane 1). Upon egg activation, Bicoid is translated, rapidly reaches high levels (lanes 4-6) and subsequently disappears (lanes 7-9). In *pum*⁻ embryos, where *bcd* with long polyA tails persist, Bicoid peaks at a later time and is produced for a longer period during embryogenesis (compare lanes 7-9 with lanes 7′-9′).

Our data strongly suggest Pumilio is indeed a factor involved in *bcd* post-transcriptional regulation.

The *bcd* NRE functions in normal *Drosophila* development

If Pumilio regulates *bcd* expression, mutating the Pumilio-binding site in the *bcd* 3′UTR should produce transcripts that are temporally independent of Pumilio. To selectively disrupt the Pumilio-*bcd* mRNA interaction while minimizing interference with known 3′UTR functions (Macdonald and Struhl, 1988; Surdej and Jacobs-Lorena, 1998), we took a minimally disruptive mutational approach. As *bcd* NREs are evolutionarily conserved (Fig. 1) and *hb*-Pumilio interaction studies have indicated that box B is most sensitive to mutation (Murata and Wharton, 1995; Wharton et al., 1998; Zamore et al., 1997), we simultaneously modified both B boxes. A UG→AC mutation was introduced in the downstream B box with either an identical (NRE1) or UA→GC (NRE2) mutation in the upstream B box (Fig. 4A). Either dinucleotide mutation introduced in the second NRE of *hb* weakened or abolished the Pumilio-RNA interaction, as assayed by u.v. crosslinking, and rendered the *hb* NRE non-functional phenotypically (Wharton et al., 1998).

By P-element transformation we generated *bcd* transgenic flies independently harboring either the mutated NRE1 or NRE2 cassettes. Corresponding wild-type transgenics (*) were also generated as matched gene dose controls for these experiments. As previous work has shown that increased *bcd* gene dose per se does not impede anterior development (Frohnhöfer and Nüsslein-Volhard, 1986; Frohnhöfer and Nüsslein-Volhard, 1987; Driever and Nüsslein-Volhard, 1988b) the wild-type* transgenics allow us to distinguish consequences of Bicoid amounts versus temporal persistence. All transgenic flies possessed a full complement of endogenous *bcd* transcript, plus the transgenic *bcd* mRNA; these species differ by only four nucleotide changes, and are indistinguishable in size, precluding independent detection by northern blot. However, if the mutant transcripts are stabilized

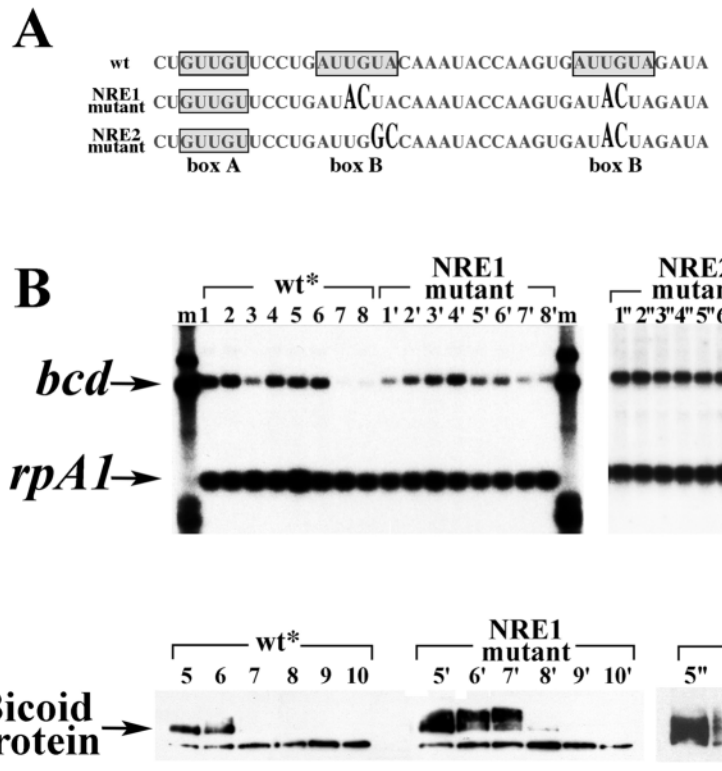

Fig. 4. Transgenic mRNAs with mutated NREs escape Pumilio regulation. (A) The *bcd* NRE (A and B boxes shaded) and NRE transgenic mutant constructs (NRE1, NRE2; dinucleotide mutations noted). (B) Northern blot of timed samples from wild-type transgenic (wt*, lanes 1-8), NRE1 (lanes 1′-8′) and NRE2 (lanes 1″-8″) embryos probed for *bcd* and *rpA1*. Both NRE1 and NRE2 transgenics contain *bcd* at later developmental times. m, size markers. (C) Western blot of wild-type transgenic (wt*, lanes 5-10), NRE1 (5′-10′) and NRE2 (5″-10″) mutant embryo extracts probed for Bicoid. The developmental period overlaps with and extends beyond Fig. 4B. The lowest band (secondary antibody crossreaction) acts as a loading control. Transgenics contain a full complement of both endogenous and transgenic *bcd* mRNAs and Bicoid protein. The wild-type* and NRE1 embryos are homozygotes; the NRE2 mutant line is heterozygote for the *bcd* transgene.

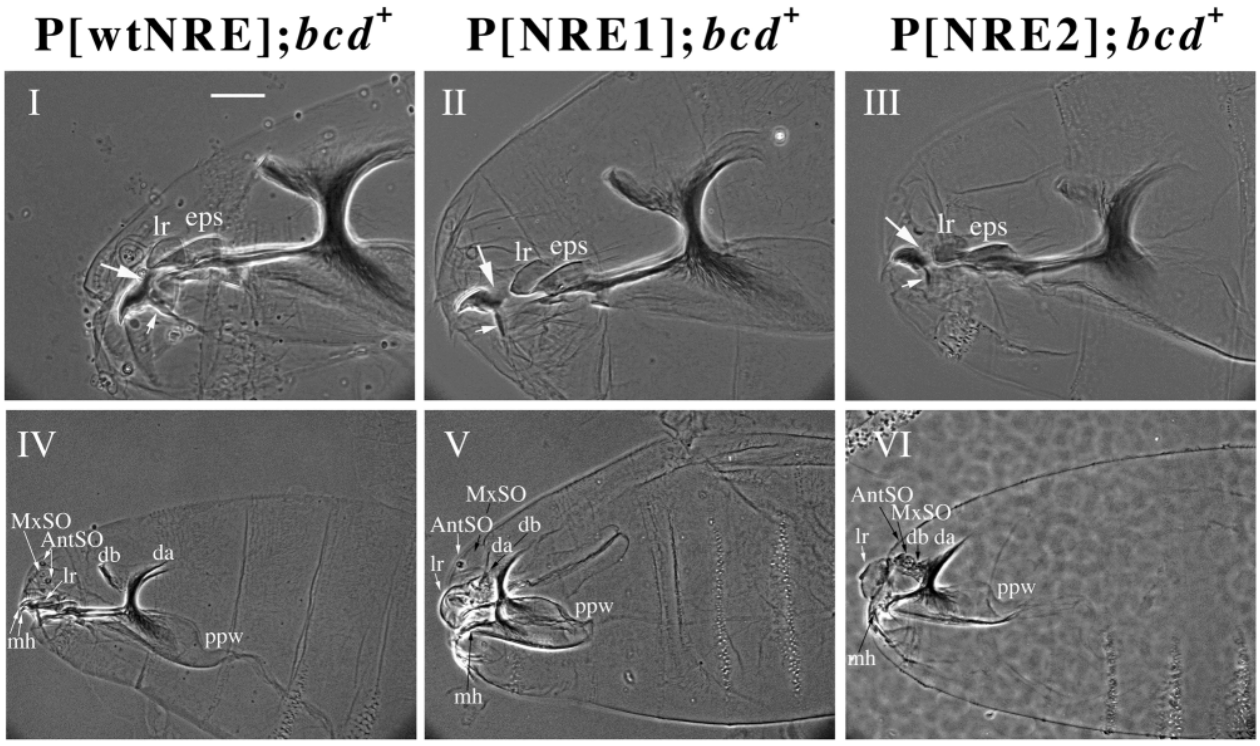

Fig. 5. Prolonged Bicoid expression dominantly interferes with head development. Head cuticles of wild-type*, NRE1 and NRE2 transgenic embryos in a bcd^+ background with maternal genotypes (phase contrast, side views, dorsal towards the top, anterior leftwards). (part I) Wild-type* tridentate mouth hooks (mh) with dorsal (large arrow) and ventral (small arrow) projections. The *labrum* (lr) and epistomal sclerite (eps) are labral segment markers. *bcd* NRE1 (part II) and NRE2 (part III) mutant transgenics fail to develop the dorsal mh projection. (Part IV) Wild-type* head with notable marked structures (Jürgens et al., 1986): mh, MxSO, AntSO, lr, dorsal arms (da), dorsal bridge (db) and posterior pharyngeal wall (ppw). Some mutant transgenic embryos with the mh defect fail to complete head involution, resulting in deformations and altered spatial relationships among cuticle structures. NRE1 (part V) and NRE2 (part VI) transgenics with a reduced head skeleton, deformed protruding lr and mh defect. Black arrows: structures residing in a different focal plane. Parts I-III visualize mh abnormality and parts IV-VI reveal head involution defects. Scale bar: 20 μm in parts I-III; 40 μm in parts IV-VI.

and/or translationally activated for a prolonged period, this effect should be dominant at the RNA and protein levels, and would be detectable by northern and western analyses.

Northern blots revealed that *bcd* is present at later times in embryos from mothers harboring either NRE1 or NRE2 transgenes, when compared with those containing the wild-type* control (Fig. 4B). By phosphorimager analyses against *rpA1*, expression levels of the transgenes were similar to endogenous *bcd* (0.6-1.3×). Endogenous hb^{mat} transcripts are not stabilized because hb^{mat} possesses wild-type NREs and both Pumilio and Nanos are unaltered (not shown).

We analyzed Bicoid temporal expression in our transgenics by western blot (Fig. 4C), focussing on later time points because our mRNA assays predicted that only these may be affected. Consistent with wild-type embryos (Driever and Nüsslein-Volhard, 1988a) our wild-type* *bcd* transgenics produce detectable Bicoid until 3-4 hours AEL. By contrast, NRE1 or NRE2 embryos, which contained the longer-lived transgenic *bcd* transcripts, produced Bicoid for a protracted time period (compare lanes 7,7′ and 7″).

Interestingly, at even later times, mutant transgenic *bcd* mRNA persisted without corresponding Bicoid protein (Fig. 4B, lanes 7′,8′,7″,8″). A slow, NRE-independent, general deadenylation might eventually generate *bcd* transcripts that are translated inefficiently. Alternatively, a fail-safe mechanism may exist that represses late *bcd* translation, either specifically or more generally by silencing maternal transcripts that escaped the major mRNA degradation preceding midblastula transition (Bashirullah et al., 1999).

From our data on both pum^{-} and *bcd* NRE mutant transgenic embryos, we conclude that mutating Pumilio or its binding site within the *bcd* 3′UTR alters *bcd* expression. Both mutations generate detectable *bcd* mRNA at later times in embryogenesis and both result in Bicoid protein persistence.

Protracted *bcd* expression induces dominant head defects

Bicoid is the major factor that specifies embryonic anterior development in the presence of *torso* repression of Hunchback at the anterior pole (Janody et al., 2000; Ronchi et al., 1993; Wimmer et al., 2000), and it acts synergistically with Hunchback in head and thorax patterning (Simpson-Brose et al., 1994). Expression of the anteriorly localized *bcd* mRNA is tightly regulated temporally, resulting in a sharp peak of Bicoid production (Berleth et al., 1988; Driever and Nüsslein-Volhard, 1988a). While disruption of *bcd* localization is known to alter head development, the consequences of perturbations to this temporal regulation are unknown. Therefore, we investigated whether Bicoid persistence at later embryonic times in pum^{-} and mutant *bcd* NRE transgenics resulted in any phenotypic alterations.

Cuticles of first instar larvae from NRE1 and NRE2 mutant embryos in a bcd^{+} background exhibited a highly penetrant, dominant mouth hook (mh) base defect. While the wild-type tridentate mh possess posterior dorsal and ventral projections (Fig. 5, part I; large and small arrow, respectively), in NRE mutants the mh dorsal projection failed to develop (98% NRE1, *n*=54; 88% NRE2, *n*=110; Fig. 5, parts II, III) and the ventral projection was often smaller. By contrast, wild-type* transgenic embryos (our gene dosage control) developed only wild-type mh.

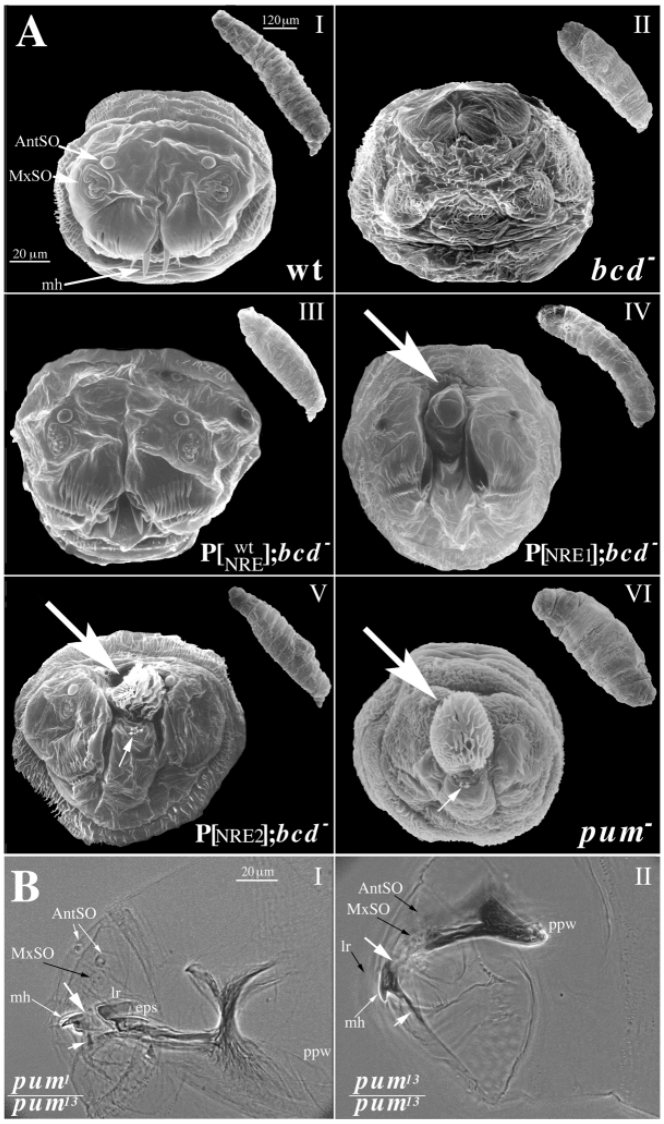

Fig. 6. Analogous head defects in *bcd* NRE and pum^{-} mutants. (A) Scanning electron microscopy of the embryonic cuticle head (frontal view) and body reference (side view) with maternal genotypes. (Part I) A wild-type head with notable structures (mh, MxSO, AntSO). (Part II) bcd^{-} null embryos have no head structures. The globular anterior structures resemble posterior ones. (Part III) A wild-type *bcd* transgene (wt*) rescues the bcd^{-} null anterior defects. NRE1 (part IV) and NRE2 (part V) mutant *bcd* transgenes rescue bcd^{-} anterior development, while additionally inducing defects in some embryos, consistent with the dominant effects in Fig. 5. Protruding structures (arrow). (Part VI) A pum^{-} (pum^{13}/pum^{13}) embryo has an analogous medial protrusion and an exposed sclerite resembling those in part V (large and small arrows, respectively). Body side views (right) show abdominal defects documented for pum^{-} mutation (Barker et al., 1992; Lehmann and Nüsslein-Volhard, 1987; Wang and Lehmann, 1991; Wharton and Struhl, 1991). (B) pum^{1}/pum^{13} (part I) and pum^{13}/pum^{13} (part II) heads (phase contrast) do not develop the mh dorsal projection (large arrow; ventral projection, small arrow). pum^{13}/pum^{13} exhibits additional head skeleton defects consistent with its relative allelic strength and its suggested dominant-negative molecular behavior (Barker et al., 1992; Wharton et al., 1998). The protruding lr (black arrow) from defective head involution is in a different focal plane. Black arrows indicate structures residing in a different focal plane. Scale bars: 120 μm in A; 20 μm in B.

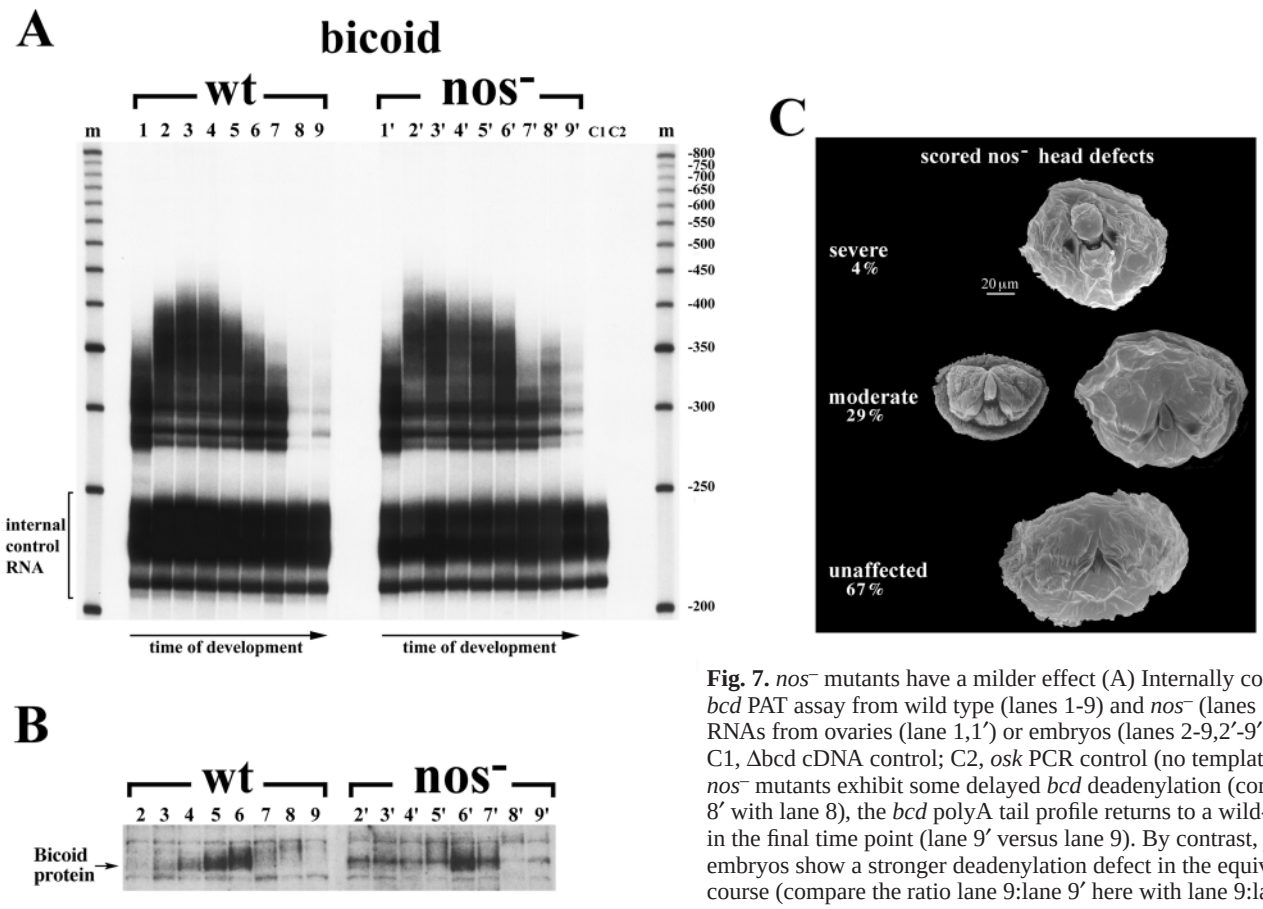

Fig. 7. *nos*⁻ mutants have a milder effect (A) Internally controlled *bcd* PAT assay from wild type (lanes 1-9) and *nos*⁻ (lanes 1′-9′) RNAs from ovaries (lane 1,1′) or embryos (lanes 2-9,2′-9′, 25°C). C1, Δbcd cDNA control; C2, *osk* PCR control (no template). While *nos*⁻ mutants exhibit some delayed *bcd* deadenylation (compare lane 8′ with lane 8), the *bcd* polyA tail profile returns to a wild-type one in the final time point (lane 9′ versus lane 9). By contrast, *pum*⁻ embryos show a stronger deadenylation defect in the equivalent time course (compare the ratio lane 9:lane 9′ here with lane 9:lane 9′ in Fig. 2B). Note, each mutant set can only be compared with its parallel wild-type collection. (B) Western blot of wild-type (lanes 2-9) and *nos*⁻ (lanes 2′-9′) extracts probed for Bicoid (time course as in A). A nonspecific crossreactive band of slightly lower mobility than Bicoid appears in *nos*⁻ extracts. (C) Scanning electron micrograph of *nos*⁻ heads (frontal view). Four percent of *nos*⁻ cuticles exhibit a protrusion analogous to *pum*⁻ (severe), 29% showed a smaller variably sized protrusion (moderate) and 67% had no such abnormality (unaffected). *n*=52. Scale bar: 20 µm.

Some NRE mutant embryos also failed to complete head involution. These cuticles showed reduced head skeleton (hs) and the deformed *labrum* (lr) remained exposed to the exterior of the cuticle (89% NRE1, *n*=96; 25% NRE2, *n*=153; Fig. 5, parts V, VI). Structures that develop independently of head intersegmental contacts (e.g. antennal sensory organ, AntSO; maxillary sensory organ, MxSO) were always recognizable. Notably, the percentage of embryos with failed head involution positively correlated with mutant transgene expression levels and was significantly higher than in our wild-type* control embryos (3% wild-type* transgenics, *n*=127; Fig. 5 IV) underscoring the specificity of this mutant NRE-mediated defect.

Mouth hooks are maxillary structures (Rogers and Kaufman, 1997), and their abnormal development suggests that maxillary segment determination is defective as a result of *bcd* NRE mutation. Higher penetrance of the mh alteration than the head involution defect implies this primary maxillary defect could interfere with the morphogenetic movements of head involution. Consistently, the mh defect always occurred in NRE mutant embryos with failed head involution (see below).

We analyzed the rescue effect of our transgenes in a bcd^{E1}/bcd^{E1} (bcd^{-}) null background, where embryos fail to develop head and thorax (Frohnhöfer and Nüsslein-Volhard, 1986). To facilitate evaluation of head rescue, we analyzed cuticles by scanning electron microscopy (SEM; Fig. 6A) noting the extent to which structures were introverted. A wild-type head (Fig. 6A, part I) shows bilateral symmetry and head structures (mh, MxSO, AntSO). *bcd* mutation abolishes head formation (Fig. 6A, part II) with structures at the anterior resembling posterior ones. One copy of a *bcd* transgene provides sufficient anterior morphogenetic potential to support embryonic anterior development in a *bcd*⁻ background (not shown, Fig. 6A, parts III-V), implying the *bcd* transgenic transcripts are properly localized and processed. While a wild-type *bcd* transgene (Fig. 6A, part III) invariantly gives rise to wild-type heads, the NRE1 or NRE2 transgenes (with prolonged *bcd* expression) support head formation, while inducing head involution defects with protruding structures (Fig. 6A, parts IV, V) analogous to Fig. 5, parts V, VI.

pum⁻ embryos exhibit head defects analogous to *bcd* NRE mutant transgenics

pumilio was originally characterized as a gene whose mutation caused posterior morphological defects (Nüsslein-Volhard, 1991; Nüsslein-Volhard et al., 1987). No anterior defects have been reported for these mutants. However, in all our molecular

assays, the *bcd* expression profile in *bcd* NRE transgenics mirrored that of *pum*⁻ embryos. This raised the strong possibility of head defects in *pum*⁻ embryos.

pum⁻ heads are indeed abnormal. SEM showed that *pum*⁻ cuticles displayed a protruding structure reminiscent of the *bcd* NRE mutants (Fig. 6A, part VI, large arrow; 30% pum^{1}/pum^{13}, n=97; 88% pum^{13}/pum^{13}, n=82). Interestingly, both its morphology and a ventral sclerite closely resemble those in the NRE2 mutant (Fig. 6A, parts VI, V, small arrows) possibly suggesting head involution arrested at the same stage. Phase contrast showed that *pum*⁻ mutants also exhibited the same highly penetrant mh defect as *bcd* NRE mutant transgenics, underscoring a maxillary segment determination defect (Fig. 6B, parts I, II; penetrance>98% pum^{1}/pum^{13}, n=57; >95% pum^{13}/pum^{13}, n=63). Analogous defects (mh 99%, head involution 81%, n=124) in an independent strong *pum*⁻ background (pum^{Msc}/pum^{FC8}, presumptive null, not shown) support the conclusion that these phenotypic alterations are specifically related to *pum* mutation.

The identification of anterior defects in *pum*⁻ mutants that mirror those induced by *bcd* NRE mutant transgenes strongly implies that in addition to its function in posterior development, Pumilio contributes to *Drosophila* anterior patterning. Pumilio allows posterior patterning by translationally repressing *hb* mRNA via the *hb* NRE and regulates anterior patterning by translationally regulating *bcd* mRNA via the *bcd* NRE.

Is Nanos the Pumilio partner that affects *bcd* expression?

Disruption of the *hb* mRNA-Pumilio-Nanos interaction in the posterior of the embryo results in *hb* translational derepression and posterior patterning defects (Lehmann and Nüsslein-Volhard, 1987; Barker et al., 1992; Wharton and Struhl, 1991). By analogy, Pumilio and Nanos might associate with the *bcd* NRE to affect *bcd* expression. However, Nanos protein is found in a gradient emanating from the posterior pole for approximately one third of the length of the embryo (Wang et al., 1994; Wang and Lehmann, 1991). Hence, the posterior domain of Nanos seems incompatible with a physiological effect on *bcd*.

Nonetheless, as Nanos is the canonical Pumilio partner, we tested whether Nanos was responsible for NRE-dependent *bcd* regulation by examining embryos from mothers homozygous for the strong nos^{BN} mutation (null, *nos*⁻) (Wang et al., 1994). If Pumilio and Nanos assemble in a ternary complex on *bcd* mRNA in vivo, mutating either should produce identical *bcd* molecular and phenotypic defects.

Parallel PAT assays (Fig. 7A) of simultaneously collected wild-type (lanes 1-9) and *nos*⁻ (lanes 1′-9′) samples revealed normal *bcd* cytoplasmic polyadenylation in both (lanes 1-4 versus lanes 1′-4′). In *nos*⁻ embryos, *bcd* deadenylation is slightly delayed (lanes 5-9 versus lanes 5′-9′) and *bcd* transcripts are slightly stabilized (lane 8 versus lane 8′). However, this profile returns to wild type at the final point (9 versus 9′), in contrast to the situation with *pum*⁻, where *bcd* mRNA with long polyA tails persist at the time course conclusion. Hence, when compared with their respective wild-type controls, PAT assay results from the *nos*⁻ and *pum*⁻ embryos are not identical: the former are milder. By western blot (Fig. 7B), the Bicoid profile in *nos*⁻ mutants seems flatter and does not reflect the clear *bcd* de-repression in *pum*⁻ embryos (Fig. 3C).

Using SEM (Fig. 7C), *nos*⁻ cuticles showed severe head involution defects in only 4% (n=52) of *nos*⁻ nulls (compared with 81% of *pum*⁻ presumptive null). In most cases where *nos*⁻ heads were morphologically aberrant (mild, 29%), embryos had a medial structure reminiscent of NRE mutant transgenics, but of smaller size. Consistent with our molecular results, these data delineate a milder anterior phenotype for *nos*⁻ than for *pum*⁻ embryos.

Partial non-overlap between the molecular and phenotypic results from *nos*⁻ and *pum*⁻ embryos is consistent with the possibility that Pumilio regulates *bcd* in part independently of Nanos. This scenario implies the existence of distinct Pumilio-dependent complexes on *bcd* and *hb* mRNAs, where an anterior (unknown) or a posterior (Nanos) factor would join the specific Pumilio-RNA complexes.

DISCUSSION

We have demonstrated that Pumilio temporally regulates *bcd* mRNA expression: its mutation causes delayed deadenylation and stabilization of the *bcd* message, resulting in protracted Bicoid protein expression. Disruption of this molecular control perturbs normal *Drosophila* head development.

Temporal versus spatial control of expression

An intricate combination of spatial and temporal controls orchestrate expression of a gene hierarchy resulting in appropriate embryonic patterning. For *bcd*, initial spatial restriction in the embryo is provided by anterior localization of translationally silent *bcd* mRNA. The RNA is then translationally deployed over a short period, resulting in a pulse of Bicoid. We found this latter process of temporal control of localized *bcd* mRNA expression is regulated by the evolutionarily conserved *bcd* NRE to ensure proper head development. Either NRE or Pumilio mutation causes protracted *bcd* translation. Resulting Bicoid found later in development would have prolonged access to its downstream targets and/or novel access to inappropriate targets from which it is temporally segregated in wild type. Either could interfere with anterior development, ultimately causing head defects.

While affected Bicoid targets are presently only speculative, we fortuitously noticed that late hb^{zyg} was increased in northern blots of *bcd* NRE mutants, hinting at one potential affected molecule (not shown). This is consistent with our *pum*⁻ data (Fig. 3B, lane 7 versus lane 7′). A second target candidate arises from the defective mh base present in both *bcd* NRE mutant transgenics and *pum*⁻ embryos. This alteration, which is suggestive of a maxillary segment defect, similarly occurs when *orthodenticle* (*otd*) is expressed ectopically (E. Wimmer personal communication) (Gallitano-Mendel and Finkelstein, 1998; Janody et al., 2000). Interestingly, Bicoid activates *otd* transcription (Gao and Finkelstein, 1998) and resulting Orthodenticle has the same DNA-binding specificity as Bicoid (Mailhos et al., 1998). Hence, the prolonged Bicoid expression in mutant *bcd* NRE transgenics and *pum*⁻ embryos may interfere with normal head development through a complex pattern of interactions.

The posterior determinant Pumilio functions in anterior patterning

Pum was originally characterized as a posterior group gene: Pumilio and Nanos cooperate to repress hb^{mat} in the posterior of the embryo, allowing abdominal patterning (Barker et al., 1992; Lehmann and Nüsslein-Volhard, 1987; Lehmann and Nüsslein-Volhard, 1991; Tautz and Pfeifle, 1989; Wharton and Struhl, 1991). However, ubiquitous expression of Pumilio (Macdonald, 1992) in excess of *hb* (Zamore et al., 1999) implies it could possess additional function(s) elsewhere. We have demonstrated that Pumilio also participates in *Drosophila* anterior embryonic patterning. *pum* embryos exhibit head defects. The Pumilio anterior function is mediated via *bcd* post-transcriptional expression, as similar anterior abnormalities occur when we mutate its presumptive *bcd* mRNA-binding site.

An alternate partner for Pumilio?

We asked if *bcd* NRE regulation required the Pumilio canonical partner Nanos. When *bcd* mRNA was injected posteriorly (Wreden et al., 1997) or Nanos was expressed anteriorly by genetic means (Wharton and Struhl, 1991; Wharton and Struhl, 1989; Gavis and Lehmann, 1992), Pumilio and Nanos could affect *bcd* expression because all co-existed. In each case, large Nanos amounts were present and head morphogenesis was inhibited.

A major Nanos role in normal head formation seems unusual because Nanos and *bcd* mRNA reside at opposite ends of the embryo. Surprisingly we found Nanos does influence *bcd* expression and subsequent anterior development to some degree. This suggests undetectable Nanos amounts may regulate *bcd* mRNA in the anterior. Analogously, a contribution of low Nanos levels in oogenesis was reported (Verrotti and Wharton, 2000).

bcd mRNA might encounter low Nanos levels via the NRE-dependent back-up mechanism postulated to repress it when it escapes localization, diffuses posteriorly and intercepts the Nanos gradient (Gavis and Lehmann, 1992; Wharton and Struhl, 1991). Alternatively, sufficient Nanos moieties might diffuse anteriorly, analogous to when enough Bicoid molecules exist in the posterior of the embryo to elicit *hairy* stripe 7 expression (Rosée et al., 1997) or to cooperate with Caudal in *knirps* activation (Rivera-Pomar et al., 1995). In a different scenario, *nos* mRNA translational repression throughout the embryo (Bergsten and Gavis, 1999; Crucs et al., 2000) may be leaky, yielding low basal Nanos levels everywhere, including the anterior. How a Pumilio-*bcd* mRNA complex can recruit enough Nanos for action and whether this involves additional (anterior?) factors to modulate Nanos activity are questions for future studies.

nos^{-} severe head involution defects occur at a significantly lower frequency than in pum^{-} cuticles (4% versus 81%; null versus presumptive null), raising the intriguing possibility that an additional partner(s) for Pumilio exists at the anterior that affects *bcd* NRE function independently of Nanos. Consistently, the sequence between the A and B boxes of the *bcd* and *hb* NREs diverges at two of the four nucleotide positions known for *hb* recruitment of Nanos (Sonoda and Wharton, 1999). While we are accustomed to thinking about Pumilio and Nanos functioning in concert, they have only partially overlapping roles in the *Drosophila* germline and may function independently in oogenesis (Forbes and Lehmann, 1998; Lin and Spradling, 1997). The alternate Pumilio partner for *bcd* might be an anterior Nanos paralog (although only one *nos* gene was found in the fly genome) or a distinct moiety. Interestingly, *S. cerevisiae* has five Puf proteins involved in mRNA metabolism (Olivas and Parker, 2000) but no Nanos homologs, suggesting some Puf proteins can function with novel partners, consistent with recent *C. elegans* data (Luitjens et al., 2000).

Function of the *bcd* NRE

Our molecular data indicate the *bcd* NREs act temporally, repressing translation in a deadenylation dependent way. We also show that mutating either Pumilio or the *bcd* NREs results in protracted Bicoid expression. Presently, we cannot distinguish if the *bcd* NREs primarily constitute a translational control element with mRNA deadenylation and instability accompanying specific repression or a regulated instability element whose downstream effects are seen at the protein level. Interestingly, in addition to detecting specific Pumilio-dependent *bcd* NRE regulation, we noticed a second effect of pum^{-} mutation: stabilization of maternal mRNAs devoid of NREs (Fig. 2C, not shown). While it is unclear whether this effect is direct, it may reflect a novel Pumilio function in general NRE-independent mRNA turnover.

Our complementary phenotypic analyses of *bcd* NRE mutant transgenes revealed prolonged Bicoid expression interferes with maxillary segment determination, which may affect head involution by altering the intersegmental contacts required for appropriate head morphogenetic movements. Incomplete overlap between the highly penetrant mh defect and the partially penetrant head involution defect might reflect the complexity of fly head development, which is subjected to redundancy and fail-safe mechanisms (Rogers and Kaufman, 1997; Schmidt-Ott, 2001).

The conservation of the *bcd* and *hb* NREs, their Pumilio association, and their ability to direct translational regulation imply functional similarity between these elements. However, the *hb* regulatory system operates on a uniformly distributed mRNA to repress its expression in the embryonic posterior where Nanos is most concentrated. By contrast, *bcd* mRNA is spatially restricted to the anterior via localization, which conceivably impacts NRE action and predicts underlying functional differences between *bcd* and *hb* NREs.

Widening roles of the Puf protein Pumilio

Puf homologs have been identified throughout the animal kingdom and analyzed members in developmental systems only contribute to early embryonic and germline development in the posterior (Parisi and Lin, 2000; Wickens et al., 2000). The novel Pumilio role in anterior development documented here raises the exciting possibility that the prototypical Puf protein Pumilio operates more generally than previously thought, regulating multiple physiological pathways in different *Drosophila* embryonic locales. Furthermore, as Pumilio is also expressed in the adult fly (Macdonald, 1992) and pum^{-} flies exhibit additional uncharacterized phenotypes (Barker et al., 1992), Pumilio may function in mRNA metabolism throughout the life of the fly.

To date, NREs have been identified in three mRNA species: *hb* (Barker et al., 1992; Tautz, 1988; Tautz and Pfeifle, 1989;

Wharton and Struhl, 1991), *bcd* (Wharton and Struhl, 1991) (this report) and *cyclin B* (Asaoka-Taguchi et al., 1999; Dalby and Glover, 1993). For each, NRE organization differs: *hb* and *bcd* contain two and 1 1/2 copies of the basic (A box-N_5-B box) NRE motif, respectively, while *cyclin B* contains one NRE motif with a larger spacer. Furthermore, hb^{mat} and hb^{zyg} mRNA have identical NREs, but hb^{zyg} mRNA seems relatively insensitive to regulation by Pumilio/Nanos. Differences among NREs combined with distinct distributions of NRE-containing mRNAs and their known effectors underlie a potential combinatorial model of NRE recognition in which a common factor (Pumilio) associates with the mRNA target sequence and subsequently recruits different (sets of) factors (e.g. Nanos, BRAT for hb^{mat} mRNA) (Sonoda and Wharton, 2001) to regulate ultimately and specifically unique target expression. How Pumilio functions on different NRE-containing mRNAs, what factor combinations are employed in distinct situations and whether Nanos homologs are involved in every case are experimental questions begging to be answered.

We especially thank R. Gutell and E. Wimmer for sharing their expertise on multiple sequence alignment and head development, respectively. Reagents were kindly supplied by T. Kaufman (*bcd* genomic clone), R. Wharton (*nos* flies), the Bloomington *Drosophila* stock center (flies) and S. Gunderson (recombinant PAP). We are grateful to J. Mendenhall (SEM), B. Göttgens (image analysis), C. Desplan, R. Finkelstein, J. Fisher, E. Goodwin, S. Haynes, P. Macdonald, A. Simcox and members of the Gottlieb laboratory (discussion and manuscript comments). This work was funded by NIH grant RO1 GM50800 to E. G.

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