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Translational control of maternal *Cyclin B* mRNA by Nanos in the *Drosophila* germline

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In the *Drosophila* embryo, Nanos and Pumilio collaborate to repress the translation of *hunchback* mRNA in the somatic cytoplasm. Both proteins are also required for repression of maternal *Cyclin B* mRNA in the germline; it has not been clear whether they act directly on *Cyclin B* mRNA, and if so, whether regulation in the presumptive somatic and germline cytoplasm proceeds by similar or fundamentally different mechanisms. In this report, we show that Pumilio and Nanos bind to an element in the 3′ UTR to repress *Cyclin B* mRNA. Regulation of *Cyclin B* and *hunchback* differ in two significant respects. First, Pumilio is dispensable for repression of *Cyclin B* (but not *hunchback*) if Nanos is tethered via an exogenous RNA-binding domain. Nanos probably acts, at least in part, by recruiting the CCR4-Pop2-NOT deadenylase complex, interacting directly with the NOT4 subunit. Second, although Nanos is the sole spatially limiting factor for regulation of *hunchback*, regulation of *Cyclin B* requires another Oskar-dependent factor in addition to Nanos. Ectopic repression of *Cyclin B* in the presumptive somatic cytoplasm causes lethal nuclear division defects. We suggest that a requirement for two spatially restricted factors is a mechanism for ensuring that *Cyclin B* regulation is strictly limited to the germline.

KEY WORDS: Nanos, Pumilio, Translational regulation, Germ cell, CCR4, Deadenylase

INTRODUCTION

Nanos (Nos) represses translation of maternal *hunchback* (*hb*) mRNA in the presumptive somatic cytoplasm of the early *Drosophila* embryo, thereby governing abdominal segmentation (Hülskamp et al., 1989; Irish et al., 1989; Struhl, 1989). Nos is recruited into a repressor complex that contains two ubiquitous factors, Pumilio (Pum) and Brain tumor (Brat). Pum nucleates formation of the complex by recognizing Nanos Response Elements (NREs) in the 3′ UTR of *hb* and recruiting Nos (Sonoda and Wharton, 1999). The subsequent recruitment of Brat to the Pum-*hb* NRE-Nos complex (Sonoda and Wharton, 2001) results in translational inhibition via both poly(A)-dependent and poly(A)-independent mechanisms (Chagnovich and Lehmann, 2001). A transient gradient of Nos is generated by Oskar (Osk)-dependent localization and translational activation of *nos* mRNA in the specialized pole plasm at the posterior of the embryo (Ephrussi and Lehmann, 1992; Smith et al., 1992). For regulation of *hb*, Nos is thought to be the sole spatially limiting factor (Gavis and Lehmann, 1992).

In addition to their role in abdominal patterning, Nos and Pum play a number of roles in the primordial germ cells (PGCs). PGCs that lack Nos or Pum function enter mitosis prematurely, fail to migrate to the somatic gonad, undergo apoptosis, and fail to maintain stem cell identity in adults (Lin and Spradling, 1997; Asaoka-Taguchi et al., 1999; Asaoka and Lin, 2004; Hayashi et al., 2004; Wang and Lin, 2004). After dissipation of the Nos gradient in the presumptive somatic cytoplasm (e.g. by nuclear division cycle 9-10), high levels of Nos are found only in the PGCs (Wang et al., 1994). The limited distribution of Nos, coupled with the study of Nos orthologs in *Caenorhabditis elegans* and *Homo sapiens*, suggests that the ancestral function of Nos is in the germline (Subramaniam and Seydoux, 1999; Jaruzelska et al., 2003; Tsuda et al., 2003).

Although the regulatory targets of Nos and Pum in the PGCs have not yet been defined, one excellent candidate is maternal *Cyclin B* (*CycB*) mRNA. PGCs cease proliferating shortly after their formation at the posterior pole of the embryo, emerging from quiescence only after migrating to, and arriving in, the presumptive gonad in late embryogenesis (Su et al., 1998). At least part of this quiescence is thought to be due to Pum- and Nos-dependent repression of *CycB* mRNA (Asaoka-Taguchi et al., 1999). CycB accumulates prematurely in the PGCs of embryos from *nos* or *pum* mutant females (hereafter, *nos* and *pum* mutant embryos). Conversely, ectopic CycB drives otherwise wild-type PGCs into premature mitosis, consistent with the idea that repression of *CycB* translation limits proliferation. However, the ectopic CycB in these experiments was derived from a transgene that directs the maternal synthesis of a chimeric mRNA (consisting of 5′ and 3′ UTRs from *nos* fused to the *CycB* ORF) under *nos* transcriptional control (Asaoka-Taguchi et al., 1999); the experiment thus provides only modest support for the idea that Pum and Nos directly target native *CycB* mRNA.

Recent experiments have provided insight into the likely functions of two components of the repressor complex assembled on *hb*-Pum and Brat. Pum is a founding member of the conserved Puf domain family of RNA-binding proteins (Zhang et al., 1997). One of the budding yeast Puf proteins, MPT5, has recently been shown to bind specific mRNA targets and regulate their stability by interacting with the Pop2 subunit of the CCR4-Pop2-NOT complex (Goldstrohm et al., 2006). This complex contains deadenylation enzymes (CCR4 and Pop2) as well as factors that promote decapping (Dhh1), and thus is able to regulate either the stability or translation (or both) of mRNAs to which it is recruited. Based on the observation that Puf proteins from *H. sapiens*, *C. elegans* and *Saccharomyces cerevisiae* interact with orthologous Pop2 subunits, Wickens and colleagues (Goldstrohm

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et al., 2006) suggested that Puf proteins generally act by recruiting the deadenylase complex. Brat appears to repress translation (at least in part) by recruiting 4E-HP, which inhibits binding of the essential initiation factor eIF-4E to the mRNA cap (Cho et al., 2006). Brat is likely to have additional repressor functions, because mutants that do not bind 4E-HP exhibit relatively minor defects in *hb* regulation. The only function attributed to Nos to date has been to assist Pum in recruiting Brat (Sonoda and Wharton, 2001).

Regulation of maternal *hb* and *CycB* mRNA differs in two important respects. First, repression of *CycB* is Brat-independent (Sonoda and Wharton, 2001). As the only known function of Nos for regulation of *hb* is in Brat recruitment, the role of Nos in regulation of *CycB* (and presumably other mRNAs in the PGCs) has been unclear. Second, repression of *hb* occurs both in the PGCs and broadly throughout the posterior of the embryo, whereas repression of *CycB* is strictly limited to the PGCs (Tautz, 1988; Asaoka-Taguchi et al., 1999). These different spatial domains of repression might be due to differential sensitivities of the *hb* and *CycB* mRNAs to the concentration of Nos, which persists at high levels only in the PGCs. However, this idea has not been critically tested.

In this report, we investigate the regulation of maternal *CycB* mRNA as a model for understanding Nos and Pum action in the germline. We first show that *CycB* indeed is directly regulated by binding of Nos and Pum to an element in its 3′ UTR. We then describe experiments that suggest Nos is primarily responsible for recruiting the CCR4-Pop2-NOT deadenylase complex to *CycB*. Finally, we show that regulation of *CycB* in the somatic cytoplasm is deleterious and that it is restricted to the germline by a dual requirement for high levels of Nos and another factor active only in the PGCs.

MATERIALS AND METHODS

Strains, reagents and microscopic methods

pum^{ET3,} *In(3R)Msc*, and *nos*^{BN} flies were from R. Lehmann (New York University, NY); *brat*^{fs1}, *Df(2L)TE37C-7, CCR4* ^{KG877}, and *Df*(3R)*crb*-F89-4 flies were from the Bloomington Stock Center; *CycB*^{2} and *CycB*^{3} flies (Jacobs et al., 1998) were from C. Lehner (University of Bayreuth, Germany). *CycB*, *hb*, *nos-bcd* and *osk-bcd* transgenes were constructed in pCasPeR derivatives and introduced into *w*^{1118} flies by standard methods. The rat anti-Hb and rabbit anti-Nos antibodies were gifts from P. Macdonald (University of Texas, Austin, TX) and A. Nakamura (RIKEN, Kobe, Japan), respectively. The F2F4 anti-CycB developed by P. O'Farrell was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa. Anti-Xpress antibodies were from Invitrogen, anti-Histone antibodies from Chemicon International (Fig. 6), and chicken anti-Vas from K. Howard (University College London, UK). Secondary antibodies were from Jackson ImmunoResearch Laboratories. The distributions of CycB, Vasa, Hb and DNA were detected in fixed embryos with a Zeiss 510 confocal microscope. The distributions of Hb and Nos were detected with peroxidase-coupled secondary antibodies with a Zeiss Axiophot microscope using either brightfield or Nomarski optics and a Spot RT digital camera. *CycB* mRNA was detected by standard in situ hybridization methods using digoxigenin-labeled DNA probes (Roche) and visualized as above for Hb and Nos. To detect transgenic *CycB* gene products, transgenes were crossed into either *CycB*^{2}/+,*CycB*^{3}/+, or *CycB*^{2}/*CycB*^{3} backgrounds so that embryos did not receive an excess of maternal gene product. For the *pum*$^{-}$ embryos in Fig. 4, *CycB* mRNA or protein from the endogenous genes would interfere with the detection of transgenic gene product, and so these experiments were performed in a *CycB*^{2}/*CycB*^{3} background.

In vitro RNA-binding experiments

RNA for all experiments was prepared by transcription of derivatives of the plasmid R4685, which contains a *Spe*I site (underlined) embedded in *hb* 3′ UTR-coding sequence (CTAAAAACTATCATAAAGACTAGTCTGGAGAAACATAAGCCCTGCA) that is inserted into Bluescript II KS–. Oligonucleotides encoding fragments of *hb* (ctagTATTATTTTGTTGTCGAAAATTGTACATAAGCC), *CycB* (ctagtcgagGCATAAAAAAGAGACGTAGACTATTTGTAATTTATATCATGTATTTCGCACATTCATACgaattcatcgatgat), or *eIF4E* (ctagGCATAATATAAAATCTATCCGCTTTTTGTAATCACTGTCAATAATGGATTAGACGGAAAAGTATATTAA) were inserted into the *Spe*I site of R4685. (The 5′ *Spe*I-overhang and, where relevant, polylinker derived nucleotides are in lower case.) ^{32}P-labeled RNA was prepared using T7 MEGAShortscript (Ambion).

Plasmids encoding GST-Pum, His6-Nos and His6-Brat are described elsewhere (Wharton et al., 1998; Sonoda and Wharton, 1999; Edwards et al., 2003). A plasmid encoding the *Drosophila* Pum RNA-binding domain was constructed in a derivative of pET-19b in which the thrombin site is replaced with a Tev cleavage site. Cleavage with Tev liberates the following fragment of *Drosophila* Pum from an N-terminal His-tag: gtRSRLL...YYlKITN (where gt are vector-encoded and l is substituted for the native M near the C-terminus) (Edwards et al., 2000). Like the RBD of human Pum, the *Drosophila* protein is monomeric at mM concentrations.

Gel mobility shift experiments were performed essentially as described (Murata and Wharton, 1995), with the following modifications. Each 10 μl reaction contained purified protein, reaction buffer [10 mM HEPES (pH 7.4), 20 mM KCl, 1 mM DTT, 0.2 mg/ml heparin, 0.05 mg/ml poly(U), 5% glycerol], and heat-denatured labeled RNA (10,000 cpm). Nos and Brat recruitment experiments were performed essentially as described (Sonoda and Wharton, 1999; Sonoda and Wharton, 2001), with the following modifications: each 40 μl reaction contained 1.5 μM GST-Pum, 0.6 μM His6-Nos, 2 μM RNA, and, for Brat recruitment, 0.4 μM His6-Brat, in reaction buffer [20 mM HEPES (pH 7.9), 5 mM $MgCl_2$, 5 μM $ZnCl_2$, 5mM DTT, 100 mM NaCl, 0.5% Tween 20, 0.1% BSA, 500 U/ml RNase Inhibitor (Roche) and 1×EDTA-free protease inhibitor (Roche)]. For the experiments shown in Fig. S3 of the supplementary material, all *CycB* RNAs were either the wild type sequence above (i.e. bearing 59 nt from the 3′ UTR) or mutant derivatives thereof.

Transgenes

CycB transgenes were derived from a modified wild-type rescuing construct (Jacobs et al., 1998) that bears an *Xba*I site immediately 3′ to nucleotides encoding the stop codon to facilitate plasmid construction. Oligonucleotides encoding the 59 nt *CycB* NRE (above), the 50 nt *CycB* NRE (ctagtAGAGACGTAGACTATTTGTAATTTATATCATGTATTTCGCACATTCATAC), the inactive 40 nt *CycB* NRE (underlined nucleotides in the 50 nt sequence deleted), two copies of the 32 nt *hb* NRE (above) and either one or two copies of the MS2hp (ctagAAACATGAGGATCACCCATGTA) were inserted into a *Spe*I site that replaces sequences deleted in the ΔI portion of the ΔI+II gene or into the deleted portion of the ΔIII gene (Fig. 1, and see Fig. S2 in the supplementary material). *hb* transgenes were derivatives of p2343 (Murata and Wharton, 1995). Derivatives of a wild-type *nos* transgene that encode three different Nos-CP fusions were constructed by inserting sequences encoding wild-type MS2 CP between wild-type Nos residues 3 and 4, residues 197 and 198, and at the C-terminus. Essentially identical regulation of *CycB*(2x MS2hp) was observed with each fusion.

Protein-protein interaction experiments

GST-pulldown experiments were performed as described (Goldstrohm et al., 2006), except that Pop2 proteins were labeled with ^{35}S-methionine during synthesis in vitro. The GST-HsPum fusion protein was as described (Goldstrohm et al., 2006); the GST-Dm-Pum protein used in these experiments contains the homologous region only (i.e. residues 1091-1433 containing the RBD but not the C-terminal tail). NOT4 clones were isolated in two different yeast interaction screens, a two-hybrid screen performed by M. Patterson and R.P.W. using DBD-Nos (full-length) as bait and a four-hybrid screen (Sonoda and Wharton, 2001). The NOT4 clones identified in the latter screen proved to interact with the Nos moiety of the three-hybrid bait. Fusions of *Drosophila* Pop2 to the DBD or AD were prepared from cDNA clone RH51274 and plasmids pGBT9, pGAD424,and pActII. Interaction between various protein pairs was tested by co-transformation with plasmids encoding Nos, Pum, Cup or NOT4 derivatives into PJ69-4A (James et al., 1996). Tethering of Pop2 via the DBD robustly stimulated transcription of the HIS3 and ADE2 reporters without co-expression of any AD-fusion, and thus the DBD-Pop2 fusion was not used further.

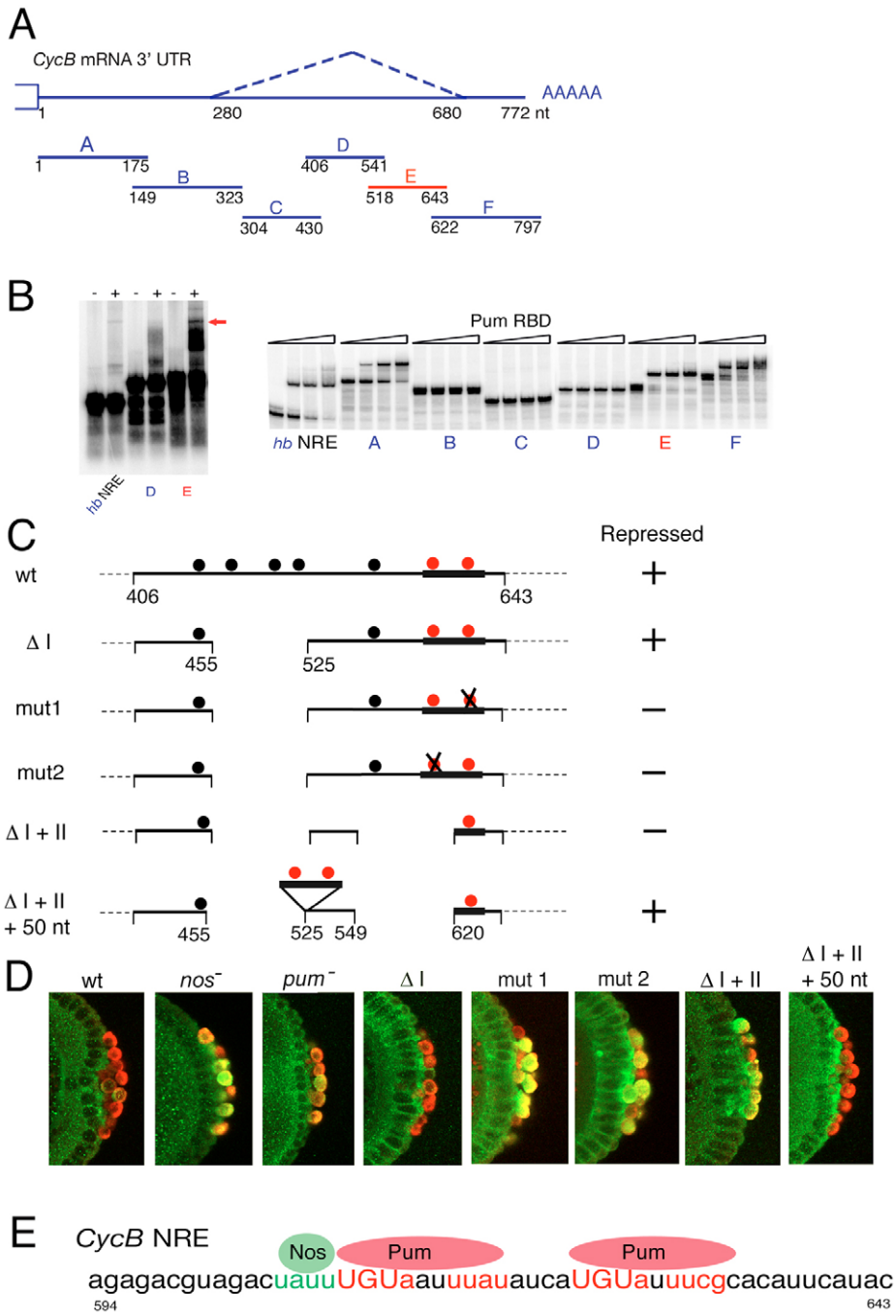

Fig. 1. Identification of a Nos Response Element in the *CycB* 3′ UTR. (**A**) Drawing to scale of the *CycB* 3′ UTR and the fragments used in the experiments below. The critical regulatory region (fragment E in red) is present in the maternal mRNA isoform but spliced out of the zygotic isoform (dashed line). Numbers identify the 3′ UTR nucleotides present in each fragment. Note that fragment F contains 25 nt encoded by genomic DNA downstream of the polyadenylation and cleavage site. (**B**) Gel mobility shift experiments with RNA bearing the *hb* NRE or various fragments of the *CycB* 3′ UTR. On the left, RNA was incubated with embryonic extract prepared as described (Murata and Wharton, 1995). On the right, the Pum RBD was incubated at concentrations of 0, 0.14, 0.42 and 1.3 μM in lanes 1-4 of each titration. The figure is a composite of two different gels. (**C**) Drawing of the region spanning fragments D and E, where each dot represents a potential Pum-binding site (UGU trinucleotide). The functionally defined 50 nt *CycB* NRE (black box, nts 594-643 of the 3′ UTR) contains two such UGU trinucleotides (red dots, see the sequence in E). Repression in the PGCs at stage 4 for various derivatives is indicated to the right. (**D**) Accumulation of CycB (green) in stage 4 embryos in which the PGCs are marked by accumulation of Vasa (red), detected by immunofluorescence and confocal microscopy. In these and all subsequent images, repression of *CycB* mRNA causes the pole cells to appear red, whereas de-repression results in co-localization of CycB and Vasa, causing the pole cells to appear yellow-orange. Embryos are either from w^{1118} (wt) nos^{BN}, or pum^{ET3}/pum^{Msc} females, or $CycB^{2}$/+ females that also bear the indicated *CycB* transgene. (**E**) Sequence of the 50 nt *CycB* NRE (nts 594-643 of the 3′ UTR). Binding sites for Nos and Pum are inferred from experiments with purified proteins and a collection of mutant RNAs. Supporting data is presented in Fig. S3 of the supplementary material.

RESULTS

Maternal *Cyclin B* mRNA regulated by binding of Pum and Nos

A sequence required for translational repression of *CycB* mRNA in the PGCs was previously mapped to nts 430-469 of the 3′ UTR (within Fragment D in Fig. 1A) by microinjection of modified mRNAs encoding epitope-tagged protein (Dalby and Glover, 1993). However, we found that neither the full-length Pum in embryonic extracts nor a purified untagged Pum fragment consisting of little more than the RBD (residues 1092-1433) bound detectably to Fragment D in gel mobility shift experiments (Fig. 1B).

We showed that nts 594-643 of the 3′ UTR comprise an NRE necessary for repression of *CycB* in the PGCs, using a combination of molecular genetic and biochemical experiments that are summarized below, with supporting evidence presented in Fig. 1C-E and see Figs S1, S2 and S3 in the supplementary material. The *CycB* NRE contains two UGUA motifs that are present in most Pum-binding sites (Gerber et al., 2006). Two complexes were detectable in gel mobility shift experiments using a short RNA substrate bearing the wild-type NRE. Analysis of Pum binding to various mutant NREs is consistent with the idea that one Pum RBD recognizes each UGUA motif (plus flanking nts). Mutations in either motif reduced Pum binding in vitro and abrogated repression in the PGCs, suggesting that normal regulation requires binding of Pum to both UGUA motifs. Nos was recruited to Pum-*CycB* NRE complexes (see Fig. S3 in the supplementary material), much as it is recruited to Pum-*hb* NRE complexes (Sonoda and Wharton, 1999). However, Brat was recruited only to the Pum-Nos

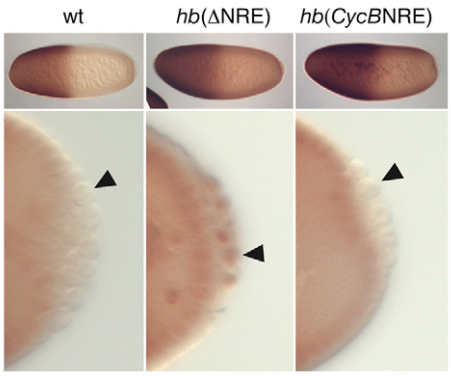

Fig. 2. The *CycB* NRE confers regulation on *hb* mRNA exclusively in the PGCs. Accumulation of Hb in embryos from wild-type (wt) females or females carrying the indicated *hb* transgene, with high magnification views (of other embryos) shown below to allow visualization of the pole cells (marked with arrowheads). Accumulation in the anterior ~50% of the embryo derives from both unrepressed maternal mRNA and zygotically transcribed mRNA; accumulation in the posterior ~50% of the embryo derives primarily from unrepressed maternal mRNA (in the transgenic embryos), with a minor contribution at the stage shown from zygotic transcription under the control of the terminal system (most easily seen in the wild-type embryo).

complex assembled on the *hb* NRE and not the corresponding complex assembled on the *CycB* NRE (see Fig. S3 in the supplementary material); this finding is consistent with the observation the *CycB* regulation is Brat-independent (Sonoda and Wharton, 2001).

We next wished to determine whether the *CycB* NRE is sufficient to confer regulation on another maternal mRNA, and if so, whether regulation is restricted to the PGCs. To this end, we constructed a transgene encoding a chimeric *hb* mRNA in which the *CycB* NRE is substituted for the native *hb* NRE, and assayed the distribution of Hb in embryos from transgenic females.

As shown in Fig. 2, the *CycB* NRE imparted *CycB*-like regulation on *hb* mRNA: protein accumulation was blocked but only in the PGCs at the posterior extreme of the embryo. Hb protein accumulated throughout the posterior of *hb*(*CycB* NRE) embryos, a distribution that was indistinguishable at all stages examined from that in *hb*(ΔNRE) embryos. The ectopic Hb in *hb*(*CycB* NRE) embryos blocked all abdominal segmentation, as described previously for *hb*(ΔNRE) embryos (Wharton and Struhl, 1991). We conclude that the *CycB* NRE mediates repression in the PGCs but is not normally functional in the prospective somatic cytoplasm, even immediately adjacent to the PGCs, where high levels of Nos accumulate (albeit transiently).

Interaction of Nos with a deadenylase complex

Although the mechanism by which *CycB* mRNA is repressed is not yet known, two lines of evidence suggested that deadenylation catalyzed by the CCR4-Pop2-NOT complex is likely to be involved. First, *CycB* mRNA is de-regulated and hyper-adenylated in ovaries from flies hemizygous for a partial loss-of-function allele of *CCR4* (also known as *twin* – Flybase) (Morris et al., 2005). Consistent with these observations, we find that *CycB* was de-repressed in the PGCs of embryos from *CCR4* mutant females (Fig. 3A). The second line of evidence comes from a recent study of budding yeast MPT5 (structurally and functionally related to Pum), which suggested that Puf domain proteins generally interact with the Pop2 subunit of the

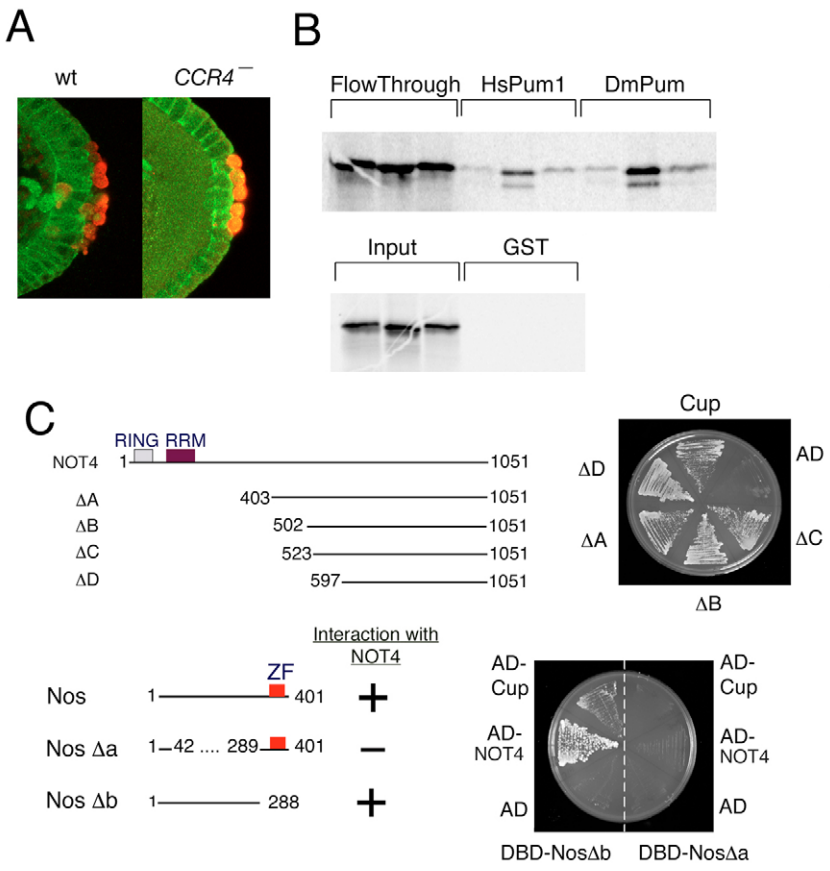

Fig. 3. Interaction of NOT4 with the N-terminal region of Nos. (**A**) CycB (green) and Vasa (red) accumulation in embryos from wild-type (wt) and *CCR4* ^{KG877}/*Df*(3R)*crb*-F89-4 females. (**B**) GST-pulldown experiments show that Hs Pum1 and Dm Pum interact in a similar fashion with three different Pop2 homologs; each GST fusion was incubated with Hs CNOT7 (lane 1), Hs CNOT8 (lane 2) and Dm Pop2 (lane 3). Note that a truncated fragment of CNOT8 generated during in vitro synthesis binds preferentially to both Pum RBDs. (**C**) Four different fragments of NOT4 interact with full-length Nos in yeast, as does Cup [supporting growth –His + 1 mM 3-aminotriazole (3-AT)]. At the bottom, interaction of NOT4 residues 597-1051 with various portions of Nos is summarized on the left, based on growth –His + 5 mM 3-AT, shown on the right. Inclusion of residues 1-42 of Nos is necessary for efficient expression of the C-terminal zinc finger domain (ZF) in yeast. The AD-Cup fusion provides a positive control and the AD encoded by the empty pGAD424 vector provides a negative control.

deadenylase complex to regulate mRNA stability or translation (Goldstrohm et al., 2006). Consistent with this idea, we found that the Pum RBD bound to *Drosophila* Pop2 in GST-pulldown experiments (Fig. 3B). As is the case for Puf proteins from *H. sapiens*, *S. cerevisiae* and *C. elegans* (Goldstrohm et al., 2006), *Drosophila* Pum also bound to heterologous Pop2 proteins (Fig. 3B).

If *Drosophila* Pum interacts directly with Pop2, then what is the role of Nos? Unlike regulation by the yeast Puf protein MPT5 (which is thought to act without any co-factor), regulation by *Drosophila* Pum is absolutely dependent on its ability to recruit Nos to the NRE. One possibility is that Nos helps recruit the deadenylase complex, stabilizing its binding to the 3′ UTR and increasing the probability of deadenylation. We did not observe binding of Pop2 to Nos in yeast two-hybrid experiments (not shown). However, we isolated four different clones encoding C-terminal fragments of NOT4 (another component of the deadenylase complex) in two different yeast interaction screens in which Nos was part of the bait (Fig. 3C). The interaction between Nos and NOT4 was somewhat more robust than the interaction between Nos and Cup, shown previously to be biologically relevant during early oogenesis (Verrotti and Wharton, 2000). Binding of NOT4 to Nos was mediated by its N-terminal region (Fig. 3C), which is essential for Nos activity, despite being very poorly conserved (Curtis et al., 1997). Taken with previous work (Sonoda and Wharton, 1999), these results suggest that Nos acts as a bridge, contacting Pum with its C-terminal zinc finger and NOT4 with its N-terminal region.

In principle, the Nos-NOT4 interaction might be sufficient to regulate *CycB*; alternatively, contacts made by Pum (to Pop2) and Nos (to NOT4) might both be required to efficiently recruit the deadenylase complex and regulate translation. To distinguish between these models, we asked whether tethering Nos via an exogenous RNA-binding domain would repress translation of *CycB* mRNA. To do so, we prepared transgenic flies that express a chimera in which Nos is fused to bacteriophage MS2 coat protein (CP) (Coller and Wickens, 2002) under the control of native *nos* regulatory signals. When crossed into the appropriate background, these *nos-CP* transgenes fully rescued various *nos*$^{-}$ phenotypes, demonstrating that the fusion protein is functional when recruited to target mRNAs via Pum. Next, we prepared transgenic flies expressing a *CycB*(2x MS2hp) derivative in which the NRE is replaced with two copies of a short hairpin to which CP binds with high affinity. We then asked whether tethering of Nos via CP can repress the *CycB*(2x MS2hp) mRNA by monitoring CycB accumulation in embryos from doubly transgenic females.

Binding of the Nos-CP chimera repressed translation of *CycB*(2x MS2hp) mRNA in the PGCs (Fig. 4A). *CycB*(2x MS2hp) mRNA was not repressed in the PGCs by wild-type Nos, presumably because its natural binding partner, the Pum-*CycB* NRE complex, could not form (in the absence of the NRE). However, *CycB*(2x MS2hp) mRNA was repressed upon co-expression of Nos-CP, even in the absence of *pum* function. Apparently, the sole role of Pum in regulation of *CycB* is to recruit Nos.

We considered the possibility that tethering Nos via the high-affinity CP-MS2hp interaction might reveal a weak intrinsic capacity of Nos for translational regulation that is normally significant only with additional contributions from bound Pum. If so, we might expect that Nos-CP would bypass the requirements for both Pum and Brat and regulate an analogous *hb*(2x MS2hp) chimeric mRNA (bearing a substitution of MS2hp sites for the endogenous NRE). As shown in Fig. 4B, we saw no evidence of such regulation: Hb accumulated uniformly throughout the posterior somatic cytoplasm

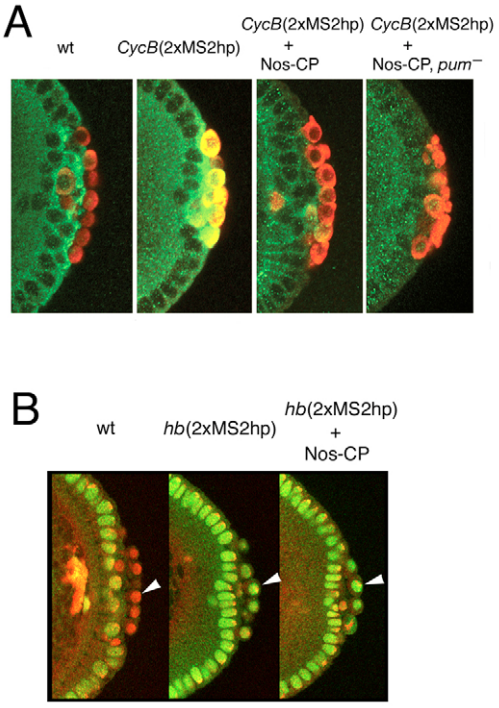

Fig. 4. Tethered Nos can repress *CycB* but not *hb*. (**A**) Accumulation of CycB (green) monitored in the primordial germ cells, marked by accumulation of Vasa (red). The relevant maternal genotypes are: (1) $CycB^{+}/CycB^{+}$ (wild type, wt), (2) $CycB^{3}/CycB^{+}$; *CycB*(2x MS2hp), (3) $CycB^{3}/CycB^{+}$, *Nos-CP*; *CycB*(2x MS2hp), (4) $CycB^{3}/CycB^{2}$, *Nos-CP*; pum^{ET3} / *CycB*(2x MS2hp), pum^{Msc} (all transgenes single-copy). (**B**) Accumulation of Hb (green) in embryos from wild-type females or females bearing the indicated transgenes, with pole cells marked by arrowheads and nuclei labeled with TOPRO3 (red). Note that the accumulation of Hb in somatic nuclei at the posterior of the transgenic embryos derives from both *torso*-dependent zygotic transcription and unrestrained translation of the maternal *hb*(2x MS2hp) mRNA, whereas wild-type embryos contain only the former source of Hb (as in Fig. 2).

and in the PGCs of embryos bearing maternal *hb*(2x MS2hp) mRNA, whether or not Nos-CP was co-expressed. The failure to observe repression of *hb*(2x MS2hp) even in the PGCs argues against the idea that repression of *CycB*(2x MS2hp) is artefactual.

In summary, the evidence described above supports the idea that, for regulation of *CycB* mRNA in the PGCs, the primary role of Pum is to recruit Nos, which subsequently recruits the deadenylase complex via direct interaction with NOT4.

A germline-restricted co-repressor for *CycB* regulation

Nos and Pum jointly repress *CycB* and (with the help of Brat) *hb* mRNAs in different regions of the embryo. *CycB* is repressed only in the PGCs at the posterior extreme, whereas *hb* is repressed broadly throughout the posterior ~50% of the embryo. How are these very different domains established? A priori, it seemed likely that the spatiotemporal gradient of Nos would be primarily responsible for setting different domain boundaries, because the other known direct regulators of either *CycB* or *hb*, Pum and Brat, are uniform along the anteroposterior axis of the early embryo. According to this idea, the *CycB* NRE is relatively insensitive, tuned to respond only to the high levels of Nos that persist in the PGCs; the *hb* NRE is more sensitive, tuned to respond to the low levels of Nos

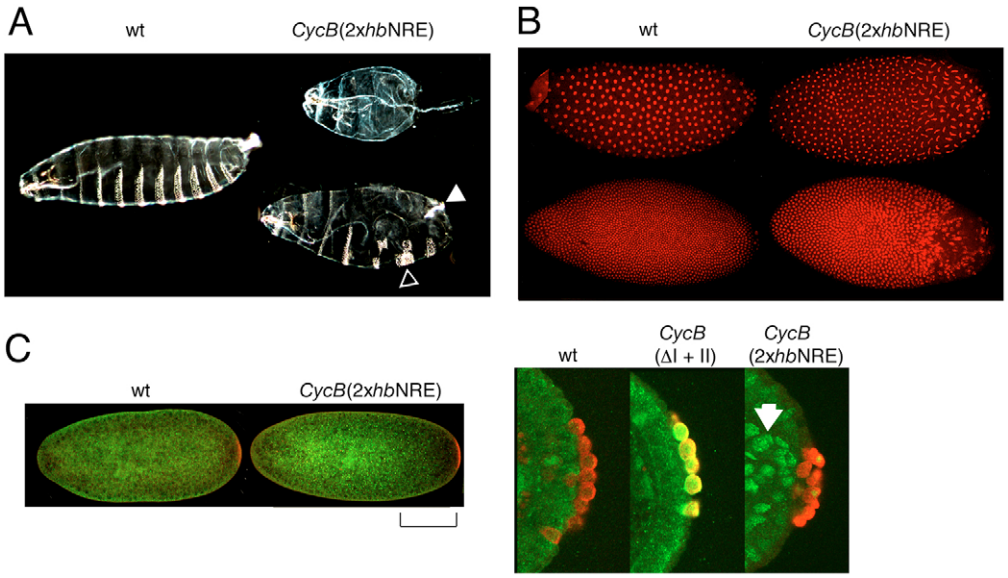

Fig. 5. Deleterious consequences of Nos-dependent repression of *CycB* mRNA outside the PGCs. (**A**) Darkfield photomicrographs of cuticle secreted by embryos from wild-type (wt) or $CycB^2/CycB^3$; *CycB*(2x *hb*NRE) females. Note the reduction of abdominal segments, each marked by a band of large denticles (e.g. black arrowhead) and the rudimentary posterior terminalia (filzkörper, white arrowhead); both features are absent in the limit *CycB* (2x *hb* NRE) phenotype (above). (**B**) Surface views of nuclei in two different cycle 10-13 embryos from females of the indicated genotype. The wild-type embryos are from $CycB^2$/+ females. Note that many nuclei in the posterior of the embryo at the lower right have fallen into the center and are not visible in the focal plane shown. (**C**) Distributions of CycB (green) and Vasa (red) in mid-line views of embryos from females of the indicated genotype. The posterior region that contains lower levels of CycB is bracketed. To the right are high magnification views of post-cycle 9 embryos, showing repression of *CycB* (2x *hb*NRE) in the PGCs. The white arrowhead highlights nuclei that have fallen into the interior. The embryo on the left is from a $CycB^2$/+ female and the embryo in the middle from a $CycB^2$/+ female bearing the *CycB* (ΔI+II) transgene.

present transiently in the somatic cytoplasm nearer the middle of the embryo. In this model, Nos is the sole spatially limiting factor that determines the domain of regulation along the anteroposterior axis of the early embryo.

As a test of this idea we asked whether overexpression of Nos in the anterior somatic cytoplasm from a *nos-bcd* chimeric mRNA results in ectopic repression of *CycB* mRNA. To our surprise, we saw no evidence of such repression (not shown), despite the ability of the ectopic Nos in these embryos to repress *hb* mRNA in the anterior (Gavis and Lehmann, 1992). We considered the possibility that maternal *CycB* mRNA is resistant to repression in the somatic cytoplasm (e.g. by virtue of a *cis*-acting activation signal that overrides repressive signals). To test this idea, we constructed a transgene encoding a chimeric *CycB* mRNA in which two copies of the *hb* NRE are substituted for the native signal. When embedded in the 3′ UTR of either *hb* or *torso* mRNAs, the *hb* NRE mediates repression broadly throughout the posterior 50% of the embryo (Wharton and Struhl, 1991).

Expression of *CycB*(2x *hb* NRE) mRNA causes no obvious dominant phenotypes in a wild-type background and rescues the oogenesis defects associated with *CycB* null alleles (Jacobs et al., 1998). However, we observed profound developmental defects in embryos in which the sole source of maternal CycB derives from the chimeric transgene. As shown in Fig. 5A, over 95% of $CycB^{-}$; *CycB*(2x *hb* NRE) embryos had defects in the abdominal segments, the posterior terminalia or both. In the extreme, embryos die with an open posterior hole and no segments following the third thoracic. These development defects appear to arise during nuclear cleavage cycles 9-13, when oscillations in Cyclin/Cdk2 activity first regulate nuclear divisions (Edgar et al., 1994). As shown in Fig. 5B, somatic nuclei in the posterior of $CycB^{-}$; *CycB*(2x *hb* NRE) embryos delayed progression through cycles 9-13 and ultimately lost contact with the embryonic cortex, falling into the yolky interior. Nuclear cycle defects were limited to the posterior and were not observed during cycles 1-8, when divisions are independent of fluctuation in Cyclin/Cdk2 activity (Edgar et al., 1994). Taken together, these observations suggest that CycB activity can be significantly repressed in the somatic cytoplasm via the *hb* NRE.

The *hb* NRE imparts *hb*-like regulation on *CycB* mRNA by another criterion: during the initial nuclear cleavages, CycB protein accumulation was repressed in the posterior of ~40% of $CycB^{-}$; *CycB*(2x *hb* NRE) embryos (Fig. 5C). After nuclear division cycle 9, however, CycB was uniform along the anteroposterior axis of all such embryos, except in the PGCs, where accumulation was efficiently repressed (Fig. 5C). The apparent difference between the response of *hb* and *CycB* mRNAs to regulation mediated by the *hb* NRE is probably due to a number of factors (e.g. the stability of repressed *CycB* mRNA, nuclear sequestration of Hb, dissipation of the somatic Nos gradient after cycle 9). Nevertheless, the salient finding is that repression of *CycB* can be observed in the somatic cytoplasm, ruling out the possibility that the mRNA is intrinsically resistant to regulation.

As repression of *CycB* in the somatic cytoplasm has a much more dramatic effect on nuclear division than on CycB protein accumulation, we re-examined the question of whether ectopic Nos in the anterior of *nos-bcd* embryos can repress *CycB*, which we did by examining nuclear morphology. As shown in Fig. 6, CycB-dependent progression through syncitial nuclear cleavages 9-13 was normal, despite the presence of high levels of persistent Nos in such embryos. The obvious difference between the ectopic Nos in the somatic anterior and the endogenous Nos at the posterior of *nos-bcd* embryos is that the latter is accompanied by germline factors localized in the PGCs.

Therefore, we asked whether ectopic Nos can repress *CycB* mRNA in the presence of pole plasm components. To do so, we examined embryos with a chimeric *oskar-bicoid* mRNA (Ephrussi and Lehmann, 1992). Translation of this chimera generated sufficient Oskar (Osk) at the anterior pole to direct the formation of pole cells, the recruitment of *nos* mRNA and accumulation of

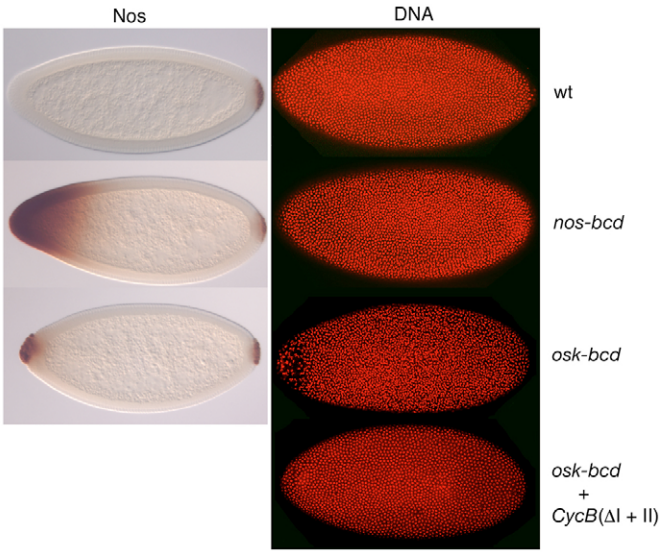

Fig. 6. Ectopic Nos not sufficient to repress wild-type *CycB* mRNA outside the pole plasm. Distributions of Nos (left) and nuclei (right) in embryos from various females, genotypes indicated at the right. Note the ectopic pole cells at the anterior of the *osk-bcd* embryo.

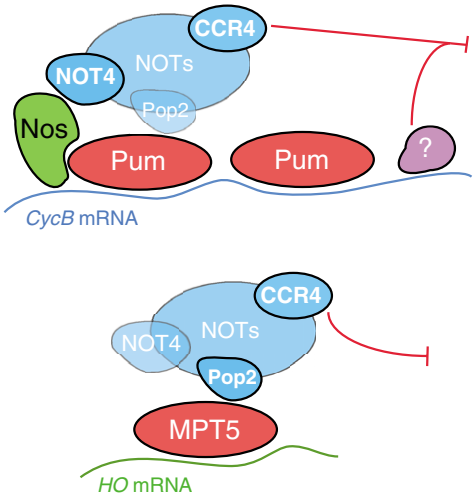

Fig. 7. Puf proteins nucleate the assembly of different repressor complexes in *Drosophila* and budding yeast. Models of the mechanism by which Pum and MPT5 repress *CycB* and *HO* mRNAs in *Drosophila* and *S. cerevisiae*, respectively. The data of Figs 2 and 4 are consistent with the idea that a germline-limited co-factor (magenta) binds directly to the *CycB* NRE; however, other roles for the co-factor are possible.

anterior Nos to levels only slightly higher than those generated in the posterior under native regulatory signals (Fig. 6). Osk also accumulates to somewhat lower levels in the adjacent somatic cytoplasm at the anterior, in contrast to the endogenous Osk at the posterior, which is confined to the PGCs (Ephrussi and Lehmann, 1992). We observed significant defects in progression through nuclear cleavages 9-13 near the anterior pole in 20% of *osk-bcd* embryos (Fig. 6). These defects were due to inappropriate repression of *CycB* mRNA, as they were rescued if the embryos contained, in addition to the wild-type *CycB* mRNA, the unrepressible ΔI+II mRNA that lacks a functional NRE (Figs 1, 6). Evidently, in 20% of *osk-bcd* embryos, the level of Osk in the anterior somatic cytoplasm was sufficient to recruit or stabilize at least one essential co-repressor that acted in conjunction with Nos (and Pum) to repress *CycB* mRNA.

In summary, Nos is not the sole spatially limiting factor for regulation of *CycB*, as it is for regulation of *hb*. Repression of *CycB* mRNA is confined to the PGCs jointly by a requirement for high levels of Nos and by at least one other (as yet unidentified) factor that is restricted to the pole plasm.

DISCUSSION

We have shown that Nos and Pum directly regulate maternal *CycB* mRNA, binding to an NRE in its 3′ UTR. As discussed below, differences in the spacing and arrangement of protein-binding sites within the *hb* and *CycB* NREs appear to account for the regulation of *hb* but not *CycB* by Brat. For regulation of *CycB*, the main function of Pum is to recruit Nos, a role that can be bypassed by tethering Nos via an exogenous RNA-binding domain. *CycB*-bound Nos is then likely to act, at least in part, by recruiting a deadenylase complex, interacting with its NOT4 subunit. Regulation of *CycB* is limited to the PGCs to avoid the deleterious consequences of repression in the presumptive somatic cytoplasm. The requirement for both Nos plus at least one additional germline-restricted factor may be part of a mechanism to ensure that *CycB* regulation is strictly limited to the PGCs.

Binding of Pum and Nos to the *CycB* and *hb* NREs

The co-crystal structure of human Pum bound to a fragment of the *hb* NRE shows that a single Pum RBD directly contacts eight nucleotides of the RNA (Wang et al., 2002b). However, Puf proteins bind with essentially wild-type affinity to many mutant sites (Bernstein et al., 2005; Cheong and Hall, 2006) (L.K., Y.H., T.L. and R.W., unpublished), suggesting that all eight nucleotides are not rigidly specified. How, then, do Puf proteins recognize specific mRNA targets in vivo?

Part of the answer appears to be that, within functional NREs, more than eight nucleotides are recognized, at least by *Drosophila* Pum. Mutations that simultaneously disrupt Pum binding in vitro and regulation in vivo are spread over 20 nts of the *hb* NRE (Wharton et al., 1998) and 18 nts of the *CycB* NRE (see Fig. S3 in the supplementary material). These extended Pum mutational ‘footprints’ are too large to be accounted for by binding of a single RBD; we suggest that two or more Pum RBDs bind each NRE, an idea supported by the detection of two RNA-protein complexes in gel mobility shift experiments using both the *CycB* and *hb* NREs (not shown). This model disagrees with earlier experiments that suggested only a single Pum RBD binds to the *hb* NRE (Zamore et al., 1999). Further biochemical and structural studies will be required to resolve the issue.

The distribution of Pum- and Nos-binding sites within the *CycB* and *hb* NREs is different. In the former, the Nos binding site lies 5′ to the Pum-binding site(s), whereas in the latter, the Nos-binding site is flanked by nucleotides recognized by Pum (Sonoda and Wharton, 1999). We assume that the different arrangement of Nos- and Pum-binding sites is responsible for the assembly of Pum-NRE-Nos complexes with different topographies, such that Brat is recruited to *hb* but not to *CycB*. Further definition of each RNP structure will ultimately be required to understand the combinatorial assembly of different repressor complexes on each NRE.

In addition to the NRE, Pum also binds with high affinity to at least two other sites in the *CycB* 3′ UTR (Fig. 1); however, binding to these sites does not mediate translational repression in the PGCs,

perhaps because neither supports recruitment of Nos. These sites may simply bind Pum fortuitously, or they may mediate Nos-independent regulation at other stages of development. Pum has been suggested to destabilize *bcd* mRNA at the anterior of the embryo in a Nos-independent manner (Gamberi et al., 2002). Another Nos-independent function of Pum is the repression of *CycB* translation throughout the prospective somatic cytoplasm during the early syncitial nuclear cleavages (Tadros et al., 2007; Vardy and Orr-Weaver, 2007). These processes might be mediated by elements in Fragments A and F of the 3′ UTR, which bind Pum but not Nos (Fig. 1, and see Fig. S1 in the supplementary material).

A molecular function for Nos: recruitment of the CCR4-Pop2-NOT deadenylase complex

Recent work from the Wickens lab has provided a general framework for understanding how Puf proteins act to control either the translation or stability of target mRNAs (Goldstrohm et al., 2006). The yeast Puf protein MPT5 interacts directly with Pop2, one of the catalytically active subunits of a large deadenylase complex. Subsequent deadenylation could either silence the mRNA or cause its degradation, depending on other signals in the transcript or the composition of the deadenylase complex (or both). The Puf-Pop2 interaction is conserved across species (including *Drosophila*, Fig. 3), supporting the idea that the mechanism uncovered for MPT5 might generally be applicable to Puf proteins.

In this context, it is surprising that Pum is dispensable if Nos is tethered to *CycB* via MS2 CP. We suggest that yeast Puf proteins both recognize target mRNAs and recruit the deadenylase, but that in the *Drosophila* germline these functions are partitioned, with Pum primarily responsible for target mRNA recognition and Nos primarily responsible for effector recruitment (Fig. 7). This model has the attraction of attributing an important role to Nos, which is essential for Puf-mediated regulation in *Drosophila*, and probably other metazoans as well (Subramaniam and Seydoux, 1999; Jaruzelska et al., 2003). What, then, might be the role of the conserved interaction between Pum and Pop2? One possibility is that it acts cooperatively with Nos to recruit the deadenylase; unlike *CycB*, other mRNA targets (e.g. *hb*) might require recruitment by both Nos and Pum to ensure efficient deadenylation. Another possibility is that it plays an essential role for mRNAs regulated by Pum but not Nos.

Deleterious Nos-dependent repression of maternal *CycB* mRNA in the somatic cytoplasm

Oscillations in CycB activity underlie normal cell cycle progression. During the early embryonic syncitial nuclear cleavages, degradation in the vicinity of the nuclei is thought to deplete CycB locally (Edgar et al., 1994; Su et al., 1998). Recent work has shown that Pum can inappropriately repress de novo translation of *CycB* mRNA during the initial nuclear cleavages if not antagonized by the PNG kinase, resulting in mitotic failure (Tadros et al., 2007; Vardy and Orr-Weaver, 2007). This early Pum-dependent repression is thought to be Nos-independent, as it occurs efficiently in the anterior, where Nos activity is undetectable.

Our results (Figs 5, 6) show that if *CycB* is inappropriately subjected to Pum+Nos-dependent repression via the *hb* NRE, CycB is locally depleted, resulting in mitotic failure during nuclear division cycles 10-13. As is thought to be the case during the early cycles (1-7), de novo synthesis of CycB apparently is required to counteract the local degradation that probably occurs during M phase of each cycle. The *CycB* NRE must therefore be precisely tuned to repress translation only in the PGCs and not in the presumptive somatic cytoplasm.

A germline-restricted co-repressor for regulation of *CycB*

Osk is the limiting factor for assembly of pole plasm in the embryo (Ephrussi and Lehmann, 1992; Smith et al., 1992); our results suggest that it stimulates the accumulation or activity of at least one factor in addition to Nos that is required for repression of *CycB* in the PGCs. The existence of a co-factor is inferred from the finding that ectopic Nos can repress *CycB* in the somatic cytoplasm only in the presence of ectopic Osk (Fig. 6). Regulation of *CycB* may depend on more than one germline-restricted factor to ensure that potentially deleterious repression does not occur in the somatic cytoplasm.

A germline Nos co-factor might act in a variety of ways. It could bind to the *CycB* NRE adjacent to Pum and Nos, substituting functionally for Brat, which is recruited to the Pum-*hb* NRE-Nos complex. The 50 nt *CycB* NRE is inactivated by a truncation at both ends that leaves the Pum- and Nos-binding sites intact, consistent with the idea that another factor binds to the element (see Figs S2 and S3 in the supplementary material). Another possibility is that the co-factor is a germline-specific component of the adenylation/deadenylation machinery, as is the case for the GLD-2 cytoplasmic poly(A)-polymerase in *C. elegans* (Wang et al., 2002a). Distinguishing among these ideas awaits identification of the co-factor.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/134/8/1519/DC1

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