The *nanos* translational control element represses translation in somatic cells by a Bearded box-like motif

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Abstract

Developmental control of translation is frequently mediated by regulatory elements that reside within 3′ untranslated regions (3′ UTRs). Two stem-loops within the *nanos* 3′ UTR translational control element (TCE) act independently to direct translational repression of maternal *nanos* mRNA in the ovary or embryo. We have previously shown that the *nanos* TCE can also function in select somatic sites. Using an ectopic expression screen, we now identify a new site of TCE function, the dorsal pouch epithelium. Analysis of TCE mutants reveals that TCE activity in the dorsal pouch does not depend on either of the stem-loops required for maternal TCE function, but instead requires a third feature—a sequence that closely matches the Bearded box, a regulatory motif found in the 3′ UTRs of several *Notch* pathway genes. In addition, we identify *pleiohomeotic* mRNA as an endogenous candidate for regulation by Bearded box-like motifs in the dorsal pouch. Together, these results suggest that the TCE has appropriated a conserved regulatory motif to expand its function to somatic tissues.
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Introduction

Control of mRNA translation plays an important role in the spatial and temporal regulation of gene expression during development. Studies of numerous maternal mRNAs, whose transcription occurs prior to fertilization, have revealed widespread use of translational control mechanisms to limit when and where the proteins they encode are synthesized. Translational regulation is not limited to maternal mRNAs, however. Sex-specific regulation of dosage compensation in *Drosophila*, cell lineage decisions during larval development in *C. elegans*, temporal regulation of organelle destruction during mammalian erythrocyte differentiation, and synaptic plasticity all rely on translational control of zygotically transcribed mRNAs (Banerjee and Slack, 2002; Klann and Dever, 2004; Kuersten and Goodwin, 2003; Steward and Schuman, 2003; Wilkie et al., 2003).

The role of translational control of maternal mRNAs in anterior–posterior patterning of the Drosophila embryo is well established. In the anterior of the embryo, Bicoid (Bcd) directs head and thorax development by activating transcription of genes like *hunchback* (*hb*) and repressing translation of *caudal* mRNA (Ephrussi and St Johnston, 2004). In the posterior, Nanos (Nos) protein represses translation of maternal *hb* mRNA to exclude Hb protein from the posterior, thereby permitting expression of genes required for abdominal development (Hülskamp et al., 1989; Tautz and Pfeifle, 1989). Conversely, synthesis of both Bcd and Hb proteins in the anterior of the embryo requires that Nos be limited to the posterior (Gavis and Lehmann, 1992, 1994; Wharton and Struhl, 1989). This restricted distribution of Nos is generated by selective translation of a subset of *nos* mRNA that is localized to the germ plasm at the posterior of the embryo coupled with translational repression of *nos* mRNA distributed throughout the bulk cytoplasm (Bergsten and Gavis, 1999; Gavis and Lehmann, 1994).

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Both posterior localization and translational repression of *nos* RNA are mediated by the *nos* 3′ untranslated region (3′ UTR) (Gavis and Lehmann, 1992, 1994). A 90-nucleotide translational control element (TCE) within the *nos* 3′ UTR confers repression through formation of two stem-loop structures, whose functions are temporally distinct (Crucs et al., 2000; Forrest et al., 2004). One stem-loop contains the binding site for Smaug (Smg), which represses *nos* translation in the early embryo (Dahanukar et al., 1999; Smibert et al., 1996, 1999). The second stem-loop mediates translational repression during oogenesis, presumably by binding to an ovarian factor (Forrest et al., 2004).

Although *nos* expression was first thought to be restricted to the germline and early embryo, recent work has revealed a role for *nos* in dendritic morphogenesis in the Drosophila peripheral nervous system (PNS). Moreover, the TCE can regulate *nos* translation in Class IV dendritic arborization (da) neurons in the PNS (Ye et al., 2004). The ability of the TCE to function in the PNS, together with evidence that Smg and other translational regulators have multiple RNA targets, suggests that translational control by TCE-like motifs may be used more widely for regulation of other mRNAs in somatic tissues. Using an ectopic expression assay, we have shown that the TCE can repress translation in cells of the central nervous system (CNS) involved in the ecdysis signaling pathway, although the regulated mRNA has not been identified in this case (Clark et al., 2002).

In a screen for somatic tissues that support TCE function, we have now identified the dorsal pouch epithelium as one such tissue. By analyzing the activity of TCE mutants in the dorsal pouch epithelium, we show that TCE function in these cells does not depend on the same sequence and structural features required for its function in the oocyte and early embryo. Rather, activity of the TCE in the dorsal pouch requires a sequence that closely matches the Bearded (Brd) box, a motif found in the 3′ UTRs of genes involved in the *Notch* signaling pathway (Leviten et al., 1997). Brd boxes have been shown to mediate both RNA degradation and translational control of a heterologous RNA (Lai and Posakony, 1997). A Brd box-like motif embedded within the second TCE stem-loop effects translational control in the dorsal pouch, without significantly affecting RNA stability. We propose that the TCE evolved as a multi-use regulatory element that functions by distinct mechanisms in different tissues.

Since *nos* is not expressed endogenously in the dorsal pouch epithelium, the finding that the TCE is capable of repression in this tissue suggests that one or more endogenous mRNAs are similarly regulated. We identify *pleiohomeotic* (*pho*) mRNA as a candidate for translational repression in the dorsal pouch by Brd box-like motifs. Thus, Brd box-like motifs may be used by a wide range of RNAs for translational regulation in somatic tissues.

Materials and methods

Fly stocks

The following mutants and GAL4 lines were used: $y\ w^{67c23}$ (Lindsley and Zimm, 1992), pum^{680} (Lehmann and Nüsslein-Volhard, 1987), smg^1 and Df(Scf^{R6}) (Dahanukar et al., 1999; Lindsley and Zimm, 1992), *h-GAL4* (Brand and Perrimon, 1993), and $GAL4^{24B}$ (Luo et al., 1994).

Construction of transgenes and transgenic lines

The *UAS-nos-tub3′UTR*, *UAS-nos-tub:nos+2*, *UAS-nosΔB-tub3′UTR*, and *UAS-luc-tub3′UTR* transgenes have been previously described (Clark et al., 2002). For technical reasons, all of the other transgenes were generated in the pUAST derivative, pUASTβ, in which the *BamHI* fragment containing the UAS, hsp70 TATA, polylinker and SV40 polyadenylation signal sequences is inverted (R. Ray, personal communication). For consistency, a new *UAS-nos-tub3′UTR* transgene was generated in pUASTβ and used in the experiments shown in Figs. 4–6. For all *UAS-nos-tub3′UTR* derivatives, DNA fragments encoding the relevant 3′ UTR sequences were inserted at a unique, engineered *NheI* site within the α-tubulin 3′ UTR sequences. The *NR* fragment includes nucleotides 329–438 of the *nos* 3′ UTR, which lack localization and translational regulatory activity. The TCE insert, which includes nucleotides 6–96 of the *nos* 3′ UTR, and the TCEIIA, IIU, IIIA, and SRE⁻ mutants have been described (Crucs et al., 2000; Gavis et al., 1996). Further mutagenesis of TCEIIIA to create TCEIIA/IIIA and mutagenesis of the B1, B2, and B3 motifs were achieved by PCR according to the method of Nelson and Long (1989). Double and triple mutants were generated sequentially. A fragment encompassing nucleotides 42–173 of the *Brd* 3′ UTR was generated by PCR from Oregon-R genomic DNA. The *pho* 3′ UTR fragment (nucleotides 1989–2378) was generated by PCR of the *pho* RE17954 cDNA (Flybase). All 3′ UTR inserts were confirmed by sequencing.

For *UAS-pho*, a 2.1-kb *BstN1–KpnI* fragment containing the *pho* coding region and 3′ UTR was excised from the *pho* RE17954 cDNA in pFLc-1 and inserted between the *EcoRI* and *KpnI* sites of pUASTβ after end-filling of the *BstNI* and *EcoRI* sites. To generate *UAS-pho-tub3′UTR*, sequences downstream of a *PsiI* site in the *pho* 3′ UTR including the Brd box-like motifs were removed by digestion of *UAS-pho* with *PsiI* and *XbaI* and replaced by a *ScaI–HindIII* fragment from pTα5′3′ that includes the α-tubulin 3′ UTR and 3′ genomic sequences.

Transgenes were introduced into $y\ w^{67c23}$ embryos by P element-mediated germline transformation (Spradling, 1986) and multiple transgenic lines were isolated and balanced for each transgene.

Generation and screening of GAL4 enhancer trap lines

New GAL4 enhancer trap lines were generated by mobilizing an X chromosome insertion (*109C1*) of *pGAL4-TRAP* (Fuerstenberg and Giniger, 1998). Mobilization followed the scheme of Bier et al. (1989) except that new insertions on the second and third chromosomes were isolated from crosses of individual males to *y w* females. Balanced stocks were established for 143 autosomal GAL4 enhancer trap lines.

Heterozygous males from balanced GAL4 enhancer trap lines were crossed to either *UAS-nos-tub3′UTR* or *UAS-nos-tub:nos+2* females at 25°C and adult progenies were scored for visible phenotypes and lethality. GAL4 lines that produced a phenotype or decreased viability at higher penetrance with *UAS-nos-tub3′UTR* than with *UAS-nos-tub:nos+2* were retested and >100 progeny scored.

Lethality assay

Heterozygous *HD34A-GAL4*/CyO or *h-GAL4*/TM3, Sb females were crossed to males carrying the appropriate UAS transgene at 25°C. Homozygous *GAL4^{24B}* females were crossed to males heterozygous for UAS transgenes and either the CyO or TM3, Sb balancer. Use of dominantly marked balancer chromosomes provided an "expected" class of viable progeny. Adult progeny were scored and lethality was calculated as $1 -$ (number of flies carrying both transgenes/number of flies in the "expected" class). For *HD34A-GAL4*, six to seven independent lines of each transgene were tested. The lethality of an individual line represents an average from two or three experiments, with 100–300 progeny scored per experiment. The lethality values of the individual lines were then used to calculate the mean lethality and standard deviation for each transgene. For *h-GAL4* and *GAL4^{24B}*, two to three independent lines per UAS transgene were tested in two different experiments.

Whole-mount in situ hybridization and antibody staining

Embryos age 12–16 h AEL were collected at 25°C, then fixed, devitellinized, and stored in methanol at −20°C. In situ hybridization was performed according to Gavis and Lehmann (1992). The antisense *nos* RNA probe was synthesized from the *nos* N5 cDNA (Wang and Lehmann, 1991). The antisense *pho* RNA probe was synthesized from the *pho* cDNA RE17954 (Flybase). Embryos were mounted in LX112 embedding medium (Ladd Research Industries, Inc.) and photographed using Nomarski optics.

For immunofluorescence, primary antibodies (1:500 mouse anti-GFP [Clontech], 1:200 rabbit anti-CncB [gift of W. McGinnis], 1:500 rabbit anti-Pho [gift of J. Kassis] were applied overnight at 4°C in BBT (PBS/0.1% BSA/

0.1% Tween 20) and secondary antibodies (1:1000 AlexaFluor 586 goat anti-rabbit, 1:1000 Oregon Green 488 goat anti-rabbit or goat anti-mouse [Molecular Probes]) were applied for 2 h at room temperature in BBT/2% normal goat serum. Embryos were mounted in Aqua Poly Mount (Polysciences) for confocal imaging. Nos protein was visualized as follows. For the *HD34A-GAL4* driver, rabbit anti-Nos antibody (gift of A. Nakamura) was used at 1:1000 as described above and detected using 1:2000 HRP-goat anti-rabbit secondary antibody (Vector Labs) and peroxidase immunohistochemistry. For the *h-GAL4* and *GAL4^{24B}* drivers, anti-Nos antibody was used at 1:500 and 1:1000, respectively, and detected with 1:2000 biotin goat anti-mouse secondary antibody (Jackson) followed by amplification with Vectastain reagent (Vector Labs) and peroxidase immunohistochemistry. Embryos were mounted in LX112 embedding medium (Ladd Industries) and photographed using Nomarski optics.

Results

Screen for tissues competent for TCE-mediated translational regulation

In the early embryo, translation of transgenic *nos-tub3′UTR* RNA, which bears *α-tubulin* 3′ UTR sequences in place of the *nos* 3′ UTR, is not subject to TCE-mediated repression (Gavis and Lehmann, 1994). By contrast, translation of transgenic *nos-tub:nos+2* RNA, a derivative that contains the *nos* TCE and adjacent sequences, is regulated (Gavis et al., 1996). To investigate TCE function in somatic tissues, we generated *UAS-nos-tub3′UTR* and *UAS-nos-tub:nos+2* transgenes (Fig. 1A) that allow expression of *nos-tub3′UTR* and *nos-tub:nos+2* RNAs under control of the GAL4 transcriptional activator. Due to the toxicity of Nos protein, ectopic expression of either RNA by a variety of GAL4 drivers results in lethality or visible phenotypes. However, we identified one GAL4 driver that produces an "adolescent" phenotype, characterized by failure of wing expansion and body cuticle hardening after eclosion, at high frequency when combined with the *UAS-nos-tub3′UTR* transgene but only at low frequency with the *UAS-nos-tub:nos+2* transgene. We showed that the TCE sensitivity of the adolescent phenotype reflects TCE function in cells that impinge upon the ecdysis signaling pathway (Clark et al., 2002).

Here, we have taken advantage of this ectopic expression assay to investigate the prevalence of TCE-mediated regulation. One hundred forty-three new GAL4 enhancer trap lines were generated and crossed to both the *UAS-nos-tub3′UTR* and *UAS-nos-tub:nos+2* transgenes. Progeny bearing both a *GAL4* enhancer trap and *UAS* transgene were screened for lethal and visible adult phenotypes. Approximately 15% of the GAL4 enhancer traps produce
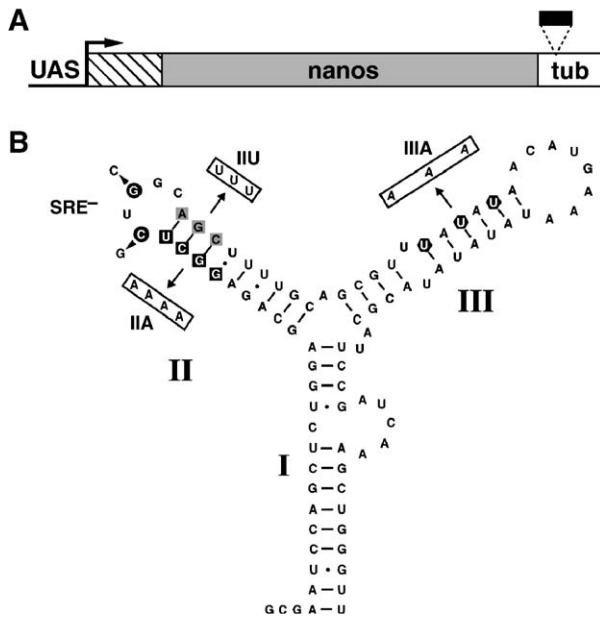
Fig. 1. (A) Structure of the *UAS-nos-tub3′UTR* transgene. The *nos* 5′ UTR (hatched) and coding region (shaded) are fused to sequences from the α-*tubulin* 3′ UTR (open box). Placement of the hybrid *nos-tub3′UTR* cDNA downstream of the yeast UAS permits transcriptional activation by GAL4. The solid box indicates insertion of the *nos* 3′ UTR +2 element, TCE, TCE*, *Brd* 3′ UTR, or *pho* 3′ UTR sequences to generate the corresponding *UAS-nos-tub3′UTR* derivatives. (B) The *nos* TCE showing nucleotide changes associated with the SRE⁻ (circles), IIA (black squares), IIU (gray squares), and IIIA (hexagons) mutations. Stem I and stem-loops II and III are indicated.

the adolescent phenotype in combination with the *UAS-nos-tub3′UTR* transgene. Three of these lines produce the adolescent phenotype at higher frequency with *UAS-nos-tub3′UTR* than with *UAS-nos-tub:TCE* and have been characterized previously (Clark et al., 2002). Approximately 70% of the new GAL4 enhancer traps result in decreased viability (>50% lethality) when combined with either *UAS-nos* transgene. One (*HD34A-GAL4*), however, produces lethality at high frequency (93%) in combination with *UAS-nos-tub3′UTR*, but at significantly reduced frequency (25%) with *UAS-nos-tub:nos+2* (Table 1). Very few other phenotypes were observed and these occur at low frequency with either transgene.

The HD34A-GAL4 enhancer trap drives expression specifically in the dorsal pouch epithelium

The expression domain of the *HD34A-GAL4* enhancer trap was determined using *UAS-lacZ*, *UAS-luciferase*, and *UAS-gfp* reporters. All three reporters show expression localized to a discrete structure in the anterior of the embryo. Immunolocalization of the pharyngeal marker CncB (McGinnis et al., 1998) together with GFP in *HD34A-GAL4/UAS-GFP* embryos revealed that the cells expressing GFP are distinct from, and appear to overlay, the CncB expressing pharyngeal cells (Fig. 2). The relative position of this structure to the pharynx and its morphology identify it as

the dorsal pouch epithelium, a transient epithelial structure that secretes part of the cephalopharyngeal (head) skeleton and contributes to the eye-antennal imaginal disk (Nassif et al., 1998). Attempts to identify the target site of the *HD34A-GAL4* P element insertion by both plasmid rescue and inverse PCR of flanking sequences were unsuccessful due to the presence of nearby repetitive DNA.

The nos TCE can repress translation in the dorsal pouch epithelium

The TCE-sensitive lethality produced by *HD34A-GAL4*, together with the *HD34A-GAL4* expression domain, suggests that translation of transcripts containing the TCE is repressed in cells of the dorsal pouch epithelium. To confirm that the observed lethality results from unregulated production of Nos protein, we generated flies carrying *HD34A-GAL4* and either of two control transgenes that do not produce a functional Nos protein: *UAS-luc-tub3′UTR*, which contains *luciferase* rather than *nos* coding sequences fused to the α-tubulin 3′ UTR, and *UAS-nosΔB-tub3′UTR*, which contains a nonsense mutation that truncates the Nos protein but does not alter RNA stability in somatic tissues (Clark et al., 2002). Neither of these transgenes produces lethality in combination with *HD34A-GAL4* (Table 1). Nos works together with Pumilio (Pum) protein in the early embryo and the PNS, but may act independently of Pum in germline stem cells (Barker et al., 1992; Forbes and Lehmann, 1998; Ye et al., 2004). Lethality due to ectopic Nos in the dorsal pouch epithelium, like the adolescent phenotype produced by ectopic Nos in the nervous system, is not dependent on *pum* function (Table 1). At present, the basis for Nos toxicity in these tissues is unknown.

In situ hybridization to *nos* RNA in *HD34A-GAL4/UAS-nos-tub3′UTR* and *HD34A-GAL4/UAS-nos-tub:nos+2* embryos showed that *nos-tub3′UTR* and *nos-tub:nos+2* transcripts accumulate to comparable levels in the dorsal pouch epithelium (Figs. 3A and B). Northern blot analysis confirmed this result (data not shown). By contrast, anti-Nos immunostaining reveals differences in Nos protein levels in the dorsal pouch epithelium between *HD34A-*

Table 1
Ectopic expression of UAS transgenes by *HD34A-GAL4*

Transgene	Lethality	
	+	pum⁻/pum⁻
UAS-nos-tub3′UTR	93%	100%
UAS-nos-tub:nos+2	25%	N.D.
UAS-luc-tub3′UTR	0%	N.D.
UAS-nosΔB-tub3′UTR	0%	N.D.

Lethality of animals carrying both the *HD34A-GAL4* driver and a UAS transgene was determined as described in Materials and methods. This dependence of the observed lethality on *pum* was tested in *HD34A-GAL4/UAS-nos-tub3′UTR* animals that were homozygous mutant for *pum*[680]. All other crosses were wild-type for *pum* (+).
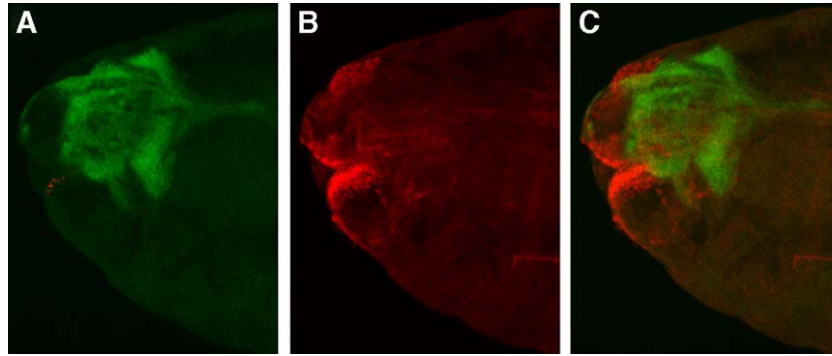
Fig. 2. *HD34A-GAL4* drives expression in the dorsal pouch epithelium. Confocal images of embryos carrying both *HD34A-GAL4* and *UAS-gfp* transgenes. (A) Anti-GFP immunofluorescence showing the expression domain of *HD34A-GAL4*. (B) Anti-CncB immunofluorescence. (C) Merge of A and B.

GAL4/UAS-nos-tub3′UTR and *HD34A-GAL4/UAS-nos-tub:nos+2* embryos. Whereas the majority of *HD34A-GAL4/UAS-nos-tub3′UTR* embryos show robust anti-Nos immunostaining in the dorsal pouch, the majority of *HD34A-GAL4/UAS-nos-tub:nos+2* embryos exhibit weak or no immunostaining (Figs. 3C and D). This TCE-dependent decrease in the amount of Nos protein, but not *nos* RNA, indicates the *nos* TCE can repress translation in the dorsal pouch epithelium. Although we do not know the threshold amount of Nos protein required to produce lethality, the correlation of decreased lethality with decreased Nos protein levels indicates that this phenotype provides a measure of TCE-mediated regulation in the dorsal pouch.

Different cis-acting requirements for TCE function in the dorsal pouch epithelium and the oocyte/early embryo

The ability of the TCE to repress maternal *nos* mRNA in the oocyte and early embryo requires both primary sequence and structural motifs (Crucs et al., 2000). Formation of stem-loop II is specifically required for *nos* repression in the embryo (Forrest and Gavis, 2003). The Smg repressor binds to nucleotides within the loop that comprise the Smaug Recognition Element (SRE). Smg binding and TCE function in the embryo are both disrupted by mutation of the SRE (SRE$^-$, Fig. 1B) or by mutations that prevent base pairing in stem-loop II (e.g., IIA, Fig. 1B) (Crucs et al., 2000; Dahanukar et al., 1999; Smibert et al., 1996). Stem-loop III acts primarily in the ovary and mutations that alter either the sequence or the structure of the stem-loop III helix (e.g., IIIA, Fig. 1B) disrupt TCE-mediated repression in the ovary (Forrest and Gavis, 2003). To determine the cis-acting requirements for TCE function in the dorsal pouch, we generated *UAS-nos-tub:TCE** transgenes containing either the wild-type TCE or a mutant TCE (TCE*) whose function had been previously assayed in the ovary and early embryo (Figs. 1A and B). Unlike *UAS-nos-tub:nos+2*, these transgenes include only the TCE segment of the *nos* 3′ UTR. As an additional control, we generated the *UAS-nos-tub:NR* transgene, which carries an insertion of an unrelated region of the *nos* 3′ UTR with no known regulatory function.
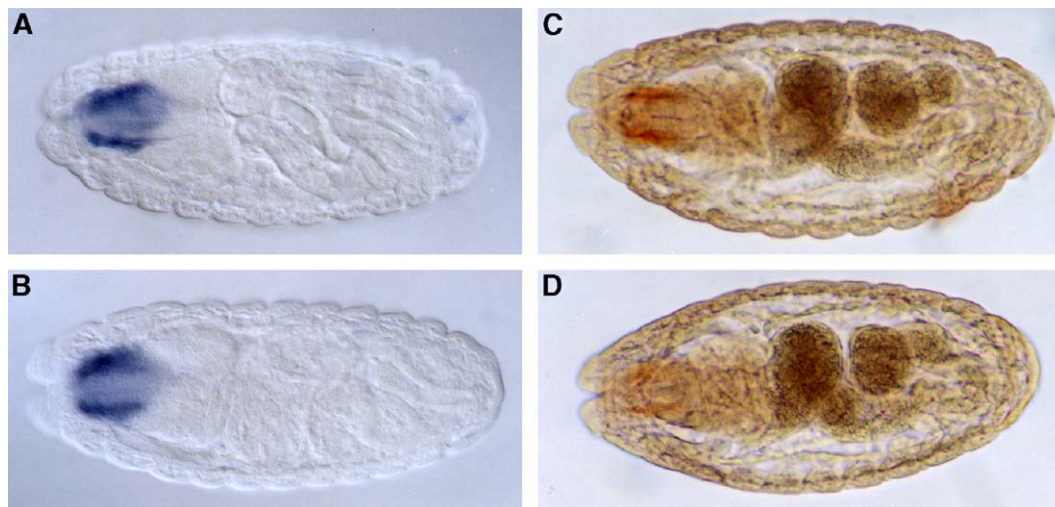

Fig. 3. Ectopic expression of *nos* RNA and protein in the dorsal pouch epithelium. Whole mount in situ hybridization to *nos* RNA in *HD34A-GAL4/UAS-nos-tub3′UTR* (A) and *HD34A-GAL4/UAS-nos-tub:nos+2* (B) embryos shows that the two transgenic RNAs are present at comparable levels in the dorsal pouch. Anti-Nos antibody staining of *HD34A-GAL4/UAS-nos-tub3′UTR* (C) and *HD34A-GAL4/UAS-nos-tub:nos+2* (D) embryos shows that Nos protein is reduced when the transcript contains TCE sequences.
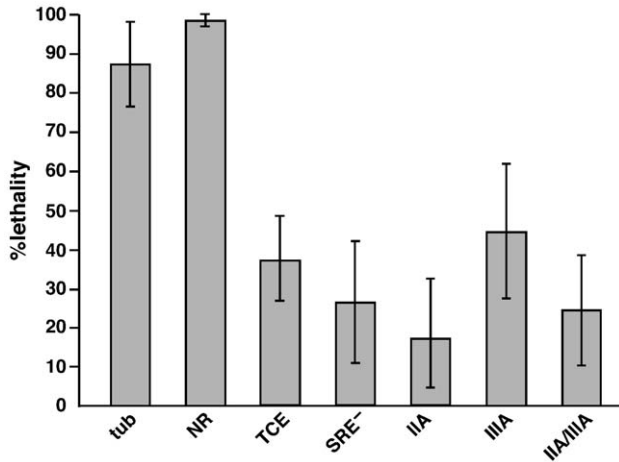
Fig. 4. Effect of TCE mutations on *nos* regulation in the dorsal pouch epithelium. Lethality caused by *HD34A-GAL4* activation of *UAS-nos-tub3′UTR*, *UAS-nos-tub:NR*, *UAS-nos-tub:TCE*, and the indicated *UAS-nos-tub:TCE** transgenes in the dorsal pouch was determined as described in Materials and methods. Error bars reflect variability among independent lines for a given transgene due to their sites of insertion.

As anticipated, the *UAS-nos-tub:NR* transgene behaves similarly to *UAS-nos-tub3′UTR*, producing high levels of lethality when combined with *HD34A-GAL4* (Fig. 4). Likewise, the *UAS-nos-tub:TCE* transgene behaves similarly to *UAS-nos-tub:nos+2*, with lethality significantly reduced in *HD34A-GAL4/UAS-nos-tub:TCE* animals. Surprisingly, *UAS-nos-tub:TCE** transgenes bearing the SRE⁻ mutation or mutations that disrupt stem-loop II (IIA) and III (IIIA) function in the oocyte and embryo also produce low levels of lethality, comparable to each other and to the wild-type *UAS-nos-tub:TCE* transgene. Likewise, a TCE mutant for both stem-loops (IIA/IIIA) behaves similarly to the wild-type TCE (Fig. 4). Northern blot analysis of RNA prepared from *HD34A-GAL4/UAS-nos-tub:TCE** embryos as well as in situ hybridization experiments showed that the behavior of these transgenes does not result from reduced transcript levels (data not shown). Furthermore, the lethality exhibited by *HD34A-GAL4/UAS-nos-tub:TCE* animals does not increase when they are mutant for *smg*, indicating that TCE function in the dorsal pouch is Smg-independent. Thus, TCE function in the dorsal pouch requires cis-acting features distinct from those required for its maternal functions.

A Brd box-like motif in the nos TCE is important for TCE function in the dorsal pouch epithelium

The failure of mutations that disrupt TCE stem-loops II and III to abrogate repression in the dorsal pouch epithelium suggests that these structural features of the TCE are not important for its function in this tissue. Intriguingly, the TCE contains three segments that closely resemble the Brd box, a nine nucleotide motif present three times within the *Brd* 3′ UTR that has been shown to mediate negative post-

transcriptional regulation of *Brd* mRNA (Lai and Posakony, 1997; Leviten et al., 1997). A seven nucleotide Brd box core element is also found in the 3′ UTRs of six *Enhancer of split* complex genes (Leviten et al., 1997). The core elements within two of the TCE's Brd box-like motifs (B2 and B3; Fig. 5A) are completely conserved between *Drosophila melanogaster* and *Drosophila virilis*.

To determine whether the Brd box-like motifs in the TCE are important for translational repression in the dorsal pouch epithelium, we generated TCE mutations
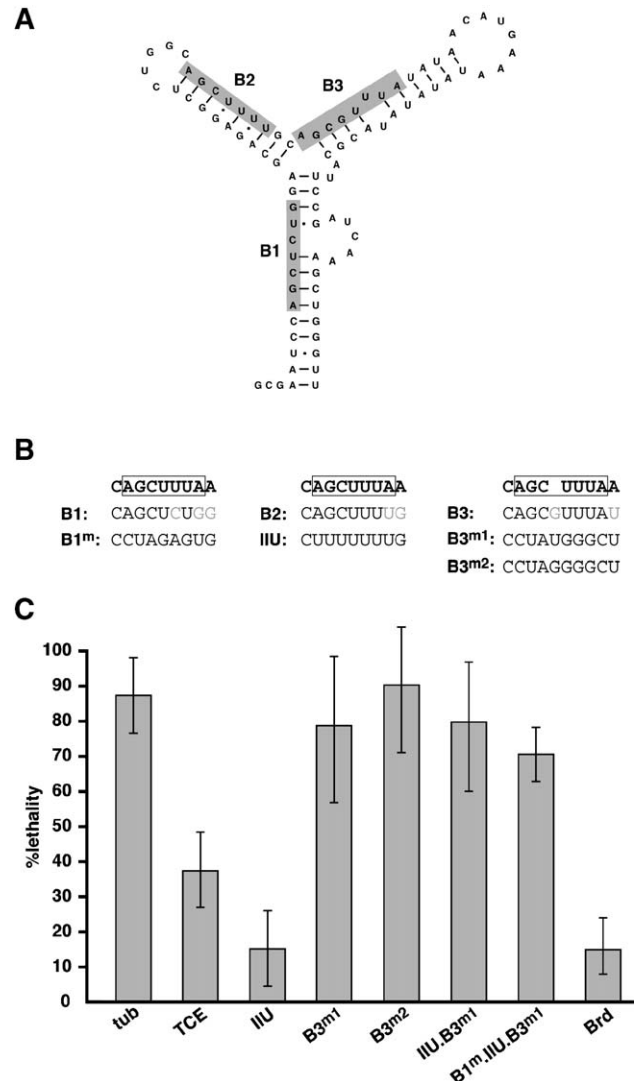

Fig. 5. Effect of mutations in the TCE Brd box-like motifs on regulation in the dorsal pouch. (A) The positions of the Brd-like motif B1, B2, and B3 core elements in the TCE are indicated. (B) Comparison of the sequences of the wild-type B1, B2, and B3 motifs to the full nine nucleotide Brd box consensus (bold face), with nucleotides that deviate from the consensus indicated in gray. The seven-nucleotide core sequence is boxed. The corresponding mutant sequences (B1ᵐ, IIU, B3ᵐ¹, and B3ᵐ²) are shown below. (C) Lethality caused by *HD34A-GAL4* activation of *UAS-nos-tub3′UTR*, *UAS-nos-tub:TCE*, and the indicated *UAS-nos-tub:TCE** transgenes in the dorsal pouch was determined as described in Materials and methods. Error bars reflect variability among independent lines for a given transgene due to their sites of insertion.

that target these motifs, either individually or in combination (Fig. 5B), and tested the effect of these mutations on TCE function in the dorsal pouch. Northern blotting and in situ hybridization experiments showed that these mutations do not affect transcript stability and that significant differences in behavior among different transgenes do not result from differences in RNA expression levels (data not shown). Mutation of the B2 motif does not affect TCE-mediated repression in the dorsal pouch, since *UAS-nos-tub:TCEIIU/HD34-GAL4* embryos show low levels of lethality (Fig. 5C). Because the IIU mutation only alters three of the seven nucleotides within the Brd box consensus, however, we cannot completely eliminate the possibility that the B2 motif contributes to TCE function. By contrast, the *UAS-nos-tub:TCEB3^{m1}* or *UAS-nos-tub:TCEB3^{m2}* transgenes, in which the B3 motif is mutated, behave similarly to *UAS-nos-tub3'UTR* and cause high levels of lethality in combination with *HD34-GAL4* (Fig. 5C). Simultaneous mutation of B2 and B3 produces the same result. Although we have not tested the B1 mutation alone, the lack of B1 element conservation and the behavior of the B1^m.IIU.B3^{m1} triple mutant suggest that the B1 motif plays little, if any, role (Fig. 5C). Taken together, these results show that the B3 motif is the primary cis-acting determinant of TCE function in the dorsal pouch.

Further evidence that Brd boxes can repress translation in the dorsal pouch is provided by the *UAS-nos-tub:Brd* transgene, which carries a portion of the *Brd* 3' UTR containing its three Brd boxes. Although the *Brd* 3' UTR contains neither an SRE or TCE stem-loop III motif, *UAS-nos-tub:brd* behaves similarly to the *UAS-nos-tub:TCE* transgene in combination with *HD34A-GAL4*. This result is most consistent with regulation by the Brd boxes (Fig. 5C).

The TCE and Brd 3' UTRs exhibit different ranges of activity

Previous ectopic expression analysis indicates that the *Brd* 3' UTR, via its Brd boxes, can repress translation in numerous tissues throughout development (Lai and Posakony, 1997). In our ectopic expression screen and previous experiments with selected GAL4 drivers (Clark et al., 2002), the *UAS-nos-tub3'UTR* and *UAS-nos-tub:nos+2* transgenes frequently behaved indistinguishably, suggesting that the TCE, unlike the *Brd* 3' UTR, does not function in most tissues. To determine whether the *nos* TCE and *Brd* 3' UTRs have different ranges of activity, we compared their ability to regulate translation of ectopic *nos* RNA in two major tissues, the embryonic ectoderm and mesoderm.

Expression of either *UAS-nos-tub3'UTR* or *UAS-nos-tub:TCE* in the ectoderm by *h-GAL4* is lethal. By contrast, expression of *UAS-nos-tub:Brd* is substantially less deleterious and the majority of *h-GAL4/UAS-nos-tub:Brd* animals survive (Table 2). Analysis of *nos* RNA

Table 2
Differential activity of the TCE and *Brd* 3' UTR in the ectoderm and mesoderm

Transgene	Lethality	
	h-GAL4	GAL4^{24B}
UAS-nos-tub3'UTR	100%	100%
UAS-nos-tub:TCE	98%	100%
UAS-nos-tub:Brd	35%	100%

The *h-GAL4* and *GAL4^{24B}* drivers were crossed to UAS transgenes to activate expression in the ectoderm and mesoderm, respectively. Lethality of *GAL4/UAS* progeny was determined as described in the Materials and methods. The values shown are the average of two independent experiments with a single line of each UAS transgene. The UAS lines used in this experiment are identical to those whose RNA and protein levels are shown in Fig. 6.

and protein levels revealed that although all three transcripts are present at comparable levels in the embryonic ectoderm, *nos-tub:Brd* RNA yields low levels of Nos protein relative to *nos-tub3'UTR* and *nos-tub:TCE* RNAs (Fig. 6A). Thus, the difference in behavior of the *UAS-nos-tub:Brd* and *UAS-nos-tub:TCE* transgenes reflects a difference in the ability of the *Brd* 3' UTR and TCE to confer translational regulation in the ectoderm.

When expressed in the mesoderm by the *GAL4^{24B}* driver, however, the *UAS-nos-tub3'UTR*, *UAS-nos-tub:TCE*, and *UAS-nos-tub:Brd* transgenes behave identically, producing complete lethality (Table 2). Consistent with this behavior, the *nos-tub3'UTR*, *nos-tub:TCE*, and *nos-tub:Brd* transcripts produce similar amounts of Nos protein (Fig. 6B). Thus, neither the TCE nor the *Brd* 3' UTR can mediate repression in the embryonic mesoderm. Moreover, the Brd 3' UTR may not function as generally as previously suggested (Lai and Posakony, 1997).

Pho is a potential target for translational regulation in the dorsal pouch

While *nos* is normally not expressed in the dorsal pouch epithelium, the ability of the dorsal pouch to support translational repression by the TCE Brd box-like motif suggests that an mRNA endogenous to the dorsal pouch may be repressed by a similar mechanism. *Pleiohomeotic* (*pho*) mRNA, whose 3' UTR contains four Brd box-like motifs (Fig. 7A), is one such candidate. *Pho* encodes a Polycomb group (Pc-G) DNA-binding protein that recruits Pc-G protein complexes to chromatin for transcriptional silencing (Brown et al., 2003; Mohd-Sarip et al., 2002). Whereas *pho* RNA is present at highest levels in a subset of cells within the dorsal pouch and in the trachea at late stages of embryogenesis (Fig. 7B), Pho protein is not detected above background in the dorsal pouch (Fig. 7C). To determine whether the *pho* 3' UTR is capable of repression in the dorsal pouch, *HD34A-GAL4* was used to drive expression of a *UAS-nos-tub:pho3'UTR* transgene. Although their RNA levels are comparable to those from *UAS-nos-*
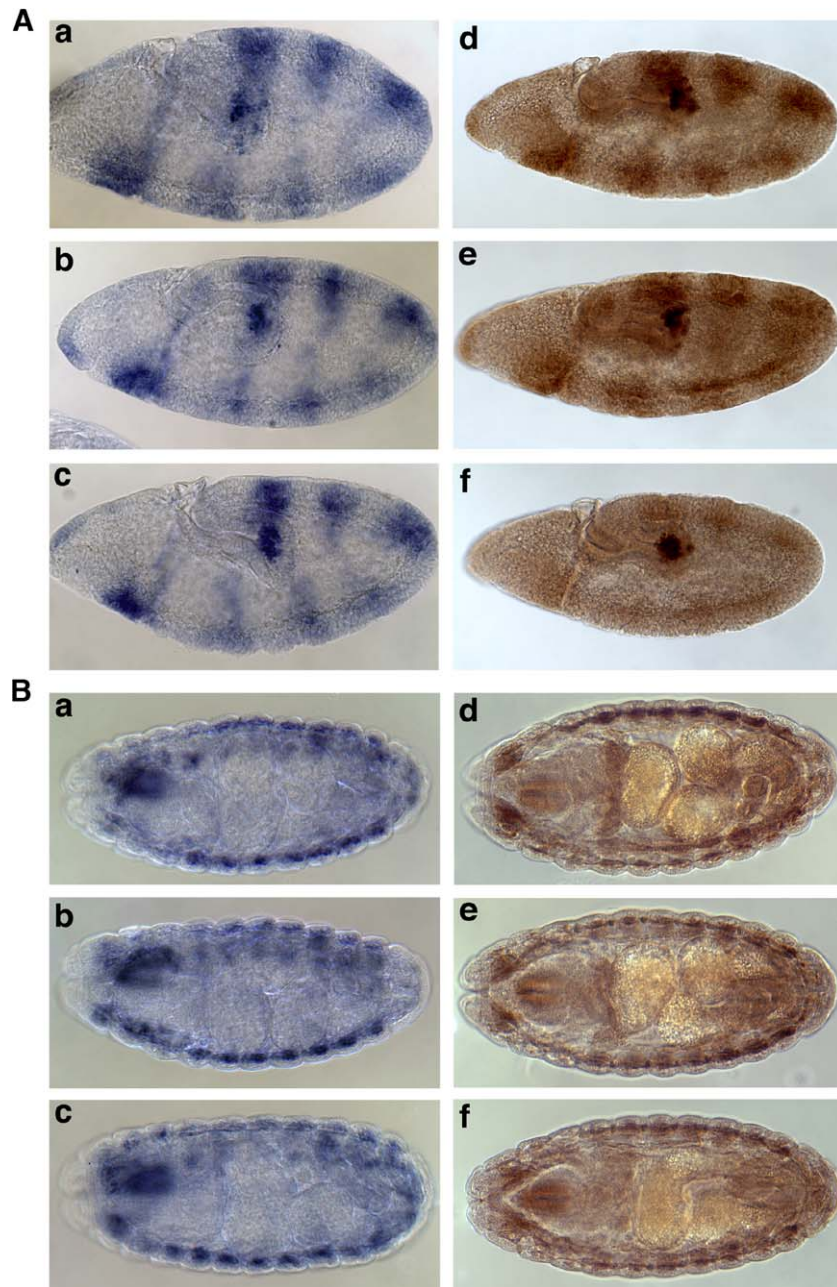
Fig. 6. *Brd* 3′ UTR and TCE activity in the embryonic ectoderm (A) and mesoderm (B). (A) Whole mount in situ hybridization to *nos* RNA (a–c) and anti-Nos antibody staining (d–f) of *h-GAL4/UAS-nos-tub3′UTR* (a and d), *h-GAL4/UAS-nos-tub:TCE* (b and e), and *h-GAL4/UAS-nos-tub:Brd* (c and f) embryos. Whereas *nos* RNA is detected similarly in embryos of all three genotypes, Nos protein is reduced in *h-GAL4/UAS-nos-tub:Brd* embryos. Nos RNA and protein are also detected in germ cells within the lumen of the invaginating posterior midgut. (B) In situ hybridization to *nos* RNA (a–c) and anti-Nos antibody staining (d–f) of *GAL4²⁴ᴮ/UAS-nos-tub3′UTR* (a and d), *GAL4²⁴ᴮ/UAS-nos-tub:TCE* (b and e), and *GAL4²⁴ᴮ/UAS-nos-tub:Brd* (c and f) embryos shows similar accumulation of *nos* RNA and Nos protein in the mesoderm. *Nos* RNA detected in the anterior of the embryo (a–c) is due to *GAL4²⁴ᴮ*-dependent expression in the pharyngeal mesoderm, not the dorsal pouch epithelium. Nos protein is produced similarly in the pharyngeal mesoderm in all three genotypes (d–f), but is out of the plane of focus.

tub3′UTR lines, multiple independent *UAS-nos-tub:pho3-′UTR* transgenic lines produced no lethality in combination with *HD34A-GAL4* (data not shown). These results are consistent with 3′ UTR-mediated repression of *pho* RNA in the dorsal pouch.

To begin to investigate the role for 3′ UTR-mediated repression of *pho*, we generated a *UAS-pho* transgene bearing the intact *pho* 3′ UTR and a UAS-*pho-tub3′UTR* transgene that lacks the distal two thirds of the *pho* 3′ UTR including the Brd-like boxes. Neither of these transgenes contain SRE or TCE stem-loop III motifs. Although both transgenes produce substantial amounts of RNA in the dorsal pouch when combined with *HD34A-GAL4* (Figs. 8A and B), neither produced a detectable phenotype.
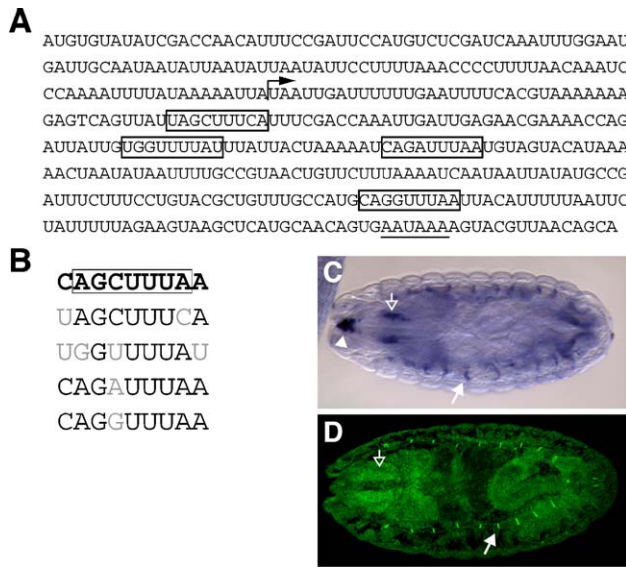
A

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AUGUGUAUAUCGACCAACAUUUCCGAUUCCAUGUCUCGAUCAAAUUUGGAAU
GAUUGCAAUAAUAUUAAUAUUAAUAUUCCUUUUAAACCCCUUUUAACAAAUC
CCAAAAUUUUAUAAAAAUUAUAAUUGAUUUUUUGAAUUUUCACGUAAAAAAA
GAGUCAGUUAUUAGCUUUCAUUUCGACCAAAUUGAUUGAGAACGAAAACCAG
AUUAUUGUGGUUUUAUUUAUUACUAAAAAUCAGAUUUAAUGUAGUACAUAAA
AACUAAUAUAAUUUUGCCGUAACUGUUCUUUAAAAAUCAAUAAUUAUAUGCCG
AUUUCUUUCCUGUACGCUGUUUUGCCAUGCAGGUUUAAUUACAUUUUUUAAUUC
UAUUUUUAGAAGUAAGCUCAUGCAACAGUGAAUAAAAGUACGUUAACAGCA
```

B

CAGCUUUAA
UAGCUUUCA
UGGUUUUAU
CAGAUUUAA
CAGGUUUAA

Fig. 7. Translational repression of *pho* in the dorsal pouch epithelium. (A) Sequence of the *pho* 3′ UTR showing the position of the Brd box-like motifs (boxes) and poly(A) signal (underlined). The portion of the *pho* 3′ UTR downstream of the arrow is deleted and replaced by α-tubulin 3′ UTR sequences in the *UAS-pho-tub3′UTR* transgene. (B) Sequences of Brd box-like motifs in the *pho* 3′ UTR shown in A compared to the Brd box consensus (bold face). The core sequence is boxed and nucleotides in the *pho* 3′ UTR that deviate from the consensus are indicated in gray. (C) Whole mount in situ hybridization showing *pho* RNA in a subset of dorsal pouch cells (open arrow), tracheal branches (solid arrow). The signal detected in the forming mouth parts (arrowhead) is nonspecific as it is also produced by a sense strand RNA probe (not shown). (D) Anti-Pho immunofluorescence shows accumulation of Pho protein in the tracheal branches (solid arrow), but not in the dorsal pouch (open arrow).

However, accumulation of Pho protein from the two transgenic RNAs differed dramatically, with substantial levels of Pho present in the dorsal pouch of *HD34A-GAL4/UAS-pho-tub3′UTR* embryos but little or none in *HD34A-*

GAL4/UAS-pho embryos (Figs. 8C and D). Together, these results show that the region of the *pho* 3′ UTR containing the Brd box-like motifs is necessary and sufficient for translational repression in the dorsal pouch, but that production of Pho protein in the dorsal pouch is not, on its own, deleterious.

Discussion

Multiple modes of TCE function

Through an enhancer trap screen for TCE-sensitive phenotypes, we found that the *nos* TCE can repress translation in cells of the dorsal pouch epithelium. Surprisingly, TCE function in dorsal pouch cells does not depend on the sequence and structural features of stem-loops II and III that are required for activity in the early embryo and oocyte, respectively. Rather, dorsal pouch activity of the TCE relies on a distinct Brd box-like sequence motif. A mutation that deletes five of the seven nucleotides within the Brd box-like B3 motif does not disrupt TCE function in the oocyte or embryo (Crucs et al., 2000), however, indicating that the B3 element is not necessary for regulation at these developmental stages. Thus, the TCE is both unique and versatile in consisting of three distinct functional elements, stem-loop II, stem-loop III, and the B3 element, which mediate different regulatory events.

Although the requirement for the B3 element in TCE function in the dorsal pouch was uncovered using an ectopic expression assay, the recent discovery that the TCE can repress *nos* translation in Class IV da neurons together with the requirement for *nos* in these neurons for dendritic morphogenesis (Ye et al., 2004) suggests a bona fide role for somatic TCE activity. In addition, roles for *oskar*, which is
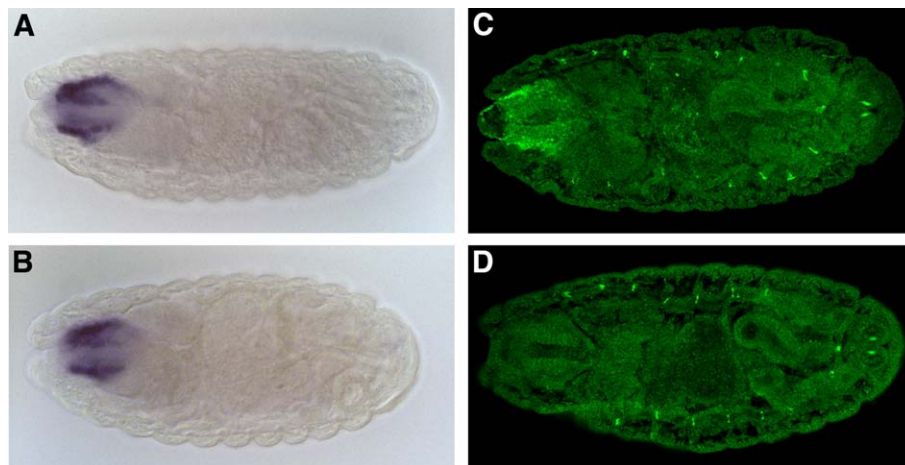

Fig. 8. Translational repression in the dorsal pouch epithelium by the *pho* 3′ UTR. Whole mount in situ hybridization to *pho* RNA in *HD34A-GAL4/UAS-pho-tub3′UTR* embryos (A) and *HD34A-GAL4/UAS-pho* embryos (B) confirming comparable transgenic RNA levels. Anti-Pho immunofluorescence showing accumulation of Pho in the dorsal pouch of *HD34A-GAL4/UAS-pho-tub3′UTR* embryos (B) but not in *HD34A-GAL4/UAS-pho* embryos (A).

required for localization of maternal *nos* RNA, and *pum* in learning and memory (Dubnau et al., 2003) suggest that *nos* may be expressed and regulated in other neurons. The B3 element may thus have evolved to adapt *nos* regulation to somatic tissues. It will be of interest to determine whether TCE function in da, and potentially other neurons, requires the B3 element.

Multiple RNAs use Brd box-like motifs

The identification of the Brd box-like motif as the effector of TCE function in the dorsal pouch led us to investigate whether an RNA normally expressed in the dorsal pouch might be similarly regulated. Evidence that *pho* RNA is expressed in a subset of dorsal pouch cells at late stages of embryogenesis without concomitant accumulation of Pho protein, together with the occurrence of four Brd box-like motifs in the *pho* 3′ UTR, makes *pho* a likely target. The demonstration that the region of the *pho* 3′ UTR containing these motifs is both necessary and sufficient for repression in the dorsal pouch suggests that one or more of these elements is responsible for regulation of *pho*. The analysis of mutations that target individual Brd box-like motifs will be required to dissect their contributions to *pho* regulation.

Unregulated expression of *pho* throughout the dorsal pouch epithelium has no phenotype, however. Thus, it is possible that repression of *pho* in the dorsal pouch is simply fortuitous. Alternatively, Pc-G proteins recruited by Pho for transcriptional silencing may be absent from the dorsal pouch epithelium. Consistent with this hypothesis, transcripts from *Sex combs on midleg*, *extra sex combs*, *polycomb*, *polycomb like*, and *posterior sex combs* each contain one or more Brd-like motifs in their 3′ UTRs (J.L.B., unpublished observation), suggesting that they, too, are repressed in the dorsal pouch.

Recognition of Brd box motifs

Results presented here, together with previous analysis of *Brd* 3′ UTR function (Lai and Posakony, 1997), indicate that *Brd* 3′ UTR regulatory activity has a broader spatial range than TCE activity. This difference may be due to differences in the sequence composition of the Brd and Brd-like boxes or to differences in the number, arrangement, or context of these sequence elements, all of which could affect interactions with trans-acting factors. Notably, the TCE B3 element differs from the Brd boxes within the *Brd* 3′ UTR by a single nucleotide insertion within the conserved core sequence. It has previously been suggested that the Brd box may be a target of the *mir-4* microRNA, as the 5′ end of *mir-4* is complementary to the core sequence (Lai et al., 2003). Complementarity between the critical 5′ seed region of *mir-4* and the TCE B3 sequence is suboptimal, however, since the insertion within the B3 core results in a bulged TCE nucleotide. Alternatively, deviation within the

core sequence could affect the interaction of B3 with protein regulatory factors. Furthermore, although it is not known whether all three Brd boxes contribute to *Brd* 3′ UTR activity (Lai et al., 2003), synergistic activity of these elements could enhance *Brd* 3′ UTR function. TCE B3 activity may therefore be limited to tissues where regulatory factors are most abundant or may require additional factors present in select tissues. Further mutational analysis will be required to distinguish among these possibilities.

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