

# A common translational control mechanism functions in axial patterning and neuroendocrine signaling in *Drosophila*

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## SUMMARY

**Translational repression of maternal *nanos* (*nos*) mRNA by a *cis*-acting Translational Control Element (TCE) in the *nos* 3'UTR is critical for anterior-posterior patterning of the *Drosophila* embryo. We show, through ectopic expression experiments, that the *nos* TCE is capable of repressing gene expression at later stages of development in neuronal cells that regulate the molting cycle. Our results predict additional targets of TCE-mediated**

**repression within the nervous system. They also suggest that mechanisms that regulate maternal mRNAs, like TCE-mediated repression, may function more widely during development to spatially or temporally control gene expression.**

Key words: *nanos*, Translational control, Neuroendocrine, 3' untranslated region, *Drosophila*

## INTRODUCTION

Translational control offers unique possibilities for spatial and temporal fidelity of gene expression. Accordingly, it plays important roles in such diverse processes as cell cycle control, erythrocyte differentiation, sex determination and embryonic axis specification (Wickens and Kimble, 1996). In the early *Drosophila* embryo, post-transcriptional regulation plays an essential role in patterning of the anteroposterior body axis by spatially regulating the synthesis of key developmental regulators from maternally supplied RNAs. Opposing gradients of Bicoid (Bcd) and Nanos (Nos) proteins are generated by translation of *bcd* and *nos* mRNAs that are localized, respectively, to the anterior and posterior poles of the early syncytial embryo (Driever and Nüsslein-Volhard, 1988; Gavis and Lehmann, 1992; Wang et al., 1994). In the anterior half of the embryo, Bcd activates zygotic transcription of anterior-specific genes like *hunchback* (*hb*) (reviewed by Driever, 1993). In addition, Bcd represses translation of maternal *caudal* (*cad*) mRNA, through a direct interaction with the *cad* 3' untranslated region (3'UTR) (Dubnau and Struhl, 1996; Rivera-Pomar et al., 1996). In the posterior half, Nos functions with Pumilio (Pum) and Brain Tumor (Brat) proteins to repress translation of maternal *hb* mRNA (reviewed by Parisi and Lin, 2000; Gavis, 2001). By repressing synthesis of the Hb transcriptional repressor, Nos permits expression of genes that direct abdominal development.

Repression of *hb* is mediated by Nanos Response Elements (NREs) in the *hb* 3'UTR (Wharton and Struhl, 1991; Murata and Wharton, 1995). As the *bcd* 3'UTR also contains an NRE, it is crucial that Nos activity be limited to the posterior half of

the embryo; ectopic Nos in the anterior results in translational repression of both *bcd* and *hb*, with consequent suppression of head and thorax development (Gavis and Lehmann, 1992; Gavis and Lehmann, 1994). The Nos gradient is generated solely from the translation of *nos* mRNA that is localized to the germ plasm at the posterior pole (Gavis and Lehmann, 1994). Localization of *nos* mRNA is not sufficient to limit synthesis of Nos protein to the posterior, however, as more than 95% of *nos* mRNA remains unlocalized (Bergsten and Gavis, 1999). Rather, translational repression of unlocalized *nos* mRNA is essential for spatially restricting Nos protein production.

A striking theme in translational control of developmental events is its frequent dependence on sequences located in the 3'UTRs of the regulated mRNAs. Translational repression of unlocalized *nos* mRNA in the *Drosophila* oocyte and embryo is mediated by a 90 nucleotide translational control element (TCE) within the *nos* 3'UTR (Dahanukar and Wharton, 1996; Smibert et al., 1996; Gavis et al., 1996a). TCE function requires formation of a bipartite secondary structure consisting of two stem-loops (Crucs et al., 2000). Smaug (Smg) protein interacts with one stem-loop and is required for repression of *nos* translation in the early embryo (Smibert et al., 1996; Dahanukar et al., 1999; Smibert et al., 1999). We have shown that TCE-mediated repression of *nos* mRNA occurs after translation has initiated (Clark et al., 2000).

Although *nos* RNA is present solely in the germline and early embryo, it is not known whether TCE-mediated regulation is restricted to these sites. Studies in *C. elegans* and *Drosophila* suggest that post-transcriptional regulatory mechanisms may be used more widely during development than is currently appreciated. Analysis of heterochronic genes

in *C. elegans* reveals that multiple RNAs are translationally regulated by a similar mechanism, at different times and in different tissues during development (reviewed by Ambros, 2000). Genetic pleiotropy of the *smg* mutant phenotype suggests that the Smg repressor regulates not only *nos*, but also one or more genes involved in the early embryonic mitoses (Dahanukar et al., 1999). Evidence that regulatory mechanisms that control specific maternal mRNAs in *Drosophila* are used again at later developmental stages comes from analysis of *stau* (*stau*) gene function. *Stau*, a double-stranded RNA binding protein, mediates localization of *bcd* and *oskar* mRNAs during oogenesis and is later required for localization of *prospero* mRNA in neuroblasts (St Johnston et al., 1991; St Johnston et al., 1992; Li et al., 1997; Broadus et al., 1998). Recently, translational control has been shown to play a role in cell fate determination in the *Drosophila* nervous system (Okabe et al., 2001).

To explore the possibility that other mRNAs may be regulated by the TCE or a TCE-like motif at different developmental stages, we investigated whether the TCE could repress translation in sites other than the *Drosophila* oocyte and early embryo. By using the GAL4/UAS system (Brand and Perrimon, 1993) to ectopically express either a regulatable *nos* mRNA, which bears the TCE, or an unregulatable *nos* mRNA, which lacks the TCE, in a variety of temporal and spatial patterns during development, we provide evidence that TCE-mediated repression can occur in a subset of cells in the central nervous system. Production of Nos from the unregulatable *nos* mRNA in these cells produces a characteristic adult phenotype. Strikingly, this phenotype is suppressed when *nos* RNA bearing the TCE is expressed in the identical cells. The phenotype caused by ectopic Nos resembles one caused by ablation of neurosecretory cells that produce eclosion hormone (EH). However, we find that TCE-mediated regulation does not occur in EH cells themselves, but in other cells that act within this complex neuroendocrine signaling pathway. The ability of the TCE to repress *nos* activity in the nervous system implies that TCE-mediated repression may be a more general mechanism for both spatial and temporal control during development.

## MATERIALS AND METHODS

### Fly stocks

The following mutants were used: *y w<sup>67c23</sup>*, *pum<sup>680</sup>*, *smg<sup>1</sup>* and *Df(Scf<sup>R6</sup>)* (Lindsley and Zimm, 1992; Dahanukar et al., 1999). GAL4 enhancer trap and other transgenic lines used in this study include *GAL4<sup>1407</sup>*, *GAL4<sup>24B</sup>* and *elav-GAL4* (Luo et al., 1994); *GAL4<sup>109-68</sup>* (Guo et al., 1996) and *h-GAL4* (Brand and Perrimon, 1993); *GMR-GAL4* (gift of Bruce Hay); *sev-GAL4<sup>332.1</sup>* (gift of Daniel Curtis); *EHups-GAL4* (McNabb et al., 1997); and *tubP-GAL80* (Lee and Luo, 1999).

### Construction of transgenes and transgenic lines

The pUAST vector (Brand and Perrimon, 1993) was used for all transgenic constructs. *UAS-nos-tub3'UTR* and *UAS-nos-tub:nos+2* contain the *nos* 5'UTR and coding sequences (Wang and Lehmann, 1991). The *nos* 3'UTR was substituted by the  $\alpha$ -*tubulin* 3'UTR as previously described (Gavis and Lehmann, 1994). For *UAS-nos-tub:nos+2*, a 180 nucleotide region of the *nos* 3'UTR defined as the +2 element was inserted into a *Bst*EII site within the  $\alpha$ -*tubulin* 3'UTR as previously described (Gavis et al., 1996b). The +2 element contains the *nos* TCE and an adjacent region of the *nos* 3'UTR harboring a

second Smg-binding site. *UAS-nos-tub:TCE* was generated similarly, by insertion of the 90 nucleotide *nos* TCE, as previously described (Crucs et al., 2000). *UAS-nos $\Delta$ B-tub3'UTR* and *UAS-nos $\Delta$ B-tub:nos+2* are identical to the respective *UAS-nos* transgenes, except that a *Bst*EII site within the *nos*-coding region (position 455 of the *nos* cDNA) was end-filled and re-ligated to create a frameshift, followed closely by a translation stop codon. The *EHups-GAL80* transgene is derived from *tubP-GAL80* (Lee and Luo, 1999). To generate *EHups-GAL80*, the *tubulin* promoter sequences were replaced with the 2.4 kb upstream region of the *EH* gene used in *EHups-GAL4* (McNabb et al., 1997).

Transgenes were introduced into *y w<sup>67c23</sup>* embryos by P element-mediated germline transformation (Spradling, 1986) and multiple independent transgenic lines were isolated and balanced for each transgene. To facilitate experiments requiring combinations of three different transgenes, the following recombinant chromosomes were generated: *EHups-GAL4*, *UAS-GFP*; *GAL4<sup>109-68</sup>*, *UAS-nos-tub3'UTR*; and *GAL4<sup>109-68</sup>*, *UAS-nos-tub:nos+2*.

### Phenotypic analysis

Crosses between flies carrying GAL4 driver transgenes and flies carrying *UAS* transgenes were carried out at 25°C. The *UAS-nos-tub3'UTR* and *UAS-nos-tub:nos+2* transgenes were tested simultaneously with each GAL4 driver. For each cross, 100-300 adult progeny were scored for visible phenotypes and lethality was calculated as described in Table 1. In order to calculate lethality, one of the transgenic chromosomes was used in heterozygous combination with a dominantly marked balancer chromosome to provide an 'expected' class of viable progeny. For analysis of the adolescent phenotype, adult progeny displaying the adolescent phenotype were collected, placed in fresh food vials and stored for at least 1 day at 25°C for rescoring. As both the adolescent phenotype and the *Cy* marker present on the second chromosome balancer affect wing morphology, flies from lines carrying second chromosome transgene insertions that were not homozygous viable were first outcrossed to place the transgene over a chromosome marked with *Pin*. Transgene/*Pin* males were then crossed to virgin females from GAL4 driver or *UAS* lines.

### Direct visualization of GFP and immunostaining

For direct visualization of GFP fluorescence, third instar larvae were picked from the walls of bottles with forceps and inverted in phosphate-buffered saline (PBS), then fixed in 2% paraformaldehyde/PBS for 30 minutes or 4% paraformaldehyde/PBS for 15 minutes at RT. CNS tissue was dissected, washed three to five times in PBS, and mounted in 70% glycerol/PBS. For immunofluorescence, inverted larvae were fixed overnight in 4% paraformaldehyde/PBS at 4°C, CNS tissue was dissected and washed five times in PBST (PBS, 0.1% Triton X-100) before blocking in 5% goat serum/PBST for 30 minutes. Tissue was incubated overnight at 4°C in primary antibody [1:150 rabbit anti-EH (McNabb et al., 1997) or 1:67 mouse anti-GFP (Clontech)]. Samples were washed for 5×10 minutes with PBST, then incubated with secondary antibody [1:500 Alexa Fluor 568 goat anti-rabbit (Molecular Probes) or 1:1000 Oregon Green 488 goat anti-mouse (Molecular Probes)] for 2 hours at RT or overnight at 4°C. Samples were washed for 5×10 minutes with PBST, mounted in Aqua Poly Mount (Polysciences) and imaged on a BioRad MRC-600 confocal microscope.

## RESULTS

### Ectopic expression and regulation of *nos* RNA

Ectopic expression of *nos* in the developing eye produces a rough eye phenotype (Wharton et al., 1998), indicating that Nos protein can cause cellular toxicity when produced outside

**Table 1. Ectopic expression of *nos-tub3'UTR* and *nos-tub:nos+2* RNAs**

GAL4 driver	Expression	Observed phenotypes	Penetrance	
			<i>UAS-nos-tub3'UTR</i>	<i>UAS-nos-tub:nos+2</i>
<i>GAL4<sup>24B</sup></i>	Embryonic mesoderm and muscle cells	Lethality	100%	100%
<i>h-GAL4</i>	Ectoderm (non-neuronal)	Lethality	100%	100%
<i>GMR-GAL4</i>	Eye	1) Rough eye 2) Black spots	1) 100% 2) ND	1) 100% 2) ND
<i>elav-GAL4</i>	Pan-neuronal	Lethality	82%	84%
<i>sev-GAL4</i>	Eye, CNS	1) Rough eye 2) Adolescent	1) 100% 2) 26%	1) 100% 2) 24%
<i>GAL4<sup>1407</sup></i>	Neuroblasts, CNS, PNS	1) Lethality 2) Adolescent	1) 68% 2) 2%	1) 61% 2) 3%
<i>GAL4<sup>109-68</sup></i>	Larval CNS, sensory organ precursors, R8 photoreceptors	1) Adolescent 2) Missing/extra bristles	1) 74% 2) ND	1) 23% 2) ND
<i>HD23A-GAL4</i>	Larval CNS, eye/antennal disc	Adolescent	85%	33%
<i>HD44A-GAL4</i>	Larval CNS, eye/antennal disc, larval fat body	Adolescent	90%	24%
<i>KF42A-GAL4</i>	Larval CNS	Adolescent	38%	0.9%

*UAS-nos-tub3'UTR* and *UAS-nos-tub:nos+2* transgenic lines were crossed to the GAL4 driver lines listed above. *HD23A-GAL4*, *HD44A-GAL4* and *KF42A-GAL4* were identified in a new enhancer trap screen (H. K. D. and E. R. G., unpublished). Values shown derive from a single *UAS-nos-tub3'UTR* and a single *UAS-nos-tub:nos+2* line, whose RNA expression levels show a ratio 1.1:1 by northern blotting. Similar results were obtained with additional independent lines. Lethality was calculated as 1-(number of flies carrying both transgenes/number of flies in 'expected' class) (see Materials and Methods). Phenotypic penetrance was calculated as number of flies with phenotype/number of flies carrying both GAL4 driver and UAS transgenes. Two phenotypes (black spots produced by *GMR-GAL4* and bristle number defects produced by *GAL4<sup>109-68</sup>*) were not rigorously quantified over a large number of flies (ND), but appeared at roughly similar frequencies for both *UAS-nos* transgenes.

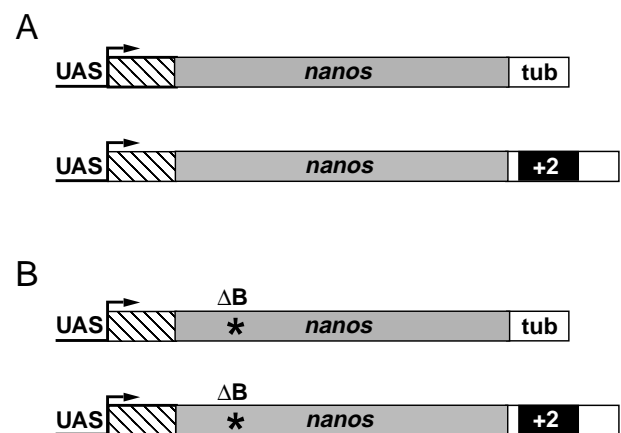
of its normal environment. We took advantage of the potential toxicity of Nos when ectopically expressed to search for cells and tissues capable of TCE-mediated regulation.

In the early embryo, the hybrid *nos-tub3'UTR* RNA, which bears  $\alpha$ -tubulin 3'UTR sequences in place of the *nos* 3'UTR, is not subject to TCE-mediated regulation and is consequently translated throughout the early embryo (Gavis and Lehmann, 1994). By contrast, *nos-tub:nos+2* RNA, which contains an insertion of the *nos* TCE and adjacent *nos* 3'UTR sequences (see Materials and Methods), is properly regulated (Gavis et al., 1996a). To assay TCE function more generally, we constructed two transgenes, *UAS-nos-tub3'UTR* and *UAS-nos-tub:nos+2*, which permit zygotic expression of either *nos-tub3'UTR* or *nos-tub:nos+2* RNA under control of the GAL4 transcriptional activator (Fig. 1A).

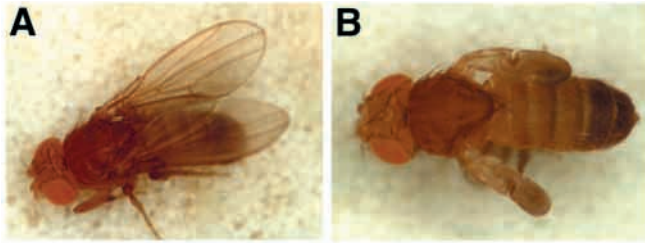
Combination of the *UAS-nos-tub3'UTR* transgene with various GAL4 drivers should result in zygotic expression of *nos-tub3'UTR* RNA and ectopic production of Nos protein. In some cases, toxicity of the ectopic Nos protein may cause a visible phenotype. For many GAL4 drivers, expression of *nos-tub:nos+2* RNA from the *UAS-nos-tub:nos+2* transgene would also be expected to result in ectopic Nos production and any associated phenotype. However, if cells where a GAL4 driver is active harbor factors capable of repressing translation via the TCE, expression of *nos-tub:nos+2* RNA in those cells will not yield ectopic Nos. For this GAL4 driver, transcription of the *UAS-nos-tub3'UTR* and *UAS-nos-tub:nos+2* transgenes in the same set of cells could lead to different phenotypic consequences, as a result of post-transcriptional repression by the TCE.

We tested a variety of existing GAL4 drivers for their ability to produce a phenotype at high frequency in combination with the *UAS-nos-tub3'UTR* transgene but at reduced frequency

with the *UAS-nos-tub:nos+2* transgene. GAL4 drivers were selected to survey the consequences of expression of the two *nos* RNA derivatives in cells within a variety of tissues and at different developmental times. A range of phenotypes, including lethality, rough eyes, and either missing or



**Fig. 1.** *Nos* reporter transgenes for ectopic expression. (A) The *nos*-coding region (shaded) and 5'UTR sequences (hatched) were fused to yeast UAS sequences to permit transcriptional activation of *nos* by GAL4. In the *UAS-nos-tub3'UTR* transgene (top), the *nos* 3'UTR is replaced by the  $\alpha$ -tubulin 3'UTR (open box). In the *UAS-nos-tub:nos+2* transgene (bottom), the  $\alpha$ -tubulin 3'UTR sequences contain an insertion of the *nos* 3'UTR +2 element (black box), which includes the *nos* TCE and an adjacent region with weak translational repression function. The *UAS-nos-tub:TCE* transgene (not shown), which carries an insertion of the TCE alone, behaves similarly. (B) Transgenes are identical to those in A, except for the presence of a stop codon mutation (\*) in the *nos*-coding region.



**Fig. 2.** Ectopic expression of *nos* by *GAL4*<sup>109-68</sup>. Adult flies, carrying both *GAL4*<sup>109-68</sup> and *UAS-nos-tub3*'UTR transgenes, 2-3 days post-eclosion. A small percentage of these flies appear wild type (A), whereas the majority show the adolescent phenotype (B).

supernumerary bristles are observed among the GAL4 drivers tested. However, these phenotypes are produced at similar frequencies by both the *UAS-nos-tub3*'UTR and *UAS-nos-tub:nos+2* transgenes (Table 1).

Several GAL4 drivers produce a striking phenotype in which adult flies fail to undergo the characteristic wing expansion and body cuticle hardening that occurs shortly after eclosion from the pupal case (Fig. 2). Although these flies are fertile, they show reduced viability and those that survive for several days resemble newly eclosed flies for the duration of their lives. We designate this phenotype as 'adolescent'. Notably, the *GAL4*<sup>109-68</sup> driver (Luo et al., 1994) produces the adolescent phenotype at high frequency (74%) when combined with the *UAS-nos-tub3*'UTR transgene but at substantially reduced frequency (23%) when combined with *UAS-nos-tub:nos+2* (Table 1). In a screen of 200 new enhancer trap lines (H. K. D. and E. R. G., unpublished), we have identified three additional GAL4 drivers that, like *GAL4*<sup>109-68</sup>, produce an adolescent phenotype at higher frequency when combined with *UAS-nos-tub3*'UTR than with *UAS-nos-tub:nos+2* (Table 1). A fourth GAL4 driver that produces a different TCE-sensitive phenotype will be described elsewhere. These enhancers are distinct from one another and from the 109-68 enhancer as determined by their expression patterns (Table 1, Fig. 3) and their chromosomal locations (data not shown).

The differences in penetrance of the adolescent phenotype are not due to position effects on expression of the *UAS-nos* transgenes as similar results were obtained using multiple independent *UAS-nos-tub3*'UTR and *UAS-nos-tub:nos+2* transgenic lines (data not shown). Furthermore, we carried out northern blot analysis to evaluate the general expression levels of *UAS-nos-tub3*'UTR and *UAS-nos-tub:nos+2* transgenes. The limited temporal and spatial expression of the GAL4 drivers that produce a TCE-sensitive adolescent phenotype (see below) makes it difficult to obtain adequate material for

quantitative RNA analysis. Therefore, expression of *UAS-nos-tub3*'UTR and *UAS-nos-tub:nos+2* transgenes was activated in the embryonic CNS and PNS with the *GAL4*<sup>1407</sup> driver (Luo et al., 1994). *nos-tub3*'UTR and *nos-tub:nos+2* RNAs are indeed present at similar levels (ratio of 1.1:1; data not shown), indicating that the difference in penetrance of the adolescent phenotype cannot be accounted for by differences in the transcriptional activity of the two transgenes. Rather, this difference must reflect a difference in the activities of the *nos-tub3*'UTR and *nos-tub:nos+2* RNAs. Furthermore, subsequent analysis of a *UAS-nos-tub:TCE* transgene, which carries only the *nos* TCE, showed that this transgene behaves similarly to the *UAS-nos-tub:nos+2* transgene (data not shown). Thus, the observed regulation can be attributed solely to the *nos* TCE.

### The adolescent phenotype is due to ectopic Nos

To determine whether the adolescent phenotype results from ectopic expression of Nos protein, we engineered a premature stop codon in the *nos*-coding sequences of the *UAS-nos-tub3*'UTR and *UAS-nos-tub:nos+2* transgenes (*UAS-nosΔB-tub3*'UTR and *UAS-nosΔB-tub:nos+2*; Fig. 1B). Translation termination at this stop codon is predicted to produce a non-functional 73 amino acid N-terminal Nos peptide. Northern blot analysis confirmed that both *nosΔB* transgenes produce stable RNAs whose expression levels are comparable with those of the unaltered *nos* transgenes when activated by the *GAL4*<sup>1407</sup> driver (data not shown). Neither the *UAS-nosΔB-tub3*'UTR nor the *UAS-nosΔB-tub:nos+2* transgene, when combined with the *GAL4*<sup>109-68</sup> driver, produces an adolescent phenotype (Table 2). These results confirm that the adolescent phenotype results from ectopic expression of Nos protein. Furthermore, they indicate that the low penetrance of the phenotype observed with the *UAS-nos-tub:nos+2* transgene results from decreased production of ectopic Nos protein. Considering these results together with those presented above, we conclude that the amelioration of the adolescent phenotype reflects post-transcriptional regulation of ectopically expressed *nos-tub:nos+2* RNA by the *nos* TCE.

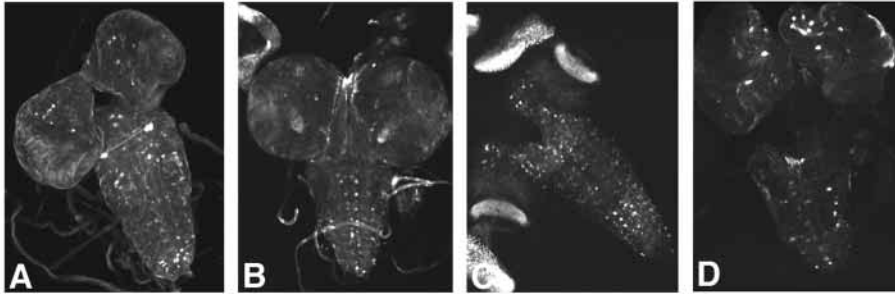
### The adolescent phenotype does not depend on *pum* or *smg*

In the precellular embryo, Nos acts together with two other proteins, Pum and Brat, to repress translation of maternal *hb* mRNA (Barker et al., 1992; Murata and Wharton, 1995; Sonoda and Wharton, 2001). After cellularization, Nos is present only in the germ cells (Wang et al., 1994), whereas Pum and Brat are more widely distributed during development (Barker et al., 1992; Wharton et al., 1998; Arama et al., 2000). Nos and Pum collaborate to regulate *cyclin B* mRNA in the germ cell precursors (Asaoka-Taguchi et al., 1999), although

**Table 2. Production of the adolescent phenotype by *UAS-nos* and *UAS-nosΔB* transgenes**

GAL4 driver	% Adolescent			
	<i>UAS-nos-tub3</i> 'UTR	<i>UAS-nos-tub:nos+2</i>	<i>UAS-nosΔB-tub3</i> 'UTR	<i>UAS-nosΔB-tub:nos+2</i>
<i>GAL4</i> <sup>109-68</sup>	74%	23%	0.4%	0.5%
<i>EHups-GAL4</i>	70%	75%	0.5%	0.4%

The penetrance of the adolescent phenotype was determined in progeny carrying either the *GAL4*<sup>109-68</sup> or *EHups-GAL4* driver combined with a *UAS-nos* or *UAS-nosΔB* transgene as described in Table 1. The *UAS-nos* transgenic lines were those used in the experiments of Table 1. Values shown for the *UAS-nosΔB* transgenes derive from one line of each that produces RNA levels comparable with each other and with the two *UAS-nos* transgenes. Similar results were obtained with additional independent lines.



**Fig. 3.** GAL4 drivers that produce a TCE-sensitive adolescent phenotype are expressed in the CNS. Confocal images of CNS dissected from wandering third instar larvae carrying both *UAS-GFP* and (A) *GAL4<sup>109-68</sup>*; (B) *HD44A-GAL4*; (C) *HD23A-GAL4*; and (D) *KF42A-GAL4* transgenes. The expression domains of each driver are revealed by direct GFP fluorescence.

Brat does not participate in this event (Sonoda and Wharton, 2001). The ability of Nos to cause a rough eye phenotype when ectopically expressed in the eye also depends on Pum (Wharton et al., 1998), although the target of Nos and Pum in the eye is not known. The dependence of the adolescent phenotype on *pum* function was assessed in *pum* mutant animals carrying both the *GAL4<sup>109-68</sup>* driver and the *UAS-nos-tub3'UTR* transgenes. These animals display the adolescent phenotype at a frequency of 76%, comparable with the frequency of 73% produced by otherwise wild-type animals carrying *GAL4<sup>109-68</sup>* and *UAS-nos-tub3'UTR*. Thus, Nos acts independently of Pum in causing the adolescent phenotype.

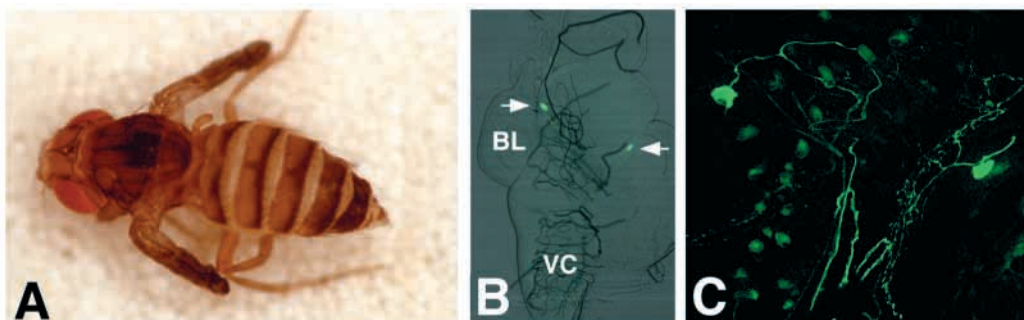
Translational repression of unlocalized *nos* mRNA in the early embryo requires the interaction of Smg protein with the TCE (Smibert et al., 1996; Dahanukar et al., 1999). However, neither Smg protein nor its activity is detectable in ovary extracts (Smibert et al., 1999). As we do not detect accumulation of Nos protein in late oocytes (E. R. G., unpublished), a different TCE-binding factor may repress translation of *nos* during oogenesis. Animals carrying both the *GAL4<sup>109-68</sup>* and *UAS-nos-tub:nos+2* transgenes show a similarly low penetrance of the adolescent phenotype regardless of whether they are wild-type or mutant for *smg* (21% versus 32% adolescent, respectively). Thus, the ability of the TCE to suppress the adolescent phenotype caused by ectopic expression of *nos* RNA is independent of Smg.

#### The adolescent phenotype resembles loss of EH cells

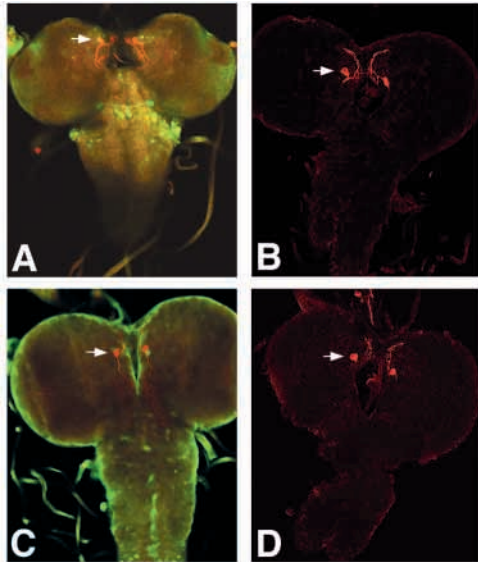
The adolescent phenotype produced by ectopic *nos* expression

resembles a phenotype produced by ablation of neurosecretory cells that secrete the neuropeptide eclosion hormone (EH) (McNabb et al., 1997). Work carried out largely in the tobacco hornworm *Manduca sexta* has shown that EH plays a central role in a complex neurosecretory network that controls ecdysis, the shedding of the cuticle at the end of each molt. EH acts within the CNS to activate peptidergic neurons as well as on peripheral targets (Ewer et al., 1997; Gammie and Truman, 1997; Gammie and Truman, 1999). In *Drosophila*, EH is produced by a single pair of neurons (EH cells) in the ventromedial region of the brain (McNabb et al., 1997; Park et al., 1999). Surprisingly, ablation of the EH cells in *Drosophila* by targeted expression of cell death genes is only semi-lethal; over 30% of animals lacking EH cells survive to adulthood. Of these, more than 70% display an adolescent phenotype (McNabb et al., 1997).

Visualization of a *UAS-GFP* reporter revealed that all of the GAL4 drivers that yield a TCE-sensitive adolescent phenotype are expressed in subsets of cells within the larval brain lobes and ventral nerve cord (Fig. 3). The similarity of the phenotypes caused by EH cell ablation and ectopic *nos* expression by *GAL4<sup>109-68</sup>*, coupled with the ability of the TCE to suppress the adolescent phenotype, suggests that EH cells might be capable of TCE-mediated repression. We therefore used the *EHups-GAL4* driver (McNabb et al., 1997) to drive expression of *UAS-nos* transgenes exclusively in EH cells. Expression of the *UAS-nos-tub3'UTR* transgene by *EHups-GAL4* produces the adolescent phenotype in 70% of flies; expression of *UAS-nos-tub:nos+2* by *EHups-GAL4* yields a similar result, with 75% of flies displaying the adolescent



**Fig. 4.** Expression of *nos* in EH cells. (A) Adult fly carrying the *EHups-GAL4* and *UAS-nos-tub3'UTR* transgenes displays the adolescent phenotype. The same phenotype is produced by the *UAS-nos-tub:TCE* transgene. (B) Confocal image of fixed larval CNS tissue with EH cells (arrow) revealed by direct GFP fluorescence. In this experiment, animals carry the *EHups-GAL4*, *UAS-GFP* and *UAS-nos-tub3'UTR* transgenes. GFP fluorescence and bright field image are visualized simultaneously for orientation (BL, brain lobe; VC, ventral nerve cord). (C) Confocal projection of larval CNS of an animal from experiment in B shown at high magnification to illustrate the EH cell processes. The morphology of the EH cells and their processes is not obviously different from that of animals carrying only the *EHups-GAL4* and *UAS-GFP* transgenes (not shown).



**Fig. 5.** EH cells are not the sites of ectopic *nos* expression. (A,C) Confocal images of CNS from third instar larvae carrying a *UAS-GFP* reporter transgene and either the *GAL4<sup>109-68</sup>* (A) or *HD44A-GAL4* (C) driver, immunostained with anti-EH antibody. The merge of anti-GFP immunofluorescence (green) and anti-EH antibody staining (red) is shown. In both cases, cells that lie below the EH cells express GFP (not readily visible in these sections) but the EH cells (arrow) do not. (B,D) Confocal images of CNS from third instar larvae carrying *UAS-nos-tub3'UTR* transgene and either *GAL4<sup>109-68</sup>* (B) or *HD44A-GAL4* (D) transgenes, immunostained with anti-EH antibody (red). EH cells, their processes and production of EH are not affected when *nos* is ectopically expressed by these drivers.

phenotype (Fig. 4A, Table 2). The phenotype depends specifically on the production of Nos protein, as it is not produced by either of the *UAS-nosΔB* transgenes (Table 2). As shown with either a *UAS-GFP* reporter or anti-EH

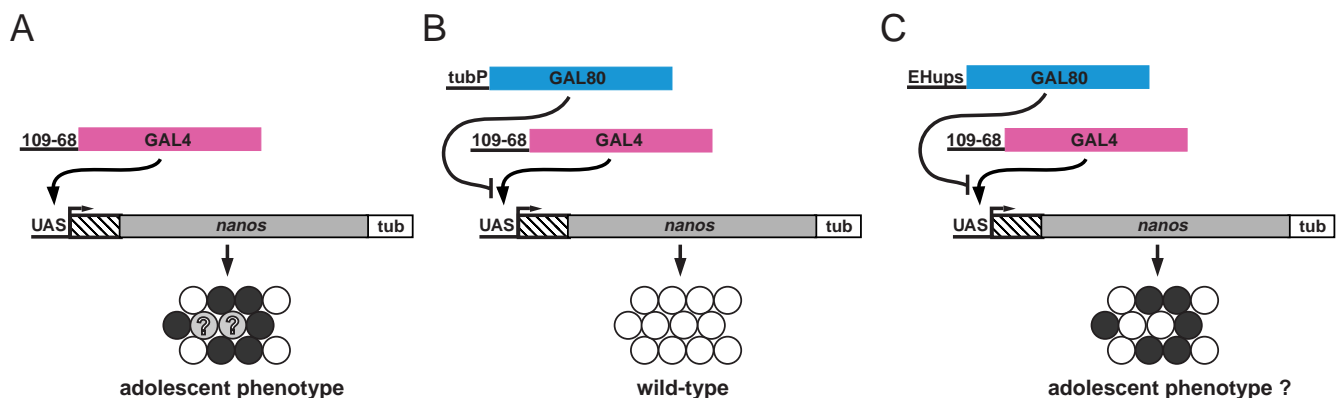
immunofluorescence, neither EH cells nor their processes are destroyed by ectopic *Nos* expression, indicating that *Nos* must interfere in some way with the function of these cells (Fig. 4B,C and data not shown).

While *Nos* can disrupt EH cell function, the failure to observe TCE-mediated repression suggests that the EH cells are not the site of the observed regulation within the *GAL4<sup>109-68</sup>* expression domain. Alternatively, differences in the levels to which the *EHups-GAL4* and *GAL4<sup>109-68</sup>* drivers activate transcription in EH cells or in their temporal profiles might account for the difference in the response of the *UAS-nos* transgenes to the two drivers. As *EHups-GAL4* initiates expression earlier in development than does *GAL4<sup>109-68</sup>* and also drives expression to a higher level (data not shown), *nos* mRNA might accumulate to substantial levels in EH cells before the regulatory machinery is in place or overwhelm the machinery.

### EH cells are not the site of TCE-mediated regulation in the CNS

We have taken two further approaches to determine if the EH cells are indeed the site of TCE-mediated regulation when *nos-tub:TCE* RNA is ectopically expressed in the CNS. First, we used anti-EH antibody to label EH cells in the larval CNS from *GAL4<sup>109-68</sup>/UAS-GFP* or *HD-44A/UAS-GFP* animals. Confocal imaging revealed that neither GAL4 driver is active in EH cells (Fig. 5A,C). In addition, EH cells and their processes appear wild type in animals expressing *nos-tub3'UTR* RNA in either the *GAL4<sup>109-68</sup>* or *HD44A-GAL4* expression domains (Fig. 5B,D). Although this experiment does not address whether the EH cells are functional, it excludes the possibility that expression of *Nos* in other neuroendocrine cells interferes with EH cell determination or viability.

In a second approach, we took advantage of the ability of the GAL80 inhibitor to block transcriptional activation by GAL4 when the two are co-expressed (Lee and Luo, 1999) to dissect the expression domain of *GAL4<sup>109-68</sup>*. We first



**Fig. 6.** Suppression of ectopic *nos* expression by GAL80. (A) *GAL4<sup>109-68</sup>* activates *UAS-nos-tub3'UTR* in a subset of cells in the CNS (dark shading). *GAL4<sup>109-68</sup>* may also activate *UAS-nos-tub3'UTR* at low levels or at unexamined times in EH cells (light shading with '?'). Ectopic synthesis of Nos in one or more cells of the *GAL4<sup>109-68</sup>* expression domain produces the adolescent phenotype. (B) Ubiquitous expression of GAL80 under control of the tubulin promoter (*tubP-GAL80*) blocks the ability of *GAL4<sup>109-68</sup>* to activate transcription of the *UAS-nos-tub3'UTR* transgene in all cells and suppresses the adolescent phenotype (Table 3). (C) The ability of GAL80 to block transcriptional activation by *GAL4<sup>109-68</sup>*, as shown in B, was used to test whether *GAL4<sup>109-68</sup>* is indeed active in EH cells. Expression of GAL80 specifically in EH cells by *EHups-GAL80* should block GAL4 activation of *UAS-nos-tub3'UTR* and suppress the adolescent phenotype only if *GAL4<sup>109-68</sup>* drives expression of *UAS-nos-tub3'UTR* in EH cells (Table 3).

**Table 3. Suppression of GAL4 activation by GAL80**

GAL4 driver	GAL80 driver	% Adolescent
<i>GAL4<sup>109-68</sup></i>	none	97%
<i>GAL4<sup>109-68</sup></i>	<i>tubP-GAL80</i>	0%
<i>GAL4<sup>109-68</sup></i>	<i>EHups-GAL80</i>	91%
<i>EHups-GAL4</i>	none	85%
<i>EHups-GAL4</i>	<i>EHups-GAL80</i>	13%

The penetrance of the adolescent phenotype for the indicated combinations of GAL4 or GAL80 drivers with the *UAS-nos-tub3'UTR* transgene was determined as described in Table 1. For the *UAS-nos-tub3'UTR*, *GAL<sup>109-68</sup>* combination, a recombinant chromosome that carries both transgenes was used. This recombinant chromosome produces the adolescent phenotype at high frequency.

confirmed that GAL80 can indeed block transcriptional activation by the *GAL4<sup>109-68</sup>* driver by expressing GAL80 ubiquitously under control of the *tubulin* promoter (*tubP-GAL80*) (Lee and Luo, 1999) (Fig. 6B). Whereas 97% of flies carrying both the *GAL4<sup>109-68</sup>* and *UAS-nos-tub3'UTR* transgenes on a recombinant chromosome display the adolescent phenotype, none of the flies carrying the *tubP-GAL80* transgene in addition to the *GAL4<sup>109-68</sup>*, *UAS-nos-tub3'UTR* chromosome appear adolescent (Table 3).

To express GAL80 selectively in EH cells, we fused GAL80 sequences to the same EH enhancer sequences present in the *EHups-GAL4* transgene (McNabb et al., 1997), creating an *EHups-GAL80* transgene. Flies carrying the *EHups-GAL80* transgene in combination with the *GAL4<sup>109-68</sup>*, *UAS-nos-tub3'UTR* chromosome (Fig. 6C) were generated and examined phenotypically. Unlike *tubP-GAL80*, the *EHups-GAL80* transgene does not suppress the adolescent phenotype caused by the *GAL4<sup>109-68</sup>*, *UAS-nos-tub3'UTR* chromosome (Table 3). To confirm that GAL80 is indeed produced and active in the EH cells, we tested whether *EHups-GAL80* could suppress the adolescent phenotype caused by the combination of *EHups-GAL4* with *UAS-nos-tub3'UTR*. Whereas 85% of *EHups-GAL4/UAS-nos-tub3'UTR* flies are adolescent, only 13% of *EHups-GAL80/+; EHups-GAL4/UAS-nos-tub3'UTR* flies show this phenotype (Table 3), indicating that *EHups-GAL80* blocks GAL4 activation in EH cells. In a second experiment, we assayed the ability of the GAL80 transgene to block GAL4 activation of the *UAS-GFP* reporter in EH cells. In *EHups-GAL4/UAS-GFP* animals, GFP fluorescence is readily detected in EH cells. By contrast, GFP fluorescence is eliminated in EH cells of *EHups-GAL4/UAS-GFP* animals that also carry the *EHups-GAL80* transgene (Fig. 7). Taken together, these results show that *GAL4<sup>109-68</sup>* does not drive expression of *UAS-nos-tub3'UTR* in EH cells to produce the adolescent phenotype. Consequently, TCE-mediated regulation must occur in a different population of cells involved in the ecdysis signaling pathway.

## DISCUSSION

The co-option of a genetic regulatory mechanism for use in different contexts, at multiple times during development, provides a powerful means to expand the developmental potential of an organism and thereby motivate evolutionary change. We now show that a post-transcriptional regulatory

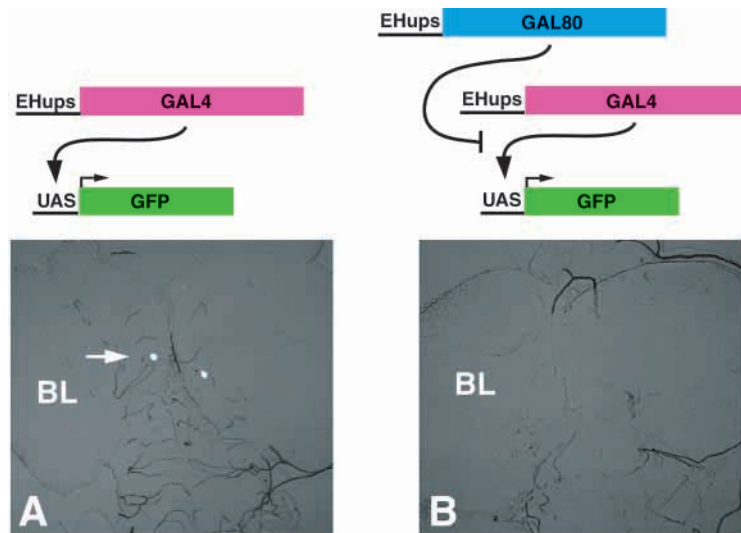
mechanism, TCE-mediated repression can operate at multiple times during development, first within the oocyte/early embryo and later within cells of the neuroendocrine signaling pathway that mediates ecdysis.

Ectopic expression of Nos protein frequently causes developmental defects or lethality. While we do not know the basis for Nos toxicity, suppression of this toxicity provides a useful assay for TCE-mediated repression in different tissues over a wide range of developmental times. The ability of a number of neuronal GAL4 drivers to produce a characteristic adolescent phenotype when combined with *UAS-nos* transgenes reveals that Nos is toxic to cells of the neuroendocrine pathway that controls ecdysis. The striking sensitivity of this specific phenotype to the TCE observed with four different Enhancer-GAL4 drivers indicates that a subset of these cells are capable of TCE-mediated repression.

Neurosecretory cells that produce EH were a prime candidate for the site of TCE function as ablation of EH cells results in a significant number of viable animals that show an adolescent phenotype (McNabb et al., 1997). However, we find no evidence that two GAL4 drivers that produce the TCE-sensitive adolescent phenotype in combination with *UAS-nos* transgenes are active in EH cells. Furthermore, EH cells are not grossly affected and continue to produce EH under conditions where ectopic Nos causes an adolescent phenotype. Thus, TCE-mediated regulation must occur in other cells that participate in or impinge on the ecdysis pathway. The GAL4 drivers that produce the TCE-sensitive adolescent phenotype are active in numerous, partially overlapping sets of cells within the nervous system. The complexity of these expression patterns coupled with the lack of adequate immunological reagents has so far precluded identification of the particular cells where TCE-mediated repression occurs. Identification of these cells may be further complicated if ectopic Nos interferes with cell viability, differentiation or migration. We are currently developing translational reporters based on fluorescent proteins to assist in identifying sites of TCE-mediated repression.

Our results potentially identify new cells in the ecdysis pathway involved in regulating post-eclosion events such as wing inflation and cuticle hardening. Physiological studies provide evidence for multiple EH targets whose cellular identities are as yet unknown (Baker et al., 1999). Wing inflation and cuticle hardening are thought to be mediated by an unidentified neuroendocrine factor, possibly bursicon. Presumably, the failure of these post-eclosion events in a large percentage of flies following EH cell ablation results from loss of EH-dependent excitation of the unidentified neuroendocrine cells. However, as ecdysis and post-eclosion events can still occur in the absence of EH, these and other EH target cells must be controlled by multiple, as yet unidentified inputs (McNabb et al., 1997).

In *C. elegans*, the hormonally controlled molting cycle includes a regulatory cascade of heterochronic genes including *lin-14* and *lin-28*. Genetic analysis suggests several similarities between temporal patterning in *C. elegans* and spatial patterning in *Drosophila* (Slack and Ruvkun, 1997). Translational repression of *lin-14* and *lin-28* mRNAs generates temporal gradients of the respective proteins. As with *nos*, translational control of these genes requires double-stranded RNA sequences in their 3'UTRs, although in contrast to



**Fig. 7.** GAL80 blocks GAL4-dependent activation in EH cells. (A) GFP fluorescence in CNS of third instar larva carrying *EHups-GAL4* and *UAS-GFP* transgenes. Bright field image is visualized simultaneously for orientation. Arrow indicates one of the two EH cells, one brain lobe (BL) is indicated for point of reference. (B) Animal as in A but carrying the *EHups-GAL80* transgene as well. No GFP fluorescence is detected.

the *nos* TCE, the RNA duplexes arise from intermolecular interactions with the small RNA *lin-4* (Lee et al., 1993; Wightman et al., 1993; Ha et al., 1996; Moss et al., 1997). Furthermore, translational repression of *nos*, *lin-14* and *lin-28* occurs after initiation of translation, suggesting similar mechanisms of regulation (Olsen and Ambros, 1999; Clark et al., 2000; Seggerson et al., 2002). The data presented here suggest that TCE-dependent repression operates during part of the *Drosophila* molting cycle and may therefore represent a widely used mechanism for modulating neuroendocrine signaling pathways.

The finding that the TCE can repress ectopic *nos* activity in the nervous system implies that TCE-mediated repression may be a more general mechanism for both spatial and temporal control during development. In both invertebrates and vertebrates, post-transcriptional mechanisms such as RNA localization and translational control are used extensively in both oocytes/embryos (Bashirullah et al., 1998) and neuronal cells (Steward, 1997; Martin et al., 2000). Translational repression may be particularly important in generating asymmetric protein distributions necessary for proper neuronal development and function. Indeed, translational repression of *tramtrack69* (*ttk69*) mRNA by Musashi (Msi) protein has recently been shown to be essential to generate asymmetry in the *Drosophila* sensory organ lineage (Okabe et al., 2001). Msi1, the murine Msi homolog, is enriched in neural progenitor cells in the developing mouse CNS (Sakakibara et al., 1996). Msi1 interacts with the 3'UTR of mammalian *numb* mRNA, whose *Drosophila* homolog is required for the asymmetric division of sensory organ precursors. Msi1 can repress translation of mammalian *numb* in cultured cells and Msi1-mediated regulation of this protein has been proposed to play a role in the self-renewal of neural stem cells (Imai et al., 2001). In higher vertebrates, post-transcriptional regulation appears to play roles in neurite regeneration (Twiss et al., 2000)

and synaptic plasticity (Martin et al., 2000; Steward and Schuman, 2001).

Support for the idea that regulatory machinery may be present in more than one tissue or cell type comes from analysis of Staufen. In *Drosophila*, Staufen protein regulates localization and/or translation in *Drosophila* oocytes, early embryos and neuroblasts (St Johnston et al., 1991; Li et al., 1997; Broadus et al., 1998). Functions of Stau in RNA localization and translational control in multiple tissues may be conserved in vertebrates, as Stau-related proteins are found in germ cells (Saunders et al., 2000) and also in mammalian hippocampal neurons, where they are implicated in dendritic RNA localization (Kiebler et al., 1999; Monshausen et al., 2001; Tang et al., 2001).

At least one TCE-binding factor appears to have multiple target RNAs. Embryos from *smg* mutant females show abnormal syncytial nuclear cleavage cycles and fail to undergo cellularization. As these defects are unrelated to *nos*, Smg must regulate one or more mRNAs in the early embryo in addition to *nos*. Whether Smg acts through a TCE or a different recognition motif in this case is not known. The suggestion that Smg may be present later during development in the ventral nerve cord and brain (Smibert et al., 1999) is consistent with TCE-mediated

translational repression in neuronal cells. However, we have not detected Smg in either the embryonic or larval CNS (H. K. D., A. N. V. and E. R. G., unpublished). Furthermore, genetic experiments indicate that *smg* function is not required for repression of ectopic *nos-tub:nos+2* RNA.

TCE function requires formation of two stem-loops, designated as II and III (Crucs et al., 2000). In the embryo, TCE-mediated repression requires binding of Smg to stem-loop II. In the absence of Smg, a different TCE binding factor may repress *nos* RNA in the ovary. Recognition of stem-loop III by at least one as yet unknown factor is also required for translational repression of maternally synthesized *nos* RNA. This factor could participate in TCE-mediated regulation in the CNS as well, possibly in combination with a unique stem-loop II binding factor. Thus, different factors present at different times or in different tissues during development may regulate translation through the TCE or a TCE-like recognition motif. Similarly, the finding that Pum protein interacts with different co-factors when binding to NREs in different mRNAs indicates that distinct regulatory complexes may be assembled on similar sequence motifs in different RNAs.

Our results suggest that one or more RNAs in addition to *nos* are regulated post-transcriptionally by a *nos*-like TCE. The importance of secondary structure to TCE function makes identification of this motif on a genome wide basis difficult. Furthermore, alternate TCEs may use only one of the two stem-loops required for function in the oocyte/early embryo or may substitute one of these with a novel stem-loop. Design of new computational and molecular methods to identify TCE-containing RNAs will provide an important challenge for understanding the role of TCE-mediated regulation during development.

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