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A conserved 90 nucleotide element mediates translational repression of *nanos* RNA

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SUMMARY

Correct formation of the *Drosophila* body plan requires restriction of *nanos* activity to the posterior of the embryo. Spatial regulation of *nanos* is achieved by a combination of RNA localization and localization-dependent translation such that only posteriorly localized *nanos* RNA is translated. *Cis*-acting sequences that mediate both RNA localization and translational regulation lie within the *nanos* 3′ untranslated region. We have identified a discrete translational control element within the *nanos* 3′ untranslated region that acts independently of the localization signal to mediate translational repression of unlocalized *nanos* RNA. Both the translational regulatory function of the *nanos* 3′UTR and the sequence of the translational control element are conserved between *D. melanogaster* and *D. virilis*. Furthermore, we show that the RNA helicase Vasa, which is required for *nanos* RNA localization, also plays a critical role in promoting *nanos* translation. Our results specifically exclude models for translational regulation of *nanos* that rely on changes in polyadenylation.

Key words: *nanos*, translational control element, translational regulation, 3′UTR, pattern formation, *Drosophila*

INTRODUCTION

The generation of body pattern during embryonic development requires temporal and spatial regulation of gene expression. In a variety of organisms such as *Drosophila*, *Caenorhabditis*, and *Xenopus*, the earliest events in embryogenesis are controlled by proteins synthesized from maternally provided mRNAs. Since these RNAs are present in oocytes prior to fertilization, their expression during development must be controlled by mechanisms other than transcriptional regulation. Numerous maternal mRNAs are stored in a translationally silent state in oocytes until after fertilization, when they become actively translated to produce proteins needed by the newly formed embryo (reviewed by Standart, 1992; Curtis et al., 1995a). In the early *C. elegans* embryo, spatially regulated translation of *glp-1* RNA restricts GLP-1 protein to the anterior blastomere of the two cell embryo, leading to the specification of anterior cell fates (Evans et al., 1994). In *Xenopus*, localization of maternal RNA molecules within the oocyte cytoplasm may play a role in limiting the distribution of proteins that induce patterning events in the early embryo (reviewed by St Johnston, 1995).

Both mRNA localization and translational regulation play important roles in the spatial restriction of key molecules in the *Drosophila* embryo. In the *Drosophila* embryo, whose early development occurs as a syncytium, gradients of regulatory proteins specify cell fates along the anterior-posterior axis. A gradient of Bicoid (Bcd) protein emanating from the anterior pole controls development of head and thoracic structures (Driever and Nüsslein-Volhard, 1988a) while a gradient of Nanos (Nos) protein emanating from the posterior pole controls development of the abdomen (Barker et al., 1992; Gavis and Lehmann, 1992). The activity of Bcd in anterior development is largely a function of its role as a concentration-dependent transcription factor (Driever et al., 1989; Struhl et al., 1989). Nos, in contrast, inhibits translation of the transcriptional repressor Hunchback in the posterior of the embryo, allowing expression of genes required for abdomen formation (reviewed by Hülskamp and Tautz, 1991). More recently, Bcd has also been shown to act as a translational regulator, inhibiting translation of Caudal protein in the anterior of the embryo where Caudal suppresses anterior development (Dubnau and Struhl, 1996; Rivera-Pomar et al., 1996). Thus, spatially restricted Bcd and Nos proteins confer spatial control of translation upon their target genes.

The generation of the Bcd and Nos protein gradients depends on the localization of the *bcd* and *nos* RNAs to the anterior and posterior poles of the embryo, respectively (Driever and Nüsslein-Volhard, 1988b; Wang et al., 1994). Posterior localization of *nos* RNA is essential for proper pattern formation, as production of Nos protein at the anterior of the embryo results in the suppression of anterior development and duplication of abdominal structures at the anterior (Wharton and Struhl, 1989; Gavis and Lehmann, 1992). In oocytes from females mutant for any one of nine genes, including *oskar* (*osk*) and *vasa* (*vas*), posterior localization of *nos* RNA does not occur (Wang et al.,

1994). The resulting embryos lack abdominal segments, a phenotype indistinguishable from the phenotype of embryos produced by *nos* mutant females (Lehmann and Nüsslein-Volhard, 1991). Thus, the failure of *nos* RNA localization results in loss of *nos* activity. We have previously shown that loss of *nos* activity in embryos defective in *nos* RNA localization results from translational repression of unlocalized *nos* RNA and that translation of *nos* RNA requires posterior localization (Gavis and Lehmann, 1994).

Among the genes required for *nos* RNA localisation, *osk* plays a key role. *Osk* activity directs formation of the pole plasm, the specialized posterior cytoplasm (Ephrussi et al., 1991; Kim-Ha et al., 1991; Ephrussi and Lehmann, 1992). Two components of pole plasm, Vasa (Vas) and Tudor (Tud) proteins, are also required for *nos* RNA localization (Wang et al., 1994) and these three factors may form a localization complex (Gavis et al., 1996). While the biochemical activities of Osk and Tud are unknown, Vas shows sequence similarity to translation initiation factor eIF-4A (Hay et al., 1988a; Lasko and Ashburner, 1988) and exhibits ATP-dependent RNA helicase activity in vitro (Liang et al., 1994). Vas is therefore an attractive candidate for a molecule involved in regulation of the activity of *nos* RNA.

Both localization of *nos* RNA and translational repression of unlocalized *nos* RNA are mediated by sequences within the *nos* 3′ untranslated region (3′UTR) (Gavis and Lehmann, 1994; Gavis et al., 1996). Replacement of the *nos* 3′UTR by 3′UTR sequences from the *α-tubulin* gene produces an unlocalized *nos* RNA that is translated throughout the embryo (Gavis and Lehmann, 1994). The *nos* RNA localization signal is large, spanning a 547 nucleotide region of the 3′UTR, and is composed of multiple partially redundant localization elements (Gavis et al., 1996). By contrast, we have now identified a discrete translational control element in the *nos* 3′UTR that is required for translational repression of unlocalized *nos* RNA. This element lies within the 5′-most portion of the RNA localization signal but functions independently of it. Sequences within the translational control element as well as the translational regulatory function of the *nos* 3′UTR are conserved between *D. melanogaster* and the related species, *D. virilis*. We show that *vas* is required to activate *nos* translation, providing a link between RNA localization and translational regulation.

MATERIALS AND METHODS

Fly stocks

The following mutant alleles were used: nos^{BN} (Wang et al., 1994), osk^{54} (Lehmann and Nüsslein-Volhard, 1991), vas^{PD} (Schüpbach and Wieschaus, 1986) vas^{D1} (Lehmann and Nüsslein-Volhard, 1991). For analysis of transgenes in a vas^{-} background, a vas^{PD}/vas^{D1} heteroallelic combination was used.

Transgenes, P element-mediated germline transformation, and analysis of transgenic lines

Construction of the *nos-tub3′UTR*, *nos-tub:nos3′UTR*, and *D. virilis nos* transgenes and the production of transgenic lines has been described by Gavis and Lehmann (1994) and Gavis et al. (1996). The *nosΔ10* transgene was constructed by removal of the 451 bp *Hpa*I-*Xmn*I fragment from the 3′UTR of the *nos* gene in pHSXgnosb^{R} (Gavis and Lehmann, 1992) followed by ligation of the remaining *Hpa*I and filled *Xmn*I ends. To create the *mel-vir3′UTR* transgene, an *Eco*RI site was introduced 5′ to the first nucleotide of the *D. virilis nos* 3′UTR (position 2506; Curtis et al., 1995b) by PCR. The integrity of the PCR-generated DNA was determined by dideoxy sequencing. The *D. virilis nos* 3′UTR and 3′ flanking genomic DNA were then joined to the *Eco*RI site at the 3′ end of the *D. melanogaster nos* coding region in pHSXgnosb^{R} (Gavis and Lehmann, 1992). For P element-mediated germline transformation (Spradling, 1986), the *mel-vir3′UTR* hybrid gene was inserted into the ry^{+} transformation vector pDM30 (Mismer and Rubin, 1987) and injected into ry^{506} embryos.

For analysis of *nos-tub:nos3′UTR* transgenes in nos^{-}, osk^{-}, and vas^{-} backgrounds, 3-9 independent lines of each transgene (except for *nos-tub:nos+3*) were tested in each background to guard against variations due to differences in RNA expression levels. Whenever possible, lines bearing insertions on the X chromosome were tested in each background to ensure uniformity. However, no X chromosome insertions were found among sixteen independent lines of *nos-tub:nos+9*, and X chromosome insertions could not be isolated for transgenes that produce dominant female sterility (*nos-tub3′UTR*, *nos-tub:nos+3*, +4, and +6). For *nos-tub3′UTR*, *nos-tub:nos+4*, +6, and +9, 3-6 second chromosome insertions were tested in both nos^{-} and osk^{-} females, whereas 3-6 third chromosome insertions were tested in vas^{-}. Only one second chromosome and one third chromosome insertion were isolated for *nos-tub:nos+3*. A second chromosome insertion of the *D. virilis nos* transgene was introduced into both nos^{-} and osk^{-} females. Eight independent insertions of the *mel-vir3′UTR* transgene were tested in nos^{-} females and four in osk^{-} females. All lines behaved similarly in these experiments.

For immunoblot analysis of embryos from nos^{-} females carrying different *nos-tub:nos3′UTR* transgenes, transgenic lines whose RNA levels had been shown to be similar (Gavis et al., 1996) were used.

Analysis of embryonic phenotypes

Embryos were collected on yeasted apple juice agar plates (Wieschaus and Nüsslein-Volhard, 1986) at room temperature and aged for >30 hours at room temperature, after which the number of hatched embryos was determined and larval cuticle preparations were made (Wieschaus and Nüsslein-Volhard, 1986). For genotypes in which any embryos developed sufficient numbers of abdominal segments to hatch, embryos were collected prior to hatching and isolated in small baskets on individual agar plates. In this way, all embryos could be recovered from all lines and independent lines could be kept segregated, regardless of the extent of their abdominal segmentation.

Extracts and immunoblotting

0- to 2-hour embryos were collected at room temperature, dechorionated with 50% bleach, washed with dH_2O, frozen in liquid nitrogen, and stored at –80°C. Frozen embryos were homogenized in 3 volumes of boiling sample buffer (0.125 M Tris-HCl pH 6.8, 4% SDS, 10 mM DTT, 20% glycerol, 5 M urea), boiled for 2-3 minutes, and spun at 14,000 ***g*** for 20 minutes. Supernatants were separated by SDS-PAGE followed by semi-dry transfer to PVDF membrane (Immobilon, Millipore). Immunoblotting was carried out with anti-Nos antiserum (Wang et al., 1994) and AP-goat anti-rabbit secondary antibody (Jackson ImmunoResearch) in 10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20, 5% nonfat dry milk. Proteins were detected using alkaline phosphatase immunochemistry (Blake et al., 1984).

Secondary structure analysis

Optimal and suboptimal secondary structure predictions were obtained at both 25°C and 37°C with the MFOLD program (Zuker, 1989; Jaeger et al., 1989a,b). Graphic representations were generated using LoopDLoop (D. Gilbert).

Analysis of poly(A) tail length

Total RNA was isolated from 0- to 2-hour embryos from wild-type, osk^{54} and vas^{PD}/vas^{D1} females. 0.1 μg of RNA was used for poly(A)

test (PAT) cDNA synthesis with oligo(dT) in a 65 μl reaction according to the method of Sallés and Strickland (1995). 1 μl of this reaction was amplified by PCR with Primer T and a *nos* specific primer. PCR reactions were phenol extracted and ethanol precipitated. Half of each reaction was digested with *Xho*I. Half of each undigested sample and half of each digested sample were separated on a 2% agarose gel, then transferred to nylon membrane (Zetaprobe, BioRad). The membrane was hybridized with a ^{32}P-labeled probe prepared from the 435 bp *Eag*I-*Xho*I fragment of the *nos* 3′UTR (Wang and Lehmann, 1991) according to the manufacturer's protocol.

Primer T sequence: 5′GCGAGCTCCGCGGCCGCGTTTTTTTTT-TTT3′
nos primer sequence: 5′GTCGTCGGCTACGCATTCATTGT3′.

RESULTS

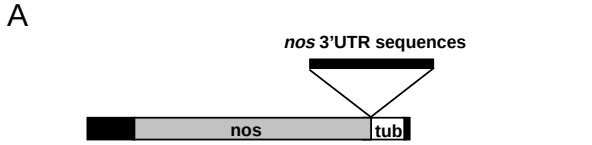
Cis-acting sequences controlling translational repression of *nos* RNA

Because the *nos* 3′UTR contains the *nos* RNA localization signal, replacement of *nos* 3′UTR sequences with sequences from the *α-tubulin* 3′UTR yields an unlocalized *nos* RNA derivative (*nos-tub3′UTR*; Gavis and Lehmann, 1994) In contrast to wild-type *nos* RNA, whose translation is repressed in the absence of localization, unlocalized *nos-tub3′UTR* RNA is translated to produce Nos protein throughout the embryo (Gavis and Lehmann, 1994). Thus, in addition to localization signal sequences, the *nos* 3′UTR contains sequences that repress translation of unlocalized *nos* RNA. To define more specifically which 3′UTR sequences mediate translational repression, we tested a set of *nos* 3′UTR deletion mutants for their ability to confer translational regulation on the unregulated *nos-tub3′UTR* RNA (Fig. 1).

nos 3′UTR deletion mutants constructed in vitro using restriction endonucleases or by PCR amplification were inserted into a single site within the *α-tubulin* 3′UTR sequences of the *nos-tub3′UTR* hybrid gene (Fig. 1A,B; Gavis et al., 1996). Transgenes bearing these *nos-tub:nos3′UTR* hybrid genes were introduced into flies by P element-mediated germline transformation. After isolation of multiple independent transgenic lines for each construct, embryos from transgenic females were analyzed for the ability of the 3′UTR fragments to confer translational repression by monitoring the levels of *nos* activity and Nos protein. Comparisons among different *nos-tub:nos3′UTR* transgenes have been made using lines that produce similar amounts of RNA (Gavis et. al., 1996 and data not shown). In addition, we have tested multiple independent lines for all transgenes except *nos-tub:nos+3* (see Materials and Methods). Therefore, differences in the behavior of these transgenes are unlikely to result from differences in the levels of expression or stabilities of the hybrid RNAs.

Because Nos protein is present throughout the embryo, the *nos-tub3′UTR* transgene causes dominant female sterility. All embryos produced by transgenic females lack head and thoracic structures and often exhibit duplication of posterior terminal structures at the anterior. These anterior defects result from the ability of Nos protein to inhibit translation of *bicoid* and maternal *hunchback* RNAs at the anterior of the embryo (Gavis and Lehmann, 1994). Since the wild-type *nos* gene is still present in these embryos, translational repression of *nos-tub3′UTR* RNA should lead to reversion of this dominant female sterility and the production of viable embryos.

For all lines tested, embryos produced by females carrying either one or two copies of the *nos-tub:nos+1* transgene show completely wild-type development, suggesting that the +1 region is sufficient to confer translational repression (Fig. 1B). 50-80% of embryos produced by females carrying one copy of the *nos-tub:nos+2′* transgene are phenotypically wild-type, whereas the remainder show reduction of head and thoracic structures. When females carry two copies of the transgene, nearly all embryos exhibit defects in formation of anterior



B

	RNA LOCALIZATION	REVERSION OF FEMALE STERILITY	TRANSLATIONAL REPRESSION
nos 3'UTR	4+	complete	strong
+1	1+	complete	strong
+2'	0.5+	partial	weak
+3	2+	no	no
+4	1+	no	no
+6	–	no	no
+2	2.5+	complete	strong
+2,3	4+	complete	strong
+9	3+	partial	weak
+10	4+	complete	weak

Fig. 1. Identification of sequences that mediate translational repression of *nos* RNA. (A) Organization of the *nos-tub:nos3′UTR* RNAs, including the *nos* 5′UTR (large filled box) and coding region (stippled box), *α-tubulin* 3′UTR sequences (open box), *nos* polyadenylation signal (small filled box), and specific *nos* 3′UTR sequences inserted at a unique site within the *α-tubulin* 3′UTR. (B) *nos* 3′UTR deletion mutants. Wild-type *nos* 3′UTR sequences (3′UTR nucleotides 6-842) used to generate deletion mutants (Gavis et al., 1996) are depicted by the open box. The sequences retained in each deletion mutant are represented by the stippled bars. The relative RNA localization function of each mutant was previously determined (Gavis et al., 1996) and is expressed on a scale of '4+' for wild-type function to '–' for no detectable posterior localization. Reversion of dominant female sterility was determined by analysis of rates of hatching, and the presence of head and thoracic structures of embryos from wild-type females carrying single copies of *nos-tub:nos3′UTR* transgenes. Lines producing embryos, all of which fail to hatch and lack head and thoracic structures, show 'no' reversion of dominant female sterility. Lines producing hatching embryos with wild-type anterior development show 'complete' reversion. Lines producing both wild-type embryos and embryos with reduced anterior structures show 'partial' reversion. 3-6 independent lines of each transgene were analyzed and >100 embryos were scored for each line. The ability of *nos* 3′UTR deletion mutants to repress translation of *nos* RNA summarizes the cumulative results of all assays described in the text.

Table 1. Activity of *nos-tub:nos3′UTR* transgenes in embryos from *nos*⁻ and *osk*⁻ females

Abdominal segments	% of embryos											
	nos-tub3′UTR		*nos-tub:nos+1*		*nos-tub:nos+2′*		*nos-tub:nos+3*		*nos-tub:nos+4*		*nos-tub:nos+6*	
	nos⁻	*osk*⁻	*nos*⁻	*osk*⁻	*nos*⁻	*osk*⁻	*nos*⁻	*osk*⁻	*nos*⁻	*osk*⁻	*nos*⁻	*osk*⁻
0	–	–	–	64	–	–	–	–	–	–	–	–
1-3	–	–	98	30	–	–	–	–	–	–	–	–
4-6	33*	30*	2	6	2	3	62*	80*	–	–	–	–
7-8	67	70	–	–	98	97	38	20	100	100	100	100
Loss of head/ thorax	100	100	–	–	50	30	100	100	100	100	100	100

Abdominal segments	% of embryos									
	nos-tub:nos+2		*nos-tub:nos+2,3*		*nos-tub:nos+9*		*nos-tub:nos+10*		*nos-tub:nos+3'UTR*	
	nos⁻	*osk*⁻	*nos*⁻	*osk*⁻	*nos*⁻	*osk*⁻	*nos*⁻	*osk*⁻	*nos*⁻	*osk*⁻
0	–	73	–	96	–	–	–	–	–	88
1-3	–	26	–	4	–	–	–	9	–	10
4-6	2	1	–	–	5	22	–	62	–	2
7-8	98		100	–	95	78	100	29	100	–
Loss of head/ thorax	–	–	–	–	30	50	–	–	–	–

nos-tub3′UTR and *nos-tub:nos3′UTR* transgenes were introduced into *nos* or *osk* mutant females. With the exception of *nos-tub:nos+3*, multiple independent lines were tested for each transgene and the same transgenic lines were tested in both genotypes. The number of abdominal segments and the loss of head and thoracic structures was determined in cuticle preparations of >100 embryos for each transgene. Numbers represent the average of two independent experiments using the same transgenic line.

*These embryos are sufficiently posteriorized that they lack anterior abdominal segments. A small number exhibit the bicaudal phenotype in which posterior structures are duplicated in mirror image symmetry at the anterior.

structures. Thus, the +2′ region confers partial repression and the decrease in activity can be overcome by increasing the transgene dosage. As expected, embryos from females carrying the *nos-tub:nos+2* transgene, which contains the +1 as well as the +2′ region, show normal development.

In contrast to the 178 nucleotide portion of the *nos* 3′UTR encompassed by the +1 and +2′ regions, the remainder of the 3′UTR does not confer translational repression (Fig. 1B). Three of the transgenes, *nos-tub:nos+3*, +4, and +6, fail to revert the dominant female sterility and behave similarly to the *nos-tub3′UTR* transgene, indicating that these regions of the *nos* 3′UTR do not contain sequences capable of mediating translational repression. Three larger portions of the 3′UTR behave as expected from the combinations of sequences they contain. *nos-tub:nos+9* behaves similarly to *nos-tub:nos+2′* in that some embryos from females heterozygous for the transgene show reduced head structures and this number is increased when females are homozygous for the transgene. *nos-tub:nos+2,3* and *nos-tub:nos+3′UTR* behave similarly to *nos-tub:nos+2*, allowing the production of phenotypically wild-type embryos. Dominant female sterility is also completely reverted by the *nos-tub:nos+10* transgene, which differs from +9 only in its 3′ extent (see below).

These results indicate that a large portion of the *nos* 3′UTR, designated by the +3, +4, and +6 regions, cannot mediate translational repression of *nos-tub3′UTR* RNA. By contrast, sequences within the +1 region severely reduce the activity of *nos-tub3′UTR* RNA in the anterior of the embryo whereas sequences within the +2′ region confer partial reduction. To provide a more sensitive assay for the amount of *nos* activity produced by the different *nos-tub:nos3′UTR* RNAs, the transgenes were introduced into females lacking *nos* function (Table 1). In these embryos, all *nos* activity is provided by the transgene. The *nos* activity produced by the *nos-tub3′UTR* transgene permits the formation of abdominal segments in *nos*⁻ females and inhibits anterior development (Gavis and Lehmann, 1994). Therefore, embryos from *nos*⁻ females carrying the *nos-tub3′UTR* transgene are indistinguishable from embryos from wild-type females carrying the transgene. By contrast, the *nos-tub:nos+3′UTR* transgene behaves like a wild-type *nos* gene and completely complements the *nos*⁻ phenotype, producing phenotypically wild-type embryos. Embryos from *nos*⁻ females carrying the *nos-tub:nos+1* transgene show little *nos* activity, developing with only 1-3 abdominal segments and wild-type head and thorax. *nos*⁻ females carrying the *nos-tub:nos+2′* transgene, however, show significant *nos* activity, producing 7-8 abdominal segments. Some of these embryos exhibit reduced formation of head and thoracic structures. In *nos*⁻ females, *nos-tub:nos+3*, +4, and +6 transgenes behave identically to *nos-tub3′UTR*, yielding embryos with eight abdominal segments but lacking anterior structures. *nos-tub:nos+9* behaves very similarly to +2′ while *nos-tub:nos+2*, +2,3, and +10 behave identically to a wild-type *nos* transgene, producing phenotypically wild-type embryos.

To determine whether the *nos* activity levels detected in this assay reflect Nos protein levels in the embryos, extracts of embryos from *nos*⁻ females carrying *nos-tub:nos3′UTR* transgenes were analyzed on immunoblots with an anti-Nos antibody (Fig. 2). In all cases, the relative amounts of Nos protein produced by the different transgenes correlates with the level of Nos activity detected phenotypically. On the basis of both types of data, we conclude that the 90 nucleotide +1 region contains sequences capable of repressing translation of *nos-tub3′UTR* RNA while the 88 nucleotide +2′ region contains sequences that provide weak repression. The *α-tubulin* 3′UTR sequences are unlikely to play a role in this

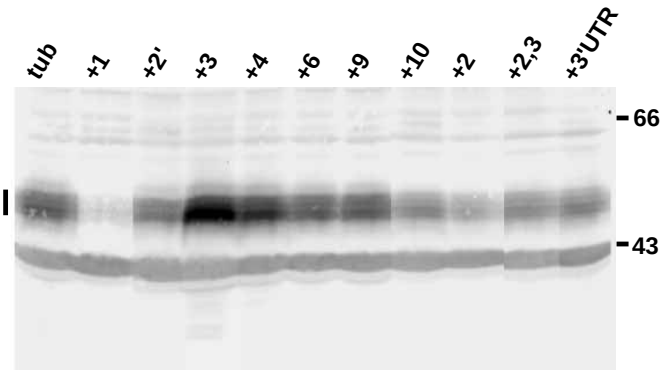


Fig. 2. Nos protein levels reflect activity of *nos-tub:nos3′UTR* RNAs. Equivalent amounts of extracts of 0- to 2-hour embryos from *nos*⁻ females carrying different *nos-tub:nos3′UTR* transgenes, as determined by Ponceau-S staining of the blot, were fractionated on a 10% SDS-polyacrylamide gel. Nos protein was detected with a polyclonal anti-Nos antiserum (Wang et al., 1994). Nos protein appears as a set of bands, indicated by the vertical bar, when *nos-tub:nos3′UTR*s are translationally active. The more rapidly migrating band present in each lane represents the abundant yolk proteins, which are detected nonspecifically by the anti-Nos antiserum. Extracts are designated by the *nos* 3′UTR region present in the respective transgenes (see Fig. 1). Relative molecular mass markers: BSA, 66×10^3; ovalbumin, 43×10^3.

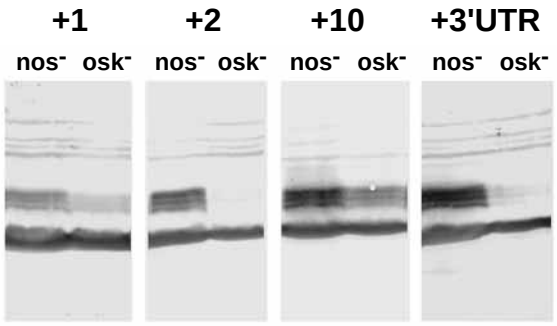


Fig. 3. Nos protein levels reflect *nos* localization and translational regulation. Individual *nos-tub:nos3′UTR* transgenes were introduced simultaneoulsy into *nos*⁻ and *osk*⁻ females. Equivalent amounts of extracts of 0- to 2-hour embryos from these females, as determined by Ponceau-S staining, were fractionated on a 10% SDS-polyacrylamide gel. Both *nos-tub:nos+1* extracts were overloaded relative to the other extracts to facilitate visualization of the small amount of Nos protein present. Nos protein was detected with a polyclonal anti-Nos antiserum. Extracts are designated by the *nos* 3′UTR region present in the respective transgenes. Relative molecular mass markers as in Fig. 2.

effect since they are present in all constructs tested. Furthermore, a *nos* deletion mutant in which the +10 region of the 3′UTR has been removed (*nosΔ10*), leaving only the +1 and +6 regions fused to each other, behaves similarly to *nos-tub:nos+1* in all assays (data not shown).

Coordinate regulation of *nos* by translational repression and RNA localization

Previous analysis of *nos-tub:nos3′UTR* and *lacZ-nos3′UTR* hybrid RNAs demonstrated that sequences within a 547 nucleotide region of the *nos* 3′UTR are capable of mediating varying levels of posterior localization (Gavis et al., 1996). Two overlapping domains, the +2,3 region and the +10 region, each provide wild-type localization function. Since localization permits translation of *nos* RNA, the activity of *nos-tub:nos3′UTR* RNAs reflects both their ability to be localized and translated and the ability of the unlocalized RNA to be translationally repressed. Therefore, to confirm that the results of the experiments described above reflect translational repression of unlocalized RNA and to evaluate the relationship between RNA localization and translational regulation in *nos* activity, we eliminated their localization by introducing *nos-tub:nos3′UTR* transgenes into females lacking *osk* function.

A key component of the localization machinery, *osk*, acts genetically through the *nos* RNA localization signal (Gavis et al., 1996). In embryos from *osk*⁻ females carrying *nos-tub:nos3′UTR* transgenes, both wild-type *nos* RNA and *nos-tub:nos3′UTR* RNAs are unlocalized (data not shown). In the absence of *osk* function, the low level of *nos* activity and Nos protein produced by *nos-tub:nos+1*, which is weakly localized, is further reduced (Table 1; Fig. 3). *nos-tub:nos+1* RNA is thus strongly repressed when unlocalized but can be translated upon posterior localization. *nos-tub:nos+2′* RNA, which has minimal localization ability, shows similar *nos* activity in embryos from either *nos*⁻ or *osk*⁻ females. *nos-tub:nos+2*, which contain both the +1 and +2′ regions, shows moderate localization. In the absence of localization, *nos* activity and protein levels produced by *nos-tub:nos+2* are significantly reduced. These results demonstrate that sequences within the +1 and +2′ regions mediate translational repression of unlocalized RNA. Activity of *nos-tub:nos+2* RNA in *nos*⁻ females results, therefore, from derepression and translation of localized RNA.

The *nos-tub3′UTR*, *nos-tub:nos+3*, +4, and +6 RNAs, which lack the +1 and +2′ regions, continue to produce high levels of *nos* activity throughout the embryo and dominant female sterility in the absence of *osk* function, confirming that these RNAs are translated regardless of their state of localization (Table 1). Since the +3 region of the *nos* 3′UTR has moderate localization function, the increased activity of *nos-tub:nos+3* at the anterior of embryos from *osk*⁻ females may reflect an increase in the amount of RNA in the anterior region of the embryo. *nos-tub:nos+9* RNA, which shows substantial posterior localization, exhibits a small increase in *nos* activity in the anterior and concomitant decrease at the posterior in embryos from *osk*⁻ females. These results are consistent with a shift from conditions in which a significant amount of *nos-tub:nos+9* RNA is localized and efficiently translated at the posterior pole while the remaining unlocalized RNA is partially repressed, to conditions in which all of the *nos-tub:nos+9* RNA is unlocalized and its translation is partially repressed. *nos-tub:nos+10* RNA, whose localization is wild-type, behaves similarly to +9 in that it shows a decrease in posterior activity and Nos protein in embryos from *osk*⁻ females, consistent with loss of localization and translation at the posterior pole. Unlocalized *nos-tub:nos+10* RNA shows somewhat less activity than *nos-tub:nos+9* RNA. Since *nos-tub:nos+10* differs from +9 in its inclusion of the +4 region, this region may be able to enhance repression by +2′ region although it fails to repress translation on its own. As expected, the activites of *nos-tub:nos+2,3* and *nos-tub:nos+3′UTR*,

Table 2. Activity of *nos-tub:nos3′UTR* RNAs in embryos from *osk*⁻ and *vas*⁻ females

Abdominal segments	% of embryos							
	nos-tub:nos+1		*nos-tub:nos+2*		*nos-tub:nos+3′UTR*		*nos-tub:nos+2′*	
	osk⁻	*vas*⁻	*osk*⁻	*vas*⁻	*osk*⁻	*vas*⁻	*osk*⁻	*vas*⁻
0	64	96	75	100	88	100	–	–
1	15	4	11	–	7	–	–	–
2	6	–	3	–	1	–	–	–
3	5	–	6	–	2	–	–	–
4	5	–	1	–	1	–	–	–
5	3	–	4	–	1	–	–	–
6	2	–	–	–	–	–	–	–
7	–	–	–	–	–	–	3	8
8	–	–	–	–	–	–	97	92

nos-tub:nos3′UTR embryos were introduced into *osk* and *vas* mutant females and the number of abdominal segments in embryos was determined in cuticle preparations of >100 embryos for each transgene. At least 3 independent lines were tested for each transgene and the same transgenic line was tested simultaneously in both genotypes. The data shown represents the average of two independent experiments, in which the line with the greatest activity was used for each transgene to optimize comparison between the two genotypes. The *osk* allele used, osk^{54}, introduces a stop codon early in the coding regions of both the long and short Osk proteins (Kim-Ha et al., 1991; Markussen et al., 1995) and behaves as a null allele (Lehmann and Nüsslein-Volhard, 1991). The vas^{PD} and vas^{D1} alleles, which do not alter the coding region, severely reduce the amount of Vas protein produced (Hay et al., 1988a; Lasko, 1990).

which contain translational repression as well as RNA localization signal sequences, are severely reduced when localization is eliminated.

Differential regulation of *nos* by *osk* and *vas*

Localization of RNAs bearing *nos* 3′UTR sequences to the posterior pole requires the wild-type function of both *osk* and *vas* (Gavis and Lehmann, 1992; Gavis et al., 1996). *nos-tub:nos3′UTR* transgenes containing the translational control element behave differently, however, when introduced into *osk*⁻ versus *vas*⁻ females. These results were confirmed in at least three experimental trials using multiple independent lines of each transgene. In these experiments, *nos-tub:nos+1*, +2, and *nos-tub:nos+3′UTR* consistently show low levels of activity in the absence of *osk* function but completely lack activity in the absence of *vas* function (Table 2). Control females mutant for the *osk* or *vas* alleles alone consistently produce embryos lacking all abdominal segments (data not shown). This difference in activity between *osk*⁻ and *vas*⁻ is not detected for the other *nos-tub:nos3′UTR* transgenes but is exhibited by the *nosΔ10* transgene, which lacks the *α-tubulin* sequences (data not shown). Since wild-type *nos* RNA levels are not affected by loss of either *osk* or *vas* function (Wang et al., 1994), and several different *nos-tub:nos3′UTR* RNAs show this effect, it is unlikely that it results from differential stability of the RNAs in these two mutants. Therefore, these results indicate that translation of *nos* RNAs containing the translational control element requires derepression by *vas*.

Evolutionary conservation of sequence of the translational control element and conservation of translational control mechanisms

Comparison of the 3′UTRs of *nos* genes from *D. melanogaster* and its sibling species *D. virilis* revealed ten discrete regions of sequence similarity (Gavis et al., 1996). Five regions with balanced base composition fall within the *nos* RNA localization signal whereas five A/T-rich regions lie to its 3′ side (Fig. 4A). One of the regions within the localization signal comprises the majority of the translational control element. This region shows 76% identity over 69 nucleotides (Fig. 4B). The adjacent region (+2′), which has weak translational repression function also contains a conserved region of 42 nucleotides with 66% identity. RNA folding algorithms (Zuker, 1989; Jaeger et al., 1989a,b) predict similar secondary structures for the sequences within the *D. melanogaster nos* translational control element and the corresponding conserved region of the *D. virilis nos* 3′UTR (Fig. 4C). In addition to the similarity in their general organization, these structures show complete sequence identity in the distal region of stem II, the loop at the end of stem II, and nearly complete identity in the composition of stem III.

Table 3. Translational regulation of *D. virilis nos* RNA in *D. melanogaster* embyros

Abdominal segments	% of embryos			
	vir nos		*mel-vir3′UTR*	
	nos⁻	*osk*⁻	*nos*⁻	*osk*⁻
0	–	72	0	95
1-3	–	23	0	5
4-6	44	4	0	0
7-8	56	1	100	0

A *D. virilis nos* transgene (*vir nos*; Curtis et al., 1995b) and the hybrid *mel-vir3′UTR* transgene were each introduced into *nos* and *osk* mutant females. The activity of *vir nos* and *mel-vir3′UTR* RNAs were assayed in the localized (*nos*⁻) and unlocalized (*osk*⁻) state by the formation of abdominal segments. >100 embryos were analyzed for each genotype.

When introduced into *D. melanogaster* females, a *D. virilis nos* transgene provides substantial *nos* activity (Table 3; Curtis et al., 1995b). *D. virilis nos* RNA shows wild-type posterior localization in embryos from transgenic *D. melanogaster* females (Gavis et al., 1996). When this transgene is introduced into *osk*⁻ females, *nos* activity is severely reduced, indicating that translation of unlocalized *D. virilis nos* RNA can be repressed in a *D. melanogaster* embryo (Table 3). The *D. virilis nos* 3′UTR is sufficient to confer translational regulation in *D. melanogaster*. Replacement of the *D. melanogaster nos* 3′UTR with the *D. virilis nos* 3′UTR produces a hybrid *mel-vir3′UTR* transgene that behaves similarly to a wild-type *nos* transgene (Table 3). A single copy of the *mel-vir3′UTR* transgene complements the abdominal defects of embryos produced by *nos*⁻ *D. melanogaster* females, permitting complete viability. In

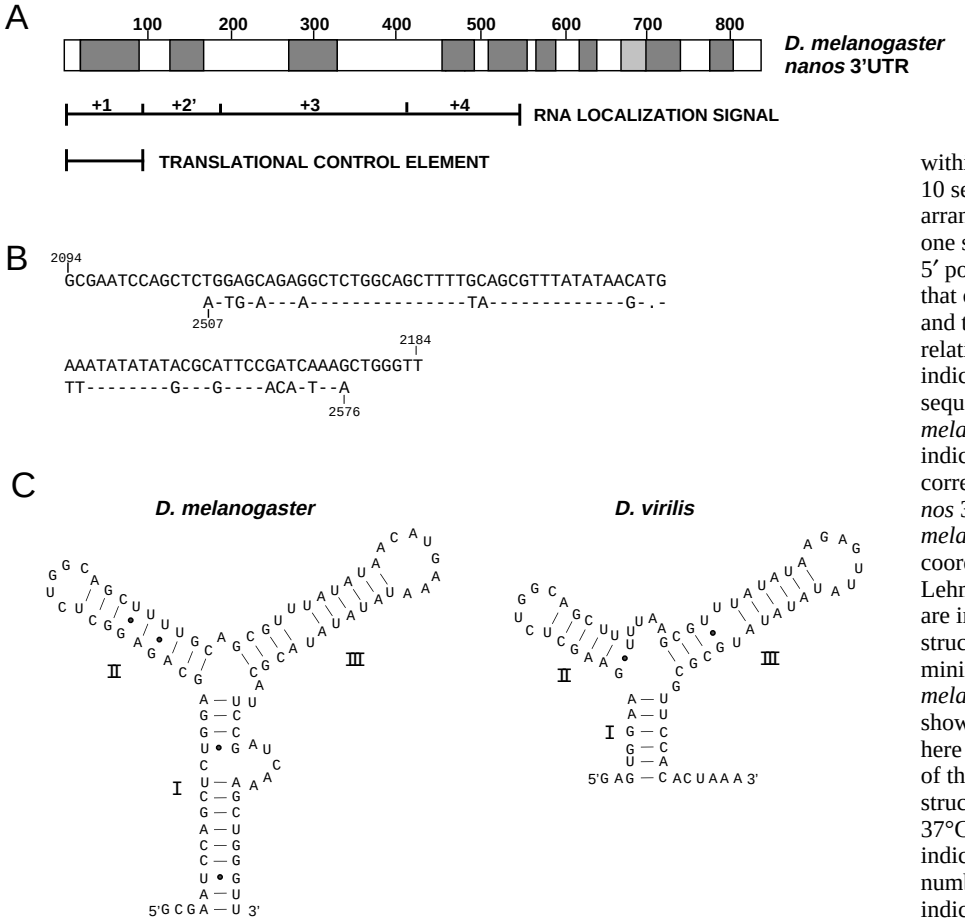


Fig. 4. Evolutionary conservation of *nos* 3′UTR sequences and structure. (A) Schematic representation of the *nos* 3′UTR of *D. melanogaster* showing the segments of similarity to sequences within the *nos* 3′UTR of *D. virilis*. Nine of the 10 segments (dark stippling) show a colinear arrangement between the two 3′UTRs while one segment (light stippling) occupies a more 5′ position in the *D. virilis* 3′UTR. Sequences that compose the *nos* RNA localization signal and translational control element and their relationship to the conserved segments are indicated. (B) Alignment of *D. virilis* 3′UTR sequence (bottom line) with the *D. melanogaster* +1 region sequence (top line) as indicated in A. The sequences shown correspond to nucleotides 2-71 of the *D. virilis nos* 3′UTR and nucleotides 6-96 of the *D. melanogaster nos* 3′UTR. Sequence coordinates (*D. melanogaster*: Wang and Lehmann, 1991; *D. virilis*: Curtis et al., 1995b) are indicated. (C) Predicted secondary structures of the sequences shown in B. The minimum energy structures predicted for the *D. melanogaster* and *D. virilis* sequences are shown. (N.B. The *D. virilis* sequence shown here begins at position 2506, the first nucleotide of the 3′UTR). The same minimum energy structures were obtained at both 25°C and 37°C. The 5′ and 3′ ends of the RNA are indicated and the corresponding stems are numbered from I-III. G-U base pairs are indicated by a filled circle.

embryos from *osk*⁻ females, the activity of *mel-vir3′UTR* RNA is severely reduced, demonstrating that the *cis*-acting translational regulatory sequences of the *nos* 3′UTR are conserved in function as well as in sequence.

Translational control is not mediated by a cytoplasmic polyadenylation-dependent mechanism

Recent analysis of a number of maternal RNAs in *Drosophila* as well as in other organisms has revealed a mechanism of translational regulation by cytoplasmic polyadenylation (reviewed by Wickens, 1992; Vassalli and Stutz, 1995). RNAs encoded by the *bcd*, *torso* and *Toll* genes are present in oocytes but are not translated until after egg activation or fertilization (Sallés et al., 1994). Activation of translation is accompanied by poly(A) tail elongation and, as shown for *bcd*, elongation is required for translational activation to occur. By contrast, the poly(A) tail length of *nos* RNA is relatively constant in ovaries and embryos (Sallés et al., 1994). These experiments did not distinguish localized from unlocalized *nos* RNA, however. To test whether polyadenylation is used to regulate translation of *nos* in a localization-dependent manner, the poly(A) tail length of *nos* RNA isolated from embryos from WT, *osk*⁻, and *vas*⁻ females was compared using an RT/PCR assay (Sallés and Strickland, 1995). No variation in *nos* poly(A) tail length was detected in *nos* RNA from each of these genotypes (Fig. 5), indicating that cytoplasmic polyadenylation does not mediate localization-dependent translational control of *nos* RNA.

DISCUSSION

Identification of the *nos* translational control element

We have identified a 90 nucleotide region of the *nos* 3′UTR (the +1 region) that mediates translational repression of unlocalized *nos* RNA and we designate this region as the *nos* translational control element (TCE). An adjacent 88 nucleotide region (+2′) shows limited ability to repress translation and acts as an auxiliary element. The small size of the TCE contrasts with the *nos* RNA localization signal, which is large and complex (Gavis et al., 1996). Recently, spatially regulated translation of several other maternal mRNAs in *Drosophila* has been shown to be mediated by sequences contained within their 3′UTRs. Like *nos*, unlocalized *osk* RNA is translationally repressed (Kim-Ha et al., 1995; Markussen et al., 1995; Rongo et al., 1995) and this repression is mediated by sequences within three small segments of the *osk* 3′UTR that are recognized by an 80×10^3 M_r ovarian protein (Kim-Ha et al., 1995). Translational repression of maternal *hunchback* (*hb*) mRNA by Nos and Pumilio proteins is mediated through small sequence motifs within the *hb* 3′UTR (Wharton and Struhl, 1991; Murata and Wharton, 1995). Sequences within the *caudal* 3′UTR mediates *bcd*-dependent translational regulation of *caudal* mRNA and are recognized by the Bcd homeodomain (Dubnau and Struhl, 1996; Rivera-Pomar et al., 1996). Thus, 3′UTR-mediated translational regulation appears to be a general means

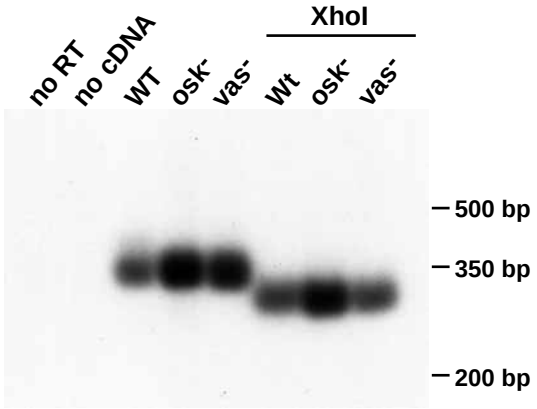


Fig. 5. *nos* poly(A) tail length does not reflect *nos* translational activity. *nos* RNA isolated from embryos from wild-type (WT), *osk*$^{-}$, and *vas*$^{-}$ females was analyzed by the PCR poly(A) test (PAT) (Sallés and Strickland, 1995). As controls, reverse transcriptase was omitted from the wild-type cDNA synthesis reaction (no RT) and wild-type cDNA was omitted from the PCR (no cDNA). Poly(A) tail-containing RT/PCR products were visualized by Southern blotting using a ^{32}P-labeled *nos* 3′UTR probe. In all genotypes, the undigested product migrates as a discrete band of ~350 bp. Since the distance from the 5′ end of the specific *nos* primer to the site of poly(A) addition is 300 bp, the maximal poly(A) tail length is approx. 50 nt, consistent with previous measurements (Sallés et al., 1994). Digestion of this product with *Xho*I, whose site is located 286 bp from the 5′ end of the *nos* primer, produces a band of the expected size. Because this assay is not quantitative, differences in the amount of the RT/PCR products generated for the different genotypes is not meaningful.

of generating spatial information during oogenesis and embryogenesis.

Independence of localization signal and translational repression

While the *nos* TCE is contained within the *nos* RNA localization signal, the TCE acts independently of the localization signal to mediate repression of unlocalized *nos* RNA. All *nos-tub:nos3′UTR* RNAs bearing the TCE show decreased levels of *nos* activity and protein when unlocalized in embryos from *osk*$^{-}$ and *vas*$^{-}$ females. Likewise, *nos* RNA localization does not require TCE sequences. *nos-tub:nos+10*, which lacks the TCE, shows completely wild-type localization (Gavis et al., 1996).

Our results show that the amount of *nos* activity in the embryo depends upon both the state of localization of the *nos* RNA, i.e. the ratio of localized to unlocalized RNA, and the ability of the unlocalized RNA to be translationally repressed. Moderate to strong localization combined with translational repression of unlocalized RNA (*nos-tub:nos+2*, *nos-tub:nos+2,3* and *nos-tub:nos+3′UTR*) leads to the production of Nos at the posterior of the embryo and the elimination of *nos* activity from the anterior of the embryo. However, once the RNA is unlocalized, only the function of the TCE determines how many abdominal segments will be formed. Weak localization of *nos-tub:nos+1* combined with repression of the large portion of the RNA that remains unlocalized leads to production of a low levels of Nos protein at the posterior pole and formation of a small number of abdominal segments. When this RNA is completely unlocalized, as in the absence of *osk* or *vas* function, little Nos protein is produced and the majority of embryos completely lack abdominal segments. Weak repression of the poorly localized *nos-tub:nos+2′* RNA leads to a uniform distribution of Nos protein at a level that is sufficient to allow abdominal segmentation but not to suppress anterior development completely. In this case, a wild-type embryo can be produced in the absence of the Nos protein gradient.

Conservation of translational control mechanism

The *nos* 3′UTRs of *D. melanogaster* and *D. virilis* show sequence similarity within ten discrete segments. The 5′-most segment in both 3′UTRs lies within the *nos* TCE and the next 3′ segment lies within the +2′ region. The ability of *D. virilis nos* 3′UTR to confer translational regulation in a *D. melanogaster* embryo demonstrates that the regulatory mechanism is evolutionarily conserved and suggests that the conserved sequences may represent targets of the translational regulatory machinery. Since the +1 and +2′ regions are also components of the RNA localization signal and localization signal function is conserved between *D. melanogaster* and *D. virilis* (Gavis et al., 1996), the same motifs may be recognized by both localization and translational regulatory machinery or different subregions of the conserved segments may be recognized independently by each. In addition to primary sequence conservation, the +1 regions of the *D. melanogaster nos* 3′UTR and the corresponding region of the *D. virilis nos* 3′UTR are predicted to form similar dual stem-loop structures. Thus, structural features of the *nos* TCE may dictate its recognition or, alternatively, provide its mechanism of action.

Mechanism of translational regulation

Models to explain translational regulation of *nos* RNA must address how the *nos* TCE mediates repression of the unlocalized RNA and how posterior localization relieves this repression. The *nos* TCE could function solely as RNA, forming a structure that inhibits translation, or through the activity of trans-acting factor(s) bound to this sequence. *nos* RNA synthesized in vitro is translated after injection into embryos, suggesting that RNA structure alone is not sufficient to maintain the RNA in a repressed state. The failure of the injected RNA to be repressed suggests that *nos* RNA becomes translationally repressed by interaction with a trans-acting repressor protein at an earlier time, perhaps when the RNA first enters the oocyte. Alternatively, this repressor protein may be present in limiting amounts in embryos and injection of large quantities of *nos* RNA may lead to its rapid titration.

One mechanism by which 3′UTR sequences have been shown to regulate translation of maternal RNAs involved in embryonic patterning in *Drosophila* is through cytoplasmic polyadenylation (Sallés et al., 1994). In these studies, elongation of the poly(A) tail was shown directly to be necessary for activation of translation of the *bcd* RNA after fertilization. Since the poly(A) tail of *nos* RNA does not change depending on its state of localization, localization-dependent translational regulation of *nos* RNA does not appear to be regulated in a similar manner. Translational regulation of RNAs that undergo cytoplasmic polyadenylation is often linked to general developmental cues such as egg activation, fertilization, or activation of zygotic transcription (reviewed by

Wickens, 1992; Vassalli and Stutz, 1995). Spatially regulated translation may be effected by a different 3′UTR-dependent mechanism that relays information from the 3′ end of the mRNA to control a step in the translation process such as initiation or elongation. In vitro studies and studies in yeast have shown that poly(A)-binding protein and the poly(A) tail stimulate translation (Schmid et al., 1983; Sieliwanowicz, 1987; Sachs and Davis, 1989; Tarun and Sachs, 1995). It is possible that TCE-mediated repression is achieved by blocking this stimulatory effect of poly(A)-binding protein. Alternatively, a TCE-protein complex may itself block translational initiation or elongation.

Efficient translation of *nos* RNA requires localization to the posterior pole of the embryo. While the mechanisms effecting localization and translational repression can be separated, derepression of *nos* RNA cannot be readily uncoupled from posterior localization. Since localization of *nos* RNA bearing its own 3′UTR to the anterior pole does not lead to efficient translation of this RNA (Gavis and Lehmann, 1994), the posterior pole plasm specifically appears to promote *nos* translation. How posterior localization permits translation is unknown. In particular, it is not known whether translation occurs by elimination of the repression mechanism or whether it requires additional activation events. Presumably, interaction of *nos* RNA with components of the localization machinery leads to elimination of the repressor molecule or its inactivation. Our results show that one component of the *nos* localization machinery, Vas protein, plays a role in overcoming TCE-mediated repression. RNAs bearing the *nos* translational control element are completely inactive in embryos from *vas*$^{-}$ females even though they show weak activity in embryos from *osk*$^{-}$ females. Thus, translation of *nos* occurs by a specific derepression mechanism, requiring Vas protein. While some Vas is present throughout the wild-type embryo, the high concentration of Vas at the posterior pole (Hay et al., 1988b; Lasko and Ashburner, 1990) or the combination of Vas with additional factors may facilitate efficient translation of *nos* RNA upon posterior localization. Because of its sequence similarity to translation initiation factor eIF-4A (Hay et al., 1988a; Lasko and Ashburner, 1988) and its in vitro RNA helicase activity (Liang et al., 1994), Vas is an attractive candidate for a translational regulatory factor. The biochemical activity of Vas and the potential of the TCE to form secondary structure suggest a mechanism by which Vas could inactivate TCE function.

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