Temporal complexity within a translational control element in the *nanos* mRNA

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Accepted 21 September 2004

Development 131, 5849-5857
Published by The Company of Biologists 2004
doi:10.1242/dev.01460

Summary

Translational control of gene expression plays a fundamental role in the early development of many organisms. In *Drosophila*, selective translation of *nanos* mRNA localized to the germ plasm at the posterior of the embryo, together with translational repression of *nanos* in the bulk cytoplasm, is essential for development of the anteroposterior body pattern. We show that both components to spatial control of *nanos* translation initiate during oogenesis and that translational repression is initially independent of Smaug, an embryonic repressor of *nanos*. Repression during oogenesis and embryogenesis are mediated by distinct stem loops within the *nanos* 3′ untranslated region; the Smaug-binding stem-loop acts strictly in the embryo, whereas a second stem-loop functions in the oocyte. Thus, independent regulatory modules with temporally distinct activities contribute to spatial regulation of *nanos* translation. We propose that *nanos* evolved to exploit two different stage-specific translational regulatory mechanisms.

Key words: *nanos*, Translational control, Translational regulation, Translational repressor, *Drosophila*, Oogenesis, Embryogenesis, Maternal mRNA

Introduction

Control of mRNA translation plays an important role in temporal and spatial control of gene expression during development in a variety of organisms (Wickens et al., 2000). Coupling of translational control to subcellular mRNA localization facilitates targeting and restriction of cytoplasmic proteins to specific cellular domains, and plays an essential role in the deployment of key patterning molecules in oocytes and embryos (Johnstone and Lasko, 2001). In the early *Drosophila* embryo, translation of *nanos* (*nos*) mRNA at the posterior pole produces a gradient of Nos protein that directs abdomen formation by repressing translation of maternal *hunchback* (*hb*) mRNA (Tautz, 1988; Gavis and Lehmann, 1992). Nos is also crucial at the posterior for germ cell function, by repressing translation of mRNAs like *cyclinB* (*cycB*) (Asaoka-Taguchi et al., 1999). Because Nos can also repress translation of the anteriorly localized *bicoid* (*bcd*) mRNA, however, it must be excluded from the anterior to allow head and thorax development (Wharton and Struhl, 1989; Gavis and Lehmann, 1992).

Synthesis of Nos at the posterior of the embryo requires localization of maternal *nos* mRNA to the posteriorly localized germ plasm (Gavis and Lehmann, 1992; Wang et al., 1994). When localization of *nos* RNA is abolished by mutations in genes necessary for formation of the germ plasm, such as *oskar* (*osk*) and *vasa* (*vas*), *nos* translation is repressed and the resulting embryos lack abdominal segments (Gavis and Lehmann, 1994). Posterior localization of *nos* is inefficient, however, as the vast majority of *nos* RNA fails to become localized and is distributed throughout the embryo (Bergsten and Gavis, 1999). Translational repression of this unlocalized pool of *nos* mRNA is thus essential to restrict production of Nos protein to the posterior.

A major theme in post-transcriptional regulation of developmentally relevant mRNAs is its reliance on cis-acting regulatory elements located within 3′ untranslated regions (3′UTRs) (Kuersten and Goodwin, 2003). The mechanisms by which many of these elements function are ill defined, however. Both posterior localization and translational repression of *nos* RNA require cis-acting sequences in the *nos* 3′UTR (Gavis and Lehmann, 1994). Translational repression of unlocalized *nos* is mediated by a 90 nucleotide translational control element (TCE), the function of which requires formation of two stem-loops (II and III) (Dahanukar and Wharton, 1996; Gavis et al., 1996; Smibert et al., 1996; Crucs et al., 2000). Stem-loop II contains a binding site for the Smaug (Smg) protein that has been designated as the Smaug Recognition Element (SRE) (Smibert et al., 1996; Crucs et al., 2000). Mutation of the SRE disrupts TCE function and loss of Smg results in ectopic *nos* activity, indicating that Smg is a repressor of *nos* translation (Dahanukar and Wharton, 1996; Smibert et al., 1996; Dahanukar et al., 1999). Although stem-loop III is also required for TCE function, existing evidence suggests that it acts independently of Smg. First, mutations that disrupt base pairing in stem-loop III disrupt TCE-mediated translational repression without affecting Smg binding. Second, the retention of TCE function when stem-loops II and III are separated by a large spacer suggests that the two regions of the TCE are recognized independently (Crucs et al., 2000).

It is not known, however, whether the two stem-loops act coordinately or make distinct contributions to TCE function.

As a maternal RNA, *nos* is synthesized by the ovarian nurse cells, and then enters the oocyte where it becomes localized to the posterior late in oogenesis (Wang et al., 1994; Forrest and Gavis, 2003). Many maternal mRNAs required for early embryonic development are maintained in a deadenylated and translationally silent state during oogenesis. Translation of these mRNAs is activated after fertilization by cytoplasmic polyadenylation (Wickens et al., 2000; Mendez and Richter, 2001). By contrast, *nos* does not undergo a fertilization-dependent change in polyA tail length (Sallés et al., 1994), suggesting that activation of *nos* translation may not be temporally regulated. Although *nos* is translated in the nurse cells (Wang et al., 1994), the issue of whether *nos* mRNA, either localized or unlocalized, is translated in the oocyte remains unresolved. As Smg accumulates only after fertilization (Dahanukar et al., 1999; Smibert et al., 1999), translational repression rather than activation of *nos* may be temporally controlled.

We have now investigated regulation of *nos* RNA during oogenesis using a GFP-Nos fusion protein to monitor Nos translation. We find that translation of *nos* RNA becomes repressed at late stages of oogenesis but is activated selectively at the oocyte posterior upon localization of *nos* to the germ plasm. Neither Smg nor the SRE in TCE stem-loop II are required for repression of unlocalized *nos* RNA in the oocyte. By contrast, this repression specifically requires TCE stem-loop III. These results demonstrate that the spatial control of *nos* translation essential for anteroposterior patterning is initiated during oogenesis and requires a distinct ovarian repressor. Furthermore, they decipher the structural complexity of the TCE by showing that the two stem-loops correspond to temporally separable regulatory functions. Finally, we provide evidence that protein degradation contributes to spatial restriction of Nos protein during oogenesis.

Materials and methods

Fly stocks

The following mutants and mutant combinations were used: *y w*^{67c23}and *ry*^{506} (Lindsley and Zimm, 1992), *nos*^{BN} (Wang et al., 1994), *vas*^{PD}/*vas*^{D1} (Schüpbach and Wieschaus, 1986; Lehmann and Nüsslein-Volhard, 1991), *smg*^{1} and *Df*(*Scf*^{R6}) (Dahanukar et al., 1999). *nos*$^{BNX2.1}$ was generated by imprecise excision of the *nos*^{BN} P element and is phenotypically indistinguishable from the original *nos*^{BN} allele. As in *nos*^{BN}, *nos* RNA is produced in the germarium of *nos*$^{BNX2.1}$ ovaries, but not in the nurse cells. The Vas-GFP line was provided by C. Yohn and R. Lehmann.

Construction of transgenes and transgenic lines

P$_{nos}$*gfp-nos* and *P*$_{nos}$*gfp-nos-tub3′UTR*

The plasmid pBS-P$_{nos}$GFP was created by joining a genomic fragment containing the *nos* promoter and complete 5′UTR, with a PCR engineered *Nco*I site at the position of the *nos* ATG, to the *Nco*I-*Not*I fragment of pEGFP-N1 (Clontech), containing the EGFP coding region, in pBS-SK (Stratagene). For fusion of *nos*-coding sequences to EGFP, a genomic fragment containing the *nos* coding region, 3′UTR, and 3′ flanking DNA, was modified by PCR to create a *Sma*I site in place of the ATG codon. The *nos* fragment was joined at this *Sma*I site to an end-filled *Bsr*GI site overlapping the final EGFP codon, connecting the final EGFP codon to the second Nos codon with the insertion of a glycine codon in between. For *P*$_{nos}$*gfp-nos-tub3′UTR*, the *nos* 3′UTR was substituted by the *α-tubulin* 3′UTR as previously described (Gavis and Lehmann, 1994). Both transgenes were inserted into the CaSpeR4 P element vector (Thummel and Pirrotta, 1992).

Transgenes were introduced into *y w*^{67c23} embryos by P element-mediated germline transformation (Spradling, 1986) and multiple independent transgenic lines were isolated. A single copy of the *gfp-nos* transgene was tested for complementation of the *nos*$^{BNX2.1}$ mutation. To generate *6× gfp-nos*, two independent second chromosome *gfp-nos* insertions were recombined. Flies homozygous for the both the recombinant chromosome and an X chromosome *gfp-nos* insertion contain six copies of the *gfp-nos* transgene.

The *nos-tub3′UTR* (Gavis and Lehmann, 1994), *nos-tub:TCE* (Gavis et al., 1996; Crucs et al., 2000), *nos-tub:TCEIIA*, *nos-tub:TCEIIIA*, *nos-tub:TCEIIIGC/GC*, and *nos-tub:TCEIIIA/U^C72* (Crucs et al., 2000) transgenes and transgenic lines have been previously described. The *nos-tub:TCE[SRE*$^{–}$*]* transgene is identical to *nos-tub:TCE*, except for the mutation of two nucleotides required for Smg binding (SRE$^{–}$) (Smibert et al., 1996).

Direct GFP imaging and immunofluorescence

All images were captured with a Zeiss LSM 510 confocal microscope. To analyze GFP-Nos distribution during oogenesis, ovaries from well-fed females carrying either two (2×) or six (6×)copies of the *gfp-nos* transgene were dissected in Schneider's insect culture medium (GIBCO-BRL). Ovaries were quickly rinsed once in PBS, fixed for 15 minutes in 4% paraformaldehyde/PBS, rinsed five times for 5 minutes in PBST (PBS/0.1% Tween-20), and then incubated in the dark for 30 minutes in 1:250 Rhodamine-Phalloidin:PBST (Molecular Probes). Stained ovaries were washed twice for 5 minutes in PBST, three times for 5 minutes in PBS, and mounted in PBS under slight pressure using a #1.5 square glass coverslip (Corning).

To visualize Vas and GFP-Nos simultaneously, ovaries from *6× gfp-nos* females were dissected as above, fixed for 15 minutes in 4% EM grade formaldehyde (Polysciences), rinsed in PBS/0.3% Triton X-100, and incubated for 3 hours in PBT (PBS/0.3% Triton X-100/1% BSA) with 4% v/v normal goat serum (NGS). The ovaries were then immunostained with 1:10,000 rabbit α-Vas antibody (gift of P. Lasko) in PBT/4% NGS overnight at 4°C, washed for 2 hours with several changes of PBT/4% NGS, and incubated for 2 hours with 1:500 Alexa-Fluor 568 goat α-rabbit antibody (Molecular Probes) in PBT/4% NGS. The secondary antibody was preabsorbed overnight against 0- to 2-hour-old embryos prior to use. Stained ovaries were washed for several hours with PBS/0.3% Triton X-100, followed by PBS. To label DNA, Hoescht dye (5 μg/ml final concentration) was added during the final PBS washes.

Northern blot analysis

Ovaries were dissected from well fed females in PBS, washed once with PBS, frozen in liquid N_2, and stored at –80°C. Extraction of RNA from frozen ovaries and northern blotting were performed according to Bergsten and Gavis (Bergsten and Gavis, 1999). The blot was probed simultaneously with ^{32}P-labeled probes for *nos* and *rp49* RNAs as previously described (Bergsten and Gavis, 1999). Labeled bands were quantitated by phosphorimaging.

Immunoblot analysis

For analysis of HA-Nos levels during oogenesis, ovaries were dissected from well-fed females in Schneider's medium and both stage 10 and stage 14 egg chambers were carefully separated. Both total ovary and isolated egg chambers were rinsed once in Schneider's medium, once in PBS, then frozen in liquid N_2 with minimal residual PBS. Embryos (0-2 hours) were dechorionated and washed thoroughly before freezing in liquid N_2. Thawed tissue was homogenized in SDS lysis buffer containing 5 M urea (Gavis et al., 1996), boiled for 5 minutes, spun for 5 minutes in a microfuge, and the supernatants resolved on a 10% SDS-PAGE gel. Proteins were transferred to PVDF membrane (Millipore) and immunoblotting was carried out in 10 mM Tris-HCl pH 7.5/150

mM NaCl/2% nonfat dry milk. Final antibody concentrations were 1:1000 rat α-HA (Roche), 1:20,000 mouse α-Snf (gift of P. Schedl), 1:2000 HRP-goat α-rat (Jackson Immunologicals) and 1:5000 HRP-sheep α-mouse (Amersham). Protein was visualized by chemiluminescence (Roche).

In vitro translation assay

Luciferase plasmids

Luciferase reporter plasmids were constructed in a derivative of pSP64poly(A) (Promega) that encodes a 25 nucleotide poly(A) sequence followed by a unique *Nsi*I site (kindly provided by D. Chagnovich and R. Lehmann). Each reporter contains the entire *nos* 5′UTR fused to the coding region of the firefly luciferase gene (Promega) and one of the following 3′UTRs: *α-tubulin* (Gavis and Lehmann, 1994), three tandem copies of a wild-type *nos* TCE (Bergsten et al., 1999) or three tandem copies of a mutant *nos* TCE (TCE:SRE$^-$) (Smibert et al., 1996); TCEIIA, or TCEIIIA (Crucs et al., 2000).

In vitro transcription

Templates for in vitro transcription were prepared by digestion of luciferase reporter plasmids with *Nsi*I, followed by treatment with T4 DNA polymerase. Capped transcripts were generated with the mMessage mMachine kit (Ambion). Unincorporated nucleotides and excess cap analog were removed by a G-50 spin column (Pharmacia), and the RNA was purified by phenol extraction and ethanol precipitation.

Translationally active embryo extract

Embryonic extracts were prepared as described previously (Clark et al., 2000). Briefly, fresh 0- to 2-hour-old embryos were homogenized on ice in 1 volume of Buffer A (10 mM HEPES pH 7.5/5 mM DTT/0.5 mM PMSF). The extract was cleared by microfuge centrifugation for 5 minutes at 4°C and supplemented with 1/9 volume of Buffer B (100 mM HEPES pH 7.5/1 M potassium acetate/10 mM magnesium acetate/50 mM DTT). After centrifugation for 10 minutes at 4°C, 0.8 U RNasin (Promega) and 200 μg creatine phosphokinase (Sigma) were added to the supernatant, which was then frozen in aliquots in liquid nitrogen and stored at –80°C.

In vitro translation reaction

Reactions (20 μl) contained 10 μl of extract, 0.1 nM luciferase reporter RNA, 15 mM creatine phosphate (Sigma) and 4 μl of 5× translation buffer (125 mM HEPES pH 7.5/7.5 mM magnesium acetate/1 mM spermidine/12.5 mM DTT/125 μM amino acids/6 mM ATP/1.5 mM GTP). Reactions were incubated for 45 minutes at 28°C. Translation reactions in rabbit reticulocyte extract (Promega) were carried out for 45 minutes using the manufacturer's protocol. Enzymatic assays for luciferase were performed using a substrate mix recommended by Promega. For each RNA, luciferase activity produced by translation in *Drosophila* embryonic extract was normalized to the value obtained after translation in reticulocyte extract, to control for differences in quantity or quality of the RNAs.

Results

Distribution of GFP-Nos in early to mid-oogenesis

Analysis of *nos* translation and its regulation during oogenesis has been hampered by the inaccessibility of late stage oocytes, where *nos* RNA becomes localized, to standard immunological detection methods. To circumvent this problem, we generated transgenic animals expressing a GFP-Nos fusion protein. Proper regulation was achieved by fusing GFP sequences to genomic *nos* coding sequences in an otherwise wild-type *nos* transgene that includes the 5′ genomic region and promoter, 5′UTR and 3′UTR (Fig. 1A). A single copy of the *gfp-nos* transgene completely rescues the abdominal defects of progeny from *nos* mutant females.

Analysis of GFP-Nos in fixed egg chambers is largely consistent with previous immunohistochemical analysis of Nos protein during early to middle stages of oogenesis (Wang et al., 1994). GFP-Nos is strongly expressed in regions 1-2 of the germarium and again later from stages 4-5 onwards (Fig. 2A, inset). Although GFP-Nos is enriched in the oocyte at earlier stages of oogenesis, its expression increases dramatically in the nurse cells during stages 8-10 (Fig. 2A-C). Under equivalent

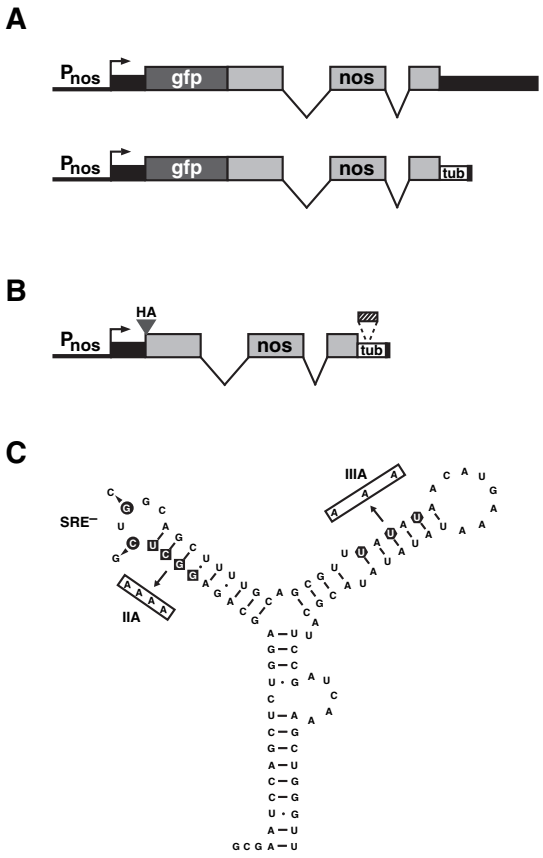

Fig. 1. Tagged *nos* and *nos-tub3′UTR* transgenes. (A) The *gfp-nos* and *gfp-nos-tub3′UTR* transgenes contain GFP sequences (dark shading) inserted at the N terminus of the genomic *nos* coding region (light shading). Both transgenes include the *nos* promoter and 5′ regulatory sequences (Pnos), 5′UTR (left black bar) and polyadenylation signal. *Gfp-nos* (top) bears the intact *nos* 3′UTR (right black bar), whereas these sequences have been replaced by *α-tubulin* 3′UTR sequences (tub) in *gfp-nos-tub3′UTR* (bottom). (B) The hemagglutinin (HA) epitope tagged *nos-tub3′UTR* transgene is identical to *gfp-nos-tub3′UTR*, except that an N-terminal HA tag replaces GFP. The *nos-tub:TCE* and TCE mutant transgenes carry insertions of wild-type and mutant TCE sequences (shown in C), respectively (hatched box). Transgenes in A and B are drawn to scale, except for introns. (C) Nucleotide changes associated with the SRE$^-$ (circles), TCEIIA (squares) and TCEIIIA (hexagons) mutations are indicated on the TCE secondary structure. The following mutations are not shown: TCEIIIA/U^C72, a compensatory mutation to TCEIIIA that restores base pairing with three A to U substitutions; and TCEIIIGC/GC, which changes the alternating U-A and A-U base pairs in the distal region of stem-loop III to alternating G-C and G-C base pairs (Crucs et al., 2000).

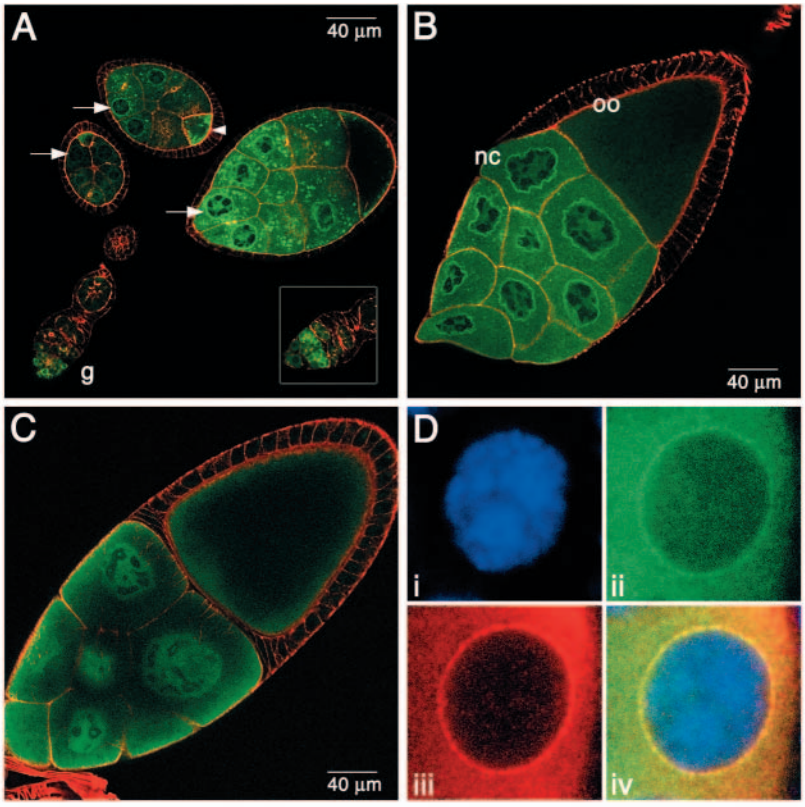

Fig. 2. GFP-Nos distribution during early to middle stages of oogenesis. (A-C) Fixed *6× gfp-nos* egg chambers, with GFP-Nos visualized directly and the actin cytoskeleton labeled red with rhodamine-phalloidin. (A) GFP-Nos (green) expression is strong in regions 1 and 2 of germarium (g, inset) and in the nurse cells during stages 5-8. In addition to forming cytoplasmic particles, GFP-Nos is enriched at the periphery of the nurse cell nuclei (arrows). GFP-Nos can also be detected in the oocyte during midoogenesis (arrowhead). (B) Stage 9 egg chamber with high levels of GFP-Nos in the nurse cells (nc) and perinuclear enrichment. Lower levels of GFP-Nos are present in the oocyte (oo). (C) Stage 10 egg chamber with loss of perinuclear GFP-Nos localization as nurse cells prepare for dumping. (D) High-magnification image of nurse cell nuclei from a stage 5 *6× gfp-nos* egg chamber showing (i) DNA stained with Hoescht's dye; (ii) GFP-Nos; (iii) anti-Vas immunofluorescence; (iv) merge of i-iii. GFP-Nos colocalizes with Vas at the nuclear periphery. A similar distribution of GFP-Nos is observed in *gfp-nos:tub3′UTR* egg chambers at the stages shown in A-C.

conditions, fluorescence is not observed in control egg chambers from females lacking the transgene (data not shown). Unlike immunohistochemical staining, direct visualization of GFP-Nos protein provides significantly higher resolution, revealing particles of GFP-Nos protein in the nurse cells and enrichment at the periphery of the nurse cell nuclei (Fig. 2A,B). GFP-Nos is also found within the nurse cell nuclei (Fig. 2A-C). Perinuclear localization of GFP-Nos is most probably due to protein targeting as it occurs in nurse cells expressing *gfp-nos-tub3′UTR* RNA (Fig. 1A; see below) that lacks the *nos* RNA localization signal. During stage 10B (Fig. 2C), perinuclear enrichment disappears, presumably owing to changes in nuclear morphology associated with the onset of nurse cell dumping.

The localization of GFP-Nos at the nuclear periphery is reminiscent of Vas protein localization to the nuage in early to mid-oogenesis (Hay et al., 1988; Liang et al., 1994). Vas, an ATP-dependent RNA helicase, is required for translational control of several maternal RNAs (Gavis and Lehmann, 1994; Markussen et al., 1995; Rongo et al., 1995; Styhler et al., 1998; Tomancak et al., 1998) and nuage, a ribonucleoprotein rich structure surrounding the nurse cell nuclei, is thought to be a site of Vas function (Hay et al., 1988; Liang et al., 1994). Although the distributions of Vas and GFP-Nos proteins overlap (Fig. 2D), perinuclear localization of GFP-Nos does not require *vas* function (see Fig. S1 in the supplementary material). Similarly, localization of Vas-GFP to nuage occurs independently of *nos* (data not shown).

Nos protein accumulates at the oocyte posterior upon localization of *nos* RNA

Synthesis of Nos protein at the posterior of the embryo requires association of *nos* RNA with the posteriorly localized germ plasm. However, localization of *nos* to the germ plasm is accomplished earlier, during stages 11-13 of oogenesis, after *nos* is transferred or 'dumped' into the oocyte by the actin-dependent contraction of the nurse cells (Forrest and Gavis, 2003). To determine whether translation of *nos* RNA is initiated during oogenesis upon localization to the posterior, or only after fertilization, we examined the distribution of GFP-Nos in late stage egg chambers (Fig. 3). During stage 11, GFP-Nos synthesized in the nurse cells is dumped along with *nos* RNA into the enlarging oocyte (Fig. 3A,D) and by stage 12, this protein is distributed uniformly throughout the oocyte (Fig. 3B,E). During stage 13 (Fig. 3C,F), the level of GFP-Nos in the bulk ooplasm decreases (compare Fig. 3C with 3B). As the oocyte volume changes little between stages 12 and 13, this decrease is probably due to protein degradation. This conclusion is indeed borne out by immunoblot analysis (see below).

Also by stage 12, a gradient of GFP-Nos emanating from the posterior pole appears (Fig. 3F; see Fig. S1 in the supplementary material). The GFP-Nos gradient is abolished when *gfp-nos* localization is abrogated, either by loss of *vas* function (Fig. S1, see supplementary material) or by removal of the *nos* 3′UTR (see below, Fig. 4), confirming that this gradient results from translation of localized RNA rather than from protein targeting. Together, these results provide evidence that following nurse cell dumping, Nos protein derived from translation of *nos* in the nurse cells is cleared from the bulk ooplasm, while *nos* RNA localized to the germ plasm at the oocyte posterior is translated, initiating formation of the Nos protein gradient.

Unlocalized *nos* RNA is translationally repressed in late oocytes

The large pool of *nos* RNA that fails to become posteriorly localized is translationally repressed in the early embryo, provided this RNA contains the *nos* TCE (Gavis and Lehmann, 1994; Dahanukar and Wharton, 1996; Gavis et al., 1996; Smibert et al., 1996). By contrast, unlocalized *nos-tub3′UTR* mRNA, which bears the *α-tubulin* 3′UTR in place of the *nos* 3′UTR, is

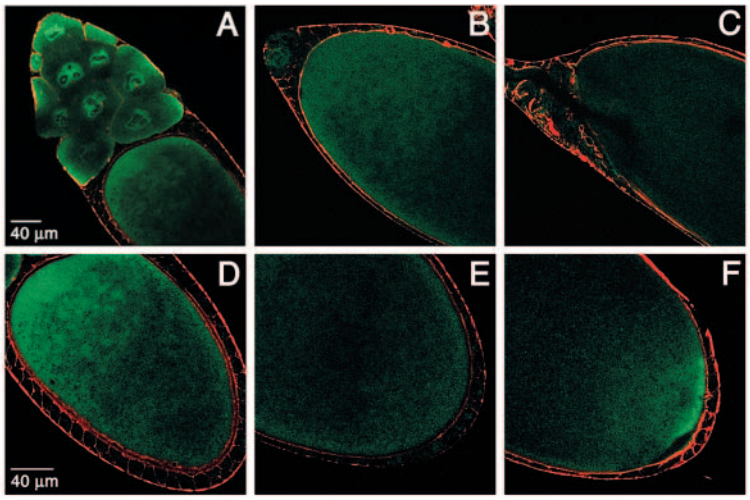

Fig. 3. GFP-Nos distribution at late stages of oogenesis. (A-C) Lower power images of the anterior region of fixed *6×* *gfp-nos* egg chambers used for staging. (D-F) Higher power images of the posterior region of egg chambers shown in A-C. (A,D) Stage 11 egg chamber, with transfer of GFP-Nos produced in the nurse cells to the oocyte during nurse cell dumping. (B,E) Stage 12 oocyte, with GFP-Nos distributed uniformly throughout the ooplasm. (C,F) Stage 13 oocyte, in which GFP-Nos has disappeared from the anterior and a gradient of GFP-Nos is observed emanating from the posterior pole. This gradient is also detected in *2×* *gfp-nos* oocytes (see Fig. S1 in the supplementary material). The actin cytoskeleton is labeled with rhodamine-phalloidin (red) in all panels.

translated throughout the embryo (Gavis and Lehmann, 1994). To determine whether the translational quiescence of unlocalized *nos* RNA initiates during oogenesis, we compared the accumulation of GFP-Nos protein from the unlocalized pool of *6×* *gfp-nos* RNA with that from *gfp-nos-tub3′UTR* RNA (Fig. 1A) in stage 13 oocytes. GFP fluorescence in the anterior half of stage 13 oocytes from *6×* *gfp-nos* females is indistinguishable from background fluorescence observed in wild-type control oocytes (Fig. 4A,B). By contrast, GFP-Nos can be readily detected throughout stage 13 oocytes of *gfp-nos-tub3′UTR* females (Fig. 4C), even though northern blot analysis shows *gfp-nos-tub3′UTR* RNA levels are tenfold lower than *6×* *gfp-nos* RNA levels (data not shown). The dramatic difference in the amount of GFP-Nos protein produced from these two RNAs in the anterior half of the oocyte indicates that translation of unlocalized *nos* RNA is repressed in the late oocyte.

To determine whether translation of unlocalized *nos* RNA in late oocytes is repressed by the *nos* TCE, we compared the amount of Nos protein in oocytes from females carrying either the *nos-tub3′UTR* or *nos-tub:TCE* transgene (Fig. 1B). These transgenes, which differ only in the presence of the *nos* TCE within their 3′UTRs, produce similar levels of unlocalized RNA encoding functional, hemagglutinin epitope-tagged Nos protein (HA-Nos; Fig. 5A). Immunoblot analysis revealed accumulation of HA-Nos protein in stage 14 oocytes from *nos-tub3′UTR* females (Fig. 5B), consistent with analysis of *gfp-nos-tub3′UTR* oocytes (Fig. 4C). By contrast, little or no HA-Nos protein is detected in stage 14 oocytes from *nos-tub:TCE* females (Fig. 5B). *nos-tub:TCE* RNA is translated at earlier stages, however, as HA-Nos protein is present in total ovarian extract and stage 10 oocytes (Fig. 5B,C). Together, these results demonstrate that the *nos* TCE mediates translational repression of unlocalized *nos* RNA in late stage oocytes.

The dramatic difference in HA-Nos protein levels between total ovarian and late oocyte extract suggests that Nos protein synthesized in the nurse cells is degraded in late oocytes. Immunoblotting of extracts from isolated, staged egg chambers shows that the high level of HA-Nos protein present at stage 10 does not persist to late oogenesis (Fig. 5C). This result, together with imaging of GFP-Nos, confirms that Nos protein synthesized in the nurse cells is degraded by late oogenesis.

TCE stem-loops act differentially during oogenesis and embryogenesis

Although TCE-mediated repression initiates during oogenesis, Smg, the only known TCE-binding factor and repressor of *nos* translation, is not present in the ovary (Dahanukar et al., 1999; Smibert et al., 1999). We have previously shown that mutations in TCE stem-loop III disrupt translational repression of unlocalized *nos* RNA without affecting the ability of Smg to bind to the SRE in stem-loop II (Crucs et al., 2000). As repression during oogenesis must be mediated by a factor other than Smg, TCE stem-loop III is a potential target for this factor. Alternatively, an ovarian repressor may also recognize the SRE or may interact with a different motif in stem-loop II.

Previous analyses of sequence and structural requirements for TCE function in vivo examined the effect of TCE mutations on *nos* regulation using phenotypic assays, in which defects in the anteroposterior pattern of the larval cuticle provide a measure of *nos* activity (Dahanukar and Wharton, 1996; Gavis et al., 1996; Smibert et al., 1996). Consequently, these studies could not distinguish TCE-mediated repression occurring during oogenesis from repression during embryogenesis. To determine how the TCE mediates repression during oogenesis, we assayed the effects of TCE mutations directly on Nos protein levels both

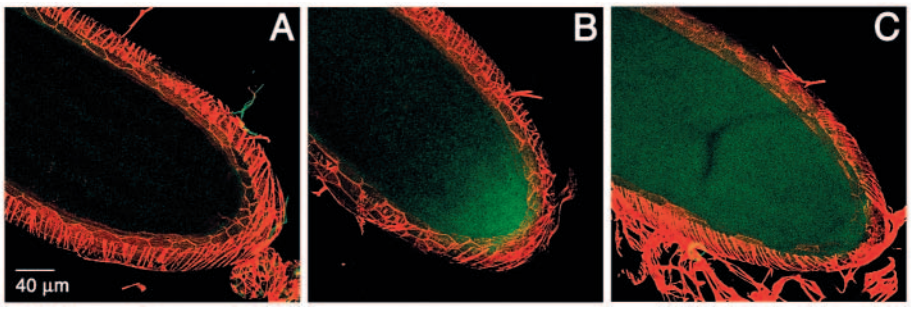

Fig. 4. Restriction of GFP-Nos to the posterior requires the *nos* 3′UTR. All images are confocal projections of fixed oocytes. The muscular sheath that encases the ovary is still attached in these preparations as revealed by staining with rhodamine-phalloidin (red). (A) Control oocyte lacking GFP-Nos. (B) Stage 13 *6×* *gfp-nos* oocyte with GFP-Nos gradient emanating from the posterior pole. (C) Stage 13 *gfp-nos:tub3′UTR* oocyte displaying a uniform distribution of GFP-Nos.

in late oocytes and in embryos, using *nos-tub:TCE* transgenes bearing mutant TCEs (Fig. 1C). For all transgenic lines used, comparable RNA expression levels were confirmed by northern blotting (Fig. 5A) (Crucs et al., 2000).

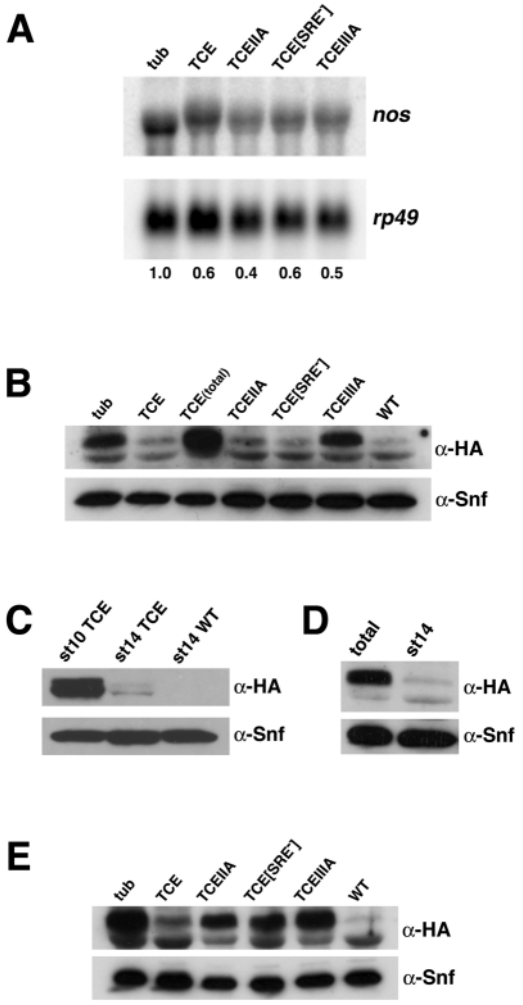

Fig. 5. Differential effect of TCE mutations on translational repression during oogenesis and embryogenesis. (A) Northern analysis of total RNA from *nos-tub3′UTR* (*tub*), *nos-tub:TCE* (TCE) or *nos-tub:TCE* mutant (TCEIIA, TCE[SRE⁻], TCEIIIA) ovaries. Transgene RNAs, detected with a *nos* probe, were normalized to the *rp49* control to determine their relative abundance, indicated below. (B) Immunoblot analysis of HA-Nos protein in extracts of stage 14 egg chambers from wild-type (WT), *nos-tub3′UTR* (*tub*), *nos-tub:TCE* (TCE) or *nos-tub:TCE* mutant derivatives (TCEIIA, TCE[SRE⁻], TCEIIIA) using an anti-HA antibody. The antibody crossreacts with a protein that co-migrates with HA-Nos, as well as a more rapidly migrating protein in all samples. Snf protein was monitored as a loading control. In stage 14 oocytes, the wild-type TCE and stem-loop II mutants (TCEIIA, TCE[SRE⁻]) prevent accumulation of HA-Nos, whereas stem-loop III mutants (TCEIIIA and others shown in Fig. S2) do not. Analysis of total ovarian extract [TCE(total)] confirms that *nos-tub:TCE* RNA is expressed and translated at earlier stages of oogenesis. (C) Immunoblot analysis of HA-Nos protein in extracts of stage 10 (st10) egg chambers and stage 14 (st14) oocytes dissected from *nos-tub:TCE* (TCE) or wild-type ovaries. (D) Immunoblot analysis of HA-Nos protein in extracts of total ovary or stage 14 (st14) oocytes from *nos-tub:TCE* females mutant for *smg* [$smg^{1}/Df(Scf^{R6})$]. (E) Immunoblot analysis of HA-Nos protein in extracts of embryos from the same transgenic and wild-type lines shown in B. HA-Nos protein is detected in early embryos for all of the TCE mutants.

Two mutations that alter stem-loop II and binding of Smg protein, TCEIIA and SRE⁻ (Smibert et al., 1996; Crucs et al., 2000), have no effect on TCE-mediated repression during oogenesis, as the mutant TCEs still prevent HA-Nos protein accumulation in late oocytes (Fig. 5B). Similarly, HA-Nos protein cannot be detected in stage 14 oocytes from *nos-tub:TCE* ovaries mutant for *smg* (Fig. 5D). By contrast, *nos-tub:TCEIIA* and *nos-tub:TCE[SRE⁻]* embryos show a dramatic increase in the amount of HA-Nos over *nos-tub:TCE* embryos (Fig. 5E) and a similar increase occurs in *nos-tub:TCE* embryos mutant for *smg* (data not shown). Thus, both stem-loop II and *smg* function are limited to embryogenesis, consistent with the restricted expression of Smg protein. Furthermore, the loss of anterior structures observed in larval cuticle preparations of *nos-tub:TCEIIA* and *nos-tub:TCE[SRE⁻]* embryos (Crucs et al., 2000) (data not shown) must result from excess Nos produced during embryogenesis.

Strikingly, mutation of stem-loop III (TCEIIIA) results in production of HA-Nos in late oocytes (Fig. 5B), indicating that translation of *nos-tub:TCEIIIA* RNA is not repressed. Phenotypic analysis showed that mutations that retain base-pairing within TCE stem-loop III but alter the sequence of the distal region of the stem (TCEIIIA/U^C72, and TCEIIIGC/GC, see Fig. 1 legend) also compromise TCE function, indicating that both the sequence and structure of stem-loop III contribute to its activity (Crucs et al., 2000). Indeed, these mutations disrupt repression of unlocalized *nos* RNA during oogenesis, as HA-Nos is detected on immunoblots of transgenic stage 14 oocytes (see Fig. S2 in the supplementary material). Thus, TCE stem-loop III acts in a sequence- and structure-dependent manner to repress translation during oogenesis. The complete lack of anterior structures observed in cuticle preparations of *nos-tub:TCEIIIA* embryos (Crucs et al., 2000) indicates that repression by stem-loop III during oogenesis is crucial for embryonic development.

TCE stem-loop III mediates *smg*-independent repression during oogenesis

Although HA-Nos protein is also present in *nos-tub:TCEIIIA* embryos (Fig. 5E), this protein may derive solely from

Table 1. Effect of TCE stem-loop II and III mutations on translational repression in vitro

3′UTR	Translation	*P*
3× TCE	1.0	–
3× SRE⁻	3.7 (±1.1)	0.02
3× TCEIIA	4.2 (±0.8)	0.01
3× TCEIIIA	1.4 (±0.04)	0.0007
tub 3′UTR	3.9 (±1.3)	0.03

Effect of TCE stem-loop II and III mutations on translational repression in vitro. Capped and polyadenylated luciferase reporter RNAs bearing the control *tub* 3′UTR or three copies of a wild-type or mutant TCE were translated in embryonic extract. For each RNA, luciferase activity was normalized to the value obtained following translation in rabbit reticulocyte extract, which does not exhibit TCE-dependent repression. The corrected values were then expressed as a ratio to the value obtained for 3× TCE, to determine the relative ability of each 3′UTR to confer translational repression. The mean and standard deviation from four independent experiments are shown. *P* values were determined using the Student's *t*-test.

unregulated translation of *nos-tub:TCEIIIA* RNA during oogenesis. To determine whether stem-loop III is required for repression during embryogenesis as well as oogenesis, we took advantage of an in vitro translation assay based on a preblastoderm embryo extract that recapitulates TCE-mediated repression (Clark et al., 2000). Capped and polyadenylated luciferase reporter RNAs bearing either the control *tub* 3′UTR, or three tandem copies of a wild-type or mutant TCE, were used to program the embryonic extract and translation was monitored using a luciferase activity assay.

As expected, we found that the SRE and stem-loop II are essential for TCE-mediated repression in vitro, as mutation of these sequences yielded luciferase levels comparable to that obtained with the *tub* 3′UTR (Table 1). By contrast, mutation of stem-loop III had little effect in this assay, indicating that the presence of HA-Nos in *nos-tub:TCEIIIA* embryos is due largely to perdurance of protein synthesized during oogenesis. Although the slight but significant decrease in repression observed for the TCEIIIA mutant suggests that stem-loop III plays a minor role during embryogenesis, this stem-loop acts primarily to promote Smg-independent translational repression of *nos* in late oocytes.

Discussion

Spatially controlled translation of *nos* mRNA during oogenesis

Selective translation of posteriorly localized *nos* mRNA achieves the restricted distribution of Nos protein required to regulate *hb* and *cycB* mRNAs in the posterior of the embryo without affecting *hb* or *bcd* translation in the anterior. Although some maternal mRNAs, including *osk* and *gurken* are translated in the oocyte (Ephrussi et al., 1991; Neuman-Silberberg and Schupbach, 1996), others, such as *hb* and *bcd*, are translationally repressed during oogenesis and activated only after fertilization (Driever and Nüsslein-Volhard, 1988; Tautz, 1988; Sallés et al., 1994). For *nos*, translational activation during oogenesis would permit accumulation of Nos protein in the posterior of the embryo early enough to block *hb* translation. However, the translational status of *nos* in late stage oocytes has remained enigmatic, owing to the impermeability of these tissues to immunostaining. Use of a *gfp-nos* fusion gene as a reporter for *nos* translation has allowed us to address this issue and establish that translation of localized *nos* mRNA indeed begins during oogenesis.

Achieving the restricted Nos protein distribution in the embryo requires that translational activity of *nos* in the oocyte be spatially regulated. However, the only known repressor of *nos* translation, Smg, is absent from the ovary (Dahanukar et al., 1999; Smibert et al., 1999). We have resolved this dilemma by showing that a distinct, Smg-independent mechanism mediates translational repression of unlocalized *nos* mRNA in late oocytes. Failure to repress *nos* in late oocytes, as exemplified by the behavior of the *nos-tub:TCEIIIA* transgene, results in unrestricted production of Nos protein that perdures to embryogenesis. The resulting embryos die, lacking anterior structures (Crucs et al., 2000). Thus, by showing that the program for spatially restricted synthesis of Nos operates during oogenesis, our results reveal how temporal demands are reconciled with spatial constraints on *nos* translation needed for embryonic patterning.

Temporal modulation of *nos* repression through differential recognition of TCE stem-loops

The elucidation of temporally distinct functions of the two TCE stem-loops explains the enigmatic structural complexity of this regulatory element. We have previously shown that both stem-loops retain function, despite their separation by a 52-nucleotide spacer, suggesting that they operate independently (Crucs et al., 2000). Indeed, phylogenetic analysis of the *nos* 3′UTR reveals that TCE stem-loops II and III are not juxtaposed in all *Drosophilid* species (R.A.J. and E.R.G., unpublished), indicating that the distance between stem II and III is not under tight evolutionary constraint.

After fertilization, Smg binds to TCE stem-loop II to mediate repression in the preblastoderm embryo (Smibert et al., 1996; Dahanukar et al., 1999; Crucs et al., 2000). We do not know if the ovarian repressor remains bound to stem-loop III in the embryo, but its function is superceded by Smg. A minor requirement for stem-loop III in the embryo suggested by our in vitro translation experiments may reflect the need to maintain the ovarian repression mechanism until Smg reaches sufficient levels in the embryo. Accordingly, the requirement for stem-loop III would decrease over time after fertilization. A more significant contribution by stem-loop III might have been missed, however, if the stem-loop III-dependent repressor is unstable in the embryonic extract.

The *smg* mutant phenotype indicates that *nos* is not the only target of Smg in the embryo (Dahanukar et al., 1999). Although the ovarian repressor has not yet been identified, we have recently isolated a candidate ovarian stem-loop III binding factor (Y. Kalifa, T. Huang and E.R.G., unpublished) that appears to regulate multiple maternal mRNAs. Thus, it seems likely that *nos* has evolved to co-opt existing stage-specific regulatory proteins for its advantage. We have previously shown that the *nos* TCE can repress translation in subsets of cells in both the central and peripheral nervous systems (Clark et al., 2002; Ye et al., 2004). Although the repressors are not known in these cases either, it is possible that the ability to interact with yet additional proteins will underlie the multifunctionality of the TCE.

Other RNAs may use a similar strategy of recognition by stage-specific factors to maintain translational regulation across developmental transitions. In the *Drosophila* oocyte, translational repression of unlocalized *osk* mRNA occurs through the interaction of Bruno (Bru) with specific sequence motifs in the *osk* 3′UTR (Kim-Ha et al., 1995). As Bru is not present in the embryo (Webster et al., 1997; Lie and Macdonald, 1999), where the majority of *osk* mRNA remains unlocalized (Bergsten and Gavis, 1999), an embryonic repressor may be required to maintain the repression initiated by Bru. Intriguingly, the existence of binding sites for multiple, distinct microRNAs within individual 3′UTRs (Lewis et al., 2003; Stark et al., 2003) suggests a similar paradigm for controlling translation through multiple developmental stages or in different tissues.

Multiple modes of regulation operate during oogenesis for spatial restriction of Nos

The translational quiescence of unlocalized *nos* in late oocytes contrasts sharply with its translational activity in the nurse cells. Deposition of both actively translated *nos* mRNA and the previously synthesized Nos protein into the oocyte during nurse cell dumping presents a challenge for restricting Nos to the posterior of the oocyte. Although we cannot determine whether

nos is repressed in oocytes prior to stage 10, our results indicate that the majority of *nos* RNA, which enters the oocyte during dumping, must switch from a translationally active state in the nurse cells to an inactive state in the oocyte. This switch could be mediated by interaction of *nos* with an ovarian repressor restricted to the oocyte. Alternatively, a repressor may bind to *nos* RNA in the nurse cells, but become activated during or after passage into the oocyte.

We have previously shown that translationally repressed *nos* RNA is associated with polysomes, indicating that repression is imposed at a late step in the translation cycle (Clark et al., 2000). However, recent evidence that Smg interacts with Cup to prevent recruitment of eIF-4G by eIF-4E suggests that translation is blocked at the initiation step (Nelson et al., 2004). The identification of a Smg-independent mechanism for translational repression during oogenesis may explain these divergent results. Indeed, a post-initiation mechanism may be ideally suited to rapidly repress polysomal *nos* RNA entering the oocyte from the nurse cells.

In addition to translationally active *nos* RNA, substantial amounts of Nos protein enter the oocyte during nurse cell dumping. Perdurance of this protein to embryogenesis would probably disrupt anterior development. We find, however, that Nos protein entering the oocyte from stage 10 nurse cells is cleared from the oocyte by stage 13. Nos protein made in the nurse cells may therefore be specifically targeted for degradation. Alternatively, Nos might have a short half-life regardless of its site of synthesis. Despite considerable effort, we have not detected ubquitinated forms of Nos protein, although the transient nature of ubquitinated intermediates may preclude detection. Similarly, we have not detected a genetic interaction between mutations in numerous components of the ubiquitin degradation pathway and *nos* transgenes. Thus, how Nos is degraded remains an unanswered question. Regardless of mechanism, however, continuous translation of wild-type *nos* RNA at the posterior pole or of unlocalized *nos-tub3′UTR* RNA throughout the oocyte would result in accumulation of Nos protein. Thus post-translational control of Nos protein stability as well as post-transcriptional regulation of *nos* RNA contribute to the correct spatial distribution of Nos in the early embryo.

We thank D. Chagnovich, C. Yohn and R. Lehmann for plasmid DNA and fly stocks; P. Lasko and P. Schedl for antibodies; Jill Penn for help constructing the *gfp-nos* transgene; and Joe Goodhouse for assistance with confocal microscopy. R.A.J. was supported by a predoctoral fellowship from the NSF. This work was supported by a grant from the NIH (GM61107).

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/131/23/5849/DC1

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