Temporal complexity within a translational control element in the \textit{nanos} mRNA

Kevin M. Forrest, Ira E. Clark, Roshan A. Jain and Elizabeth R. Gavis*

Department of Molecular Biology, Princeton University, Princeton, NJ 08544, USA
*Author for correspondence (e-mail: lgavis@molbio.princeton.edu)

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Summary

Translational control of gene expression plays a fundamental role in the early development of many organisms. In \textit{Drosophila}, selective translation of \textit{nanos} mRNA localized to the germ plasm at the posterior of the embryo, together with translational repression of \textit{nanos} in the bulk cytoplasm, is essential for development of the anteroposterior body pattern. We show that both components to spatial control of \textit{nanos} translation initiate during oogenesis and that translational repression is initially independent of Smaug, an embryonic repressor of \textit{nanos}. Repression during oogenesis and embryogenesis are mediated by distinct stem loops within the \textit{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 \textit{nanos} translation. We propose that \textit{nanos} evolved to exploit two different stage-specific translational regulatory mechanisms.

Key words: \textit{nanos}, Translational control, Translational regulation, Translational repressor, \textit{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 \textit{Drosophila} embryo, translation of \textit{nanos} (\textit{nos}) mRNA at the posterior pole produces a gradient of Nos protein that directs abdomen formation by repressing translation of maternal \textit{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 \textit{cyclinB (cycB)} (Asaoka-Taguchi et al., 1999). Because Nos can also repress translation of the anteriorly localized \textit{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 \textit{nos} mRNA to the posteriorly localized germ plasm (Gavis and Lehmann, 1992; Wang et al., 1994). When localization of \textit{nos} RNA is abolished by mutations in genes necessary for formation of the germ plasm, such as \textit{oskar (osk)} and \textit{vasa (vas)}, \textit{nos} translation is repressed and the resulting embryos lack abdominal segments (Gavis and Lehmann, 1994). Posterior localization of \textit{nos} is inefficient, however, as the vast majority of \textit{nos} RNA fails to become localized and is distributed throughout the embryo (Bergsten and Gavis, 1999). Translational repression of this unlocalized pool of \textit{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 \textit{nos} RNA require cis-acting sequences in the \textit{nos} 3′UTR (Gavis and Lehmann, 1994). Translational repression of unlocalized \textit{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 \textit{nos} activity, indicating that Smg is a repressor of \textit{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 (Sallès 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^{67C2} and ry^{36} (Lindsley and Zimm, 1992), nos^{39} (Wang et al., 1994), vas^{531}vas^{506} (Schüpbach and Wieschaus, 1986; Lehmann and Nüsslein-Volhard, 1991), smg^{1} and Df(Nos^{BPB}) (Dahanukar et al., 1999). nos^{BN2.1} 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-tub}UTR

The plasmid pBS-NosGFP was created by joining a genomic fragment containing the nos promoter and complete 5'UTR, with a PCR engineered Ncol site at the position of the nos ATG, to the Ncol-Ncol 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 Smal site in place of the ATG codon. The nos fragment was joined at this Smal site to an end-coded BstGI 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-tub}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 CaiSpeR4 P element vector (Thummel and Pirrotta, 1992).

Transgenes were introduced into y w^{67C2} 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; Crocs et al., 2000), nos-tub:TCEIIA, nos-tub:TCEIIA, nos-tub:TCEIIIGG/C, and nos-tub:TCEIIA/U^C72 (Crocs 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, Hoechst 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 N2, 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 32P-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 N2 with minimal residual PBS. Embryos (0-2 hours) were dechorionated and washed thoroughly before freezing in liquid N2. 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
nM NaCl/2% nonfat dry milk. Final antibody concentrations were 1:1000 rat α-HA (Roche), 1:20,000 mouse α-Snf (gift of P. Schell), 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 NsiI 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).

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, the RNA was purified by phenol extraction and ethanol precipitation.

In vitro transcription
Templates for in vitro transcription were prepared by digestion of luciferase reporter plasmids with NsiI, 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.

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 gerarium 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
ATP-dependent RNA helicase, is required for translational mid-oogenesis (Hay et al., 1988; Liang et al., 1994). Vas, an reminiscent of Vas protein localization to the nuage in early to morphology associated with the onset of nurse cell dumping. enrichment disappears, presumably owing to changes in nuclear localization signal. During stage 10B (Fig. 2C), perinuclear tub3 protein targeting as it occurs in nurse cells expressing Perinuclear localization of GFP-Nos is most probably due to is also found within the nurse cell nuclei (Fig. 2A-C). GFP-Nos protein provides significantly higher resolution, revealing immunohistochemical staining, direct visualization of GFP-Nos from females lacking the transgene (data not shown). Unlike 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 gfp-tub3'UTR mRNA, which bears the α-tubulin 3'UTR in place of the nos 3'UTR, is
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

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.

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.

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
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).

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 (TCEIIA/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.

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

<table>
<thead>
<tr>
<th>3′UTR</th>
<th>Translation</th>
<th>P</th>
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<tbody>
<tr>
<td>3× TCE</td>
<td>1.0</td>
<td>–</td>
</tr>
<tr>
<td>3× SRE-</td>
<td>3.7 (±1.1)</td>
<td>0.02</td>
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<tr>
<td>3× TCEIIA</td>
<td>4.2 (±0.8)</td>
<td>0.01</td>
</tr>
<tr>
<td>3× TCEIIIA</td>
<td>1.4 (±0.04)</td>
<td>0.0007</td>
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<tr>
<td>tub 3′UTR</td>
<td>3.9 (±1.3)</td>
<td>0.03</td>
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Effect of TCE stem-loop II and III mutations on translational repression in vitro. Closed 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., 1999; Dahanukar et al., 1999; Cruc 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 ubiquitinated forms of Nos protein, although the transient nature of ubiquitinated 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.

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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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