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Smaug, a novel and conserved protein, contributes to repression of *nanos* mRNA translation in vitro

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ABSTRACT

Proper deployment of Nanos protein at the posterior of the *Drosophila* embryo, where it directs posterior development, requires a combination of RNA localization and translational controls. These controls ensure that only the posteriorly-localized *nanos* mRNA is translated, whereas unlocalized *nanos* mRNA is translationally repressed. Here we describe cloning of the gene encoding Smaug, an RNA-binding protein that interacts with the sequences, SREs, in the *nanos* mRNA that mediate translational repression. Using an in vitro translation assay, we demonstrate that SRE-dependent repression occurs in extracts from early stage embryos. Immunodepletion of Smaug from the extracts eliminates repression, consistent with the notion that Smaug is involved. Smaug is a novel gene and the existence of potential mammalian Smaug homologs raises the possibility that Smaug represents a new class of conserved translational repressor.

Keywords: *Drosophila*; embryo; RNA-binding protein

INTRODUCTION

Specification of various cell and tissue types relies on differential patterns of gene expression, which are often generated at the level of transcription. Regulation, however, does not cease once transcripts are made. Instead, a variety of posttranscriptional control events may also contribute to proper expression patterns. The prevalence and specificity of posttranscriptional control mechanisms has been illustrated by the analysis of maternal mRNAs, which are contributed to the developing egg by the mother. For example, the bulk of maternal mRNAs in the eggs of some species are translationally silent until fertilization results in a general activation of protein synthesis. In addition, some maternal mRNAs are also localized to subdomains within the egg, placing spatial restrictions on their activities. Both types of posttranscriptional control are used in other situations and may prove to be relatively common features of regulated gene expression.

Several of the most prominent examples of posttranscriptional controls were revealed by studies aimed at understanding early development in *Drosophila* (reviewed by Curtis et al., 1995; St Johnston, 1995; Macdonald & Smibert, 1996; Bashirullah et al., 1998). In the *Drosophila* egg, and later the embryo, a cascade of posttranscriptional regulatory events specifies pattern along the anteroposterior body axis. During oogenesis the *oskar* (*osk*) mRNA is first localized to the posterior pole of the oocyte (Ephrussi et al., 1991; Kim-Ha et al., 1991). Repression of *osk* translation is relieved upon localization (Kim-Ha et al., 1995), and the resulting Osk protein then recruits *nanos* (*nos*) mRNA to the posterior pole of the embryo (Wang & Lehmann, 1991; Gavis & Lehmann, 1992; Wang et al., 1994). Translation of unlocalized *nos* mRNA is repressed, and Nos protein is only synthesized at the posterior of the embryo (Gavis & Lehmann, 1994). This Nos protein then acts in local repression of *hunchback* mRNA, allowing development of the posterior parts of the embryo (Tautz, 1988; Wharton & Struhl, 1991; Gavis & Lehmann, 1992). Both *osk* and *nos* mRNAs contain regulatory elements in their 3' untranslated regions (UTR) that control translation as well as localization (Gavis & Lehmann, 1992, 1994; Kim-Ha et al., 1993, 1995; Dahanukar & Wharton, 1996; Gavis et al., 1996a, 1996b; Smibert et al., 1996). Learn-

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ing how these elements function may provide general insights into mechanisms of translational control and reveal how mRNA localization and translation are coordinated.

At present, detailed mechanisms of 3' UTR mediated translational control are incompletely defined. Often, elements within the 3' UTR mediate changes in the length of the poly (A) tail in the cytoplasm, and these changes correlate with changes in translational activity. In general, activation is associated with an increase in poly (A) tail length whereas translational repression correlates with a decrease in poly (A) tail length (Richter, 1996). Discrete *cis*-acting sequences that direct shortening or lengthening of the poly (A) tail have been identified, as have some of the *trans*-acting factors that bind these elements. However, the precise molecular mechanisms by which changes in poly (A) tail length influence translation are not understood (Fox et al., 1989; McGrew et al., 1989; Sallés et al., 1992; Bouvet et al., 1994; Hake & Richter, 1994; Sheets et al., 1994; Simon & Richter, 1994).

Other forms of 3' UTR-mediated translational control do not appear to involve the poly (A) tail. In some cases, exemplified by the *osk* and *nos* mRNAs (Sallés et al., 1994; Gavis et al., 1996b) as well as the lipoxxygenase mRNA from rabbit reticulocytes (Ostareck-Lederer et al., 1994; Ostareck et al., 1997), poly(A) tail length remains constant independent of translational status. For each of these mRNAs, regulatory elements in the 3' UTR that are responsible for translational repression have been identified, with no apparent overlap in their makeup. The elements from the *lipoxxygenase* and *osk* mRNAs have no obvious secondary structure and each is characterized by multiple repeated copies of a different sequence motif (Ostareck-Lederer et al., 1994; Kim-Ha et al., 1995; Ostareck et al., 1997). In contrast, the elements from *nos* mRNA are predicted to form a stem-loop structure, for which there is both experimental and evolutionary support (Dahanukar & Wharton, 1996; Gavis et al., 1996b; Smibert et al., 1996). Given the diversity in *cis*-acting elements it seems likely that there are different classes of translational repressors that act independent of the poly(A) tail. This is certainly true for the *lipoxxygenase* and *osk* mRNAs; the binding factors that mediate repression have been characterized and are different (Webster et al., 1997; Ostareck et al., 1997).

The identity of the protein that mediates repression of unlocalized *nos* mRNA was suggested from RNA-binding experiments (Smibert et al., 1996). The *cis*-acting sequences that repress translation of unlocalized *nos* mRNA are specifically bound by the ~135-kDa Smaug (Smg) protein in UV cross-linking experiments, and have been designated as SREs (Smg Recognition Elements). In testing a collection of SRE point mutants, a perfect correlation was observed between *in vitro* Smg binding and *in vivo* repression of translation: SRE mutants defective in translational regulation fail to bind

Smg. These data suggested that the binding of Smg to *nos* mRNA represses its translation. However, it remained possible that Smg binding was fortuitous, and that some other protein with a similar binding specificity was the true repressor. Here we describe the isolation and characterization of Smg. Using an *in vitro* translation system prepared from *Drosophila* embryos we show that SRE-dependent regulation can be recapitulated *in vitro*. Immunodepletion of Smg abolishes repression of *nos* translation, as expected if Smg acts in repression. The existence of related proteins from humans and mice suggests that Smg may define a novel class of evolutionarily conserved translational repressors.

RESULTS

Identification of the *smg* cDNA

To further characterize Smg, and to more definitively test its role in regulation of *nos* mRNA translation, we isolated the protein for sequence analysis. Embryos collected 0–3 h post-egg laying were used as the source of Smg protein, as Smg RNA-binding activity is greatest during this stage of development (Smibert et al., 1996). The final step in our purification strategy (described in detail in Materials and Methods) involved binding of partially purified protein fractions to Cibacron Blue 3GA resin. Smg RNA-binding activity was eluted with RNA bearing three copies of the SRE (3×SRE+), the Smg-binding site from the *nos* mRNA 3' UTR (Fig. 1A). Comparison of proteins eluted from the resin with tRNA, with 3×SRE– RNA (a mutated form of the SREs unable to bind Smg), or with 3×SRE+ RNA revealed several proteins that elute under all conditions. However, a single protein of about 130 kDa was eluted exclusively with 3×SRE+ RNA (Fig. 1B). The size and specific elution of the protein suggests that it is Smg.

The purified protein was subjected to amino-terminal sequence analysis. In addition, mass spectrometry was used to determine the masses of peptides produced by cleavage of the purified protein with trypsin. Both types of data identified a class of cDNA from a collection of expressed sequence tags (EST) generated by the Berkeley *Drosophila* Genome Project (BDGP). We initially identified cDNAs predicted to encode the amino-terminal residues of the purified protein, MKYATGTD NAM. Subsequently, complete DNA sequence analysis of the open reading frame associated with these cDNAs (see below) identified nine peptides characterized by mass spectrometry (see Materials and Methods).

To determine if the identified class of cDNAs do in fact encode Smg, an EST clone that includes the complete open reading frame was used to express protein in a rabbit reticulocyte-coupled transcription/translation extract. The protein synthesized in this extract has an apparent size similar to that of the protein purified from

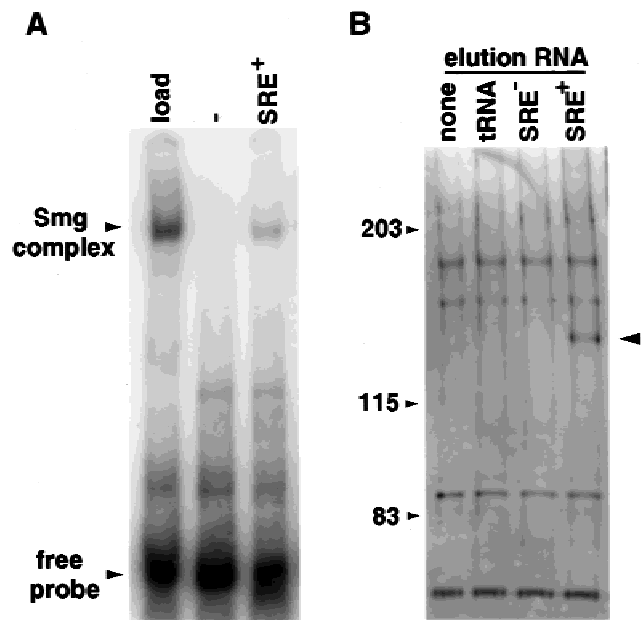


FIGURE 1. Purification of Smaug. **A:** Partially purified Smaug protein was mixed with Cibacron Blue 3GA beads, washed, and eluted with buffer containing either no RNA (–; lane 2) or labeled 3×SRE+ RNA (SRE+; lane 3). Smaug RNA-binding activity was monitored in the load material (load; lane 1) and eluates using a gel retardation assay that employed 8 ng of RNA per lane (see Materials and Methods). The mobilities of 3×SRE+ RNA and the Smaug/3×SRE+ complex are indicated. **B:** Proteins eluted from the Cibacron Blue 3GA resin with different RNAs (none, tRNA, 3×SRE– RNA, and 3×SRE+ RNA) were resolved on a 6% SDS-polyacrylamide gel and detected by silver staining. The arrowhead on the right indicates the Smaug protein; positions of molecular mass markers are indicated on the left. A protein the size of Smaug is specifically eluted by the 3×SRE+ RNA.

embryos (Fig. 2A). RNA-binding properties of the in vitro synthesized candidate Smaug protein were evaluated by UV cross-linking assays. Notably, the protein made from the cDNA displays RNA binding identical to that of bona fide Smaug protein in embryonic extracts: both proteins bind specifically to 3×SRE+ RNA, producing adducts identical in size, but neither protein binds detectably to 3×SRE– RNA (Fig. 2B). We conclude that the cDNA encodes Smaug.

DNA sequence analysis of the *smg* cDNA (GenBank AF132213) identified an open reading frame predicted to encode a protein of 999 amino acids, with a molecular weight of 109 kDa (Fig. 3A). A number of RNA-binding domains have been characterized in other proteins and can be recognized by sequence comparisons (Burd & Dreyfuss, 1994); however, Smaug contains none of these. Therefore, the Smaug RNA-binding domain appears to be novel. Database comparisons do reveal three regions of homology with human and mouse genomic DNA sequences (Fig. 3B,C). In each case the regions of sequence similarity are contained within single cosmid clones, consistent with the expectation that they are from the same protein coding region. Two of the conserved regions have no defined function and

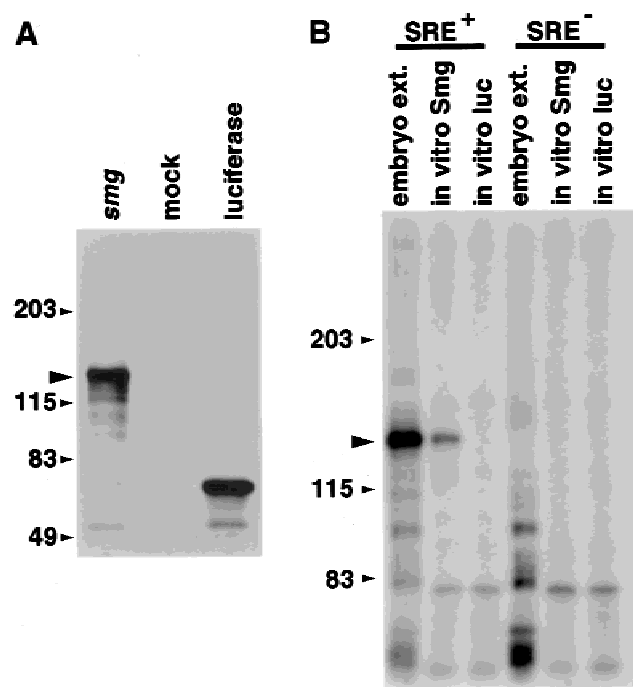


FIGURE 2. Smaug protein translated in vitro functions as a sequence-specific RNA-binding protein. **A:** A candidate *smg* cDNA (LD21155 of the Berkeley *Drosophila* Genome Project; GenBank accession number AA735347) was expressed in a coupled transcription/translation system in the presence of ³⁵S-methionine. Parallel reactions were programmed with either a luciferase cDNA or no DNA (mock), and the products resolved on a 6% SDS-polyacrylamide gel. A protein of ~130 kDa is expressed from the candidate *smg* cDNA. **B:** Unlabeled proteins were synthesized in coupled transcription/translations programmed with either the candidate *smg* cDNA or a luciferase cDNA. The products were assayed for RNA-binding activity using a UV-cross-linking assay and labeled 3×SRE+ and 3×SRE– RNAs as probes. An embryo extract collected 0–3 h post-egg laying was used as a bona fide source of Smaug RNA-binding activity. Reticulocyte lysate programmed with the *smg* EST shows an RNA-binding activity that comigrates with Smaug protein in embryo extracts, and this protein binds to wild-type 3×SRE+ RNA and not to 3×SRE– RNA. In both **A** and **B** Smaug is indicated by large arrowheads, and the positions of molecular mass markers are indicated with small arrowheads.

have been designated Smaug Similarity Region (SSR) 1 and SSR2. In contrast, the third region has been observed in other proteins and is a SAM (Sterile Alpha Mating) domain (reviewed by Schultz et al., 1997), which mediates homo- and hetero-oligomerization with other SAM domains (reviewed in Thanos et al., 1999). In addition, some SAM domains can interact with proteins that lack the SAM homology (reviewed in Thanos et al., 1999) and in one case, phosphorylation of a conserved tyrosine residue within the SAM domain allows for binding of an SH2 domain-containing protein and a low-molecular-weight phosphotyrosine phosphatase (Stein et al., 1998).

nos translational regulation in vitro

Confirming the role of Smaug in control of *nos* mRNA translation could involve either genetic or biochemical

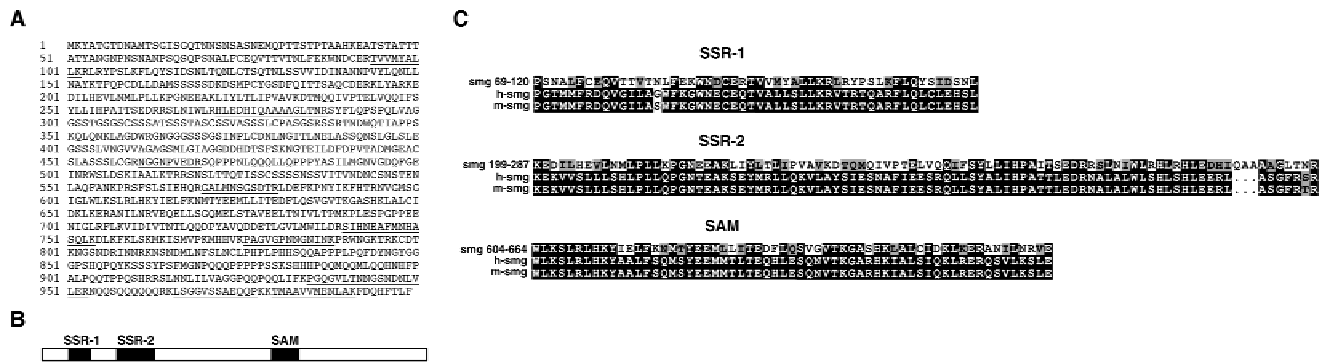


FIGURE 3. Sequence and domain structure of the Smg protein. **A:** Sequencing of the *smg* EST cDNA (LD21155; GenBank accession number AA735347) revealed a single large open reading frame predicted to encode a 999-amino-acid protein of 109 kDa. The nine underlined regions of the protein matched mass spectroscopy data of tryptic peptides derived from purified Smg protein. A portion of the *smg* cDNA, encoding residues 573–792 (GenBank accession number U03277), was previously cloned based on its ability to arrest the cell cycle when expressed in *Schizosaccharomyces pombe*; the relevance of this activity to Smg function as a translational repressor is unclear. Database comparisons reveal potential human and mouse proteins predicted by genomic sequence (GenBank accession numbers AC005239 and AC002327, respectively) that share three regions of similarity to Smg. Two of these regions have no defined function and have been designated SSR1 and SSR2. The third region is a SAM domain which has been shown to mediate protein/protein interactions. **B:** The positions of SSR1, SSR2, and the SAM domain are indicated on a schematic of the Smg protein. **C:** Alignments of the human, mouse, and *Drosophila* Smg SSR1, SSR2, and SAM domains are shown with identical residues highlighted in black and similar residues shaded grey.

experiments. We have not yet identified mutants in the *smg* gene, a prerequisite for standard genetic analyses, and so biochemical approaches have been pursued instead. As a first step in this work, we established an *in vitro* translation system from *Drosophila* embryos (see Materials and Methods), using a modification of protocols designed for production of *in vitro* translation extracts from other organisms (Tarun & Sachs, 1995; Iizuka & Sarnow, 1997). Briefly, embryos are lysed and treated with micrococcal nuclease to remove endogenous mRNAs, and the nuclease is inactivated by addition of EGTA. Translational activity of the processed extracts is monitored using exogenous mRNAs encoding firefly luciferase.

The ability of the extracts to support SRE-mediated translational regulation was tested using transcripts bearing three copies of the SRE stem/loop structure (Smibert et al., 1996) in the 3' UTR. In one transcript, luc3×SRE+, all copies of the SRE are wild type. This 3×SRE+ element was shown previously to mediate translational repression *in vivo*, and binds Smg *in vitro* (Smibert et al., 1996). In a second transcript, luc3×SRE−, each copy of the SRE was point mutated in a manner that eliminates both Smg binding *in vitro* and translational repressive activity *in vivo* (Smibert et al., 1996). Both transcripts are translated with similar efficiencies in rabbit reticulocyte lysates, demonstrating that there are no inherent differences in their translatability (Fig. 4A). However, translation in extracts prepared from very early stage embryos (harvested 0–2 h after egg laying, when Smg-binding activity is most abundant) is markedly different for the two transcripts, with the luc3×SRE− mRNA translated much

more efficiently than the luc3×SRE+ mRNA (Fig. 4A). Because these assays were performed within the linear range of input mRNA (determined using the lucSRE− mRNA; see Fig. 4B), we were able to estimate that the lucSRE− mRNA was translated roughly eightfold more efficiently than the lucSRE+ mRNA. This difference is not a consequence of differential mRNA stability, as a quantitative analysis of the levels of full-length mRNA during the course of the assay reveals that decay of the two mRNAs is essentially the same (Fig. 4B). We conclude that early embryo extracts support SRE-dependent translational repression. To confirm the relevance of these results to *nos* translation regulation, we also tested the ability of the bona fide *nos* 3' UTR to repress translation in early-stage embryo extracts. These experiments employed luciferase mRNAs bearing either the wild-type *nos* 3' UTR (lucnos3'UTR+) or a point-mutated *nos* 3' UTR (lucnos3'UTR−) that fails to repress translation *in vivo* (Smibert et al., 1996). Similar to results obtained with SRE sequences alone, lucnos3'UTR− mRNA was translated more efficiently (approximately 3.7-fold) when compared to the lucnos3'UTR+ mRNA (Fig. 4D). Although the magnitude of the repression mediated by the intact *nos* 3' UTR is less when compared to the 3×SRE sequence (likely reflecting the presence of only two SREs in the *nos* 3' UTR), these results confirm that early embryo extracts support SRE-dependent translational repression.

If the SRE-dependent repression requires Smg protein, extracts lacking the protein should translate luc3×SRE+ and luc3×SRE− mRNAs with similar efficiencies. Two related experiments were performed

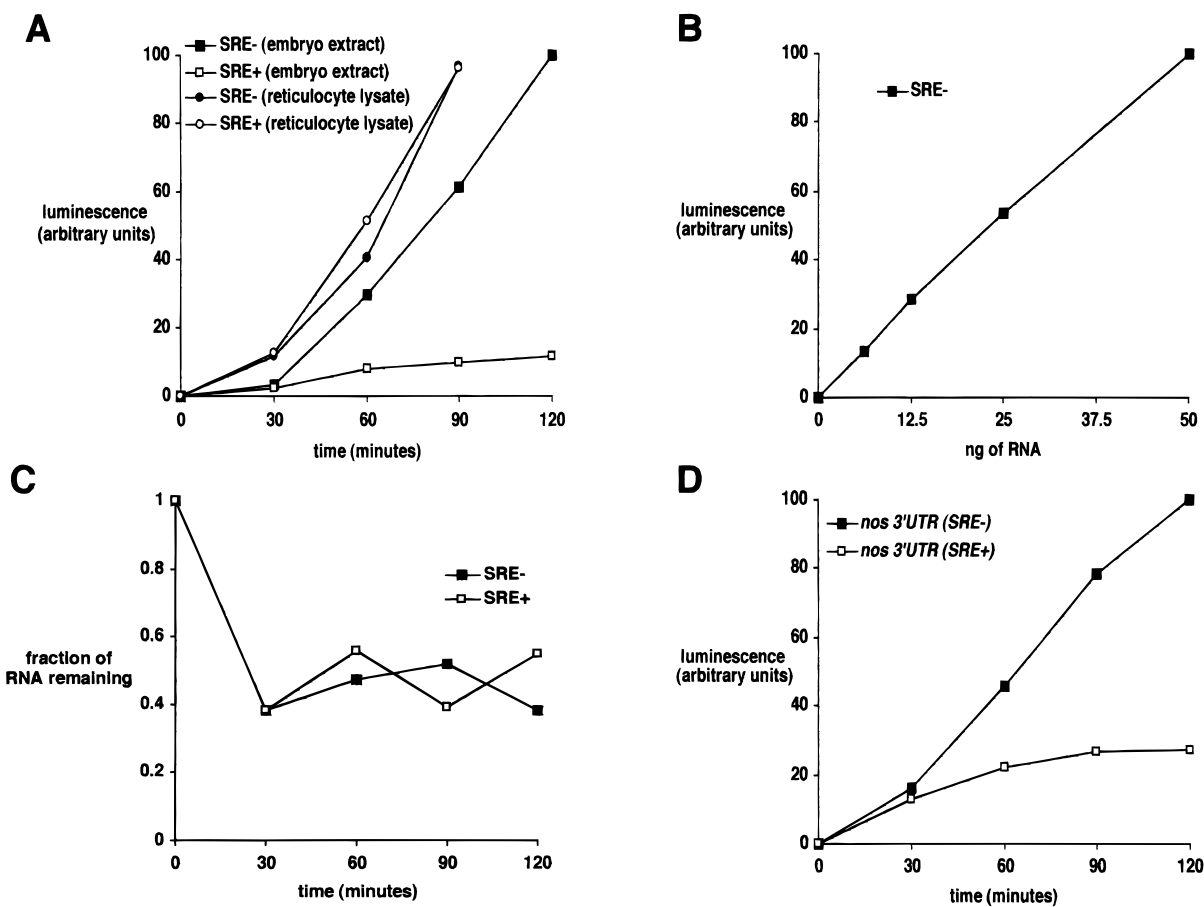


FIGURE 4. In vitro translation extracts prepared from 0–2-h embryos recapitulate SRE-dependent translational repression. **A:** Rabbit reticulocyte lysate or in vitro translation extracts derived from early embryos (collected 0–2 h post-egg-laying) were programmed with luciferase RNAs that carried either three wild-type SREs (SRE+) or three point mutated SREs (SRE–). Luciferase activity was assayed at 30, 60, 90, and 120 min after initiation of the translation reaction, revealing translational repression of SRE+ RNA in the embryo extracts. Graphed are the results of one experiment where luc3×SRE– mRNA was translated 8.0-fold more efficiently. In two additional independent experiments, the luc3×SRE– mRNA was translated 7.8- and 9.4-fold more efficiently. **B:** Early embryo extract was programmed with 6.25, 12.5, 25, and 50 ng of luc3×SRE– mRNA and luciferase activity was assayed at 120 min after initiation of the translation reaction, demonstrating a linear response as a function of input mRNA concentration. **C:** The stability of luc3×SRE+ and luc3×SRE– RNAs in 0–2-h embryo extracts was determined by assaying the RNA levels at 30, 60, 90, and 120 min. RNA was extracted and resolved on a denaturing gel, and levels of full-length RNAs were quantitated by phosphorimager analysis. **D:** Early embryo extract was programmed with luciferase RNAs that carried either the wild-type *nos* 3' UTR (luc*nos*3'UTR+) or a *nos* 3' UTR with point mutated SREs (luc*nos*3'UTR–). Luciferase activity was assayed at 30, 60, 90, and 120 min after initiation of the translation reaction, revealing translational repression of the RNA carrying the wild-type *nos* 3' UTR.

to test this prediction. First, extracts were prepared from later-stage embryos (harvested 4–6 h after egg-laying), in which Smaug protein levels are substantially reduced (see Fig. 6C). These extracts displayed little difference in the translation efficiencies of the luc3×SRE+ and luc3×SRE– mRNAs (Fig. 5A), consistent with the notion that Smaug is required for repression. Second, immunodepletion experiments were used to test the requirement for Smaug in the translational repression observed in 0–2-h embryo extracts. Mock depletion using antibodies from normal rat serum had no effect on SRE-dependent repression. In contrast, an extract depleted with purified anti-Smaug antibodies translated luc3×SRE+ and luc3×SRE– mRNAs at similar efficiencies (Fig. 5B). Western blot analysis confirmed

the depletion of Smaug protein from the extract (Fig. 5C). Ideally, we would extend this experiment by determining if addition of recombinant Smaug protein can restore repression. However, we have not yet succeeded in expressing Smaug protein and are thus unable to do the experiment. The immunodepletion data support the conclusion that Smaug protein acts as a repressor of *nos* mRNA translation, although in the absence of the add-back experiment these data do not prove that Smaug is the repressor (see Discussion).

In embryos, SRE-dependent translational repression is coupled to mRNA degradation. A careful comparison of the temporal patterns of translation and stability of mRNAs bearing wild-type and mutated SREs revealed that mRNAs whose translation is repressed via SREs

are rapidly degraded after a short lag period (Smibert et al., 1996). This aspect of the regulation of *nos* mRNA fate is not reproduced in the in vitro system, as the stabilities of *luc3*×SRE+ and *luc3*×SRE- mRNAs are similar (Fig. 4B).

Smg protein in embryos

Smg RNA-binding activity first appears very early in embryogenesis prior to the onset of zygotic transcription, indicating that the Smg protein is synthesized from

a maternal mRNA. Nevertheless, Smg RNA binding cannot be detected in ovaries (Smibert et al., 1996). Thus either the protein is absent or the RNA-binding activity is inhibited. To distinguish between these possibilities for restriction of Smg activity, the expression of Smg protein and mRNA was monitored in ovaries and early embryos. As expected, *smg* mRNA is expressed in ovaries and persists at high levels in early embryos (Fig. 6A). Despite the ovarian transcription, no Smg protein can be detected in ovaries (Fig. 6B), and we therefore find no evidence that Smg RNA-binding activity is regulated posttranslationally in ovaries. Instead, the absence of Smg protein in ovaries could reflect either translational regulation of *smg* mRNA or rapid degradation of Smg protein.

Although Smg protein levels are high during early embryogenesis, we are unable to detect Smg protein by Western blot analysis at later times (Fig. 6C). In early embryos, Smg protein is initially present uniformly and at high levels (Fig. 7A). This distribution is expected because SRE-dependent translational repression occurs throughout the embryo (Smibert et al., 1996), consistent with the hypothesis that Smg represses the translation of unlocalized *nos* mRNA. Smg protein persists at high levels during the early cleavage stages and is highly enriched in cytoplasm as compared to nuclei (Fig. 7B,H). By the time of cellularization, the level of unlocalized Smg protein decreases. In parallel, a posterior region of Smg protein localization appears, with Smg concentrated in pole cells and in a region immediately anterior to the pole cells (Fig. 7C,D). The presence of Smg in the pole cells continues, and the protein can be detected in the invaginated pole cells as late as stage 8 (Fig. 7F and data not shown; stages according to Campos-Ortega & Hartenstein, 1985).

The pattern of Smg protein accumulation at the posterior of the embryo closely resembles that of *nos*

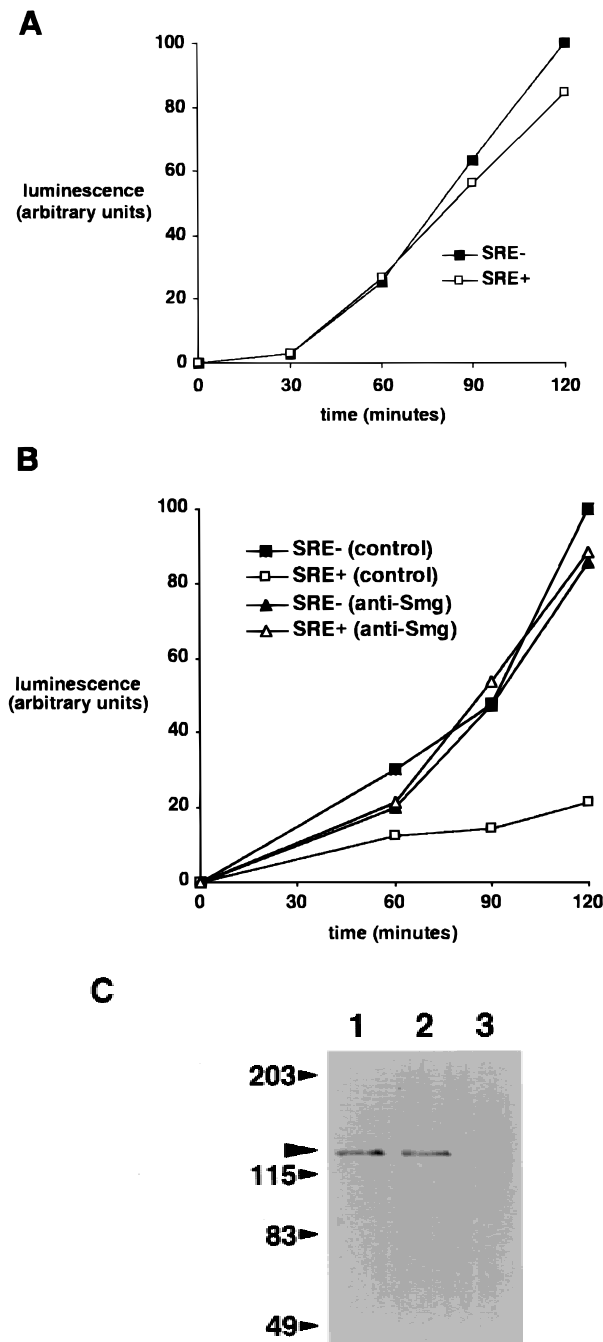


FIGURE 5. Correlation of SRE-dependent translational repression with the presence of Smg. **A:** In vitro translation extracts prepared from embryos collected 4–6 h post-egg laying [when Smg RNA-binding activity is substantially reduced (Smibert et al., 1996)] were programmed with *luc3*×SRE+ (SRE+) and *luc3*×SRE- (SRE-) RNAs. Luciferase activity was assayed at 30, 60, 90, and 120 min after initiation of the translation reactions, revealing no difference in the translation of the two RNAs. **B:** In vitro translation extracts prepared from embryos collected 0–2 h post-egg laying were immunodepleted with anti-Smg or control antibodies and programmed with *luc3*×SRE+ and 3×SRE- RNAs. Luciferase activity was assayed at 60, 90, and 120 min after initiation of the translation reaction. The control antibodies had no effect on relative translation of the two RNAs. In contrast, immunodepletion with the anti-Smg antibodies eliminated translational repression of the *luc3*×SRE+ RNA. **C:** Confirmation that immunodepletion removes Smg. Western blot detection of Smg was performed on three samples: in vitro translation extract (lane 1); extract depleted with control antibodies (lane 2); extract depleted with anti-Smg antibodies (lane 3). Smg is efficiently removed from the extracts by this procedure. Equivalent protein loading in each lane was ensured by Ponceau S staining of the blot (data not shown).

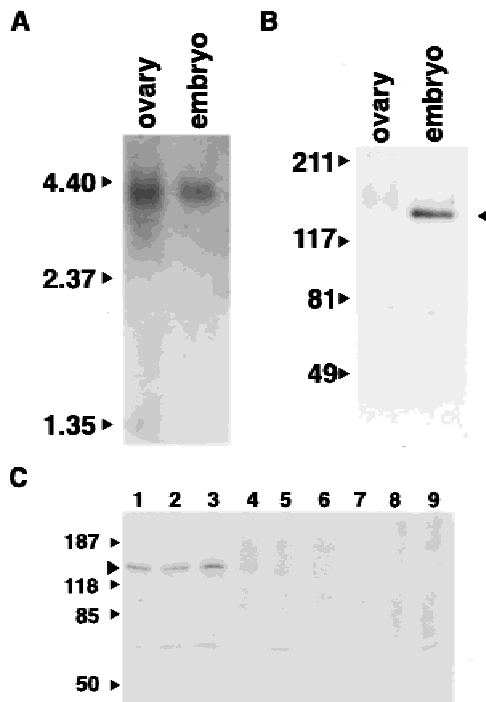


FIGURE 6. *Smg* expression is regulated during early development. **A:** Northern blot analysis of *smg* mRNA. An approximately 4.2-kb *smg* RNA was detected in both ovaries and 0–3-h embryos, indicating that *smg* is transcribed maternally. **B:** Western blot analysis of Smg protein. Smg protein is present in early embryos, but not detectable in ovaries. These results suggest that the accumulation of Smg protein is regulated at the level of either protein stability or translation. In both **A** and **B** the positions of molecular mass markers are indicated. Equivalent protein loading in each lane was ensured by Ponceau S staining of the blot (data not shown). **C:** Time course of Smg expression throughout embryogenesis. Embryos were collected at 0–1 (lane 1), 1–2 (lane 2), 2–3 (lane 3), 3–4 (lane 4), 4–6 (lane 5), 6–10 (lane 6), 10–14 (lane 7), 14–18 (lane 8), and 18–21 (lane 9) h post-egg laying and assayed for Smg expression by Western blot analysis. Smg can only be detected in early embryos. Equivalent protein loading in each lane was ensured by Ponceau S staining of the blot (data not shown).

mRNA, raising the possibility that Smg becomes localized because of its association with *nos* mRNA. To test this model, we examined Smg protein in embryos from *nos*^{BN} mutant mothers. These embryos lack *nos* mRNA (Wang et al., 1994), but retain the normal pattern of Smg protein localization (Fig. 7E). Thus, posterior localization of Smg protein does not depend on association with *nos* mRNA.

The distribution of *smg* mRNA is similar to that of Smg protein: uniform throughout the early embryo and concentrated at the posterior pole by the time of cellularization (Fig. 7I). However, *smg* mRNA is not present in the pole cells (Fig. 7J). Thus the localization of the Smg protein at the posterior could be a consequence of localization of the *smg* mRNA. Recruitment of mRNAs and proteins to the posterior of the *Drosophila* embryo often requires the *osk* gene product (Ephrussi et al., 1991; Kim-Ha et al., 1991; Webster et al., 1994). Not surprisingly, we find that localization of both Smg protein

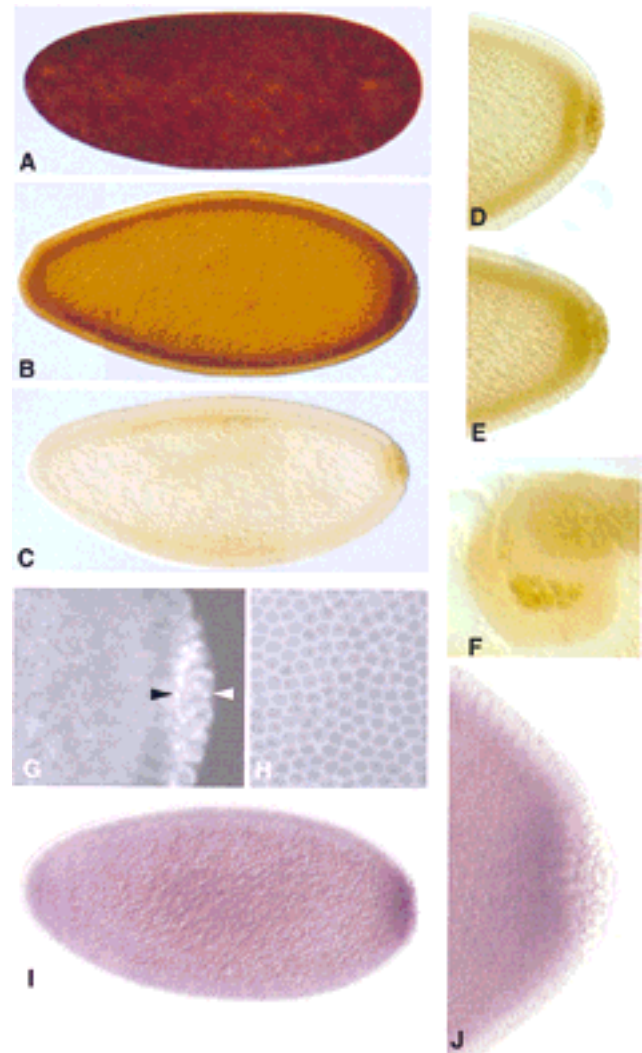


FIGURE 7. Smg protein and mRNA distribution in embryogenesis. Smg protein distribution was detected by immunohistochemistry (**A–F**) or confocal microscopy (**G,H**). All images are of wild-type embryos except for **E**, which is an embryo derived from a *nos*^{BN} mutant mother. **A,B:** Smg protein appears and persists at high levels during early cleavage stages and is distributed ubiquitously throughout the embryo. **C:** By the time of cellularization, Smg protein levels are reduced throughout most of the embryo, except for a concentration of the protein in the pole cells and in a region just anterior to the pole cells. **D,E:** Concentration of Smg at the posterior of the embryo can be detected in embryos derived from both wild-type (**D**) and *nos*^{BN} mutant mothers (**E**). Because the latter embryos contain no *nos* mRNA, Smg becomes localized by a mechanism not requiring association with *nos* mRNA. **F:** Smg protein persists in the pole cells as they invaginate into the embryo. **G:** Smg protein in the pole cells shows a diffuse cytoplasmic staining, although there are foci in which Smg is more concentrated (white arrowhead). Within the layer of cytoplasm just anterior to the pole cells the punctate distribution of Smg is much more prominent (black arrowhead). During all stages Smg is partitioned to the cytoplasm. In addition to cytoplasmic staining in the pole cells (**G**), notice the strongly staining cytoplasm observed in the blastoderm of a cleavage-stage embryo compared to the weakly staining nuclei (**H**). By the time of cellularization *smg* mRNA is concentrated at the posterior of the embryo, similar to the Smg protein, but is not present in the pole cells (**I,J**).

and mRNA to the posterior, as well as the concentration of Smg in the posterior foci described below, does not occur in an *osk* mutant embryos (data not shown).

Within the posterior zone, different patterns of Smg protein distribution can be visualized by confocal microscopy (Fig. 7G). Smg protein in the pole cells is generally diffuse and cytoplasmic, although there are foci in which Smg is more concentrated. This punctate distribution is greatly enhanced in the layer of cytoplasm lying immediately anterior to the pole cells; the level of diffuse cytoplasmic protein is similar to that of the pole cells, but Smg is much more concentrated in the foci. These regions of the embryo (pole cells and underlying cytoplasm) contain polar granules, large ribonucleoprotein structures implicated in germ cell specification (Mahowald, 1962; Illmensee & Mahowald, 1974; Frohnhofner et al., 1986), raising the possibility that Smg protein distribution results from association with polar granules.

DISCUSSION

Nos protein is deployed exclusively at the posterior of the *Drosophila* embryo, where it directs posterior development. Nos protein is synthesized from an mRNA that is both localized (to the posterior pole of the embryo) and translationally regulated. Both forms of control are mediated by the *nos* 3' UTR, as revealed by the behavior of mutants in which the entire region is deleted or replaced (Gavis & Lehmann, 1992, 1994). Translational control is essential: a subtly mutated version of the 3' UTR that retains normal mRNA localization but fails to support translational repression causes lethality (Smibert et al., 1996). The importance of mRNA localization remains uncertain, as it has proven difficult to eliminate *nos* mRNA localization while retaining normal translational control.

Progress towards understanding the mechanisms of *nos* mRNA localization and translational control has relied, in large part, on the analysis of mutated forms of the 3' UTR. Related work from several laboratories has led to similar conclusions about the sequences involved in repression of *nos* translation (Dahanukar & Wharton, 1996; Gavis et al., 1996b; Smibert et al., 1996; Bergsten & Gavis, 1999). However, there is substantial disagreement about many aspects of mRNA localization and translational activation, as well as the relationship between these processes and translational repression. Resolution of the differences will likely be made possible by isolation and characterization of the factors involved.

Evidence that translational repression of *nos* mRNA is mediated by Smg

The sequences that control translational repression of *nos* mRNA have been mapped, independently by different workers, to the first 200 nt of the *nos* 3' UTR

(Dahanukar & Wharton, 1996; Gavis et al., 1996b; Smibert et al., 1996; Bergsten & Gavis, 1999). The most precise mapping experiments identified two copies of the SRE or Smg recognition element (Smibert et al., 1996), and all available data are consistent with the conclusion that the SREs serve as the primary mediators of repression (Dahanukar & Wharton, 1996; Gavis et al., 1996b; Smibert et al., 1996; Bergsten & Gavis, 1999). In this earlier work we identified a protein, Smg, that interacts with the SREs. In addition, we established a correlation between Smg binding to the SREs in vitro and the ability of the SREs to repress translation in vivo. These results suggested but did not prove that Smg represses *nos* translation through the SREs (Smibert et al., 1996). Here, using an in vitro translation system that recapitulates SRE-dependent translational regulation of *nos* mRNA, we have provided another type of evidence supporting the conclusion that Smg is a repressor of *nos* translation. Our new evidence comes from experiments in which translation extracts immunodepleted of Smg are tested for SRE-dependent repression of *nos* mRNA. Definitive proof that Smg acts in translational repression is not possible from the new experiments, as we have as yet been unable to add pure Smg protein to the immunodepleted extracts and determine if repression is restored. However, the limitations of the two types of experiments are different, and taken together they strongly suggest that Smg plays a role in translational repression of *nos* mRNA. Specifically, in the earlier experiments the correlation between the ability of SREs to bind Smg in vitro and mediate translational repression in vivo could be misleading if the true repressor has the same binding specificity as Smg. The new experiments are limited in that a physical interaction between Smg and the true repressor could be responsible for the immunodepletion of repressor activity. When the two types of data are considered together, the possible interpretations are (1) that Smg acts as a repressor, or (2) that Smg is not the repressor but is associated with an RNA-binding protein that shares the same binding specificity and is the repressor. The latter scenario is possible, but not likely.

The sequence of Smg provides no obvious suggestion of how it may act in translational repression as a consequence of binding to the *nos* 3' UTR. This is not surprising, as very few translational repressors have been characterized and the only domains whose function is understood are those involved in RNA binding (for examples, see Murata & Wharton, 1995; Dubnau & Struhl, 1996; Rivera-Pomar et al., 1996; Bashaw & Baker, 1997; Ostareck et al., 1997; Webster et al., 1997; Zhang et al., 1997; Wharton et al., 1998; Jan et al., 1999).

At present, *nos* is the only known transcript with a recognizable SRE, despite sequence inspection of numerous other maternally expressed genes. Neverthe-

less, there may well be other targets, as the ubiquitous appearance of Smg in early embryos should allow it to act on other mRNAs. In addition, Smg may also act later in development, when *nos* mRNA is no longer present. Although by Western blot analysis we can only detect Smg protein in early embryos, this method might not reveal the expression of Smg in small populations of cells at the later stages. Indeed, whole-mount antibody stains suggest that the Smg protein appears in the region of the ventral nerve cord as well as the brain during embryogenesis (C.A. Smibert, Y.S. Lie, W. Shillinglaw, W.J. Henzel, P.M. Macdonald, unpubl. data), and a *smg* EST cDNA clone has been identified in a *Drosophila* head cDNA library (BDGP; GenBank accession number A1134156). Identification of potential target mRNAs, those that contain SRE-like sequences, will soon be possible on a genome-wide scale when sequencing of the genome is complete.

Coordination of *nanos* mRNA localization and translation

In wild-type embryos, the posterior localization and translation of *nos* mRNA appears to be coordinated: unlocalized mRNA is translationally repressed, and localization of the mRNA correlates with its translation. However, the cause-and-effect relationship between mRNA localization and translational activation has been difficult to establish. Given Smg presence at the posterior of the embryo, activation of *nos* translation at least requires the inactivation of Smg protein present at the posterior. Different models for how *nos* mRNA is regulated center on different roles played by *cis*-acting regulatory sequences. In one model, the SREs are proposed to mediate all features of control: repression, localization, and activation (Dahanukar & Wharton, 1996). Other models postulate that different regulatory elements are required for different functions (Smibert et al., 1996; Bergsten & Gavis, 1999). Attempts to reconcile apparently conflicting data that support different models have been hampered for at least two reasons. First, the sequences responsible for mRNA localization and translational activation are at least partially redundant and dispersed in the *nos* 3' UTR (Gavis et al., 1996a). Consequently, the results of an experiment may reflect a peculiarity of the exact portion of the 3' UTR used. Second, many of the mutant versions of the *nos* 3' UTR that have been tested include substantial deletions, multimerized sequences, or foreign structural elements; it would therefore be difficult to predict, with any degree of confidence, that these changes would have no unforeseen effects on RNA structure, function, or both. Thus, based on current data, any predictions about the molecular mechanisms that underlie the coordination of localization and translational activation would entail a fair degree of speculation.

Although the work presented here does not resolve all of these problems, the isolation of one of the regulatory factors will now allow specific questions to be posed about its function. Moreover, the *in vitro* system used here to demonstrate translational repression by Smg can serve as the basis for an assay to ask if this activity of Smg can be modulated by interaction with other factors, one possible mechanism for activation of *nos* translation. Our finding that Smg is itself concentrated, in a *nos* mRNA-independent fashion, at the posterior pole of the embryo raises the possibility that Smg contributes not only to translational repression, but also to mRNA localization (consistent with the model proposed by Dahanukar & Wharton, 1996). Specifically, Smg could bind and retain *nos* mRNA at the posterior pole, although the spatial specificity for this reaction would have to be provided by some other factor, as Smg is uniformly distributed during the early embryonic stages when localization is initiated. Mutation of the SREs, in the context of an otherwise intact *nos* gene, has no detectable effect on localization of the mRNA (Smibert et al., 1996), arguing against a role for the SREs in *nos* mRNA localization. However, this result does not rule out such a possibility, given the redundancy of the localization signals (Gavis et al., 1996a). If the SREs do support mRNA localization, then localization and translational activation must be separable processes, because a fragment of the *nos* 3' UTR bearing one SRE is not competent for activation of translation (Smibert et al., 1996).

Smg may represent a new class of conserved translational repressor

The sequence of the *smg* gene has revealed potential proteins, predicted by human and mouse genomic sequences, that share three regions of similarity to Smg. The presence of these multiple similar domains raises the possibility that these proteins share similar functions. Thus, Smg may represent the first member of a conserved family of translational repressors. One motif that is shared with the potential mammalian homologs is a SAM domain, which mediates protein/protein interactions (reviewed by Schultz et al., 1997 and Thanos et al., 1999). The other two conserved regions are uncharacterized. These domains may be required for one or more Smg functions, including specific RNA binding and the ability to repress translation. In addition, Smg may have to interact with factors at the posterior of the embryo that block Smg function and allow for the translation of localized *nos* mRNA. The presence of the SAM domain within Smg, which contains a conserved tyrosine residue that is phosphorylated in at least one SAM-containing protein (Stein et al., 1998), raises the possibility that tyrosine phosphorylation may play some role in Smg function. Determining the domains of protein required for translational repression and RNA

binding will necessitate testing the function of mutant versions of the Smg protein in RNA-binding assays and in the *in vitro* translation system described here.

MATERIALS AND METHODS

Generation of SRE RNAs

3×SRE+ and 3×SRE− RNAs are described by Smibert et al. (1996). High-specific-activity [α -³²P]-UTP-labeled RNAs for elution experiments detailed in Figure 1A and for UV cross-linking were generated as previously described using SP6 RNA polymerase (Macdonald et al., 1995). Unlabeled 3×SRE+ and 3×SRE− RNAs were generated using T7 RNA polymerase as described by Sampson & Saks (1993) with the exception that GMP was omitted from the reaction.

Smg purification

Approximately 77 g of embryos (representing ~7 g of total soluble protein) were collected 0–3 h post-egg laying from a large scale *Drosophila* culture that was maintained as described by Shaffer et al. (1994). Embryos were dechorionated with bleach and washed extensively with 0.1% Triton X-100. All subsequent steps were carried out on ice or at 4 °C. Embryos were washed with and lysed by Dounce homogenization in 2.5 vol 150-mM NaCl, 20 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol (DTT), and a cocktail of protease inhibitors that was used throughout the purification (1 mM Pefabloc (Boehringer Mannheim), 0.2 mM EDTA, 2 mM benzamide, 2 μ g/mL leupeptin, and 2 μ g/mL pepstatin). The lysate was spun at 17,000 \times g for 20 min, and the resulting supernatant was filtered through Miracloth (Calbiochem) and raised to 600 mM NaCl. Polyethylenimine was added to 0.1% and after mixing for 15 min, the resulting precipitate was removed by centrifugation at 17,000 \times g for 15 min. The supernatant, which contains Smg, was slowly brought to 25% saturation with solid NH₄SO₄ and the precipitate was pelleted by centrifugation at 17,000 \times g for 15 min. The resulting pellet was resuspended in and extensively dialyzed against 200 mM NaCl, 30 mM Tris-HCl, pH 7.5, 30% glycerol, 1 mM DTT, and the protease inhibitor mixture. The resulting dialysate (~13 mL at a concentration of 71.5 mg/mL) was mixed with an equal volume of 8 M urea, 30 mM Tris-HCl, pH 7.5, 10% glycerol, 0.02% Triton X-100, and 0.2 mM EDTA, and loaded onto a 15-mL SP-Sepharose column (created by joining three 5-mL SP-Sepharose HiTrap columns (Pharmacia) in series) pre-equilibrated with buffer A plus 100 mM NaCl (buffer A is 4 M urea, 30 mM Tris, pH 7.5, 30% glycerol, 0.02% Triton X-100, 1 mM DTT, and the protease inhibitor mixture). Smg was eluted with a linear NaCl gradient from 100 mM to 500 mM. Fractions containing peak Smg RNA-binding activity (as detected by a gel-retardation assay) were pooled (giving ~20 mL of protein at ~230 mM NaCl), diluted with 2.3 vol buffer A, and combined with 8 mL of Cibacron Blue 3GA beads (Sigma) equilibrated with buffer A plus 100 mM NaCl. After mixing for 3 h, the beads were loaded into a column, allowed to settle, and washed extensively with buffer A plus 100 mM NaCl. The beads were resuspended in an equal volume of buffer A containing 100 mM NaCl and

~25 μ g/mL of 3×SRE+ RNA and mixed for 15 min. After recovering the beads by low-speed centrifugation, the resulting supernatant was concentrated by ultrafiltration.

Pilot elution of Smg RNA from Cibacron Blue 3GA resin

Pilot experiments showed that the Smg activity contained in material partially purified by polyethylenimine and NH₄SO₄ precipitations and SP-Sepharose chromatography was depleted when mixed with Cibacron Blue 3GA resin. To determine if Smg RNA-binding activity could be eluted from Cibacron Blue 3GA resin with RNA carrying Smg-binding sites, 30 μ L of the dye beads that had been exposed to partially purified Smg (and extensively washed with buffer A plus 100 mM NaCl) were incubated with 50 μ L of buffer A containing 100 mM NaCl, ~100 ng of [α -³²P]-UTP-labeled 3×SRE RNA (representing ~1 \times 10⁸ cpm) for 1 h at 4 °C. Mock elution was performed in parallel with buffer lacking the 3×SRE RNA. After beads were pelleted by centrifugation, 0.25 μ L of 200-mg/mL tRNA was added to 4 μ L of each supernatant as well as to 4 μ L of the load material. To assay for Smg activity in the mock eluate and in the load material ~8 ng of [α -³²P]-UTP-labeled 3×SRE RNA was also added. After a 10 min incubation at room temperature, samples were loaded onto a 3.5% polyacrylamide gel (acrylamide:bis 37.5:1) containing 0.25 \times TBE and run in 0.25 \times TBE at 300 V for ~2 h at 4 °C. Subsequently, the gel was dried and exposed to film.

To examine the protein eluted from the Cibacron Blue 3GA beads, elution experiments were carried out as described for elution with labeled 3×SRE RNA. Elutions employed either no RNA or 100 ng of unlabeled yeast tRNA, 3×SRE− RNA, or 3×SRE+ RNA. Eluted proteins were resolved on a 6% SDS-polyacrylamide gel and detected by silver staining.

Protein sequencing and mass spectrometry

Purified Smg was resolved by electrophoresis on an SDS-polyacrylamide gel and transferred onto a Millipore Immobilon-PSQ membrane at 250 mA constant current in a Bio-Rad Trans-Blot transfer cell for 1 h (Matsudaira, 1987). The PVDF membrane was stained with 0.1% Coomassie Blue R-250 in 50% methanol for 0.5 min and destained for 2–3 min with 10% acetic acid in 50% methanol. The membrane was thoroughly washed with water and allowed to dry before storage at −20 °C.

Automated protein sequencing was performed on a PE-Applied Biosystems, Procise 494 cLC protein sequencer. The coupling buffer was *N*-methylpiperidine in *N*-propanol and water (25:60:15) supplied by PE-Applied Biosystems. Peaks were integrated with Justice Innovation software using Nelson Analytical 760 interfaces. Sequence interpretation was performed on a DEC Alpha (Henzel et al., 1987).

To generate tryptic peptides of Smg, the protein was excised from the PVDF membrane and wetted with 1 μ L of methanol. The bands were reduced and alkylated with isopropylacetamide (Kruttsch & Inman, 1993), followed by digestion in 20 μ L of 0.05 M ammonium bicarbonate containing 0.5% Zwittergent 3–16 (Calbiochem) and 0.2 μ g of trypsin (Frozen Promega Modified) at 37 °C for 17 h (Lui et al., 1996).

Smaug protein represses nanos translation

Peptides were then separated on a C18 0.18- \times -150-mm capillary column (LC Packing, Inc.). The high performance liquid chromatography (HPLC) consisted of a prototype capillary gradient HPLC system (Waters Associates) and a model 783 ultraviolet (UV) detector equipped with a Z-shaped flow cell (LC Packings, Inc.). A 30-cm length of 0.025-mm ID glass capillary was connected to the outlet of the Z-shaped cell inside the detector housing to minimize the delay volume (Henzel & Stults, 1995). Solvent A was 0.1% aqueous trifluoroacetic acid (TFA) and solvent B was acetonitrile containing 0.08% TFA. Peptides were eluted using a linear gradient of 0–80% B in 60 min and detected at 195 nm. Fractions were collected automatically by a BAI Probot onto premade spots of matrix (0.5 μ L of 20 mg/mL α -cyano-4-hydroxycinnamic acid + 5 mg/mL nitrocellulose in 50% acetone/50% 2-propanol) (Shevchenko et al., 1996) on the target plate. Ions were formed by matrix-assisted laser desorption/ionization with a nitrogen laser, 337 nm. Spectra were acquired with a PerSeptive Biosystems Voyager Elite time-of-flight mass spectrometer, operated in reflector delayed extraction mode.

Peptides detected by MALDI-TOF MS were subjected to collision-induced dissociation (CID) in an ion-trap mass spectrometer (LCQ, Finnigan MAT). A 1- μ L aliquot (5%) of the tryptic digest was loaded onto a 100- μ m i.d., 360- μ m o.d., 30-cm length of fused silica capillary packed with 15 cm of POROS 10R2 reverse phase beads (PerSeptive Biosystems). Peptides were eluted with 15 min acetonitrile gradient at a flow rate of 500 nL/min as previously described (Arnott et al., 1998a). A data-dependent experiment was performed to obtain structural information for selected peptides. Ions with m/z values corresponding to peptides observed by MALDI-TOF MS were monitored in full mass range scans and automatically subjected to CID as each eluted from the capillary column.

Peptide masses and selected b and y series fragment ions were used to search an in-house protein and DNA sequence database with an enhanced version of FRAGFIT (Henzel et al., 1993; Arnott et al., 1998b) and SEQUEST (Eng et al., 1994).

Coupled transcription/translation and UV cross-linking

Smaug protein was generated in rabbit reticulocyte lysate using the TNT-coupled transcription/translation system (Promega) according to the manufacturer's instructions. UV cross-linking assays were performed using 3 \times SRE+ and 3 \times SRE– RNAs as described previously (Smibert et al., 1996) with reactions consisting of 7 μ L of TNT lysate or 7 μ L of crude embryo extract (prepared as described above), 1 μ L of 20-mg/mL yeast tRNA, 1 μ L of 100-mM EDTA, and 1 μ L of probe RNA (5×10^6 cpm).

Production of in vitro translation extracts from *Drosophila* embryos

Embryos collected at the indicated times from our large-scale *Drosophila* culture were dechorionated with bleach and washed extensively with 0.1% Triton X-100. All subsequent steps were carried out at 4 °C or on ice. After washing the embryos extensively with 10 mM HEPES-KOH, pH 7.4,

15 mM KCl, 1.5 mM Mg(OAc)₂, 2 mM DTT, and 0.5 mM Pefabloc (Boehringer Mannheim), they were allowed to settle in a Dounce homogenizer and excess buffer was removed. After embryo disruption, the lysate was cleared by two centrifugations at 39,000 $\times g$ in a SS-34 Sorval rotor for 5 min. Extracts were then processed by passage over a Sephadex G-25 Superfine resin (Pharmacia) spun column. Poly-Prep columns (Bio-Rad) were prepared with resin equilibrated in buffer B [30 mM HEPES-KOH, pH 7.4, 100 mM KOAc, 2 mM Mg(OAc)₂, 2 mM DTT, 0.5 mM Pefabloc (Boehringer Mannheim)] as follows: 4 mL of a 1:1 buffer:resin slurry was centrifuged for 3 min at 850 rpm in a JS-4.2 rotor ($\sim 150 \times g$), followed by the addition of 2 mL of buffer B and centrifugation under the same conditions. Embryo lysate (0.25 mL) was then applied to the column and centrifuged for 2 min at 800 rpm ($\sim 120 \times g$). After discarding the flow through, 0.25 mL of buffer B was applied and the column spun at 850 rpm for 3 min. CaCl₂ and micrococcal nuclease were then added to the resulting effluent to final concentrations of 0.5 mM and 0.18 μ /L respectively. Following a 5-min incubation at room temperature, the nuclease was inactivated by the addition of EGTA to 2 mM. The extract was then stored at -80°C in small aliquots.

In vitro translation using embryo extracts

The luciferase construct T7lucpA (P. Sarnow, unpubl.) is based on the pGEM-4 vector (Promega) and contains a *Bam*HI site that was used to insert the 3 \times SRE+ and 3 \times SRE– sequences (Smibert et al., 1996) as well as nt 1–844 of the intact nos 3' UTR. The point mutated version of the intact nos 3' UTR that was used is described by Smibert et al. (1996). In vitro transcription with T7 RNA polymerase of plasmids linearized with *Hpa*I results in an RNA that carries a 3' 30-nt poly A tail. Transcriptions were performed as described by Sampson & Saks (1993), with the exception that they included the cap analog G(5')ppp(5')G (NEB) at a final concentration of 7 mM and the GTP concentration was reduced to 1.25 mM. Also GMP was omitted from the reaction and trace quantities of [α -³²P]-UTP were included. Transcriptions were terminated by the addition of 0.5 μ /L of RNase-free DNase I (Boehringer Mannheim) and incubation at 37 °C for 15 min. The resulting RNA was then purified using a Sephadex G-50 (Pharmacia) spun column followed by extraction with phenol:chloroform and chloroform and by ethanol precipitation.

Fifteen microliters in vitro translation reactions using embryo extracts consisted of 7.5 μ L of embryo extract, 2.5 μ L of 6 \times translation buffer (132 mM HEPES-KOH, pH 7.4, 9 mM Mg(OAc)₂, 4.5 mM ATP, 0.6 mM GTP, 150 mM creatine phosphate (Boehringer Mannheim), 10.2 mM DTT), 0.6 μ L 1-mM complete amino acids (Promega), 0.4 μ L 10-mg/mL creatine phosphokinase, 0.5 μ L RNasin (Promega), and 3.5 μ L of the indicated luciferase RNA. All assays utilized 25 ng of luciferase mRNA except for the immunodepletion experiments, which employed 5 ng of RNA. Reactions were incubated at room temperature and at the indicated time points, 2- μ L aliquots were removed from the reaction and added to 10 μ L of 10-mM EDTA. Luciferase activity was assayed using the Promega luciferase assay reagent and a Monolight 2001 luminometer. To assay the stability of RNAs in these extracts, the reaction described above was doubled and 5- μ L aliquots were transferred at the indicated times to 400 μ L of 7 M urea, 0.35 M

NaCl, 0.01 M Tris-Cl, pH 7.8, 1% SDS containing 20 μ g of yeast tRNA. After extensive phenol:chloroform extraction, RNA was precipitated with ethanol, resuspended in 95% formamide, 0.5 mM EDTA, 0.025% xylene cyanol, 0.025% bromophenol blue, and 0.025% SDS, and heated at 95 °C for 3 min. RNA was then resolved on a 1.0% agarose gel containing 1.1% formaldehyde and, after electrophoresis, the gel was dried and RNA levels quantified using a GS-363 Molecular Imager PhosphorImager and Molecular Analyst software (Bio-Rad).

Translation of luciferase mRNAs (25 ng) in rabbit reticulocyte lysate (Promega) were carried out according to the manufacturer's instructions.

Immunodepletion

An anti-Smg antibody was raised in rats by Josman Laboratories (Napa, California) against residues 221–646 of Smg expressed in *Escherichia coli*. Immunodepletion experiments employed anti-Smg antibody and normal rat serum (Sigma) purified over protein G agarose (Boehringer Mannheim) as described by Lane & Harlow (1988). Purified antibody was precipitated by the addition of NH_4SO_4 to 50% saturation and the resulting precipitated was resuspended in $\sim 1/10$ the starting volume of crude serum. Purified antibody was then dialyzed against buffer B.

Immunodepletion involved combining 15 μ L of embryo in vitro translation extract with 10 μ L buffer B, 10 μ L of purified antibody, and 30 μ L of protein G beads that had been equilibrated with buffer B. After mixing end over end for 3 h at 4 °C, the beads were pelleted by centrifugation and 7.5 μ L of the supernatant was removed for use in an in vitro translation assay.

Analysis of Smg expression

Whole-mount antibody stains were done with anti-Smg antibodies at a 1:300 dilution as previously described (Macdonald et al., 1991). Secondary antibodies for signal detection were a goat anti-rat horseradish peroxidase conjugate or a goat anti-rat Cy3 conjugate (Jackson ImmunoResearch Laboratories). Western blots (which employed anti-Smg antibody at a dilution of 1/20,000) and RNA extractions were performed as described by Smibert et al. (1996) and Northern blot analysis as described by Selden (1987). Whole-mount RNA *in situ* hybridization of embryos was performed according to Tautz & Pfeifle (1989) with modification as previously described (Kim-Ha et al., 1991) using an in vitro-transcribed antisense RNA probe representing nucleotides 1–620 of the *smg* open reading frame made with digoxigenin-labeled UTP (Boehringer Mannheim).

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NOTE ADDED IN PROOF

Dahanukar et al. (*Mol Cell* 4:209–218) have independently cloned the *smg* gene and have isolated an *smg* mutant. Consistent with our in vitro results suggesting that *smg* represses *nos* translation, this *smg* mutant displays ectopic *nos* activity.

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