smaug protein represses translation of unlocalized *nanos* mRNA in the *Drosophila* embryo

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nanos mRNA, which encodes the localized component of the *Drosophila* posterior body patterning determinant, is normally translated only at the posterior pole of the embryo, where the mRNA is concentrated. Here we identify two similar *cis*-acting sequences in the *nanos* mRNA 3' untranslated region that mediate translational repression. These sequences bind an embryonic protein of 135 kD, *smaug*, and we refer to them as *smaug* recognition elements (SREs). Analysis of point mutations in the SREs reveals a strong correlation between *smaug* binding and translational repression; mutants unable to bind *smaug* in vitro are not repressed translationally in vivo, whereas mutants that do bind *smaug* remain repressed translationally. These results strongly suggest that *smaug* acts in translational repression of unlocalized *nanos* mRNA. Translational repression is essential, as embryos expressing a *nanos* mRNA with mutated SREs develop with anterior body patterning defects and die, despite correct localization of the RNA.

[Key Words: *nanos*; *smaug* protein; *Drosophila* embryo; translational regulation]

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Early development is often programmed by maternally provided mRNAs (Davidson 1986). The activities of these mRNAs must be regulated to ensure an orderly progression from one developmental stage to the next. Two mechanisms with prominent roles in controlling the activities of maternal mRNAs are translational regulation and mRNA localization; frequently, these post-transcriptional controls act together to restrict narrowly the expression of a particular mRNA. Striking examples of these phenomena are found in *Drosophila* where the basic body plan is established in large part by proteins whose distributions are governed by both mRNA localization and translational control (for review, see Curtis et al. 1995).

Posterior body patterning in *Drosophila* involves the sequential actions of two cytoplasmic determinants encoded by the *oskar* (*osk*) and *nanos* (*nos*) genes. Both determinants are localized to the posterior of the oocyte or embryo, and this localization is essential; if either protein is expressed ectopically, body patterning is reprogrammed with lethal consequences (Ephrussi and Lehmann 1992; Gavis and Lehmann 1992; Smith et al. 1992; Webster et al. 1994). Localization of each determinant requires prelocalization of the corresponding mRNA. *osk* mRNA is localized to the posterior pole of the oocyte during mid-oogenesis (Ephrussi et al. 1991; Kim-Ha et al. 1991), whereas *nos* mRNA becomes concentrated at the

same site much later in oogenesis (Gavis and Lehmann 1992; Wang et al. 1994). Both mRNAs are also subject to translational regulation (Gavis and Lehmann 1994; Kim-Ha et al. 1995; Markussen et al. 1995; Rongo et al. 1995), as might be expected given the consequences of ectopic *osk* or *nos* expression. In principle, translational control could cooperate with mRNA localization to tightly restrict expression of an RNA in two ways: (1) by restraining protein synthesis until localization is complete, and (2) by blocking translation of transcripts that escape the localization process. Both of these roles for translational control require a functional link between localization and translation, a property first established by Gavis and Lehmann (1994), who demonstrated that activation of *nos* translation requires posterior localization of the *nos* mRNA. In the case of *osk* mRNA, where such a link also exists, one demonstrated role for translational repression is in inactivating transiently the mRNA until localization is complete (Kim-Ha et al. 1995). This repression requires regulatory sequences, *bruno* response elements (BREs), in the *osk* mRNA 3' untranslated region (UTR), which bind to the *bruno* RNA-binding protein (Kim-Ha et al. 1995). It is not yet clear whether the other role for translational repression, namely to block expression from mRNA that escapes localization, is used to limit the activity of *osk* mRNA. Translational repression of *nos* mRNA has been characterized less extensively, and the factors and precise sequences involved in translational control have not been identified. Moreover, the nature of the link between translation and mRNA local-

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ization is not understood. However, it is known that *nos* translation is repressed by the *nos* 3' UTR, and activation of *nos* translation requires *osk* (Gavis and Lehmann 1994).

Here we show that translational regulation of *nos* mRNA serves specifically to repress translation of *nos* transcripts that fail to localize to the posterior. Repression triggers degradation, a logical outcome to ensure that this unlocalized mRNA is never translated. We identify and characterize the *cis*-acting regulatory elements [designated *smaug* recognition elements (SREs)] that mediate these events. The SREs bind the RNA-binding protein *smaug* (smg). Failure to bind smg leads to translational derepression, suggesting that smg represses *nos* translation through the SREs. The ensuing ectopic expression of *nos* protein is lethal, demonstrating that repression of *nos* translation is essential. Furthermore, our results suggest that the role of *nos* mRNA localization in translational activation is to overcome smg-mediated repression. Thus, smg action would establish the link between *nos* RNA localization and *nos* translation.

Results

To search for translational control elements in the *nos* mRNA we used a *nos/bcd* transgene similar to one described by Gavis and Lehmann (1992). In this transgene the *nos* 3' UTR, which includes a posterior mRNA localization signal (Gavis and Lehmann 1992), has been replaced by the *bcd* 3' UTR, which contains an anterior localization signal (Macdonald and Struhl 1988). Consequently, the hybrid transcript encoded by the transgene is mislocalized to the anterior pole of the embryo, and ectopic *nos* protein causes the embryos to display a bicaudal phenotype in which anterior structures are replaced by a mirror image duplication of posterior structures (Gavis and Lehmann 1992).

We inserted regions of the *nos* 3' UTR into the *nos/bcd* transgene to determine whether any would suppress the bicaudal phenotype, suggesting they might mediate translational repression. Insertion of the first 96 nucleotides (nucleotides 1–96) of the 874-nucleotide *nos* 3' UTR largely reversed the *nos/bcd* bicaudal phenotype, restoring the normal polarity along the anteroposterior axis of the embryos as well as the normal number of abdominal and thoracic segments. This derivative of the *nos/bcd* transgene retained some anterior *nos* activity, as the embryos displayed head defects (Table 1; see Fig. 4 for a comparable cuticular phenotype). As expected for an RNA regulatory element, insertion of an antisense copy of the same region (nucleotides 96–1) into the *nos/bcd* transgene had no effect on the bicaudal phenotype. Suppression of the *nos/bcd* bicaudal phenotype by the 96-nucleotide region did not involve changes in mRNA localization, as the transgenic mRNA remained localized to the anterior pole of embryos (data not shown). Instead, the 96-nucleotide element mediated translational repression, as demonstrated below.

To determine whether the 96-nucleotide region could inhibit expression of a completely heterologous gene, we

Table 1. Mapping translational repression elements in the *nos* 3' UTR

Transgene ^a	Insertion ^b	Translational repression ^c	Phenotype ^d
<i>nos/bcd</i>	none	no	Bicaudal
	96–1	no	Bicaudal
	1–96	yes	Head defects
	1–80	yes	Head defects
	1–64	no	Bicaudal
	1–48	no	Bicaudal
	1–32	no	Bicaudal
	1–16	no	Bicaudal
	21–96	yes	Head defects
	41–96	no	Bicaudal
	61–96	no	Bicaudal
	none	no	Bicaudal
<i>nosHA/bcd</i>	1–96	yes	Head defects
	96–1	no	Bicaudal
	21–80	yes	Head defects
<i>torso</i>	96–1	no	Wild-type
	1–96	yes	<i>torso</i> ^{−e}

^aTransgenes are as described in Materials and Methods.

^bInsertion refers to sequences from the *nos* 3' UTR, numbered as noted in the text. Insertion in each case was at the start of the 3' UTR.

^cTranslational repression is demonstrated for the *nosHA/bcd* transgenes later in the paper (Fig. 1), and is inferred in other cases based on the phenotypes associated with the transgenes.

^dPhenotypes were scored in an otherwise wild-type background for the *nos/bcd* and *nosHA/bcd* transgenes, and in a *torso*[−] background for the *torso* transgenes.

^eThis transgene provided low-level *tor* activity to a *tor*[−] mutant at both the anterior and posterior of the embryo.

introduced sense or antisense copies into a *torso* (*tor*) transgene. The maternal *tor* transcript is distributed throughout the embryo and specifies the terminal-most anterior and posterior structures (Schüpbach and Wieschaus 1986; Sprenger et al. 1989). An antisense copy of the 96-nucleotide region had no effect on the ability of the *tor* transgene to rescue the defects of *tor*[−] mutants (Table 1). In contrast, a *tor* transgene carrying a sense copy of the 96-nucleotide element provided only a low-level *tor* activity at both the anterior and posterior of the embryo. Thus, the 96-nucleotide region was sufficient to inhibit expression of a heterologous gene in the absence of other *nos* sequences, and was active at both poles of the embryo.

To define more precisely the *nos* 3' UTR sequences required to repress *nos/bcd* expression, truncated forms of the 96-nucleotide region were inserted into the *nos/bcd* transgene and tested for body patterning phenotypes (Table 1). The ability to suppress the bicaudal phenotype was not affected when either the first 20 or last 16 nucleotides were deleted, indicating that those regions were dispensable for the inhibitory effect of the element. In contrast, further deletions from either end led to a loss of bicaudal suppression. Thus, the inhibitory activity was contained within a 60-nucleotide fragment, nucleotides 21–80 of the *nos* 3' UTR.

Translational repression and mRNA degradation

The observed suppression of the *nos/bcd* bicaudal phenotype by the 96-nucleotide region could involve effects on mRNA synthesis, mRNA stability, protein synthesis, or some combination of these options. To distinguish among the possibilities, an assay was required to detect directly the *nos* protein made from the *nos/bcd* transgene. The *nos/bcd* transgene was modified by addition of two carboxy-terminal influenza hemagglutinin epitope tags, giving *nosHA/bcd*. The epitope tags decreased the mobility of the *nos* protein in SDS–polyacrylamide gels, allowing us to resolve the tagged and endogenous *nos* proteins, both of which can be detected in Western blots probed with anti-*nos* sera (Fig. 1). Addition of the epitope tag did not impair detectably the *nos* activity encoded by the *nosHA/bcd* transgene; females expressing the transgene produced bicaudal embryos (Table 1). Just as for the *nos/bcd* transgene, insertion of *nos* 3' UTR nucleotides 1–96 into the *nosHA/bcd* transgene suppressed the bicaudal phenotype (Table 1). The same result was obtained by inserting nucleotides 21–80, demonstrating that this minimal region was indeed sufficient to mediate suppression.

Preliminary experiments to monitor expression of the *nosHA/bcd* transgene and its derivatives revealed that substantial changes in mRNA levels occurred during the first 3 hr of embryogenesis. Consequently, all analyses were performed with staged samples, corresponding to embryos 0–1, 1–2, or 2–3 hr after egg laying, to define accurately differences in mRNA and protein levels. The unmodified *nosHA/bcd* transgene-directed synthesis of *nosHA* protein that appeared at lower levels in the 0- to 1-hr embryos, and at higher levels in the 1- to 2- and 2- to 3-hr embryos (Fig. 1, top). Parallel RNase protection assays showed that the *nosHA/bcd* mRNA was present at high levels initially, and detected somewhat less at 2–3 hr (Fig. 1, middle). A similar pattern was observed when the *nosHA/bcd* transgene was modified by insertion of an antisense copy of the 96-nucleotide region. In contrast, insertion of a sense copy of the entire 96-nucleotide region, or just nucleotides 21–80, eliminated detectable *nosHA* protein at all times, although substantial levels of the mRNAs were present at the onset of embryogenesis. Thus, the regulatory element within nucleotides 21–80 repressed *nos* expression at the level of translation. In addition, the presence of this regulatory element had a delayed effect on mRNA stability, as the *nosHA/bcd* mRNAs carrying the nucleotides 1–96 or 21–80 insertions decayed prematurely (Fig. 1, middle). We conclude that the initial function of the regulatory element was to repress translation, and subsequently it promoted degradation of the *nosHA/bcd* transcript.

Note that endogenous *nos* mRNA is translated, although it contains the same sequences that mediate translational repression of the *nosHA/bcd* mRNA. As described below, our results suggest that the translation of endogenous *nos* mRNA requires an activation event that overcomes this repression.

Figure 1. A region of the *nos* 3' UTR mediates translational repression. Samples used in each set of three lanes were from embryos of progressively more advanced stages (0–1, 1–2, and 2–3 hr after egg laying). Transgenic females from which the embryos were obtained expressed the *nosHA/bcd* transgene (–), or derivatives carrying insertions of the *nos* 3' UTR: nucleotides 1–96 in antisense (nucleotides 96–1) or sense (nucleotides 1–96) orientations, or nucleotides 21–80 in the sense orientation (nucleotides 21–80). (Top) A Western blot probed with anti-*nos* serum showing time courses of the appearance of endogenous *nos* and transgenic *nosHA* proteins (the *nos* protein serves as a loading control). (Middle and bottom) RNase-protection assays monitoring the *nosHA/bcd* mRNA, as well as a control for RNA loading, the *rp49* mRNA. The presence of the sense version of either nucleotides 1–96 or 21–80 of the *nos* 3' UTR eliminated detectable *nosHA* protein, and led to premature degradation of the encoding mRNA. Notably, when the transgenic *nosHA* protein did appear, it was always delayed relative to endogenous *nos*. We suspect that this reflects a form of translational control normally exerted on the *bcd* mRNA; translation only begins after the poly(A) tail is extended shortly after egg laying (Sallés et al. 1994). Because cytoplasmic polyadenylation of *bcd* mRNA is controlled by the *bcd* mRNA 3' UTR (Sallés et al. 1994), which appears in the *nosHA/bcd* mRNA, extension of the poly(A) tail is presumably a prerequisite for translation of the *nosHA/bcd* mRNA. In contrast, *nos* mRNA does not require poly(A) tail extension for translation, and appears to be translated from the onset of embryogenesis. We note that repression by 3' UTR sequences can override activation conferred by cytoplasmic polyadenylation, if that is indeed the event responsible for the delay in *nosHA* translation. A similar effect is observed when the 96-nucleotide region of the *nos* mRNA 3' UTR is inserted into the *tor* mRNA, which is normally activated translationally by cytoplasmic polyadenylation (Sallés et al. 1994); repression still occurs (Table 1). It will be of interest to learn whether repression prevents cytoplasmic polyadenylation.

A 135-kD protein binds specifically to the *nos* mRNA

A UV cross-linking assay was used to test for the presence of proteins that interact specifically with nucleotides 21–80 of the *nos* 3' UTR, and potentially mediate translational repression. A number of proteins bound in a UV-dependent and extract-dependent manner to an RNA containing the 60-nucleotide region (Fig. 2, lane 1). However, competition binding experiments revealed that only one of these proteins bound specifically. Binding of a protein with an apparent molecular mass of 135-kD was greatly reduced in the presence of excess specific

Figure 2. Protein binding to nucleotides 21–80 of the *nos* mRNA 3' UTR. UV cross-linking assays were used to monitor binding of embryonic proteins to a labeled RNA containing nucleotides 21–80 of the *nos* 3' UTR. Results of typical assays (lanes 1,4). Most of the bands correspond to bound proteins, as demonstrated by their absence when the UV treatment (lane 2) or the protein extract (lane 3) is omitted from the assay. (Lanes 5–13). Competition binding experiments to demonstrate specificity of binding. Increasing amounts of unlabeled competitor RNAs (10 $\times$, 30 $\times$, and 100 $\times$ molar excess over probe) were included in the binding reactions. (Lanes 5–7) A specific competitor; (lanes 8–10) BLE1 RNA competitor; and (lanes 11–13) BRE RNA competitor (see Materials and Methods). The binding of only one detectable protein of ~135 kD (indicated by the large arrowhead) was lost in the presence of the specific competitor but not in the presence of the nonspecific competitors. Note that neither the slight decrease in the 135-kD protein in the presence of the nonspecific competitors or the apparent competition of other species at high concentration of specific competitor were reproducible. The positions at which molecular mass markers migrate are indicated.

competitor RNA (Fig. 2, lanes 5–7), but not in the presence of either of two nonspecific competitor RNAs (Fig. 2, lanes 8–13). No other protein displayed this behavior. We have designated the specifically bound protein smg. To determine when smg binding activity is present, extracts were prepared from different developmental stages. The binding activity was absent from ovary extracts, or extracts enriched for late stage eggs, and first appeared early in embryogenesis, peaked shortly thereafter, and was largely gone by 4 hr after egg-laying (Fig. 3).

smg binding correlates with translational repression

If smg mediates translational repression through the 60-nucleotide region, then its binding should correlate with the ability of the element to repress translation *in vivo*. This can be tested by studying the properties of point mutations in the 60-nucleotide region that do or do not affect binding. To identify the region of the 60-nucleotide to which smg binds, we first generated mutant RNAs bearing multiple random point mutations, and

smg represses translation of unlocalized nos mRNA

Figure 3. Time course of appearance of smg binding activity. Standard binding reactions were performed on staged extracts, using a labeled 3 $\times$ SRE RNA probe (see Fig. 6) to enhance detection of smg. Extracts were prepared from whole ovaries (lane 1), late-stage oocytes (lane 2), and embryos aged 0–1, 1–2, 2–3, 3–4, and 4–5 hr after fertilization (lanes 3–7, respectively). smg binding activity is indicated by an arrowhead.

tested them using the binding assay. Results from these experiments (data not shown) suggested that the smg binding site was located within nucleotides 25–40 of the *nos* 3' UTR, a region that has the potential to form a stem-loop structure (Fig. 4A). Eight RNAs bearing single point mutations in the implicated region were tested for binding to smg. Four mutations blocked smg binding, as assayed by UV cross-linking, whereas four mutations did not (Fig. 4B).

Each point mutation was also tested for its effect on the ability of the 60-nucleotide region to repress translation of the *nosHA/bcd* mRNA. Each of the four mutants that retained smg binding suppressed the bicaudal phenotype, suggesting that they remained competent to mediate translational repression (Fig. 4E,F,H,I). In contrast, the four mutants unable to bind smg failed to suppress the bicaudal phenotype, suggesting that they were unable to mediate translational repression (Fig. 4G). To confirm that these body patterning phenotypes reflected effects on translation, protein and mRNA levels were tested for four selected mutations that did or did not prevent detectable smg binding (Fig. 5). The two point mutations competent for smg binding displayed the translational repression and mRNA degradation associated with the intact 60-nucleotide region, whereas the two point mutations that blocked smg binding were defective in both of these processes. These results suggest strongly that smg binding mediated translational repression and, perhaps indirectly, the subsequent mRNA degradation. We refer to the smg binding site as SRE. This site is referred to as SRE1, as a second SRE appears elsewhere in the *nos* mRNA (below).

Sequence inspection suggests that SRE1 is a stem-loop structure. Mutations that prevented smg binding are in the loop itself, or in the adjacent base pairs of the stem, showing that these regions were essential for binding. Mutations in the lower part of the predicted stem did not affect *in vitro* binding to smg, or the ability of the SRE to mediate substantial repression of translation. Nevertheless, for the latter mutations subtle changes in the extent of translational repression, as revealed by the extremely sensitive body patterning assay, were consistent with the folding of SRE1 into a stem-loop structure. Mutation U42C, which strengthened a potential weak G-U base pair, retained full SRE activity (Fig. 4I). Mutations A27U and U41A, which both disrupted the same potential A-U

Figure 4. smg binding mediates suppression of the bicaudal phenotype by the SRE. (A) Sequence of the SRE1-containing region of the *nos* mRNA, drawn to indicate potential secondary structure. Point mutations used in the analysis are indicated, together with nucleotide position in the *nos* 3' UTR. (B) Binding assays using labeled point mutant RNAs as probes. The position of smg is indicated by a large arrowhead. Mutants C32G, G34C, A37U, and G38C did not bind smg detectably, whereas all other mutants bound smg much like the wild-type RNA (+). (C–I). Cuticular phenotypes of embryos obtained from mothers expressing *nosHA/bcd* transgenes. In C, the transgene lacked further *nos* 3' UTR sequences. In D–I the transgenes carried insertions of *nos* 3' UTR nucleotides 21–80, either wild-type (D) or bearing single point mutations: A27U (E), G28C (F), C32G, G34C, A37U, or G38C (G); U41A (H); U42C (I). For mutations that prevented smg binding, the cuticular phenotypes (G) were indistinguishable from those obtained when the transgene lacked *nos* 3' UTR nucleotides 21–80 (C). Mutations A27U and U41A, which affected two nucleotides that have the potential to base-pair, led to slightly increased levels of *nos* activity; the cuticles lacked thoracic segments (E,H), which would otherwise be present (D). Mutation U42C had no effect on the cuticular pattern (I). Mutation G28C leads to mixed results. Some embryos had no change in the cuticular pattern (F), whereas others lacked thoracic segments (data not shown).

base pair, displayed a modest reduction in SRE function. The level of ectopic *nos* activity produced by transgenes was now sufficient to interfere with thoracic segmentation (Fig. 4E and 4H, respectively), whereas the anterior *nos* activity present with the intact SRE only caused head defects (Fig. 4D). Finally, mutation G28C, which disrupted a potential weak G–U base pair, gave a mixture of embryos; some retained full SRE activity (Fig. 4F), whereas others had phenotypes similar to those of mutations A27U and U41A (data not shown). Thus, there is a correlation between the destabilization of the predicted stem and the strength of SRE activity.

Figure 5. smg binding mediates translational repression by the SRE. Data on protein and mRNA levels are presented as in Fig. 1. Samples were from embryos of transgenic mothers expressing the *nosHA/bcd* gene with insertion of nucleotides 21–80 of the *nos* 3' UTR. The nucleotides 21–80 region carried the point mutations indicated above each set of lanes. Mutations U42C and G28C, which did not detectably impair smg binding in vitro, retained full SRE function; *nosHA* protein does not appear, and the encoding mRNAs are degraded prematurely. In contrast, mutations G34C and G38C, which failed to detectably bind smg, lacked all SRE activity (cf. Fig. 1).

Our analysis strongly suggests that binding of smg to the SRE is required for translational repression of the *nosHA/bcd* mRNA. Deletion analysis of the 96-nucleotide region of the *nos* 3' UTR (Table 1) also indicated that the SRE-containing region was required, but identified additional essential sequences. For example, deletion of nucleotides 65–96, which leaves the SRE intact, also inactivated the element. It is possible that these deletions affected indirectly SRE function, perhaps through changes in RNA secondary structure; alternatively, the deleted region may contain the binding site for an additional factor required for translational repression. Given this ambiguity we wished to determine whether smg binding alone is sufficient to confer translational repression on the *nosHA/bcd* transgene. For these experiments we made use of an RNA containing three copies of the SRE (3× SRE). One copy corresponded to SRE1, a second to SRE2 (see below), and a third SRE was synthetic, consisting of the loop sequence shared by SRE1 and SRE2, and a stem with novel sequences. A control RNA (3× SRE[−]) was identical, except for carrying mutations in each of the predicted loops. 3× SRE RNA binds strongly to smg, whereas the 3× SRE[−] RNA did not (Fig. 6A).

The in vivo activities of the 3× SRE and 3× SRE[−] RNAs were monitored as described above using *nosHA/bcd* transgenes. The 3× SRE RNA supported full translational repression, as revealed indirectly in the body patterning assay (Fig. 6C) and directly by monitoring protein and RNA levels (Fig. 6B). In contrast, the 3× SRE[−] RNA was defective in translational repression (Fig. 6B,D). These results strongly suggest that smg binding is sufficient to confer both translational repression and mRNA degradation to the *nosHA/bcd* mRNA.

Figure 6. SREs are sufficient to confer translational repression on the *nosHA/bcd* mRNA. (A) Binding assays using labeled 3× SRE and 3× SRE[−] RNAs as probes. smg, indicated by a large arrowhead, binds strongly to the 3× SRE RNA and not detectably to the 3× SRE[−]. We confirmed that this protein was smg on the basis of its comigration with smg cross-linked to the original 60-nucleotide probe, and by competition binding experiments (data not shown). (B) Data on protein and mRNA levels are presented as in Fig. 1. Samples were from embryos of transgenic mothers expressing the *nosHA/bcd* gene with insertion of the 3× SRE or 3× SRE[−]. The presence of the 3× SRE in the *nosHA/bcd* mRNA prevented translation of nosHA protein and led to premature degradation of the encoding mRNA, suppressing the bicaudal phenotype of the unmodified *nosHA/bcd* transgene (C). In contrast, inclusion of the 3× SRE[−] affected neither translation nor mRNA stability, and failed to suppress the bicaudal phenotype (D).

SREs repress the translation of bona fide *nos*

Our results have used artificial reporter genes to investigate the role of the SREs and smg in *nos* translation. A simple approach to testing the role of these components in regulation of endogenous *nos* is to create a *nos* transgene in which all smg binding sites have been mutated, and then monitor its properties in vivo. To perform such an experiment, we first searched for additional SREs in the *nos* 3′ UTR. Competition binding experiments suggested that the *nos* 3′ UTR contained one additional smg binding site located between nucleotides 97–185 (data not shown). Inspection of this sequence revealed a potential stem-loop structure (nucleotides 130–144) where the loop sequence CUGGC is identical to the loop in SRE1, whereas the stem sequence is different. To confirm that this second stem-loop, which we call SRE2, was a smg binding site, we introduced point mutations into the loops of SRE1, SRE2, or both, in the context of an RNA spanning nucleotides 1–185 of the *nos* 3′ UTR. Both SRE1 and SRE2 must be mutated to eliminate binding of smg to this 185-nucleotide RNA (Fig. 7A). Thus, both sites bind smg, and there appears to be no other smg binding sites in the *nos* 3′ UTR (or in the remainder of the *nos* mRNA, as judged by sequence inspection).

Both mutations were inserted into an otherwise wild-type *nos* transgene to make *nosSRE[−]*. Mothers expressing a wild-type *nos* transgene produced only wild-type

Figure 7. Action of the SREs in the *nos* mRNA. (A) Binding assays using labeled RNA probes of wild-type *nos* 3′ UTR nucleotides 1–185 (lane 1), or derivatives in which SRE1, SRE2, or both SRE1 and SRE2 were mutated (lanes 2–4, respectively). Wild-type SRE1 sequence CTGGCAG was mutated to CTG-cagG. Wild-type SRE2 sequence CTGGCG was mutated to CT-GcaG. When both SREs were mutated, smg binding was no longer detected. (B,C) (phase contrast) Anterior cuticular regions of embryos from mothers expressing a wild-type *nos* transgene (B) or the *nosSRE[−]* transgene (C). The presence of the *nosSRE[−]* mRNA led to head defects (indicated by the reduced head skeleton; arrowheads). (D–F) (dark field illumination) Localization of *nos* mRNA is dispensable in the absence of smg-mediated translational repression. (D) Cuticle from an embryo of an *osk[−]* mother. In the absence of *osk* activity, *nos* mRNA is neither localized nor translated, and the embryos lacked all abdominal structures. (E) Cuticle from an embryo of an *osk[−]* mother expressing a wild-type *nos* transgene. (F) Cuticle from an embryo of an *osk[−]* mother expressing a *nosSRE[−]* transgene. The abdominal segments formed normally, indicating that *osk* activity and hence, *nos* mRNA localization, were not required for *nos* translation or posterior body patterning if smg-mediated repression was eliminated. (G) Sequence and predicted structure of SRE2.

embryos (Fig. 7B), whereas mothers expressing *nosSRE[−]* produced embryos lacking normal head structures (Fig. 7C). This phenotype is consistent with overexpression of *nos* protein and demonstrates that SRE-mediated repression of *nos* expression is essential for normal development. This phenotype did not result from aberrant localization of the *nosSRE[−]* mRNA as demonstrated by in situ hybridization (data not shown).

We then investigated the possibility that the SREs could mediate translational repression of *nos* mRNA that fails to localize to the posterior of the embryo. Such a model is consistent with previous work showing that *nos* mRNA is not translated if it is not localized to the posterior (Gavis and Lehmann 1994). To test this possibility, we assayed the levels of *nos* activity in an *osk[−]* mutant carrying the *nosSRE[−]* transgene. Because *osk* is required to localize (and therefore translate) the *nos* mRNA (Ephrussi et al. 1991), *osk[−]* mutants lack *nos* activity, which is reflected by a lack of abdominal segmentation (Lehmann and Nüsslein-Volhard 1986) (Fig. 7D). However, the *nosSRE[−]* transgene rescued completely abdominal segmentation in an *osk[−]* mutant (Fig. 7F, note that the *nosSRE[−]* transgene still induces head defects in these embryos), whereas a wild-type *nos* trans-

gene did not (Fig. 7E). Thus, the activity of the *nosSRE*⁻ transgene in wild-type and an *osk*⁻ mutant indicate that the phenotypic defects associated with the *nosSRE*⁻ transgene result from translation of unlocalized *nos* mRNA. Consistent with these results, we detect a low uniform level of unlocalized *nos* protein in embryos carrying the *nosSRE*⁻ transgene (data not shown).

Discussion

We have investigated the translational control of *nos* mRNA, a localized maternal transcript that directs posterior development (Wang and Lehmann 1991). We have characterized *cis*-acting elements SREs that mediated repression of translation, and have identified a protein, smg, that binds specifically to these sites. Mutations that eliminated smg binding also inactivated the function of the SREs, whereas mutations that retained smg binding retained substantial SRE function. These results suggest strongly that smg acted as a translational repressor. In our investigations we assayed SRE function in hybrid reporter genes and in the *nos* mRNA itself. The results from these approaches were both complementary and consistent. Each approach also provided additional information about *nos* translational control. For example, analysis of the hybrid reporter genes revealed that the SREs were active throughout the embryo. Also, a distinct activation event was required for posterior translation of *nos* mRNA (see below). Furthermore, the use of hybrid transgenes suggested that smg-mediated translational repression was associated with degradation of target mRNAs. Testing the role of SREs in their natural context, the *nos* mRNA allowed us to address the importance of translational repression to normal development; when the SREs were nonfunctional *nos* activity was no longer restricted to the posterior, despite normal localization of the *nos* mRNA. This ectopic *nos* expression resulted in lethal body patterning defects. These results suggest that mutants with altered regulation of *nos* mRNA translation will be defective in body patterning, facilitating a genetic analysis of this process.

SREs mediate translational repression of unlocalized nos mRNA in the embryo

Expression of *nos* protein outside its normal posterior domain leads to body patterning defects (Ephrussi and Lehmann 1992; Gavis and Lehmann 1992; Smith et al. 1992). Such a situation might be prevented by translational repression at either of two different phases. First, repression could prevent accumulation of *nos* protein in the oocyte before posterior localization of *nos* mRNA, which occurs only late in oogenesis. Because *nos* protein cannot be detected in late-stage oocytes (Wang et al. 1994), *nos* mRNA must be repressed translationally or the *nos* protein must be unstable. In a similar situation earlier in oogenesis, translation of *osk* mRNA is repressed before its localization to the posterior pole of the

oocyte (Kim-Ha et al. 1995). However, smg did not appear to suppress translation of *nos* mRNA before its localization within the oocyte, as we did not detect smg binding activity until early embryogenesis. There is some reason to suspect that protein instability may be responsible for the absence of *nos* protein in the oocyte. *nos* protein is present at high levels in late-stage nurse cells (Wang et al. 1994) that empty their contents into the oocyte. The failure to detect *nos* protein after this transfer suggests that it may be degraded upon entry into the oocyte. With such a protein degradation mechanism in place, there would be no need for translational repression during oogenesis.

A second opportunity for translational repression of *nos* mRNA occurs in the embryo. High levels of *nos* mRNA are localized to the posterior (Wang and Lehmann 1991), but substantial levels of the transcript are also distributed throughout the remainder of the embryo (our unpublished observations). Repression could limit the synthesis of *nos* protein from the unlocalized *nos* mRNA, and our results strongly suggest that this is the function of smg. When the SREs were mutated in an otherwise intact *nos* mRNA, *nos* activity was no longer limited to the posterior, as demonstrated by the anterior defects associated with the *nosSRE*⁻ transgene. Localization of the *nosSRE*⁻ mRNA is not affected, but *nos* protein appears throughout the embryo, indicating that the unlocalized mRNA is being translated.

A role for smg in repressing unlocalized *nos* mRNA demands that smg activity be found throughout the embryo; our results using a *tor* transgene suggest that this is the case. *tor* activity normally appears at both poles of the embryo, and *tor* transgenes carrying an antisense (and therefore, nonfunctional) copy of an SRE provided complete *tor* function. However, *tor* transgenes carrying a sense copy of an SRE failed to provide *tor* activity at either end of the embryo, suggesting that smg is present at both locations. This result also bears on the posterior activation of *nos* translation, as discussed below.

Much of our analysis of the SREs was performed in the presence of additional *nos* mRNA sequences, which could also contribute to translational repression. This includes our experiment that used the *tor* transgene where the SRE was contained within a 96-nucleotide fragment of the *nos* 3' UTR. Thus, our data show that the SREs are necessary for repression of endogenous *nos*, but they do not reveal whether the normal complement of two SREs is sufficient for repression. The possibility that the *nos* message contains additional sequences that are required to repress *nos* translation will require further analysis of the *nos* transcript. However, our demonstration that insertion of a 3× SRE cassette into the *nosHA/bcd* transgene conferred translational repression does demonstrate that multiple SREs can function in the absence of other *nos* 3' UTR sequences.

All of the above data are consistent with the ability of the SREs to function independently and therefore, raise the possibility that SRE-like sequences may act to repress translation of other maternal mRNAs. However, our search of ~45 mRNAs that are expressed in the early

embryo has not revealed any similar stem-loop structures (data not shown).

Activation of *nos* translation

Translation of *nos* mRNA is activated as a consequence of localization to the posterior pole of the embryo. In mutants defective in *nos* mRNA localization, such as *osk*[−], no *nos* protein can be detected (Gavis and Lehmann 1994). Given our model that smg represses translation throughout the embryo and that *nos* mRNA is normally only translated when localized to the posterior, it seems likely that the role of localization in *nos* translation is to overcome smg-mediated repression. If so, then localization should no longer be a prerequisite for *nos* translation if the SREs are mutated and nonfunctional. This is indeed the case, as *osk*[−] mutants carrying the *nosSRE*[−] transgene displayed normal abdominal segmentation, revealing the presence of substantial levels of *nos* activity. The failure to translate wild-type *nos* mRNA in an *osk*[−] mutant, whereas *nosSRE*[−] mRNA was translated, further strengthens our suggestion that smg serves to repress the translation of unlocalized *nos* mRNA (as detailed above).

How might localization overcome smg-dependent repression? Properties of the *tor* transgene carrying an SRE are instructive. The presence of the SRE led to loss of *tor* activity at both poles of the embryo. The absence of posterior *tor* activity indicates that the SRE by itself did not mediate translational activation at the posterior pole. This result has two important implications. First, it shows that an additional *cis*-acting regulatory element is required for activation. Our preliminary work indicates that the *nos* 3' UTR does indeed contain sequences outside of nucleotides 1–96 (the site of SRE1) that mediate translational activation (C.A. Smibert and P.M. Macdonald, unpubl.). These results are consistent with work from Dahanukar and Wharton (this issue), who have shown that a segment of the *nos* mRNA encompassing SRE1 and SRE2 (nucleotides 1–184 of the 3' UTR) mediates both repression and activation of translation. Second, it indicates that the role of *osk* in activation cannot be limited to a direct effect on smg itself; if so, we would expect that the SRE/smg complex would be a suitable substrate for *osk*-mediated translational activation. This was not the case, as *osk* did not activate the expression of the posteriorly located *tor* mRNA-carrying SRE1.

The role of *osk* in posterior development likely involves the recruitment and maintenance of a ribonucleoprotein complex at the posterior that is responsible for posterior development (Ephrussi et al. 1991; Kim-Ha et al. 1991; Webster et al. 1994). Therefore, we speculate that *osk* recruits some factor to the posterior that overcomes smg-mediated repression, thus allowing for posterior-specific translation of *nos* mRNA. This model is consistent with the link between *nos* translation and *nos* RNA localization observed by Gavis and Lehmann (1994), although it invokes a different mechanism; they proposed that the act of localization itself activated translation.

smg binding is tightly correlated with translational repression and mRNA degradation

Our work with the *nos/bcd* hybrid transgenes suggests that translational repression occurred together with mRNA degradation. Time-course experiments show that repression precedes degradation. Two results imply that smg binding to SREs mediated both processes. First, SRE point mutations that prevented smg binding blocked both events. Second, hybrid transgenic mRNAs carrying the three tandem SREs were repressed translationally and degraded subsequently. These results join the growing body of evidence that translational control and RNA stability can be intimately linked (for review, see Theodorakis and Cleveland 1996). It is possible that smg mediates both processes; alternatively, smg may repress specifically translation, and degradation occurs simply because the mRNA is not being translated.

Our results linking translational repression and mRNA degradation prompted us to re-examine the behavior of the endogenous *nos* mRNA. We found that substantial levels of unlocalized *nos* mRNA were present very early in embryogenesis, and that they were degraded before cellularization. If smg regulates the stability of this unlocalized *nos* mRNA, then we would expect unlocalized *nosSRE*[−] mRNA to persist longer than unlocalized wild-type *nos* mRNA. We found that, although the bulk of *nosSRE*[−] was degraded like wild type, a small fraction of the *nosSRE*[−] mRNA persisted into cellularization (data not shown). These results suggest that more than one mechanism degrades *nos* mRNA. It would not be surprising if a more general system also destabilizes *nos* transcripts, as many maternal mRNAs are degraded rapidly early in embryogenesis.

Why is nos expression so carefully regulated?

One intriguing aspect of our results is that the elaborate system, involving at least 10 gene products, for localizing molecules to the posterior of the embryo (St. Johnston and Nüsslein-Volhard 1992) becomes dispensable for posterior body patterning if *nos* translational repression is alleviated by mutations in the SREs. Specifically, *osk*[−] mutants are completely defective in the posterior localization of a number of molecules, including the *nos* gene products, and lack any trace of posterior body patterning activity (Lehmann and Nüsslein-Volhard 1986; Hay et al. 1990; Lasko and Ashburner 1990; Raff et al. 1990; Jongens et al. 1992; Wang et al. 1994). However, complete posterior body patterning was restored in the presence of the *nosSRE*[−] transgene, which allowed translation of unlocalized *nos* mRNA. The only negative effect associated with this promiscuous translation was the disruption of head formation. These head defects resulted from the *nos*-dependent translational repression of the *bcd* mRNA, which is mediated by *nos* response elements (NREs) in the *bcd* mRNA 3' UTR (Wharton and Struhl 1991). We have found that such defects can be eliminated completely in animals expressing a form of the *bcd* mRNA from which the NREs has

been deleted (Webster et al. 1994). Thus, it would seem that the requirements for *nos* mRNA localization and translational repression are imposed by the presence of the NRE in the *bcd* mRNA.

Materials and methods

Extract preparation

All extracts were prepared from *w¹¹¹⁸* animals. Embryos (0- to 1-hr after egg laying unless otherwise indicated) collected on yeasted apple juice plates were dechorionated in bleach for 1 min, washed extensively with 0.1% Triton X-100, and transferred to a 1.5-ml microfuge tube (this and all subsequent steps were performed on ice or at 4°C). The volume of the embryos was estimated and an equal volume of lysis buffer [50 mM Tris HCl (pH 7.5), 20 mM NaCl, 0.1% Triton X-100, 0.5 mM EDTA, 1 mM DTT, 1 mM PMSF, 2 mM benzamidine, 2 µg/ml of leupeptin, and 2 µg/ml of pepstatin] was added. Embryos were homogenized with a plastic pestle and centrifuged in a refrigerated microfuge at 15,000 rpm for 15 min. Glycerol was then added to the supernatant to a final concentration of 10%, and extracts were stored at -70°C. Ovaries from well-fed 3- to 4-day-old females were hand-dissected into ice cold 0.1% Triton X-100 and extracts were prepared as above. To prepare extracts enriched for late-stage eggs, well-fed 4-day-old virgin females were used (Wyman 1979); the enrichment was verified by visual inspection.

UV cross-linking assay

Probe synthesis and UV cross-linking assays were performed as described by Macdonald et al. (1995) with the following modifications: 3–6 µL of protein extract were combined in a 10-µL reaction with 20 µg of yeast tRNA, 1 µL of 60 mM HEPES-OH (pH 7.9), 20 mM MgCl₂, and 3 × 10⁶ cpm of ³²P-labeled RNA. RNase digestion was done at room temperature using 50 µg of RNase. Proteins were resolved on a 6% SDS-polyacrylamide gel. Probes were synthesized in vitro using either SP6 or T7 RNA polymerase (New England Biolabs) as per the manufacturer's instructions, using plasmid subclones as templates. For the *nos* 3' UTR nucleotides 21–80 probe, the subclone contained that region of *nos* DNA inserted in the *Bgl*III and *Bam*HI sites of pSP72 (Promega). To estimate the position of the smg binding site, this DNA was amplified by error-prone PCR (Cadwell and Joyce 1992), and a collection of subclones were sequenced. Fourteen subclones, each bearing 1- to 5-point mutations (covering 22 of the 60 nucleotides of the probe), were used for probe synthesis and binding reactions. Directed point mutations were introduced by PCR into the wild-type DNA. Competition binding was performed as described (Macdonald et al. 1995). The specific competitor RNA was simply the probe RNA prepared without incorporation of the radioactive label. The nonspecific competitor RNAs carried either one copy of the *bcd* RNA localization signal BLE1 (Macdonald et al. 1995) or four tandem bruno binding sites (Kim-Ha et al. 1995). Templates for the 3× SRE and 3× SRE⁻ RNAs were constructed by assembly of multiple oligonucleotides into the *Bgl*III and *Sal*I sites of pSP73 (Promega). The 3× SRE sequence was 5'-GATCTGCA-GAGGCTCTGGCAGCTTTTGCCCCGTAAGCGCCTGGC-GCGTTTCTAGAAATAATCAGCTGGCCTGIATTAG-3'. The 3× SRE⁻ sequence differed in that each underlined C or G residue was converted to a G or C, respectively. The template for synthesis of the RNA probe containing both SRE1 and SRE2 was a *Bam*HI–*Bgl*III fragment corresponding to nucle-

otides 1–185 of the *nos* 3' UTR inserted into pGEM2 (Promega). Point mutations in the SREs were introduced by PCR.

Transgenic flies

Transgenic flies were generated by P-element-mediated transformation using *w¹¹¹⁸* embryos as recipients (Spradling and Rubin 1982). The *nos/bcd* transgene used in these experiments consisted of three genomic DNA segments, all incorporated into the CaSpeR transformation vector (Pirota 1988): (1) a *nos* DNA fragment including 1.2 kb of 5' flanking sequences and the entire *nos* open reading frame, with a *Bam*HI site introduced by PCR immediately after the stop codon; (2) a 0.6-kb *Eco*RV–*Stu*I (sites converted to *Bam*HI and *Sal*I, respectively) fragment of the *bcd* 3' UTR that contains all of the *bcd* RNA localization signals but lacks the *bcd* polyadenylation signal; and (3) 3.5-kb of 3' flanking sequences from the *nos* gene beginning at an *Xho*I site immediately 5' to the polyadenylation signal. The *nosHA/bcd* transgene differed only by addition at the end of the *nos* coding region of a synthetic DNA encoding two copies of the hemagglutinin epitope tag (Kolodziej and Young 1991). Additional DNA fragments from the *nos* 3' UTR were inserted into both constructs at the unique *Bam*HI site separating the *nos* coding sequence and the *bcd* 3' UTR sequence. The *nos* 3' UTR fragments (all identified by sequence coordinates in the *nos* 3' UTR) were prepared by PCR, and incorporated 5' *Bam*HI and 3' *Bgl*III sites to facilitate cloning. The wild-type *nos* and *nosSRE⁻* transgenes consisted of the same *nos* 5' and coding sequences described for *nos/bcd*, joined to a 4.2-kb *nos* genomic fragment beginning with a *Bam*HI restriction site introduced by PCR immediately after the stop codon. For the *nosSRE⁻* transgene, PCR was used to introduce the nucleotide changes in the SRE sequences noted in the legend to Figure 7. The *tor* transgene was as described previously (Wharton and Struhl 1991) and nucleotides 1–96 of the *nos* 3' UTR flanked by an *Xba*I site on one side and an *Spe*I site on the other were inserted into the *Spe*I insertion site in the *tor* 3' UTR. Transgenic lines were used in the *w¹¹¹⁸* background. For testing in mutant backgrounds, transgenic lines on the appropriate chromosome were identified by segregation tests, doubly balanced, and crossed into the appropriate background. For analysis in *osk⁻* mutants, flies were transheterozygous for *osk³⁴* and *osk¹⁶⁶*. For analysis in *tor⁻* mutants, flies were homozygous for *tor^{PM}*.

RNA and protein analysis

Embryo extracts for Western blot analysis were prepared as for UV cross-linking assays with the exception that embryos were homogenized in 2× protein sample buffer plus 1 mM PMSF and the extracts were not centrifuged. After boiling for 5 min, proteins were resolved on 10% SDS polyacrylamide gels and then transferred to nitrocellulose using a Genie Blotter (Idea Scientific) as per the manufacturer's instructions. *nos* proteins were detected using rat anti-*nos* antisera [provided by Robin Wharton (Duke University Medical Center, Durham, NC)] and the Western Light protein detection kit (Tropix). RNase protection was performed as described previously (Macdonald and Struhl 1988), using the same *rp49* probe. For detection of *nosHA/bcd* mRNA a probe corresponding to the 3' end of the *nosHA/bcd* open reading frame (including the epitope tags) was generated to differentiate endogenous *nos* RNA from *nosHA/bcd* mRNA.

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