Role for mRNA localization in translational activation but not spatial restriction of *nanos* RNA

Sherri Evans Bergsten and Elizabeth R. Gavis*

Department of Molecular Biology, Princeton University, Princeton, NJ 08544, USA

*Author for correspondence (e-mail: lgavis@molbio.princeton.edu)

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SUMMARY

Patterning of the anterior-posterior body axis during *Drosophila* development depends on the restriction of Nanos protein to the posterior of the early embryo. Synthesis of Nanos occurs only when maternally provided *nanos* RNA is localized to the posterior pole by a large, cis-acting signal in the *nanos* 3′ untranslated region (3′UTR); translation of unlocalized *nanos* RNA is repressed by a 90 nucleotide Translational Control Element (TCE), also in the 3′UTR. We now show quantitatively that the majority of *nanos* RNA in the embryo is not localized to the posterior pole but is distributed throughout the cytoplasm, indicating that translational repression is the primary mechanism for restricting production of Nanos protein to the posterior. Through an analysis of transgenes bearing multiple copies of *nanos* 3′UTR regulatory sequences, we provide evidence that localization of *nanos* RNA by components of the posteriorly localized germ plasm activates its translation by preventing interaction of *nanos* RNA with translational repressors. This mutually exclusive relationship between translational repression and RNA localization is mediated by a 180 nucleotide region of the *nanos* localization signal, containing the TCE. These studies suggest that the ability of RNA localization to direct wild-type body patterning also requires recognition of multiple, unique elements within the *nanos* localization signal by novel factors. Finally, we propose that differences in the efficiencies with which different RNAs are localized result from the use of temporally distinct localization pathways during oogenesis.

Key words: *nanos*, RNA localization, 3′ untranslated region, Translational regulation, *Drosophila melanogaster*

INTRODUCTION

Asymmetric distribution of cytoplasmic components in oocytes from organisms as diverse as *Drosophila* and *Xenopus* underlies patterning of the body axes during embryonic development and the formation of specialized tissues like the germline. Proper patterning of the anterior-posterior (A-P) axis of the early embryo is initiated by key regulatory proteins distributed asymmetrically along the A-P axis. Maternally provided mRNAs localized to opposite poles of the oocyte, and subsequent embryo, direct polarized synthesis of these proteins in the early pre-cellular embryo.

Anterior localization of *bicoid* (*bcd*) RNA leads to production of an anterior-to-posterior gradient of Bcd protein (Berleth et al., 1988; Driever and Nüsslein-Volhard, 1988) while posterior localization of *nanos* (*nos*) RNA generates an opposing posterior-to-anterior gradient of Nos protein (Gavis and Lehmann, 1992; Wang et al., 1994). Bcd and Nos proteins regulate genes differentially along the A-P axis, directing the formation of anterior (head/thorax) and posterior (abdomen) structures, respectively. Bcd functions as both a transcriptional activator (Driever et al., 1989; Struhl et al., 1989) and translational repressor (Dubnau and Struhl, 1996; Rivera-Pomar et al., 1996) while Nos represses translation of the Hunchback transcriptional repressor in the posterior (Tautz and Pfeifle, 1989), allowing expression of genes required for abdominal development (reviewed by Hülskamp and Tautz, 1991). *nos* RNA and protein are also incorporated into the germ line progenitor cells, or pole cells, where *nos* is required for proper pole cell migration (Kobayashi et al., 1996; Forbes and Lehmann, 1998).

Analysis of maternally acting genes required for *bcd* and *nos* localization and the dynamics of their localization reveals the presence of distinct anterior and posterior localization pathways. Anterior localization of *bcd* RNA occurs in a stepwise process, requiring the functions of several genes that act at different steps (St Johnston et al., 1989; Macdonald et al., 1993). Posterior localization of *nos* occurs late in oogenesis and requires prior posterior localization of the germ plasm components, Oskar (Osk), Vasa (Vas) and Tudor (Tud) proteins (Wang et al., 1994). Germ plasm assembly itself requires posterior localization of *osk* RNA (Ephrussi et al., 1991; Kim-Ha et al., 1991). Osk protein produced from localized *osk* RNA recruits Vas and Tud proteins to the posterior pole, where all three are required for the subsequent localization of *nos* RNA (reviewed in Lehmann and Rongo, 1993).

While RNA localization generates a concentrated source for protein synthesis, coupling of translation to RNA localization ensures spatial restriction of protein synthesis. We have previously shown that *nos* activity is regulated by both RNA
localization and localization-dependent translational regulation (Gavis and Lehmann, 1992, 1994). Unlocalized nos RNA is translationally repressed while translation of nos requires posterior localization. Thus, the nos-like abdominal defects of embryos produced by maternal-effect mutations in osk, vas and tud, which disrupt localization of nos RNA, result from translational repression of the unlocalized nos RNA and the consequent lack of Nos protein. By contrast, inappropriate translation of unlocalized nos RNA to produce Nos protein throughout the embryo causes suppression of anterior development and duplication of posterior structures at the anterior (Gavis and Lehmann, 1994). This phenotype results from the ability of ectopic Nos to repress translation of both bcd and hb RNAs in the anterior (Wharton and Struhl, 1991; Gavis and Lehmann, 1992).

Sequences that mediate both localization and translational regulation of nos RNA reside within the nos 3' untranslated region (3'UTR). The RNA localization signal, which spans approx. 540 nucleotides, can be subdivided into several partially functional localization elements, each containing sequence segments that are highly conserved between D. melanogaster and D. virilis (Gavis et al., 1996a). While the functional significance of the organization of the nos localization signal is not known, the large size and complexity of this localization signal is characteristic of the signals that localize bcd (Macdonald et al., 1993; Macdonald and Kerr, 1997), osk (Kim-Ha et al., 1993), and oocyte RNA-binding (orb) (Lantz and Schedl, 1994) RNAs in Drosophila oocytes, and Vg1 (Deshler et al., 1997; Gautreau et al., 1997) and Xcat-2 (Zhou and King, 1996) RNAs in Xenopus oocytes. Translational repression of unlocalized nos RNA is mediated by a 90 nucleotide translational control element (TCE) that overlaps the localization signal but functions independently (Gavis et al., 1996b; Dahanukar and Wharton, 1996; Smibert et al., 1996). Smaug, an embryonic protein, binds to sequences within the TCE that are required for translational repression, suggesting that Smaug may be a translational repressor of nos (Smibert et al., 1996).

While the mechanism by which localization enables translation of nos is unknown, several models have been suggested to address the linkage between localization and translation (Gavis et al., 1996b; Dahanukar and Wharton, 1996; Smibert et al., 1996). Interaction of nos with components of the cellular localization machinery may directly interfere with repression by Smaug and/or other factors, permitting translation of localized nos RNA. Alternatively, components in the germ plasm not themselves responsible for nos localization may inactivate the repression mechanism at the posterior pole. Finally, a limiting amount of repressor may be exceeded at the posterior pole by the high local concentration of nos RNA.

We now report the surprising result that only a small proportion of all nos RNA in an embryo is localized to the posterior pole. In light of this result, we have further investigated the requirements for translational repression and RNA localization in the spatial restriction of nos activity and the relationship between these mechanisms. By multimerization of nos 3'UTR elements to increase the number of potential binding sites for translational repressors and localization factors, we provide evidence that translational repression and localization of nos are mutually exclusive and that RNA localization activates translation of nos RNA by preventing interaction of nos RNA with translational repressors. The distinct behavior of different localization elements upon multimerization suggests that multiple recognition events are required to ensure effective localization and maximal nos activity. Finally, these studies reveal temporally distinct localization pathways during oogenesis, which may account for differences in the efficiencies with which different RNAs are localized.

### MATERIALS AND METHODS

#### Fly stocks

The following mutant alleles were used: nosBn (Wang et al., 1994), vasPd (Schüpbach and Wieschaus, 1986), vasD1 (Lehmann and Nüsslein-Volhard, 1991), oskD4 (Lehmann and Nüsslein-Volhard, 1991), ry106 (Lindsay and Zimm, 1992). For analysis of transgenes in a vas- background, a vasPD/vasD1 heteroallelic combination was used. The nos-tub3’UTR transgenic line has been described (Gavis and Lehmann, 1994). The wild-type OreR strain was used in preparation of RNA from embryo halves.

#### Construction and analysis of nos-tub: nos3’UTR multimer transgenes

The nos-tub3’UTR transgene, in which nos 3’UTR sequences have been replaced by sequences from the α-tubulin 3’UTR, has been described previously (Gavis and Lehmann, 1994). Tandem repeats of localization signal elements were generated from nos 3’UTR fragments described by Gavis et al. (1996a). Individual, gel-isolated nos 3’UTR fragments were allowed to self-ligate, and multimers were isolated from 1.5% agarose gels. Fragments containing two or three direct repeats of the nos 3’UTR elements were end-filled and inserted into an end-filled NheI site engineered to replace the BorEII site within the α-tubulin 3’UTR. The nos-tub:nos3’UTR hybrid genes were constructed in the ry+ P element vector pDM30 (Mismer and Rubin, 1987). The copy number and the orientation of each repeat were confirmed by restriction digests and by PCR using one internal primer and one external primer. Injection of the pDM3nos-tub:nos3’UTR plasmids into ry106 embryos was done according to the method of Spradling (1986). Two to six independent lines for each construct were analyzed by genomic PCR, RNase protection and/or northern blotting, cuticle analysis in a nosBn background, and in situ hybridization.

#### Construction and analysis of the mel-vir3’UTR transgene

Production of mel-vir3’UTR transgenic lines has been described by Gavis et al. (1996b). The cuticular phenotypes of larvae from 10 independent mel-vir3’UTR transgenic lines were analyzed in a nos- background. Embryos from two representative transgenic lines were analyzed by in situ hybridization.

#### Analysis of embryonic phenotypes

Females from different transgenic lines were placed in individual tubes of multi-tube collection blocks (Wieschaus and Nüsslein-Volhard, 1986) and embryos were collected on yeast apple juice agar plates at room temperature. Embryos were aged for >24 hours at room temperature, after which larval cuticle preparations were made (Wieschaus and Nüsslein-Volhard, 1986). For genotypes in which any embryos developed to hatching, embryos were transferred to individual agar plates prior to hatching. In this way, all progeny could be recovered from all lines and independent lines could be kept segregated.

#### Whole-mount in situ hybridization and antibody staining

Embryos

Fixation, in situ hybridization with digoxigenin-labeled RNA probes, and antibody staining with anti-Nos antibody and horseradish
peroxidase immunohistochemistry were performed according to the methods of Gavis and Lehmann (1992). The antisense D. melanogaster nos RNA probe was synthesized from the nos N5 cDNA (Wang and Lehmann, 1991). Anti-Nos antibody (Wang et al., 1994) was kindly provided by C. Wang and R. Lehmann. Embryos were mounted in LX112 embedding medium (Ladd Research Industries, Inc.) and photographed using a Zeiss Axioplan microscope using Nomarski optics.

In situ hybridization was performed in parallel on embryos from ry506, nos-tub3'UTR/+; nos8BN, and nos8BN females. Alkaline phosphatase reactions were stopped at the same time for all three genotypes.

In situ hybridization was carried out in parallel to embryos from nos-tub:nos3'UTR monomer (Gavis et al., 1996a) and multimer (2X, 3X) lines for each localization signal element. At least two lines were tested for each multimer construct. In situ hybridization was also done in parallel to embryos from representative 3X lines for each element and embryos from nos8BN/+ females. In each case, the alkaline phosphatase staining reactions were stopped when the intensity of the uniform cytoplasmic staining of embryos from different lines had reached comparable levels. Anti-Nos antibody staining was performed in parallel on embryos from nos-tub:nos+2/+; nos8BN and nos-tub:nos+2-3X/+; nos8BN females.

In situ hybridization to embryos from two independent mel-vir3'UTR lines was performed in parallel with embryos from nos8BN/+ females. In one experiment, staining reactions were stopped when the uniform cytoplasmic staining levels were comparable. In a second experiment, staining reactions were allowed to proceed for the same amount of time.

Ovaries
Ovaries from females fed with yeast for 2-3 days at 25°C were dissected into cold PBS, washed, and fixed in 4% parafomaldehyde in PBS/0.2% Tween/10% DMSO and 3 volumes of heptane for 20 minutes at room temperature. Whole-mount in situ hybridization with an antisense nos RNA probe was carried out according to a modified version of the method of Tautz and Pfiffle (1989) using 55°C as the hybridization temperature. Ovaries were mounted in glycerol/PBS and photographed as above.

Isolation of RNA from anterior and posterior embryo halves
Embryos were collected for 30 minute intervals from well-fed rapidly laying females, dechorionated in 50% bleach, washed well with distilled H2O, and positioned in a row on a glass slide in the same anterior-posterior orientation with their centers aligned. After being covered with a thin coat of Halocarbon oil (HC-56; Halocarbon Products, Hackensack, NJ) the embryos were transferred to a small guillotine (Ding and Lipshitz, 1993) and were frozen on dry ice. Dechorionation and alignment took 30 minutes so that embryos were 30-60 minutes old at freezing. Frozen embryos were cut midway between anterior and posterior poles under a dissecting microscope with a frozen, teflon-coated razor blade. Anterior and posterior halves were removed to Eppendorf tubes and stored on dry ice until a total of 250 anterior and posterior halves were obtained. Only sets of embryos judged to be cut into equal portions were used.

RNA was prepared by homogenization of frozen tissue in 10 mM Tris-HCl pH 8.0/0.1 M NaCl/20 mM EDTA/1% sarkosyl, followed by phenol extraction and ethanol precipitation. Resuspended RNA was treated with 10 units RNase-free DNasel (Boehringer), 40 units RNAsin (Promega) in 1x transcription buffer (Boehringer) for 15 minutes at 37°C, followed by phenol:chloroform extraction and ethanol precipitation.

Northern blot analysis
Half of each RNA sample was denatured and separated on a 1.2% agarose/formaldehyde gel according to standard methods (Sambrook et al., 1989). Northern blotting was carried out using Zeta-Probe membrane (BioRad) according to the manufacturer’s protocol. Two duplicate blots were generated. Each blot was hybridized sequentially with probes for nos, bcd, osk and rp49 RNAs. 32P-labeled probes were generated by random hexamer labeling of gel-isolated fragments containing the nos N5 (Wang and Lehmann, 1991), bcd c53.46.6 (Berleth et al., 1988), and osk (Ephrussi et al., 1991) cDNAs and the entire rp49-containing plasmid, HR0.6 (O’Connell and Roshbash, 1984). Labeled bands were quantitated using a Molecular Dynamics Phosphorimager; subsequently autoradiography was performed (shown).

RESULTS
Posterior RNA localization is inefficient
In situ hybridization to early Drosophila embryos reveals that nos RNA is highly concentrated at the posterior pole (Fig. 1A). The signal within the bulk cytoplasm, however, is significantly higher than that detected in embryos from nos8BN mutant females (Fig. 1C), which lack nos RNA (Wang et al., 1994). In fact, the level of bulk cytoplasmic staining in wild-type embryos is similar to that observed for embryos from nos8BN females containing a completely unlocalized nos RNA derivative, nos-tub3'UTR RNA (Fig. 1B). These results suggest that embryos from wild-type females contain both unlocalized and localized nos RNA.

To determine the relative amounts of unlocalized and localized nos RNA in a wild-type embryo, RNA was prepared from anterior and posterior halves of bisected embryos and analyzed by northern blotting. Quantitative data were obtained from analysis of anterior and posterior RNA prepared from four independent sets of 250 embryos (two sets shown in Fig. 1B).
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<th>A</th>
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<tbody>
<tr>
<td><strong>bcd</strong></td>
<td></td>
<td><strong>osk</strong></td>
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<tr>
<td><strong>nos</strong></td>
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<td><strong>rp49</strong></td>
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2). Whereas 98±1.2% of all **bcd** RNA within the embryo is found in the anterior half, only 52±1.5% of all **nos** RNA is located in the posterior half. **osk** RNA shows a slightly higher enrichment, with 59±4% in the posterior half. These results indicate that as little as 4% of all **nos** and only approximately 18% of all **osk** RNA in an embryo is localized to the posterior pole, whereas the majority remains dispersed throughout the cytoplasm. The inefficiency in the mechanism for posterior localization underscores the importance of additional modes for regulation of **nos** expression.

**Uncoupling RNA localization from localized translation**

Since the majority of **nos** RNA in an embryo remains unlocalized, translational repression of this RNA must be robust. Equally important, however, is the ability of localized **nos** RNA to escape repression and be translated at the posterior pole. While RNA localization is a prerequisite to translation of **nos** RNA, the mechanism by which localization and translational derepression are linked is not known. The distinct states of translationally repressed RNA and localized, translated RNA could result if translational repressors and localization factors compete for binding to **nos** 3′UTR sequences such that RNA can be bound by either one or the other but not by both simultaneously. Alternatively, localized RNA may interact simultaneously with both localization factors and translational repressors, but the repressors would be inactivated by additional factors present within the germ plasm at the posterior pole or titrated out by the high local concentration of **nos** RNA there. These possibilities can be distinguished by varying the number of copies of 3′UTR regulatory elements that mediate localization and translational repression in **nos** RNA.

Translational repression of unlocalized RNA and RNA localization are largely mediated by the **nos** 3′UTR +2 element, composed of the +1 and +2′ subelements (Fig. 3). The **nos** TCE, which is contained within the +1 element, is sufficient to repress translation of unlocalized **nos** RNA, while the +2′ element displays limited translational repression function (Gavis et al., 1996b). Both of these elements contain binding sites for Smaug protein (Smibert et al., 1996). On their own, the +1 and +2′ elements provide only weak localization function, but their combination in +2 permits substantial, although not wild-type localization (Gavis et al., 1996a). Thus, the +2 element has the ability to interact with translational repressors as well as with localization factors. To distinguish among models for translational activation of localized RNA, we compared the ability of one, two (2X), or three (3X) direct repeats of the +2 element (Fig. 3) to confer regulation on the unregulated **nos-tub3′UTR** transgene, which lacks all **nos** 3′UTR regulatory sequences (Gavis and Lehmann, 1994). **nos-tub3′UTR** RNA serves as a control and is unlocalized and translated throughout the embryo. Nos protein produced from this transgene in the posterior half of embryos from **nos** mutant females restores abdominal segmentation while the ectopic Nos protein produced in the anterior half causes deletions of head and thorax (Gavis and Lehmann, 1994).

When the **nos-tub:nos+2** transgene, containing a single +2 element, is introduced into **nos** mutant females, 98% of embryos produced develop wild-type anterior structures and 7-8 abdominal segments (Gavis et al., 1996b; Table 1). This phenotype indicates that translation of unlocalized **nos-tub:nos+2** RNA is repressed, permitting wild-type anterior development, but that **nos-tub:nos+2** RNA localized to the posterior produces enough Nos protein to direct abdominal development. Whole-mount in situ hybridization shows that the +2 element confers significant posterior localization (Fig. 4A). Translation of this posteriorly localized **nos-tub:nos+2** RNA accounts for its ability to produce abdominal segments since no segments are produced when the **nos-tub:nos+2** transgene is introduced into females mutant for **vas** (Gavis et
with both. When the +2 element is reiterated in the nos-tub:nos+2-2X and +2-3X RNAs, localization factors and translational repressors can interact simultaneously with different copies of the +2 element in a single RNA molecule (Fig. 4B). In this case, localized RNA is still subject to translational repression. The continuous reduction in translational activity as the number of copies of the +2 element is increased and the ability of secondary structures predicted to form within the +2 element to form within the +2-2X and +2-3X multimers (data not shown) support this hypothesis and argue against the alternative possibility that multimerization of the +2 element impairs sequences required for a unique translational activation step without disrupting sequences required for localization or translational repression.

**Behavior of the +2 element depends on the TCE**

The +2 element can be subdivided into the +1 element, which contains the TCE, and the +2* element. Because the +2* element shows a limited ability to repress translation (Gavis et al., 1996b), we examined whether translational repression of nos-tub:nos+2-2X and +2-3X RNAs at the posterior pole depends on the TCE. Multimerization of the +2* element lacking the TCE results in a dramatic increase in localization function. Three copies of +2* achieve a level of localization similar to that of a single +2 element (Fig. 5). Multimerization of the +2* element also improves its ability to repress translation of unlocalized nos RNA. Two copies of +2* repress translation sufficiently to prevent nos RNA that fails to localize from causing anterior defects (compare +2* and +2-2X in nos−, Table 2). Furthermore, when localization of nos-tub:nos+2* RNA is abolished by vas or osk mutations, the number of abdominal segments produced decreases as the number of +2* elements increases (Table 2 and data not shown).

Sequences in the +2* element cannot, however, explain the translational repression of localized RNA observed for multimers of the +2 element. In contrast to the behavior of nos-tub:nos+2 RNA, localized nos-tub:nos+2* RNA continues to be translated as the number of +2* elements is increased. Localization of very small amounts of nos-tub:nos+2* RNA leads to production of detectable nos activity in the posterior: nos-tub:nos+2* RNA produces an increase in abdominal segments in embryos from nos− females, where it is localized, as compared to embryos produced by vas− or osk− females, where it is unlocalized (Table 2 and data not shown).

The greatly improved localization of nos-tub:nos+2* RNA results in substantially higher nos levels and 98% of embryos from nos− females carrying the nos-tub:nos+2* transgene develop with 7-8 abdominal segments (Table 2). Since repression by the +2* element is less effective than that by the TCE, we cannot exclude the possibility that +2* may repress translation at the posterior in higher copy number. However, in contrast to the results observed with the +2 element multimers, the amount of nos RNA localized by +2* multimers directly determines the amount of nos activity at the posterior. This indicates that the TCE is critical to the continued repression of localized nos-tub:nos+2-2X and +2-3X RNA.

**Localization signal elements contribute uniquely to localization signal function**

Although the +2 element shows significant posterior localization, wild-type localization and the ability to

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**Table 1. Activity of nos-tub:nos+2 multimer transgenes in embryos from nos− and vas− females**

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<thead>
<tr>
<th>Abdominal segments</th>
<th>% of embryos</th>
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<tr>
<td></td>
<td>nos−</td>
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<tr>
<td></td>
<td>+2</td>
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<tr>
<td>0</td>
<td>100</td>
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<tr>
<td>1-3</td>
<td>2</td>
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<tr>
<td>4-6</td>
<td>98</td>
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Loss of head/thorax:

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<th>% of embryos</th>
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complement the nos mutant phenotype completely requires combination of the +2 and +3 elements. The fact that either combination of +2 and +3 or of +2', +3, and +4 elements is sufficient for wild-type localization indicates that localization elements are partially redundant in function (Gavis, 1996a). To determine whether the number or type of element is critical for wild-type localization signal function, we compared the effects of multimerization of the +2 element. As described above, three copies of the +2 element produce a dramatic improvement in localization function over one copy. Two copies of the +3 element (+3-2X) produce substantial improvement in function over a single +3 element and three copies (+3-3X) produce a small improvement over two (Fig. 5). Localization of nos-tub:nos+4 and +4-2X RNAs shows significant variability within a given transgenic line. Localization of +4-3X, by contrast, is consistent and improved among individual embryos (Fig. 5). Since the +3 and +4 elements lack sequences that mediate translational repression, nos activity is produced from nos-tub:nos+3-2X, +3-3X, +4-2X, and +4-3X RNAs regardless of their state of localization (Table 2 and data not shown), precluding analysis of the activity of localized RNA.

Although multiple copies of these elements improve localization, in no case does multimerization of an individual element achieve wild-type localization function (Fig. 5). Thus, reiteration of the sequences within individual localization signal elements can only partially compensate for the loss of other elements. This analysis reveals that different localization elements display unique behaviors upon multimerization. The +2' element appears to act cooperatively upon multimerization while the +3 element shows a more graded improvement with +3-2X showing substantial improvement in function over a single +3 element and +3-3X showing further improvement over +3-2X (Fig. 5). Taken together, these results indicate that individual localization elements make unique contributions to localization signal function and that the unique sequences present within different elements may be critical to wild-type function.

Localization function of the nos 3’UTR is evolutionarily conserved

Each of the nos 3’UTR elements (+1, +2', +3, and +4) contains at least one segment that shows significant sequence conservation to a similarly positioned segment in the D. virilis nos 3’UTR (Gavis et al., 1996a). Transgenic D. virilis nos RNA is produced at wild-type levels and is posteriorly localized in D. melanogaster embryos (Gavis et al., 1996a), but does not completely complement the nos mutant abdominal phenotype.
Table 2. Activity of nos-tub:nos3’ UTR multimer transgenes in embryos from nos− and vas− females

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<th>% of embryos</th>
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<tr>
<td></td>
<td>+2’</td>
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<tr>
<td><strong>nos−</strong></td>
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<tr>
<td>Abdominal segments</td>
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<td>0-3</td>
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<td>4-6</td>
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<td>7-8</td>
<td>98</td>
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<tr>
<td>Loss of head/thorax</td>
<td>50</td>
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<tr>
<td><strong>vas−</strong></td>
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<tr>
<td>Abdominal segments</td>
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<td>0-3</td>
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<td>4-6</td>
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<td>7-8</td>
<td>98</td>
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<tr>
<td>Loss of head/thorax</td>
<td>58</td>
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*Excess nos activity in these embryos results in deletion of anterior abdominal segments in addition to head and thorax.

nos-tub:nos+2’, +3, and +4 multimer transgenes were introduced into nos mutant females; nos-tub:nos+2’ transgenes were also introduced into vas mutant females. The number of abdominal segments and the loss of head and thoracic structures were determined in cuticle preparations of 50-100 embryos. The values shown represent the averages from analysis of 2-5 independent transgenic lines. The values for the monomer transgenes were previously determined (Gavis et al., 1996b). At least two lines for each transgene were also analyzed by genomic PCR, northern blotting and/or RNase protection assays, and in situ hybridization.

Comparison of the number of segments produced by nos-tub:nos+2’ transgenes in nos− versus vas− indicates the amount of nos activity produced from translation of localized nos-tub:nos+2’ RNAs. Since the +3 and +4 elements lack translational repression function, nos-tub:nos+3 and +4 RNAs are translated throughout the embryo, regardless of their localization state. For unknown reasons, the nos-tub:nos+3 transgene consistently produces more nos activity than that produced by the unregulated nos-tub3’ UTR transgene (Gavis et al., 1996b). The nos-tub:nos+3-2X, +3-3X, and all nos-tub:nos+4 transgenes behave similarly to nos-tub3’ UTR.

Fig. 5. Whole-mount in situ hybridization to embryos from nos-tub:nos3’ UTR multimer lines. Since transgenes were introduced into nosRN females, the nos-specific probe detects only RNA produced by the transgenes. Representative embryos from nos-tub:nos+2’, +3, and +4 transgenic lines are shown alongside embryos from females that carry one copy of the endogenous nos gene (nos−/+). Results of RNase protection assays and northern blot analysis indicate that the observed differences in localization by different oligomeric states of each element, as well as the differences among the elements, do not result from differences in transgene expression or RNA stability (data not shown). Embryos are oriented anterior to the left, dorsal up.
(Curtis et al., 1995). To determine whether the reduced function observed for D. virilis nos RNA results from inadequate 3′UTR localization function, incomplete translational activation, or reduced activity of D. virilis Nos protein in D. melanogaster embryos, we constructed a D. melanogaster nos transgene bearing the D. virilis nos 3′UTR. This mel-vir3′UTR transgene completely complements the abdominal defects of embryos produced by D. melanogaster nos mutant females (Gavis et al., 1996b) and mel-vir3′UTR RNA shows wild-type localization (Fig. 6). The ability of the nos 3′UTR from D. virilis to substitute functionally for the D. melanogaster nos 3′UTR therefore suggests that the conserved segments define functionally relevant localization sequences.

**Posterior localization of nos RNA is not dependent on its localization during early oogenesis**

Accumulation of nos RNA at the posterior pole occurs late in oogenesis, following a dynamic pattern of localization (Wang et al., 1994). nos RNA is synthesized by the 15 nurse cells that lie anterior to the oocyte. The polyploid nurse cells remain connected by cytoplasmic bridges to each other and to the oocyte at its anterior end as a result of incomplete oogonial divisions (for review of oogenesis see Spradling, 1993). nos RNA accumulates preferentially in the oocyte during the first half of the 14 morphologically defined stages of oogenesis (Fig. 7). During stages 8-10, nos becomes enriched at the anterior margin of the oocyte. At the end of stage 10, transcription of nos RNA in the nurse cells increases dramatically. This RNA is deposited into the oocyte at the end of stage 10, when the nurse cells contract and extrude their cytoplasm into the oocyte. nos RNA becomes dispersed within the oocyte by the microtubule-dependent streaming of the oocyte cytoplasm (Gutzeit and Koppa, 1982; Theurkauf et al., 1992) that follows. Posterior localization of the nos transcript can first be detected at stage 12 (Wang et al., 1994).

The early accumulation of nos RNA in the oocyte and its enrichment at the anterior during stages 8-10 require the combined function of the +2′ and +3 elements (Gavis, 1996a) and are thought to be intermediate steps in nos localization. Because posterior localization does not occur until late in oogenesis, after a substantial amount of additional nos RNA enters the oocyte from the nurse cells, the relevance of these earlier events to posterior localization is unclear. We were able to address the significance of these earlier events by analyzing the distribution of nos-tub: nos+2′-3X RNA during oogenesis. In contrast to wild-type nos RNA, nos-tub: nos+2′-3X RNA does not accumulate preferentially in the early oocyte or become enriched at the anterior margin during stages 8-10 (Fig. 7). This RNA is, however, expressed at high levels in the stage 10 nurse cells. The significant posterior localization of nos-tub: nos+2′-3X RNA in the absence of these earlier events indicates that they are not necessary intermediates in the process of nos localization. Furthermore, the ability of nos− females carrying the nos-tub: nos+2′-3X transgene to produce wild-type embryos shows that these early events are not necessary for nos regulation.

**DISCUSSION**

We have shown that mechanisms for posterior localization of RNAs during oogenesis in Drosophila fail to localize the majority of these RNAs. The high concentration of nos RNA at the posterior pole revealed by whole-mount in situ hybridization reflects the distribution of localized nos RNA in a small and superficial volume at the oocyte cortex while the unlocalized RNA is distributed throughout the entire volume of the egg cytoplasm. Consequently, enrichment of even small amounts of localized RNA can readily be detected by this technique.

The fact that only a small proportion of nos RNA is localized to the posterior indicates that RNA localization does not provide an effective mechanism to sequester nos RNA within the embryo and underscores the importance of localization-dependent translational regulation in spatially restricting nos activity. By uncoupling RNA localization from localized translation, we provide evidence to support a testable molecular model for spatial regulation of nos RNA in which RNA localization and translational repression are normally mutually exclusive (see below). These studies also show that abdominal segmentation is highly sensitive to posterior localization. To this end, the unique contributions of multiple conserved nos 3′UTR elements to localization signal function may ensure sufficient association with germ plasm components to permit wild-type abdominal development in the face of an inefficient localization mechanism.

**Model for linkage of translation and RNA localization**

By creating a situation in which localized RNA remains translationally repressed, we have gained insight into the mechanism by which posterior localization and translational activation of wild-type nos RNA are linked. Results presented here support a model in which localization and translational repression of nos are normally mutually exclusive due to competition between localization factors and translational repressors for interaction with nos 3′UTR sequences. Translation of nos can occur when translational repressors are excluded from interacting with nos 3′UTR sequences by binding of localization factors. The behavior of the +2 multimers rules out a mechanism for translational activation in which limiting amounts of translational repressors are titrated out by the high local concentration of binding sites at the posterior. Our results also argue against models in which the repressors are inactivated by direct interaction with an antagonist that is also localized in the germ plasm (Dahunakar and Wharton, 1996; Smibert et al., 1996), although we cannot rule out the possibility that an inefficient activation mechanism would be sensitive to the number of repressors bound to the RNA. Lack of translational activation is furthermore unlikely to result from titration of a limiting amount of a localized activator by repressor-bound RNA molecules, since doubling the amount of wild-type nos RNA at the posterior pole results in a linear increase in the amount of Nos protein produced (Gavis and Lehmann, 1994).

We propose that additional activators are not required for translation of nos RNA at the posterior pole and that interaction with localization factors is sufficient to activate translation. The difference in behavior of multimers of the +2 and +2′ elements also suggests that other factors in addition to Smaug are likely to be required for TCE-mediated translational repression. Test of the biochemical predictions of this model will require identification and purification of factors that interact with
sequences within the +2 element and the development of a regulated in vitro translation assay.

**Significance of nos localization signal organization**

While combinations of three individual nos 3’UTR elements are sufficient for wild-type localization, three copies of an individual element cannot completely compensate for the loss of the other two. In addition, different elements behave uniquely when multimerized. The limited posterior localization conferred by individual localization elements requires osk-dependent assembly of germ plasm (Gavis et al., 1996a), suggesting that germ plasm components can recognize each element to some extent. The fact that osk, vas and tud mutations do not produce consistent partial localization phenotypes characteristic of nos 3’UTR deletion mutants (Gavis et al., 1996a) further suggests that Osk, Vas and Tud recognize the localization signal as a complex, rather than by interactions of individual proteins with individual elements. Recognition of localization signal elements by these germ plasm components, therefore, cannot easily explain the differential behavior of different elements upon multimerization and the requirement for several different elements in wild-type localization.

Conservation in sequence and function of nos localization elements between *D. melanogaster* and *D. virilis* predicts that these elements contain recognition sites for localization factors common to both species. With the exception of +1 and +2’, which both contain binding sites for the Smaug protein proposed to mediate translational repression (Smibert et al., 1996), there is no significant similarity among the conserved segments of different elements, with respect to both primary sequence and predicted secondary structure. Taken together, our results suggest that different elements are recognized uniquely by different cytoplasmic factors, not yet identified, that provide an interface between nos RNA and the germ plasm components. Preliminary evidence from UV-crosslinking experiments indicates that embryo extracts contain several proteins that interact differentially with the localization elements (S. E. B. and E. R. G., unpublished data). The ability of the +2’ element to act cooperatively, either with itself or with the +1 element, points to an important role for these sequences in assembling a localization complex.

**Early and late localization pathways**

In addition to nos, developmentally important RNAs including BicaudalD (Suter et al., 1989), egalitarian (Mach and Lehmann, 1997), fs(1)K10 (K10) (Cheung et al., 1992), gurken (grk) (Neuman-Silberberg and Schüpbach, 1993), orb (Lantz et al., 1992), bcd (Berleth et al., 1988), and osk (Ephrussi et al., 1991; Kim-Ha et al., 1991) have been shown to accumulate in the oocyte during stages 1-7 and at the anterior margin during stages 8-9. Anterior accumulation of grk (Neuman-Silberberg and Schüpbach, 1993) and osk (Ephrussi et al., 1991; Kim-Ha et al., 1991) occurs just prior to or coincident with movement to their respective anterior-dorsal and posterior destinations. In addition, mutations in staufen (Ephrussi et al., 1991; Kim-Ha et al., 1991), tropomyosin II (Erdélyi et al., 1995), and mago nashi (Micklem et al., 1997) enhance the anterior accumulation of osk RNA while preventing its posterior localization. These results suggest that anterior accumulation may be an important intermediate in localization of some RNAs.

Analysis of several of these RNAs has demonstrated a...
requirement for microtubules in their distributions during early and mid-oogenesis (Theurkauf et al., 1993; Pokrywka and Stephenson, 1995). Anterior localization of bcd and posterior localization of osk have been proposed to occur by active processes that involve movement of these RNAs in opposite directions within the oocyte during meroogenesis (reviewed in Glotzer and Ephrussi, 1996). In support of this model, a polarized microtubule network can be detected in oocytes from stages 7-10 (Theurkauf et al., 1992).

Polarization of the microtubule cytoskeleton is no longer apparent during later stages of oogenesis when posterior localization of osk RNA can first be detected, suggesting that translocation of nos RNA to the posterior occurs by a distinct cellular mechanism. Our results demonstrate that localization of nos RNA can occur without accumulation in the early oocyte or at the anterior margin. We propose that nos RNA, deposited in the oocyte as the nurse cells contract at the end of stage 10, becomes distributed throughout the cytoplasm by cytoplasmic streaming, and is ultimately trapped at the posterior pole by a preassembled germ plasm anchor. The fact that nos RNA can be mislocalized by the ectopic production of germ plasm at the anterior of the oocyte (Ephrussi and Lehmann, 1992) provides further support for this model. In contrast to bcd (Macdonald et al., 1993) and osk, (Kim-Ha et al., 1993) the nos localization signal does not contain discrete elements that mediate early oocyte or anterior margin accumulation (Gavis et al., 1996a), consistent with a ‘one step’ mechanism for nos RNA localization. As in the case of nos, accumulation of K10 RNA at the anterior margin of the oocyte during stages 8-10 is not required for K10 function (Serano and Cohen, 1995), supporting the idea that the anterior margin may be a default site for numerous RNAs that accumulate in the early oocyte. The machinery that targets RNAs to the early oocyte and to the anterior margin must recognize an as yet unidentified feature of multiple RNAs or be degenerate in its recognition specificity.

Inefficiency of posterior versus anterior localization mechanisms

Comparison of the proportions of bcd, nos and osk RNAs localized in an embryo indicates that anterior and posterior localization mechanisms differ greatly in how effectively they localize RNA. This difference could result from differences in the affinities of the RNAs for components of the localization machinery. Alternatively, it may reflect the fact that while all three RNAs enter the oocyte at its anterior end, nos and osk must traverse the oocyte to reach their posterior destination.

The evidence that different mechanisms operate in the early and late localization pathways points to the potential for different efficiencies inherent in these mechanisms. In this light, inefficient distribution or binding of nos RNA:protein complexes to the posterior anchor during or after cytoplasmic streaming could reduce the efficiency of nos localization. Differences between bcd and osk localization could additionally result from temporal changes in oocyte cytoskeletal organization. A kinesin-β-galactosidase fusion protein, which provides a marker for the plus ends of microtubules, is localized to the posterior pole during stages 8-9 but becomes uniformly distributed when cytoplasmic streaming begins during stage 10 (Clark et al., 1994). By contrast, the reciprocal anterior localization of a minus end-directed Nod-β-galactosidase fusion protein is maintained (Clark et al., 1997). The persistence of an anteriorly directed transport system would enable bcd RNA entering the oocyte throughout stage 10 to be continuously transported. In contrast to the active transport of osk RNA during stages 8-9, osk RNA deposited after stage 10 must rely on a diffusion/trapping mechanism. Late localization of osk RNA by cytoplasmic streaming can indeed occur when osk RNA is injected into cultured stage 10 and 11 oocytes (Glotzer et al., 1997).

During oogenesis, osk RNA is translationally repressed prior to posterior localization (Kim-Ha et al., 1995; Markussen et al., 1995; Rongo et al., 1995). Translational repression of osk during oogenesis is mediated by osk 3′UTR sequences that are recognized by Bruno, the product of the arrest gene (Kim-Ha et al., 1995; Webster et al., 1997). Since Bruno is present only in oocytes (Kim-Ha et al., 1995; Webster et al., 1997), the presence of unlocalized osk RNA in the embryo, as shown here, suggests that additional factors may be required to maintain repression of osk in the early embryo. Our results predict that, unlike nos and osk, localization of bcd is sufficient to restrict its activity within the embryo. Consistent with this idea, translation of bcd does not require anterior localization. Furthermore, since Nos protein can repress translation of bcd RNA through Nanos Response Elements (NREs) in the bcd 3′UTR (Wharton and Struhl, 1991), a mechanism is in place to prevent translation of bcd RNA in the posterior should localization be compromised.

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