Development 126, 659-669 (1999)
Printed in Great Britain © The Company of Biologists Limited 1999
DEV7673

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)

Accepted 19 November 1998; published on WWW 20 January 1999

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) body axis of the *Drosophila* 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 germline 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: nos^{BN} (Wang et al., 1994), vas^{PD} (Schüpbach and Wieschaus, 1986), vas^{D1} (Lehmann and Nüsslein-Volhard, 1991), osk^{54} (Lehmann and Nüsslein-Volhard, 1991), ry^{506} (Lindsley and Zimm, 1992). For analysis of transgenes in a vas^{-} background, a vas^{PD}/vas^{D1} 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 *Nhe*I site engineered to replace the *Bst*EII 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 pDM30*nos-tub:nos3′UTR* plasmids into ry^{506} 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 nos^{BN} 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 yeasted 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 ry^{506}, *nos-tub3′UTR*/+; nos^{BN}, and nos^{BN} 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 nos^{BN}/+ 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*/+; nos^{BN} and *nos-tub:nos+2-3X*/+; nos^{BN} females.

In situ hybridization to embryos from two independent *mel-vir3′UTR* lines was performed in parallel with embryos from nos^{BN}/+ 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% paraformaldehyde 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 Pfeifle (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 H_2O, 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 DNaseI (Boehringer), 40 units RNasin (Promega) in 1× 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. ^{32}P-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 nos^{BN} 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 nos^{BN} 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.

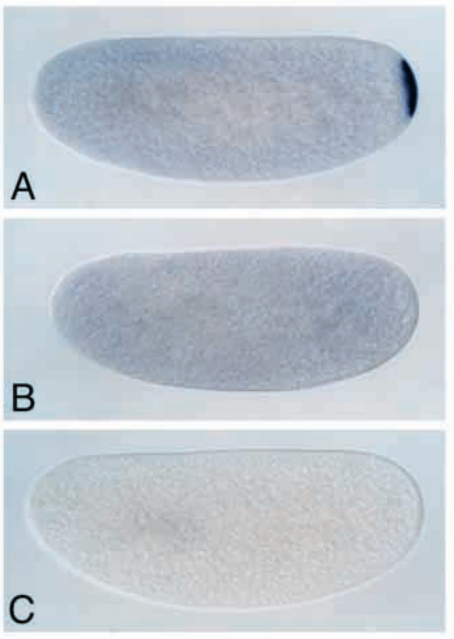

Fig. 1. Distribution of *nos* RNA detected by whole-mount in situ hybridization. In situ hybridization to *nos* RNA was performed in parallel on embryos from (A) wild-type, (B) *nos-tub3′UTR/+;* nos^{BN} and (C) nos^{BN} females. Prolonged alkaline phosphatase staining of nos^{BN} embryos produced results identical to those shown in C.

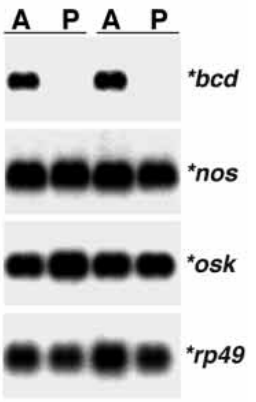

Fig. 2. Distribution of RNAs in anterior and posterior embryo halves. Northern blot analysis of total RNA prepared from 250 anterior (A) and 250 posterior (P) halves. The results from two independent experiments with 250 embryos each are shown. Hybridization signals obtained with probes for *bcd* (**bcd*), *nos* (**nos*), and *osk* (**osk*) were normalized to the unlocalized *rp49* (**rp49*) control, permitting calculation of the relative amount of each RNA in anterior versus posterior halves. Unlocalized RNA was assumed to be distributed uniformly and the proportion of RNA localized was calculated for each RNA using the equation |(A/A+P – P/A+P)|.

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 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

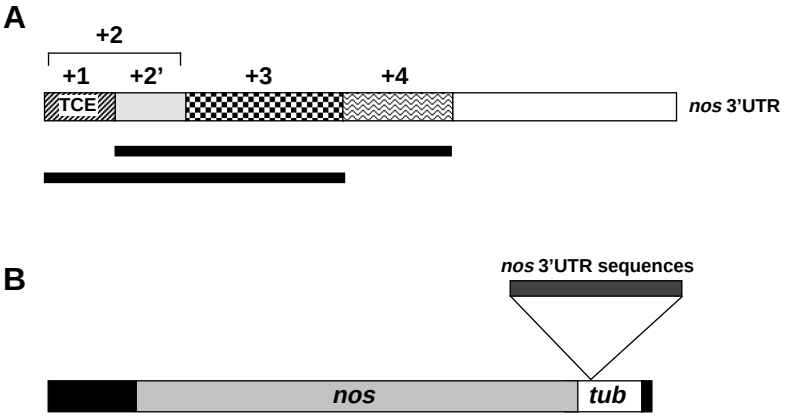

Fig. 3. Construction of *nos-tub:nos3′UTR* multimer transgenes. (A) The relative positions of *nos* localization signal elements are shown: +1 (nts 6-96 of the *nos* 3′UTR; see Gavis et al., 1996a), +2′ (nts 97-185), +3 (nts 189-408), +4 (nts 403-553). The +2 region includes both the +1 and +2′ elements. Combination of the +2′, +3 and +4 or +1, +2′, and +3 elements (filled bars) is sufficient for wild-type localization function. The +1 element contains the *nos* Translational Control Element (TCE); the +2′ element displays limited ability to repress translation and is considered an auxiliary element. (B) The +2′, +2, +3, and +4 localization signal elements were inserted as single elements, dimers, or trimers (indicated as *nos* 3′UTR sequences) into a *nos-tub3′UTR* reporter transgene that contains the *nos* 5′UTR (large filled box) and coding region (grey box), *α-tubulin* 3′UTR sequences (open box), and the *nos* polyadenylation signal (small filled box).

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

	% of embryos					
	nos⁻			*vas*⁻		
	+2	+2-2X	+2-3X	+2	+2-2X	+2-3X
Abdominal segments						
0	–	–	–	100	N.D.	100
1-3	–	2	9	–	N.D.	–
4-6	2	67	84	–	N.D.	–
7-8	98	31	7	–	N.D.	–
Loss of head/ thorax	–	–	–	–	N.D.	–

nos-tub:nos+2 multimer transgenes were introduced into *nos* and *vas* mutant females. Embryos from transgenic females mutant for *nos* contain both localized and unlocalized transgene RNA, whereas embryos from transgenic females mutant for *vas* contain only unlocalized transgene RNA. Thus, comparison of the number of segments in *nos*⁻ versus *vas*⁻ indicates the contribution made by translation of localized RNA to the activity of *nos-tub:nos+2* RNAs.

The number of abdominal segments and the loss of head and thoracic structures were determined in cuticle preparations of >100 embryos. The values shown represent the averages from analysis of 2-5 independent transgenic lines. The values for the *nos-tub:nos+2* transgene were previously determined (Gavis et al., 1996b). Representative lines for each transgene were also analyzed by genomic PCR, RNase protection assays, and in situ hybridization.

al., 1996b; Table 1), which abolishes localization of *nos* and *nos-tub+2* RNA (Wang et al., 1994; Gavis et al., 1996b).

The translational activity of localized *nos-tub:nos+2* RNA indicates that specific *nos* 3′UTR sequences outside of this region are not required for translational activation at the posterior pole. While *nos-tub:nos+2*, *+2-2X*, and *+2-3X* RNAs are localized to similar extents (Fig. 4A), embryos from *nos* mutant females carrying *nos-tub:nos+2-2X* and *+2-3X* transgenes exhibit increasingly reduced abdominal segmentation compared to embryos from *nos* mutant females carrying a *nos-tub:nos+2* transgene (Table 1). The defects reflect an increasing failure to release localized *nos* RNA from translational repression as Nos protein levels are substantially reduced in embryos from *nos*⁻ females carrying the *nos-tub:nos+2-3X* transgene (Fig. 4A). All *nos* activity detected for *nos-tub:nos+2-2X* and *+2-3X* RNAs results from translation of localized RNA since *nos* activity is completely abolished when these transgenes are introduced into *vas*⁻ or *osk*⁻ females (Table 1 and data not shown).

Additional copies of the +2 element prevent translational activation of localized RNA only when present on the same RNA molecule. By contrast, when the number of +2 elements is doubled by doubling the amount of wild-type *nos* RNA at the posterior pole, production of Nos protein increases proportionally (Gavis and Lehmann, 1994). The failure to translate localized RNA containing multiple +2 elements indicates that this RNA is still bound by translational repressors even when localized (Fig. 4B). The fact that this effect is only observed in the presence of multiple copies of the +2 element in *cis* suggests that the ability of *nos* RNA to interact with translational repression factors and localization factors is normally mutually exclusive. When only one +2 element is present, this element can interact with either translational repression factors or localization factors but not simultaneously 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 possiblity 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′-2X* RNA leads to production of detectable *nos* activity in the posterior: *nos-tub:nos+2′-2X* 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′-3X* RNA results in substantially higher *nos* levels and 98% of embryos from *nos*⁻ females carrying the *nos-tub:nos+2′-3X* 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 possiblity 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 the +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

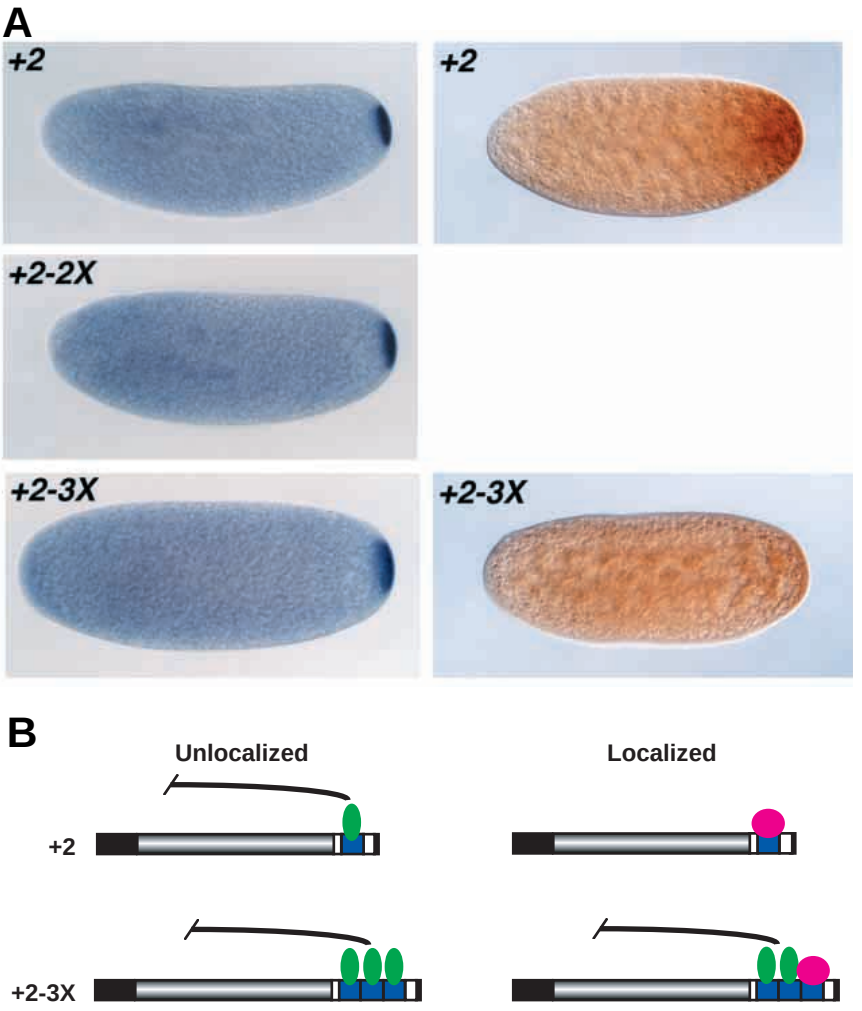

Fig. 4. Effect of multimerization of *nos* 3′UTR +2 element on RNA localization and Nos protein production. (A) Whole-mount in situ hybridization to *nos* RNA (left panels) was carried out in parallel to embryos from nos^{BN} females carrying *nos-tub:nos+2*, *+2-2X*, and *+2-3X* transgenes. Nos protein (right panels) was detected in embryos from nos^{BN} females carrying *nos-tub:nos+2* and *+2-3X* transgenes using an anti-Nos antibody. Since embryos from nos^{BN} females lack endogenous *nos* RNA, only RNA and protein produced by the transgenes are detected by the *nos* probe and anti-Nos antibody, respectively. Results of RNase protection assays indicate that the observed differences in localization by different oligomeric states of the +2 element do not result from differences in transgene expression or RNA stability (data not shown). Embryos are oriented anterior left, dorsal up. (B) Model for linkage between RNA localization and localized translation. *nos-tub:nos+2* and *+2-3X* RNAs are shown in the unlocalized state, in which translational repressors (shown collectively as a green oval) are bound to the +2 elements (blue) and translation is inhibited. In the localized state, localization factors (shown collectively as a magenta circle) interact with a similar or overlapping region of the single +2 element in *nos-tub:nos+2* RNA, eliminating repression. Localization factors and translational repressors bind to different +2 elements provided in *nos-tub:nos+2-3X* RNA so that translation remains inhibited.

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′, +3, and +4 elements. 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

	% of embryos								
	+2′	+2′-2X	+2′-3X	+3	+3-2X	+3-3X	+4	+4-2X	+4-3X
nos⁻									
Abdominal segments									
0-3	–	4	–	–	–	–	–	–	—
4-6	2	26	2	62*	8*	4*	–	4*	1*
7-8	98	70	98	38	92	96	100	96	99
Loss of head/thorax	50	2	2	100	100	100	100	100	97
vas⁻									
Abdominal segments									
0-3	–	16	72	N.D.					
4-6	2	51	28						
7-8	98	34	–						
Loss of head/thorax	58	–	–						

*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 RNAase 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.*

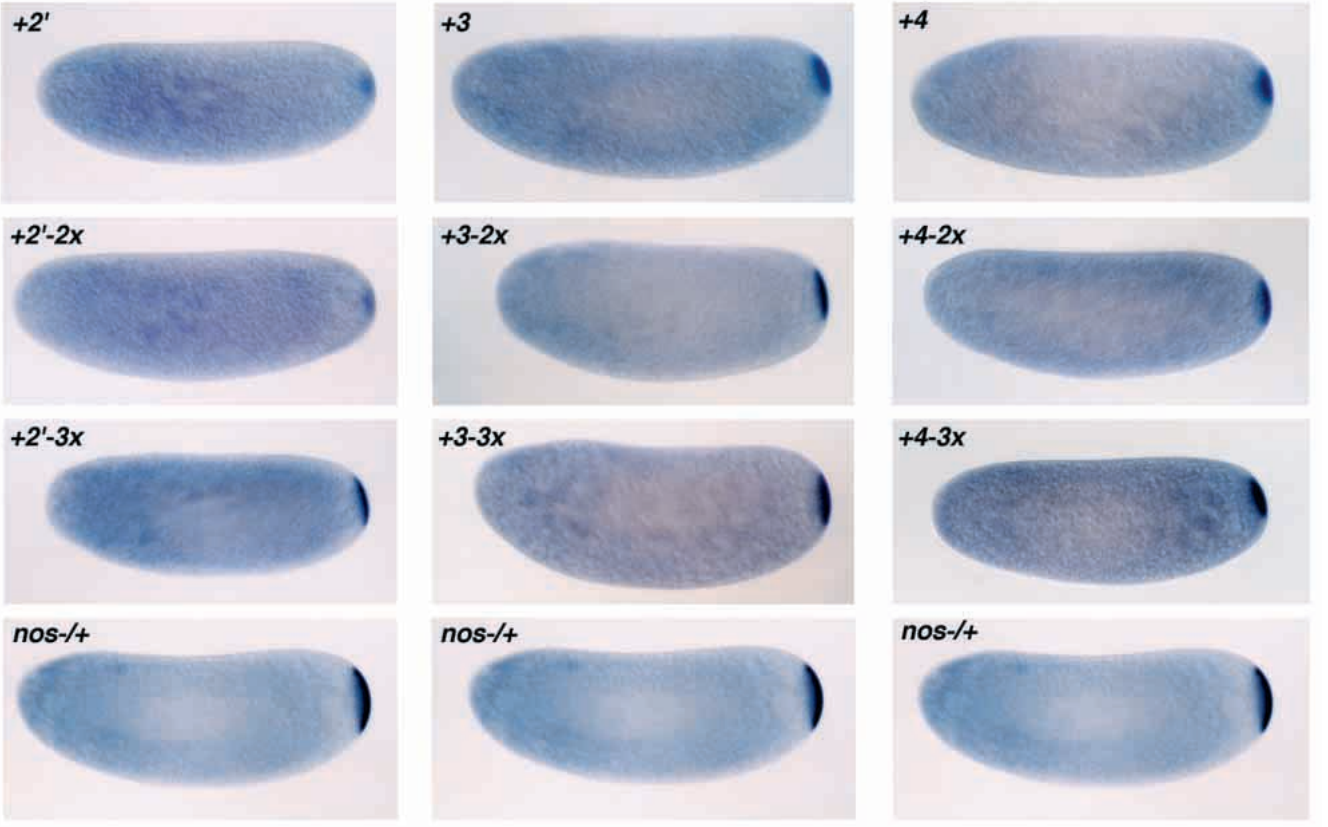

Fig. 5. Whole-mount in situ hybridization to embryos from *nos-tub:nos3′UTR* multimer lines. Since transgenes were introduced into nos^{BN} females, the *nos*-specific probe detects only RNA produced by the transgenes. Representative embryos from *nos-tub:nos+2′, +3,* and *+4* transgenic lines are shown 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* trangene 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

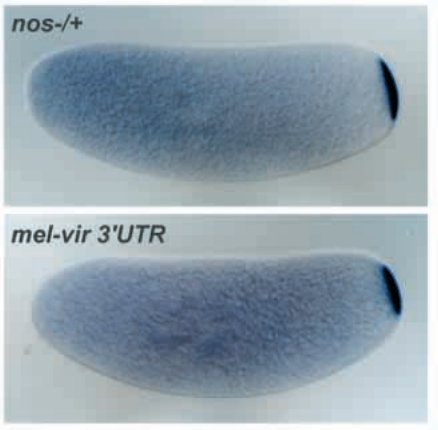

Fig. 6. Whole-mount in situ hybridization to embryos from *mel-vir3′UTR* transgenic females. In situ hybridization was performed on two representative transgenic lines (one shown here as *mel-vir3′UTR*) in parallel with embryos from females carrying one copy of the endogenous *nos* gene (*nos*–/+). Because the *mel-vir3′UTR* transgene was introduced into nos^{BN} females, the *nos* probe detects only RNA produced by the *mel-vir3′UTR* transgene. Embryos are oriented anterior to the left, dorsal up.

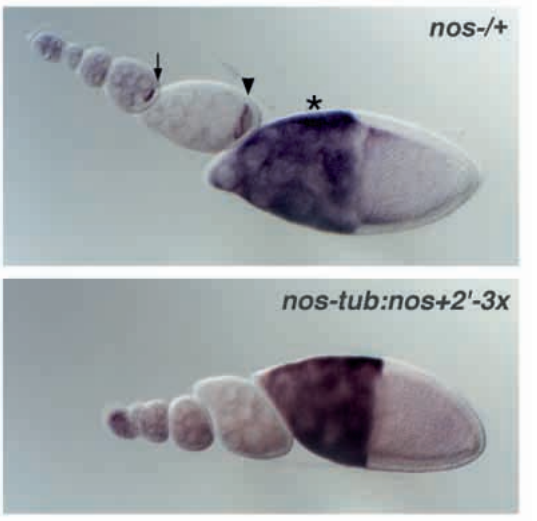

Fig. 7. Distribution of *nos-tub:nos+2′-3X* RNA during oogenesis. *nos-tub:nos+2′-3X* RNA was detected by whole-mount in situ hybridization to ovaries from nos^{BN} females carrying a *nos-tub:nos+2′-3X* transgene using a probe for *nos* sequences. Ovaries from females carrying one copy of the endogenous *nos* gene (*nos*–/+) were analyzed in parallel. Ovarioles containing egg chambers of successive stages are shown, with earliest stages toward the left. Each egg chamber consists of an oocyte situated posteriorly, 15 anterior nurse cells, and a surrounding layer of somatic follicle cells. Endogenous *nos* RNA accumulates in the oocyte during stages 3-7 (arrow) and is subsequently detected at the anterior margin of the oocyte during stages 8-9 (arrowhead). These early stages of localization are not observed in ovaries from *nos-tub:nos+2′-3X* females. Both *+2′-3X* and endogenous *nos* RNA are highly abundant in the nurse cells at stage 10 (asterisk). This RNA cannot be detected in the oocyte until after stage 10 when the nurse cells empty their contents into the oocyte (not shown).

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 midoogenesis (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 *nos* 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 efficiences 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.

We are very grateful to Dali Ding and Howard Lipshitz for providing the embryo guillotine and for advice on cutting embryos. Tripti Gupta provided invaluable assistance in establishing a working procedure for cutting embryos and preparing RNA from embryo halves. We thank Ira Clark, Fred Hughson, Ruth Lehmann, Trudi Schüpbach, and Eric Wieschaus for critical comments on the manuscript, and members of the Gavis lab and Eric Wieschaus for helpful discussions. S. E. B. was supported by an NSF predoctoral fellowship. This work was supported by a grant from the American Cancer Society and an NSF Early Career Development Award to E. R. G. E. R. G. is a Beckman Foundation Young Investigator.

REFERENCES

Berleth, T., Burri, M., Thoma, G., Bopp, D., Richstein, S., Frigerio, G., Noll, M. and Nüsslein-Volhard, C. (1988). The role of localization of *bicoid* RNA in organizing the anterior pattern of the *Drosophila* embryo. *EMBO J.* **7**, 1749-1756.

Cheung, H.-K., Serano, T. L. and Cohen, R. S. (1992). Evidence for a highly selective RNA transport system and its role in establishing the dorsoventral axis of the *Drosophila* egg. *Development* **114**, 653-661.

Clark, I., Giniger, E., Ruohola-Baker, H., Jan, L. Y. and Jan, Y. N. (1994). Transient posterior localization of a kinesin fusion protein reflects anteroposterior polarity of the *Drosophila* oocyte. *Curr. Biol.* **4**, 289-300.

Clark, I. E., Jan, L. Y. and Jan, Y. N. (1997). Reciprocal localization of Nod and kinesin fusion proteins indicates microtubule polarity in the *Drosophila* oocyte, epithelium, neuron, and muscle. *Development* **124**, 461-470.

Curtis, D., Apfeld, J. and Lehmann, R. (1995). *nanos* is an evolutionarily conserved organizer of anterior-posterior polarity. *Development* **121**, 1899-1910.

Dahanukar, A. and Wharton, R. P. (1996). The Nanos gradient in *Drosophila* embryos is generated by translational regulation. *Genes Dev.* **10**, 2610-2620.

Deshler, J. O., Highett, M. I. and Schnapp, B. J. (1997). Localization of *Xenopus* Vg1 mRNA by Vera protein and the endoplasmic reticulum. *Science* **276**, 1128-1131.

Ding, D. and Lipshitz, H. D. (1993). A molecular screen for polar-localized maternal RNAs in the early embryo of *Drosophila*. *Zygote* **1**, 257-271.

Driever, W. and Nüsslein-Volhard, C. (1988). A gradient of *bicoid* protein in *Drosophila* embryos. *Cell* **54**, 83-93.

Driever, W., Thoma, G. and Nüsslein-Volhard, C. (1989). Determination of spatial domains of zygotic gene expression in the *Drosophila* embryo by the affinity of binding sites for the bicoid morphogen. *Nature* **340**, 363-367.

Dubnau, J. and Struhl, G. (1996). RNA recognition and translational regulation by a homeodomain protein. *Nature* **379**, 694-699.

Ephrussi, A., Dickinson, L. K. and Lehmann, R. (1991). *oska*r organizes the germ plasm and directs localization of the posterior determinant *nano*s. *Cell* **66**, 37-50.

Ephrussi, A. and Lehmann, R. (1992). Induction of germ cell formation by *oskar*. *Nature* **358**, 387-392.

Erdélyi, M., Michon, A.-M., Guichet, A., Glotzer, J. B. and Ephrussi, A. (1995). Requirement for *Drosophila* cytoplasmic tropomyosin in *oskar* mRNA localization. *Nature* **377**, 524-527.

Forbes, A. and Lehmann, R. (1998). Nanos and Pumilio have critical roles in the development and function of *Drosophila* germline stem cells. *Development* **125**, 679-690.

Gautreau, D., Cote, C. A. and Mowry, K. L. (1997). Two copies of a subelement from the Vg1 RNA localization sequence are sufficient to direct vegetal localization in *Xenopus* oocytes. *Development* **124**, 5013-5020.

Gavis, E. R., Curtis, D. and Lehmann, R. (1996a). Identification of cis-acting sequences that control *nanos* RNA localization. *Dev. Biol.* **176**, 36-50.

Gavis, E. R. and Lehmann, R. (1992). Localization of *nano*s RNA controls embryonic polarity. *Cell* **71**, 301-313.

Gavis, E. R. and Lehmann, R. (1994). Translational regulation of *nanos* by RNA localization. *Nature* **369**, 315-318.

Gavis, E. R., Lunsford, L., Bergsten, S. E. and Lehmann, R. (1996b). A conserved 90 nucleotide element mediates translational repression of *nanos* RNA. *Development* **122**, 2791-2800.

Glotzer, J. B. and Ephrussi, A. (1996). mRNA localization and the cytoskeleton. *Sem. Cell. Dev. Biol.* **7**, 357-365.

Glotzer, J. B., Saffrich, R., Glotzer, M. and Ephrussi, A. (1997). Cytoplasmic flows localize injected *oskar* RNA in *Drosophila* oocytes. *Curr. Biol.* **7**, 326-337.

Gutzeit, H. O. and Koppa, R. (1982). Time-lapse film analysis of cytoplasmic streaming during late oogenesis of *Drosophila*. *J. Embryol. Exp. Morph.* **67**, 101-111.

Hülskamp, M. and Tautz, D. (1991). Gap genes and gradients – the logic behind the gaps. *BioEssays* **13**, 261-268.

Kim-Ha, J., Kerr, K. and Macdonald, P. M. (1995). Translational regulation of *oskar* mRNA by Bruno, an ovarian RNA-binding protein, is essential. *Cell* **81**, 403-412.

Kim-Ha, J., Smith, J. L. and Macdonald, P. M. (1991). *oskar* mRNA is localized to the posterior pole of the *Drosophila* oocyte. *Cell* **66**, 23-35.

Kim-Ha, J., Webster, P. J., Smith, J. L. and Macdonald, P. M. (1993). Multiple RNA regulatory elements mediate distinct steps in localization of *oskar* mRNA. *Development* **119**, 169-178.

Kobayashi, S., Yamada, M., Asaoka, M. and Kitamura, T. (1996). Essential role of the posterior morphogen *nanos* for germline development in *Drosophila*. *Nature* **380**, 708-711.

Lantz, V., Ambrosio, L. and Schedl, P. (1992). The *Drosophila orb* gene is predicted to encode sex-specific germline RNA-binding proteins and has localized transcripts in ovaries and early embryos. *Development* **115**, 75-88.

Lantz, V. and Schedl, P. (1994). Multiple cis-acting target sequences are required for *orb* mRNA localization during *Drosophila* oogenesis. *Mol. Cell. Biol.* **14**, 2235-2242.

Lehmann, R. and Nüsslein-Volhard, C. (1991). The maternal gene *nanos* has a central role in posterior pattern formation of the *Drosophila* embryo. *Development* **112**, 679-691.

Lehmann, R. and Rongo, C. (1993). Germ plasm formation and germ cell determination. *Sem. Dev. Biol.* **4**, 149-159.

Lindsley, D. L. and Zimm, G. G. (1992). The genome of *Drosophila melanogaster*. San Diego: Academic Press.

Macdonald, P. M. and Kerr, K. (1997). Redundant RNA recognition events in *bicoid* mRNA localization. *RNA* **3**, 1413-1420.

Macdonald, P. M., Kerr, K., Smith, J. L. and Leask, A. (1993). RNA regulatory element BLE1 directs the early steps of *bicoid* mRNA localization. *Development* **118**, 1233-1243.

Mach, J. M. and Lehmann, R. (1997). An Egalitarian-BicaudalD complex is essential for oocyte specification and axis determination in *Drosophila*. *Genes Dev.* **11**, 423-435.

Markussen, F.-H., Michon, A.-M., Breitwieser, W. and Ephrussi, A. (1995). Translational control of *oskar* generates Short OSK, the isoform that induces pole plasm assembly. *Development* **121**, 3723-3732.

Micklem, D. R., Dasgupta, R., Elliot, H., Gergely, F., Davidson, C., Brand, A., González-Reyes, A. and St Johnston, D. (1997). The *mago nashi* gene is required for the polarization of the oocyte and the formation of perpendicular axes in *Drosophila*. *Curr. Biol.* **7**, 468-478.

Mismer, D. and Rubin, G. M. (1987). Analysis of the promoter of the *nina* E opsin gene in *Drosophila melanogaster*. *Genetics* **116**, 565-578.

Neuman-Silberberg, F. S. and Schüpbach, T. (1993). The *Drosophila* dorsoventral patterning gene *gurken* produces a dorsally localized RNA and encodes a TGFα-like protein. *Cell* **75**, 165-174.

O'Connell, P. and Roshbash, M. (1984). Sequence, structure, and codon preference of the *Drosophila* ribosomal protein 49 gene. *Nucleic Acids Res.* **12**, 5495-5513.

Pokrywka, N. J. and Stephenson, E. C. (1995). Microtubules are a general component of mRNA localization systems in *Drosophila* oocytes. *Dev. Biol.* **167**, 363-370.

Rivera-Pomar, R., Niessing, D., Schmidt-Ott, U., Gehring, W. J. and Jäckle, H. (1996). RNA binding and translational suppression by bicoid. *Nature* **379**, 746-749.

Rongo, C., Gavis, E. R. and Lehmann, R. (1995). Localization of *oskar* RNA regulates *oskar* translation and requires Oskar protein. *Development* **121**, 2737-2746.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular cloning: A Laboratory Manual.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Schüpbach, T. and Wieschaus, E. (1986). Maternal-effect mutations altering the anterior-posterior pattern of the *Drosophila* embryo. *Wilhelm Roux's Archiv. Dev. Biol.* **195**, 302-317.

Serano, T. L. and Cohen, R. S. (1995). Gratuitous mRNA localization in the *Drosophila* oocyte. *Development* **121**, 3013-3021.

Smibert, C. A., Wilson, J. E., Kerr, K. and Macdonald, P. M. (1996). smaug protein represses translation of unlocalized *nanos* mRNA in the *Drosophila* embryo. *Genes Dev.* **1996**, 2600-2609.

Spradling, A. C. (1986). P element-mediated transformation. In *Drosophila: A Practical Approach*. (ed. D. B. Roberts), pp. 175-197. Oxford: IRL Press.

Spradling, A. C. (1993). Developmental genetics of oogenesis. In *The Development of Drosophila melanogaster* vol. I. (ed. M. Bate and A. Martinez Arias), pp. 1-70. Cold Spring Harbor, NY: Cold Spring Harbor Press.

St Johnston, D., Driever, W., Berleth, T., Richstein, S. and Nüsslein-Volhard, C. (1989). Multiple steps in the localization of *bicoid* RNA to the anterior pole of the *Drosophila* oocyte. *Development* **Suppl. 107**, 13-19.

Struhl, G., Struhl, K. and Macdonald, P. M. (1989). The gradient morphogen *bicoid* is a concentration-dependent transcriptional activator. *Cell* **57**, 1259-1273.

Suter, B., Romberg, L. M. and Steward, R. (1989). *Bicaudal-D*, a *Drosophila* gene involved in developmental asymmetry: Localized transcript accumulation in ovaries and sequence similarity to myosin heavy chain tail domains. *Genes Dev.* **3**, 1957-1968.

Tautz, D. and Pfeifle, C. (1989). A non-radioactive in situ hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals a translational control of the segmentation gene *hunchback. Chromosoma* **98**, 81-85.

Theurkauf, W. E., Alberts, B. M., Jan, Y. N. and Jongens, T. A. (1993). A central role for microtubules in the differentiation of *Drosophila* oocytes. *Development* **118**, 1169-1180.

Theurkauf, W. E., Smiley, S., Wong, M. L. and Alberts, B. M. (1992). Reorganization of the cytoskeleton during *Drosophila* oogenesis: implications for axis specification and intercellular transport. *Development* **115**, 923-936.

Wang, C., Dickinson, L. K. and Lehmann, R. (1994). Genetics of *nanos* localization in *Drosophila*. *Dev. Dynam.* **199**, 103-115.

Wang, C. and Lehmann, R. (1991). *Nanos* is the localized posterior determinant in *Drosophila*. *Cell* **66**, 637-647.

Webster, P. J., Liang, L., Berg, C. A., Lasko, P. and Macdonald, P. M. (1997). Translational repressor *bruno* plays multiple roles in development and is widely conserved. *Genes Dev.* **11**, 2510-2521.

Wharton, R. P. and Struhl, G. (1991). RNA regulatory elements mediate control of *Drosophila* body pattern by the posterior morphogen *nanos. Cell* **67**, 955-967.

Wieschaus, E. F. and Nüsslein-Volhard, C. (1986). Looking at embryos. In *Drosophila: A Practical Approach* (ed. D. B. Roberts), pp. 199-227. Oxford: IRL Press.

Zhou, Y. and King, M. L. (1996). RNA transport to the vegetal cortex of *Xenopus* oocytes. *Dev. Biol.* **179**, 173-183.