Recognition and long-range interactions of a minimal *nanos* RNA localization signal element

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**SUMMARY**

Localization of *nanos* (*nos*) mRNA to the germ plasm at the posterior pole of the *Drosophila* embryo is essential to activate *nos* translation and thereby generate abdominal segments. *nos* RNA localization is mediated by a large cis-acting localization signal composed of multiple, partially redundant elements within the *nos* 3′ untranslated region. We identify a protein of ~75 kDa (p75) that interacts specifically with the *nos* +2′ localization signal element. We show that the function of this element can be delimited to a 41 nucleotide domain that is conserved between *D. melanogaster* and *D. virilis*, and confers near wild-type localization when present in three copies. Two small mutations within this domain eliminate both +2′ element localization function and p75 binding, consistent with a role for p75 in *nos* RNA localization. In the intact localization signal, the +2′ element collaborates with adjacent localization elements. We show that different +2′ element mutations not only abolish collaboration between the +2′ and adjacent +1 element but also produce long-range deleterious effects on localization signal function. Our results suggest that higher order structural interactions within the localization signal, which requires factors such as p75, are necessary for association of *nos* mRNA with the germ plasm.

Key words: *Drosophila*, Nanos, Localization element, RNA, Germ plasm, mRNA localization

**INTRODUCTION**

Subcellular mRNA localization is a conserved mechanism for spatially restricting protein synthesis. This method of post-transcriptional control is used to regulate the distributions of proteins synthesized from maternal mRNAs in oocytes and early embryos (reviewed by Bashirullah et al., 1998) and to generate protein asymmetries necessary for polarized function in differentiated cells (reviewed by Hazerligg, 1998; Bassell et al., 1999). In some cases, translational control is coupled to RNA localization to prevent ectopic protein synthesis, either prior to localization or from RNA that escapes localization (Bashirullah et al., 1998; Hazerligg, 1998).

RNA localization plays a key role in generating the restricted distributions of regulatory proteins necessary for patterning the embryonic body axes during development in *Drosophila*. Restriction of Nanos (Nos) protein to the posterior of the embryo is essential for the establishment of anterior-posterior polarity. While Nos protein is required in the posterior of the embryo for abdominal development, Nos protein synthesis must be prevented in the anterior, in order to permit head and thorax development (Wharton and Struhl, 1989; Gavis and Lehmann, 1992; Gavis and Lehmann, 1994). Spatial regulation of Nos synthesis is achieved by a combination of RNA localization and translational control. Whereas the majority of *nos* mRNA is uniformly distributed throughout the cytoplasm, a small subset of *nos* mRNA is localized to the posterior pole of the embryo where it provides a concentrated source for the synthesis of Nos protein (Bergsten and Gavis, 1999). Posterior localization activates translation of *nos* mRNA while the unlocalized *nos* mRNA remains translationally repressed (Gavis and Lehmann, 1994).

Cis-acting signals that direct subcellular localization have been identified in numerous localized mRNAs, primarily within their 3′ untranslated regions (3′UTRs) (Bashirullah et al., 1998; Hazelrigg, 1998). Both primary sequence and structural motifs have been implicated in localization signal recognition. Localization of Vg1 RNA in *Xenopus* oocytes is mediated by a number of small, redundant sequence motifs (Gautreau et al., 1997; Deshler et al., 1998). Two proteins have been identified biochemically that interact with different Vg1 localization element motifs and are likely to play roles in Vg1 RNA localization (Deshler et al., 1998; Havin et al., 1998; Cote et al., 1999). Function of the *Drosophila* bicoid (*bcd*) and yeast Ash1 localization signals requires formation of complex structural motifs that also exhibit redundancy (Macdonald and Kerr, 1997; Macdonald and Kerr, 1998; Chartrand et al., 1999; Gonzalez et al., 1999). The double-stranded RNA-binding activity of Staufen (Stau) protein is required for *bcd* localization (Ramos et al., 2000) and an indirect assay suggests that Stau associates with a helical region of the *bcd* localization signal (Ferrandon et al., 1997).
Posterior localization of nos mRNA is mediated by a large cis-acting localization signal within the nos 3′UTR that is composed of multiple, partially redundant localization elements (Gavis et al., 1996a; Bergsten et al., 1999; Fig. 1A). Genetic analysis implicates the germ plasm components, Oskar (Osk), Vasa (Vas) and Tudor (Tud) in a localization complex that anchors nos RNA to the posterior pole (reviewed by Gavis, 1997). Our previous studies suggest that binding of nos 3′UTR localization elements by a set of distinct factors promotes the association of nos RNA with the germ plasm components (Bergsten and Gavis, 1999). However, no factor that interacts directly with a nos localization signal element has been identified.

To dissect minimal requirements for nos localization signal function, we have focused on the 88 nucleotide +2′ element (Fig. 1A,B). Although this element has very weak localization function on its own, three tandem copies confer substantial localization (Bergsten and Gavis, 1999). Furthermore, the +2′ element acts synergistically with adjacent localization elements. In particular, combination of the +2′ element with the weakly localizing +1 element produces the near wild-type localization function of the +2 element (Bergsten and Gavis, 1999, Fig. 1A). The +1 element is coincident with the nos translational control element (TCE), which mediates translational repression of unlocalized nos mRNA (Gavis et al., 1996b; Smibert et al., 1996; Dahankar and Wharton, 1996; Fig. 1A). TCE function requires the formation of two stem-loop structures (Cruc et al., 2000). We have shown that the synergistic interaction between the +2′ and +1 elements requires +1 element motifs that overlap but are distinct from the TCE structural motifs (Cruc et al., 2000). While our data indicate that the +2′ element plays an important role in assembling a localization complex, nothing is known about how the +2′ element is recognized by components of the localization machinery or the basis for its ability to collaborate with the +1 element.

Here, we show that the function of the +2′ element can be further delimited to a 41 nucleotide region that is conserved between D. melanogaster and D. viridis. Linker scanning mutations distributed throughout this conserved domain disrupt +2′ element localization function. We identify a protein present in ovaries and embryos that specifically recognizes the +2′ element. Mutations that disrupt +2′ element binding also disrupt localization in vivo, consistent with a role for this protein in nos RNA localization. Surprisingly, we find that +2′ element mutations abolish the contributions of both the +1 and +2′ elements to +2 element localization function, while leaving translational repression of unlocalized nos RNA by the TCE intact. Our results suggest that contributions of both +1 and +2′ element sequences or structure to a higher order structure underlies localization signal function. Furthermore, this localization signal structure must form alternately to that of the TCE.

MATERIALS AND METHODS

Partial purification of p75

All manipulations during purification were performed at 4°C. Oregon R embryos were collected overnight at 25°C, dechorionated, washed extensively with distilled H2O and blotted dry. Embryos were homogenized in an equal volume of lysis buffer (10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 2.5 mM MgCl2, 0.5% Triton X-100, 1 mM DTT) supplemented with protease inhibitors (0.1 mM benzamidine, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 0.1 mM PMSF). The lysate was centrifuged twice at 17,000 g to remove insoluble material and stored at −80°C in the presence of 20% glycerol.

For partial purification, thawed embryonic lysate was brought to 600 mM NaCl and polyethyleneimine (pH 7.9) was added to a final concentration of 0.1%. After mixing for 30 minutes, the resulting precipitate was removed by centrifugation for 30 minutes at 17,000 g. The supernatant was collected and subjected to (NH4)2SO4 fractionation. Protein precipitated at increasing (NH4)2SO4 saturation was recovered by centrifugation at 17,000 g, resuspended in Buffer A (100 mM KCl, 25 mM HEPES (pH 7.9), 0.5 mM EDTA, 10% glycerol, 1 mM DTT, 0.5 mM PMSF) containing 5% (NH4)2SO4, and desalted using a PD-10 gel filtration column (Amersham Pharmacia) equilibrated with Buffer A. Partially purified protein was frozen in aliquots at −80°C.

UV crosslinking assay

Bluescript plasmids containing a single insertion of either the +1/TCE, +2′, +2′(*) or +3 localization element sequences were used to generate templates for RNA synthesis. In all cases, polylinker sequences between the T7 or T3 promoter and the localization element sequences were deleted to decrease nonspecific binding. 32P-labeled RNAs were synthesized in vitro from 1 μg of linearized DNA template using T7 or T3 RNA polymerase transcription mix (Roche Molecular Biochemicals). Unlabeled RNAs for competition assays were transcribed using the AmpliScribe high yield transcription kit (Epicentre Technologies). Following treatment with RNase-free DNase to remove the DNA template, RNAs were purified through Pharmacia ProbeQuant G50 spin columns. 32P-labeled RNAs were quantified by scintillation counting and unlabeled mRNAs by spectrophotometry.

Protein was mixed with 1×106 dpbm of 32P-labeled RNA in 1× binding buffer (5 mM HEPES (pH 7.4), 2 mM MgCl2, 150 mM KCl, 1 mM DTT, 0.2 mg/ml yeast tRNA, 5 mg/ml heparin, and 40 units of RNasin (Promega)) in a 15 μl reaction volume. For competition assays, unlabeled competitor RNAs were mixed with the 32P-labeled RNA probe before the protein extract was added. Binding reactions were incubated for 10 minutes on ice, then crosslinked on ice for 10 minutes in a Stratalinker (Stratagene). Subsequently, 5 units of RNase One (Promega) were added and the reaction was incubated for 15 minutes at 37°C. Proteins were then boiled in SDS-PAGE sample buffer and resolved on a 12% SDS-polyacrylamide gel and visualized by autoradiography.

Construction of transgenes and generation of transgenic lines

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 (Gavis and Lehmann, 1994). The nos-tub:nos+2′ and nos-tub:nos+2′(*) transgenes are described by Gavis et al. (Gavis et al., 1996a) and the nos-tub:nos+2′:3X transgene is described by Bergsten and Gavis (Bergsten and Gavis, 1999). The nos-tub:nos+2′ transgene (Gavis and Lehmann, 1996a) is identical to the nos-tub:TCE transgene (Cruc et al., 2000) and is referred to here as nos-tub:nos+1/TCE. For all of the nos-tub3′UTR transgene derivatives described below, with the exception of nos-tub:nos+2′(*), wild-type or mutant nos 3′UTR sequences were inserted into a unique NheI site engineered within the α-tubulin 3′UTR sequences of the nos-tub3′UTR transgene (Bergsten and Gavis, 1999). The nos-tub:nos+2′:ME-3X transgene contains three direct repeats of nucleotides 117-158 of the nos 3′UTR. The nos-tub:nos+2′:3O transgene contains +2′ sequences (nucleotides 97-185 of the nos 3′UTR) followed by the +1 element sequences (nucleotides 6-96 of the nos 3′UTR). To generate nos-tub:nos+2′(*) transgene sequences were inserted into a BsaAI site downstream of the +1
element within the α-tubulin 3'UTR of the nos-tub:nos+1/TCE transgene. As a result, the +1 and +2' elements are separated by 130 nucleotides of α-tubulin 3'UTR sequence.

Mutations in the +2' element (+2'(*)) were engineered using PCR to replace six nucleotide segments within the wild-type +2' element sequence with an AvrII restriction site. Each nos-tub:nos+2'(−)*-3X transgene contains three direct repeats of the corresponding +2' mutant. To introduce +2' mutations into the larger +2 element (nucleotides 6-185 of the nos 3'UTR), +1 element sequences were joined directly to mutant +2' elements preserving the natural junction between these elements. Each nos-tub:nos+2(*) transgene contains one copy of the corresponding +2 mutant.

Transgenes were constructed in the ry+ P-element vector pDM30 (Misser and Rubin, 1987) and the resulting P-element plasmids were injected into ry<sup>60</sup> embryos (Lindsley and Zimm, 1992) according to Spradling (Spradling, 1986).

**Analysis of embryonic phenotypes**

Transgenes were introduced in single copy into females homozygous for the nos<sup>Bn</sup> allele (Wang et al., 1994) or transheterozygous for the var<sup>Dh</sup> and vas<sup>Dl</sup> alleles (Schüpbach and Wieschaus, 1986; Lehmann and Nüsslein-Volhard, 1991). In most cases, multiple independent lines were analyzed for each transgene (see Table 1). Females of the appropriate genotype were placed in individual tubes of multi-tube collection blocks (Wieschaus and Nüsslein-Volhard, 1986) and embryos were collected on yeast cake 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**

In situ hybridization to 0-1.5 hour embryos produced by homozygous nos<sup>Bn</sup> females that carried one copy of a given transgene was performed according to Gavis and Lehmann (Gavis and Lehmann, 1992). Since embryos from nos<sup>Bn</sup> females lack nos RNA, the only nos RNA detected by the digoxigenin-labeled antisense nos RNA probe derives from the transgene. In situ hybridizations were performed in parallel on embryos from one to two lines for each transgene, chosen as representative from the larger set analyzed for embryonic phenotypes. In all cases, comparisons were made using lines whose RNA levels were determined to be comparable during the first 90 minutes of embryogenesis. The antisense digoxigenin-labeled nos RNA probe was synthesized from the nos N5 cDNA (Wang and Lehmann, 1991). Embryos were mounted in LX1122 embedding medium (Ladd Research Industries, Inc.) and photographed with a Zeiss Axioplan microscope using Nomarski optics.

**RESULTS**

**Recognition of the +2' element by a ~75 kDa protein**

The ability of three copies of the +2' element (+2'−3X) to confer localization indicates that the +2' element contains information sufficient for recognition by localization factors. Furthermore, the small size of this element makes it an ideal substrate for biochemical assays to identify these factors. Using a UV crosslinking assay, we detect a protein with a Mr of approx. 75×10<sup>3</sup> (p75) that interacts specifically with the +2' element. p75 binding activity is present in crude extracts of ovaries and preblastoderm embryos, consistent with a requirement in nos localization (Fig. 2A). In addition, p75 binding activity is detected in extracts of 6-8-hour-old embryos, suggesting that it is present throughout embryogenesis (data not shown). Since Osk protein is not present at these developmental stages (Rongo et al., 1995), p75 binding must be independent of the germ plasm. Partial purification by ammonium sulfate fractionation yields an approx. 40-fold increase in specific activity (Fig. 2B and data not shown). In contrast to its ability to bind to the +2' element, partially purified p75 does not bind to the +1 or +4 localization elements, or to the region of the nos 3'UTR that lacks localization function (Fig. 2C and data not shown). In addition, p75 binds only weakly to the +3 localization element that lies downstream of +2' in the nos 3'UTR (Fig. 2C). Binding to the radiolabeled +2' target RNA is effectively competed by an excess of unlabeled +2' RNA but not with a 400-fold molar excess of unlabeled +1/TCE or +3 element RNA (Fig. 2D and data not shown), confirming that p75 interacts specifically with +2' sequences.

**A conserved domain mediates +2' element localization function**

To define the requirements for recognition of the +2' element by p75 and the functional significance of this recognition event, we investigated the sequence requirements for +2' localization. The +2' element contains a region of 41 nucleotides that is highly conserved between D. melanogaster and D. virilis (Gavis et al., 1996a; +2'ME in Fig. 1B). To determine whether the localization function of this element is mediated by the conserved domain, we tested its ability to localize nos mRNA when present in three copies. Three direct repeats of the conserved region (+2'ME-3X) were inserted into the 3'UTR of the nos-tub3'UTR transgene (Fig. 1C). Because it lacks nos 3'UTR sequences, the hybrid nos-tub3'UTR RNA is unlocalized (Gavis and Lehmann, 1994). By contrast, nos-tub:nos+2'ME-3X RNA shows substantial posterior localization, similar to that of nos-tub:nos+2'−3X RNA (Fig. 3A,B). Thus, the conserved domain of the +2' element recapitulates localization activity of the intact +2' element. Consequently, we designate this domain as the +2' minimal element (+2'ME).

**Mutations throughout +2'ME disrupt +2' element localization function**

The localization function of the conserved +2'ME indicates that this region contains one or more recognition motifs for localization factors. RNA folding algorithms do not reveal a preferred structural motif conserved between D. melanogaster and D. virilis (E. R. G., unpublished observations). Therefore, we carried out linker-scanning mutagenesis to investigate the sequence requirements for +2' element function. Six nearly contiguous six-nucleotide segments spanning +2'ME were individually substituted by a six-nucleotide restriction site. As a result, two to six nucleotides of each segment were altered (Fig. 4A). Each mutated +2' element (+2'(*)) was inserted in three copies into the α-tubulin 3'UTR sequences of the nos-tub3'UTR transgene, generating a set of nos-tub:nos+2*−3X transgenes. Localization of nos-tub:nos+2(*) RNAs, all of which were stably expressed, was assayed by in situ hybridization.

Strikingly, trimers of five +2' element mutants are devoid of localization function (Fig. 3C-G). The sixth, +2'(F), retains only minimal function (Fig. 3H). Thus, no single sequence
motif within the +2 element mediates its localization function. Rather, recognition of this element requires the contribution of sequences distributed throughout a 38 nucleotide region. This result indicates that the +2 element must be recognized simultaneously by multiple localization factors or that one or more factors recognize features of a complex structure formed by the +2 element.

Two non-contiguous +2 element mutations disrupt binding by p75

To determine how +2 mutations affect recognition by p75, the ability of p75 to bind to each of the six +2 mutants was assessed using a UV crosslinking competition assay (Fig. 4B).

Two mutations, +2(A) and +2(D), fail to compete with the wild-type +2 probe RNA for p75 binding, indicating that the A and D mutations disrupt recognition by p75. +2(E) and +2(F) behave similarly to the wild-type +2 element in their ability to compete for p75 binding. +2(B) and +2(C) are also bound by p75, although less efficiently than +2(E) and +2(F). Thus, the regions identified by mutations A and D are critical for recognition of the +2 element by p75. Both of the six nucleotide regions targeted by these mutations contain the sequence motif CGUU, suggesting that this sequence may be the recognition motif for p75. Alternatively, these two regions of the +2 element may compose a single recognition motif within the context of an RNA structure.

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**Fig. 1.** nos 3'UTR localization elements and construction of nos-tub3'UTR transgene derivatives. (A) The nos 3'UTR is shown, with its localization signal elements indicated (as defined in Gavis et al., 1996a). The +1 element and TCE are coincident. The +1 and +2 elements together comprise the +2 element. (B) Enlargement of the +2 region shown in A, with the 41 nucleotide +2 minimal element (+2'ME), which is conserved between D. melanogaster and D. virilis, indicated by light-gray shading. The sequence of this element is shown (Gavis et al., 1996a); nucleotides within this region that differ in the D. virilis sequence are indicated in gray above. (C) Organization of the nos-tub3'UTR transgene (Gavis and Lehmann, 1994), in which nos 3'UTR sequences are replaced by α-tubulin 3'UTR sequences (tub), is shown. Black boxes at the 5' and 3' ends represent the nos 5'UTR and poly(A) signal, respectively. The nos coding region and introns are indicated by the shaded box. Wild-type and mutant localization element sequences (dotted pattern) were inserted into the α-tubulin 3'UTR as indicated.

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**Fig. 2.** Detection of p75 binding activity by UV crosslinking. (A) UV crosslinking of crude ovarian (O) or embryonic extract (E) to a 32P-labeled +2 RNA probe. An approximately 75 kDa protein (arrowhead) is detected in both extracts. (B) Partial purification of p75 activity by ammonium sulfate fractionation. UV crosslinking to the 32P-labeled +2 RNA probe was carried out using either crude embryonic extract (E) or protein isolated in sequential 0-15%, 15-20%, 20-30%, 30-35%, 35-40%, 40-45% and 45-50% ammonium sulfate fractions. Binding reactions contained volume equivalents of either crude extract or ammonium sulfate fractions. p75 activity peaks at 25-30% ammonium sulfate saturation. This fraction contained 40 times less total protein than the crude extract, as determined by Bradford assay. (C) UV crosslinking of partially purified protein from embryonic extract to 32P-labeled RNA probes encompassing either the +2', the +1/TCE, or the +3 localization elements. (D) UV crosslinking of partially purified protein to the 32P-labeled +2 RNA probe was carried out in the absence (ø) or presence of a 50-, 100-, 200- or 400-fold molar excess of unlabeled +2' or +1/TCE RNA. Molecular mass standards are indicated for each experiment.
Mutations in the +2' element disrupt +2'-3X localization function and its ability to interact with a candidate localization factor. In the context of the nos 3'UTR, however, the +2' element functions together with adjacent localization elements. Strikingly, the combination of the +1 and +2' elements (designated as the +2 element, Fig. 1A) produces near wild-type localization, whereas each element on its own confers only weak localization (Gavis et al., 1996a). To determine how the +2' element contributes to the interaction with the +1 element, we assayed the effect of three +2' element mutations, A, C and E, on +2' element localization function. For this analysis, +2 element sequences bearing either the A, C or E mutation (Fig. 5) were inserted into the nos-tub 3'UTR transgene. Localization of the resulting nos-tub:nos+2(A), +2(C) and +2(E) RNAs was compared with that of the nos-tub:nos+1/TCE and nos-tub:nos+2 RNAs, which bear the wild-type +1 and +2 elements, respectively. All three mutations abolish the interaction between the +2' and +1 elements (Fig. 5). Surprisingly, the localization function of the +2(A), +2(C) and +2(E) mutants is less than that of the +1 element alone, with +2(A) and +2(E) most severely affected. These results indicate that small mutations distributed throughout the +2' element not only affect +2'-3X localization but also the ability of the +2' element to collaborate with the +1 element. Furthermore, these mutations have a long range effect on localization function within the intact +2' element.

**Fig. 3.** Localization by the +2'ME and +2' element mutants. (A,B) Whole-mount in situ hybridization to nos RNA in embryos from nosBN homozygous females that carry the nos-tub:nos+2'-3X (A) and nos-tub:nos+2 ME-3X transgenes (B). The nos localization element sequences present in each transgene are indicated above. (C-H) Whole-mount in situ hybridization to nos RNA in embryos from nosBN homozygous females that carry nos-tub:nos+2(*)-3X transgenes: (C) nos-tub:nos+2(A)-3X; (D) nos-tub:nos+2(B)-3X; (E) nos-tub:nos+2(C)-3X; (F) nos-tub:nos+2(D)-3X; (G) nos-tub:nos+2(E)-3X; (H) nos-tub:nos+2(F)-3X. Since embryos from nosBN females lack endogenous nos RNA (Wang et al., 1994), the nos-specific probe detects only nos RNA produced by the transgenes. Embryos are oriented anterior to the left, dorsal side upwards.
Effect of +2′ mutations on translational regulation

The +2′ element contains a sequence motif (CUGGC) that is also found in the loop of TCE stem-loop II. This motif includes nucleotides that are recognized by the translational repressor Smaug (Smg) and are essential for TCE function (Dahanukar and Wharton, 1996; Smibert et al., 1996; Crucs et al., 2000). While TCE stem-loop II alone is not sufficient for translational repression, three copies of this stem-loop confer repression (Smibert et al., 1996). Similarly, whereas a single +2′ element shows very limited ability to repress translation of unlocalized +2′ RNA, +2′-3X confers repression significantly, indicating that +2′-3X confers significant repression (Bergsten and Gavis, 1999; see below).

Translational repression of nos RNA derivatives can be assayed by the amount of nos activity they produce in embryos from nos or vas mutant females (referred to as nos or vas mutant embryos), which lack nos activity and, consequently, abdominal segments (Gavis et al., 1996b; see Table 1). When completely unlocalized in vas mutant embryos, nos-tub:nos+2′-3X and nos-tub:nos+2′ME-3X RNAs most often produce fewer than four abdominal segments (Table 1), indicating that +2′ME retains the translational repression function of the intact +2′ element. When localized, as in nos mutant embryos, translation of these mRNAs can produce eight abdominal segments (Table 1). Thus, three copies of the 41 nucleotide domain activate translation sufficiently for wild-type development.

Mutations that disrupt +1 element localization function but not TCE-mediated translational repression show that these two functions are separable (Crucs et al., 2000). We therefore examined the effect of +2′ mutations on translational repression by +2′-3X or the intact +2 element. Two mutations, E and F, have little or no effect on +2′-3X repression function while mutations A, B, C and D are deleterious (Table 1). The nos-tub:nos+2′(A)-3X and +2′(D)-3X RNAs produce less nos activity (i.e. significantly fewer embryos with anterior defects) than do nos-tub:nos+2′(B)-3X and +2′(C)-3X, however, indicating that the A and D mutations affect repression less severely than either B or C. The lack of correspondence between the effects of mutations on localization and repression indicate that these functions of the +2′ element, like those of the +1/TCE, are separable.

Inactivation of both the TCE and the +2′ element results in complete loss of translational repression (Dahanukar and Wharton, 1996; Gavis et al., 1996b; Smibert et al., 1996). The failure of the nos-tub:nos+2′(A), +2′(C), and +2′(E) transgenes to promote abdominal segmentation or suppress anterior development in nos mutant embryos demonstrates that unlocalized nos-tub:nos+2′, +2′(C) and +2′(E) RNAs are translationally repressed (Table 1). Taken together, these results show that the +2′ element acts redundantly to the TCE. Furthermore, while all three of these +2′ element mutations show a long range effect on the localization function of the +2 element, this effect does not extend to the translational repression function of the TCE. In addition, TCE-mediated repression is not affected by altered spacing or relative position of the +1 and +2′ elements as neither the nos-tub:nos+2′(A) nor
the nos-tub:nos+2(RO) transgene promotes abdominal segmentation in vas mutant embryos. Both transgenes do produce abdominal segments in nos mutant embryos, however, commensurate with their RNA localization.

DISCUSSION

We have shown that the central 41 nucleotides of the nos 3'UTR +2' element are sufficient for its RNA localization and translational regulatory activities. Remarkably, three copies of this minimal element achieve a sufficient balance between translational repression and RNA localization to permit wild-type development. Small mutations distributed throughout the

| Table 1. Effect of +2' mutations and +2 element organization on translational regulation |
|---------------------------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| % of embryos                    | nos-tub3'UTR | +1/TCE | +2 | +2'3X | +2'ME-3X | +2'(A)-3X | +2'(B)-3X |
| Abdominal segments              | nos' | vas' | nos' | nos' | vas' | nos' | nos' | nos' |
| 0                               | 96   | 100  | --  | --   | 2    | --   | --   | --   |
| 1-3                             | 98   | 72   | 53  | --   | --   | --   | --   | --   |
| 4-6                             | 28   | 2    | 43  | 3    | --   | --   | --   | --   |
| 7-8                             | 98   | 98   | 2   | 97   | 100  | 100  |     |     |
| Anterior defects                | 100  | 2    | 2   | 100  | 100  |     |     |     |
| +2'(C)-3X                       | 92   | 64   | 8   | 79   | 44   |     |     |     |
| +2'(D)-3X                       | 72   | 34   | --  | --   | --   |     |     |     |
| +2'(E)-3X                       | 5    | --   | --  | --   | --   |     |     |     |
| +2'(F)-3X                       | 2    | 2    | --  | --   | --   |     |     |     |
| +2'(A)                          | 21   | 8    | --  | --   | --   |     |     |     |
| +2'(C)                          | 21   | 8    | --  | --   | --   |     |     |     |
| +2'(E)                          | 21   | 8    | --  | --   | --   |     |     |     |
| Abdominal segments              | nos' | nos' | nos' | nos' | nos' | nos' | nos' | nos' |
| 0                               | --   | --   | --  | --   | --   | --   | --   | --   |
| 1-3                             | 28   | 64   | 8   | 79   | 44   |     |     |     |
| 4-6                             | 67   | 34   | --  | --   | --   |     |     |     |
| 7-8                             | 100  | 100  | 5   | --   | --   |     |     |     |
| Anterior defects                | 94   | 6    | --  | --   | --   |     |     |     |
| +2(A)                           | 21   | 8    | --  | --   | --   |     |     |     |
| +2(C)                           | 21   | 8    | --  | --   | --   |     |     |     |
| +2(E)                           | 21   | 8    | --  | --   | --   |     |     |     |
| +2'(A)                          | 21   | 8    | --  | --   | --   |     |     |     |
| +2'(C)                          | 21   | 8    | --  | --   | --   |     |     |     |
| +2'(E)                          | 21   | 8    | --  | --   | --   |     |     |     |
| Abdominal segments              | nos' | nos' | nos' | nos' | nos' | nos' | nos' | nos' |
| 0                               | 100  | 100  | 100 | 100  | 100  | 100  | 100  | 100  |
| 1-3                             | 94   | 94   | 94  | 94   | 94   | 94   | 94   | 94   |
| 4-6                             | 6    | 6    | 6   | 6    | 6    | 6    | 6    | 6    |
| 7-8                             | --   | --   | --  | --   | --   | --   | --   | --   |
| Anterior defects                | --   | --   | --  | --   | --   | --   | --   | --   |

The ability of nos-tub3'UTR derivatives to promote abdominal segmentation and anterior defects in nos mutant embryos reflects the contribution of both localized, translationally active RNA and unlocalized RNA to Nos protein synthesis. The contribution of localized RNA is eliminated, however, when these transgenes are assayed in vas mutants, where nos RNA localization is abolished (Wang et al., 1994; Gavis et al., 1996b). The unregulated nos-tub3'UTR transgene produces eight abdominal segments and suppresses anterior development in nos and vas mutant embryos (Gavis and Lehmann, 1994). By contrast, the regulated nos-tub:nos+1/TCE and nos-tub:nos+2 transgenes fail to promote abdominal segmentation or anterior defects in vas mutant embryos. In nos mutants, these transgenes promote abdominal segmentation commensurate with the extent to which their mRNAs are localized (Gavis et al., 1996b; this table).

Cuticular phenotypes of embryos produced by vas or nos mutant females expressing the indicated nos-tub3'UTR transgene derivatives were analyzed in preparations of >100 embryos for each transgene. The values for nos-tub3'UTR, nos-tub:nos+1/TCE, and nos-tub:nos+2 transgenes are taken from Gavis et al. (Gavis et al., 1996b) and for nos-tub:nos+2(3X) from Bergsten and Gavis (Bergsten and Gavis, 1999). In most cases, the values shown for the occurrence of abdominal segments and anterior defects represent the average percentages obtained from analysis of two to five independent lines in each mutant background. Only one third chromosome insertion was obtained for +2'(A)-3X and only one second chromosome insertion for +2'(B)-3X. Consequently, the values for +2'(A)-3X in vas' and for +2'(B)-3X in nos' are derived from single lines. Expression of the nos-tub:nos+2'(B)-3X transgene, whose RNA levels can be assayed in nos' embryos, is comparable with that of other transgenes used in this analysis.
Surprisingly, mutations distributed throughout the +2' minimal element have a long range effect within the +2 element. Alteration of as few as two nucleotides nearly or completely eliminates +2 element localization function and the ability of the +1 element alone to interact with the localization machinery. This result indicates that although the +1 element can interact independently, albeit weakly, with the localization machinery, this independent function is lost in the +2 element and the intact localization signal. Rather, sequences or local structures within the +1 element may normally participate in formation of a higher order structure with sequences or structures from the +2' element. +2' mutations may disrupt +2 element function by disrupting subdomains of +2 element structure or the interaction of a +2 element-protein complex with germ plasm components, without disrupting participation of +1 element sequences/structures. Consistent with the contribution of +1 and +2' element sequences to a larger structure, our previous analysis showed that the combination of mutations in two different regions of the +1 element affects collaboration of the +1 and +2' elements (Crucs et al., 2000). In addition, this idea is supported by the results of altering the spacing and relative positions of the +1 and +2' elements. The separation of +1 and +2' element sequences could still permit secondary or tertiary interactions to occur, whereas altering their relative positions would not.

The ability of multiple mutations spanning the conserved +2'ME to disrupt localization suggests that localization depends on either the simultaneous binding of multiple proteins to distinct sequence motifs or the binding of one or more proteins to a complex structural motif. Recognition of the +2' element by p75 requires the integrity of two non-contiguous sets of nucleotides. p75 may bind as an obligate dimer, with each molecule contacting one binding site. Folding of the RNA may be necessary to bring the sites into close proximity or, alternatively, may create a single binding motif within a larger secondary structure. The fact that mutations B and C, which lie between A and D, have some effect on p75 binding while mutations E and F, which lie outside this region, behave as wild-type is consistent with an interaction dependent on structural features of the RNA. The purification of p75 and the generation of additional +2' element mutations will facilitate the quantitative biochemical analysis required to distinguish between these possibilities. However, the ability of mutations to disrupt localization without affecting binding by p75 indicates that at least one other factor is required for +2' localization function in vivo.

The sensitivity of the +2' element to mutation resembles that of the +1 element (Crucs et al., 2000). While the secondary structure of the TCE is well conserved, analysis of mutations that disrupt +1 element localization function does not support a requirement for this structural motif in localization, and RNA folding algorithms do not predict alternative structures that might mediate localization (Crucs et al., 2000). Similarly, secondary structure requirements for +2' element localization function do not appear to be readily predicted or assayed by mutagenesis. Multiple isoenergetic structures predicted for the +2' element by RNA folding algorithms reveal little similarity between structures predicted for the D. melanogaster and D. virilis +2' sequences (E. R. G., unpublished observations). If formation of specific RNA structures is indeed required for localization function, these alternate structures may be driven or stabilized by the binding of localization factors such as p75 and, thus, would not be readily calculated.

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