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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 Hazelrigg, 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; Hazelrigg, 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; Dahanukar and Wharton, 1996; Fig. 1A). TCE function requires the formation of two stem-loop structures (Crucs 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 (Crucs 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. virilis*. 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 H_2O 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 $MgCl_2$, 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 polyethylenimine (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 $(NH_4)_2SO_4$ fractionation. Protein precipitated at increasing $(NH_4)_2SO_4$ 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% $(NH_4)_2SO_4$, 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. ^{32}P-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. ^{32}P-labeled RNAs were quantified by scintillation counting and unlabeled mRNAs by spectophotometry.

Protein was mixed with 1×10^6 dpm of ^{32}P-labeled RNA in 1× binding buffer (5 mM HEPES (pH 7.4), 2 mM $MgCl_2$, 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 ^{32}P-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+1* transgene (Gavis and Lehmann, 1996a) is identical to the *nos-tub:TCE* transgene (Crucs 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(RO)* 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(^)*, +2′ element sequences were inserted into a *Bsa*AI 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 *Avr*II 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 (Mismer and Rubin, 1987) and the resulting P-element plasmids were injected into ry^{506} 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^{BN} allele (Wang et al., 1994) or transheterozygous for the vas^{PD} and vas^{D1} 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 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

In situ hybridization to 0-1.5 hour embryos produced by homozygous nos^{BN} females that carried one copy of a given transgene was performed according to Gavis and Lehmann (Gavis and Lehmann, 1992). Since embryos from nos^{BN} 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 LX112 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 M_r of approx. 75×10^3 (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

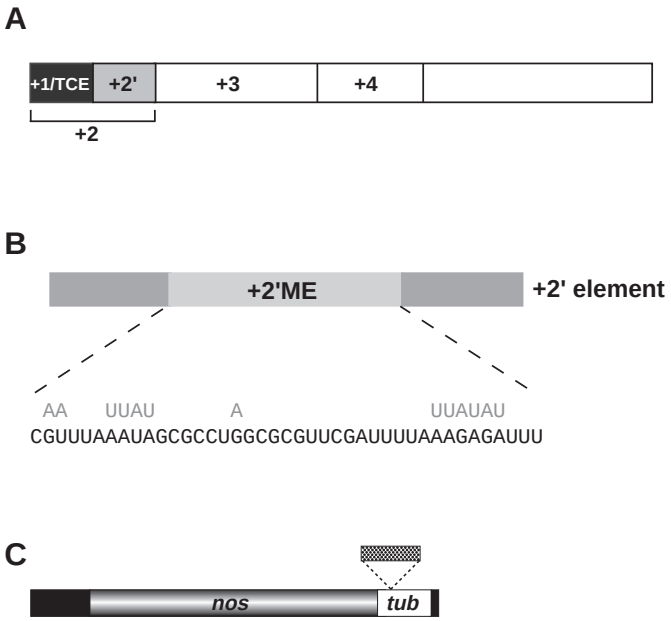

Fig. 1. *nos* 3′UTR localization elements and construction of *nos-tub3′UTR* transgene derivatives. (A) The *nos* 3′UTR is shown, with its localization signal elements indicated (as defined in Gavis et al., 1996a). The +1 element and TCE are coincident. The +1 and +2′ elements together comprise the +2 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.

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.

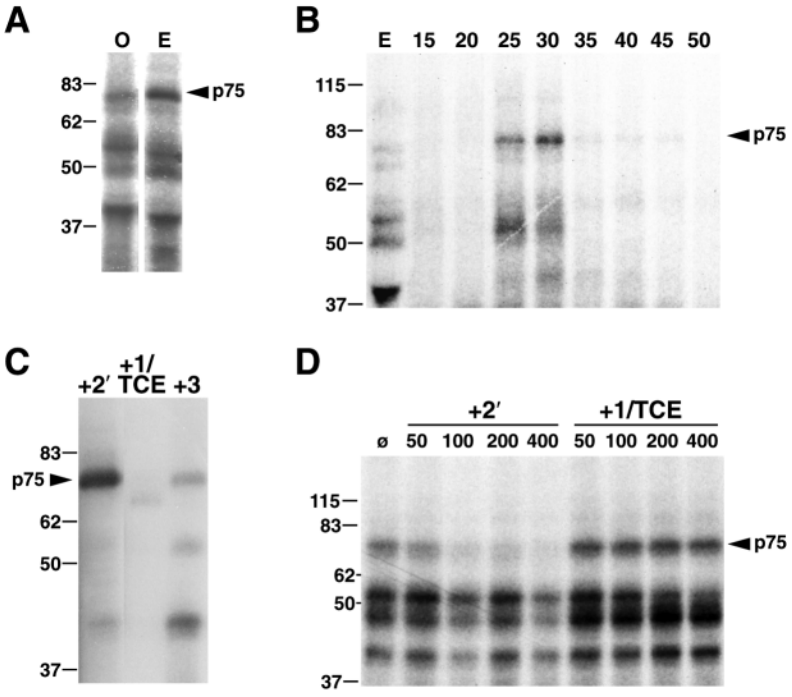

Fig. 2. Detection of p75 binding activity by UV crosslinking. (A) UV crosslinking of crude ovarian (O) or embryonic extract (E) to a ^{32}P-labeled +2′ RNA probe. An approximately 75 kDa protein (arrowhead) is detected in both extracts. (B) Partial purification of p75 activity by ammonium sulfate fractionation. UV crosslinking to the ^{32}P-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 ^{32}P-labeled RNA probes encompassing either the +2′, the +1/TCE, or the +3 localization elements. (D) UV crosslinking of partially purified protein to the ^{32}P-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.

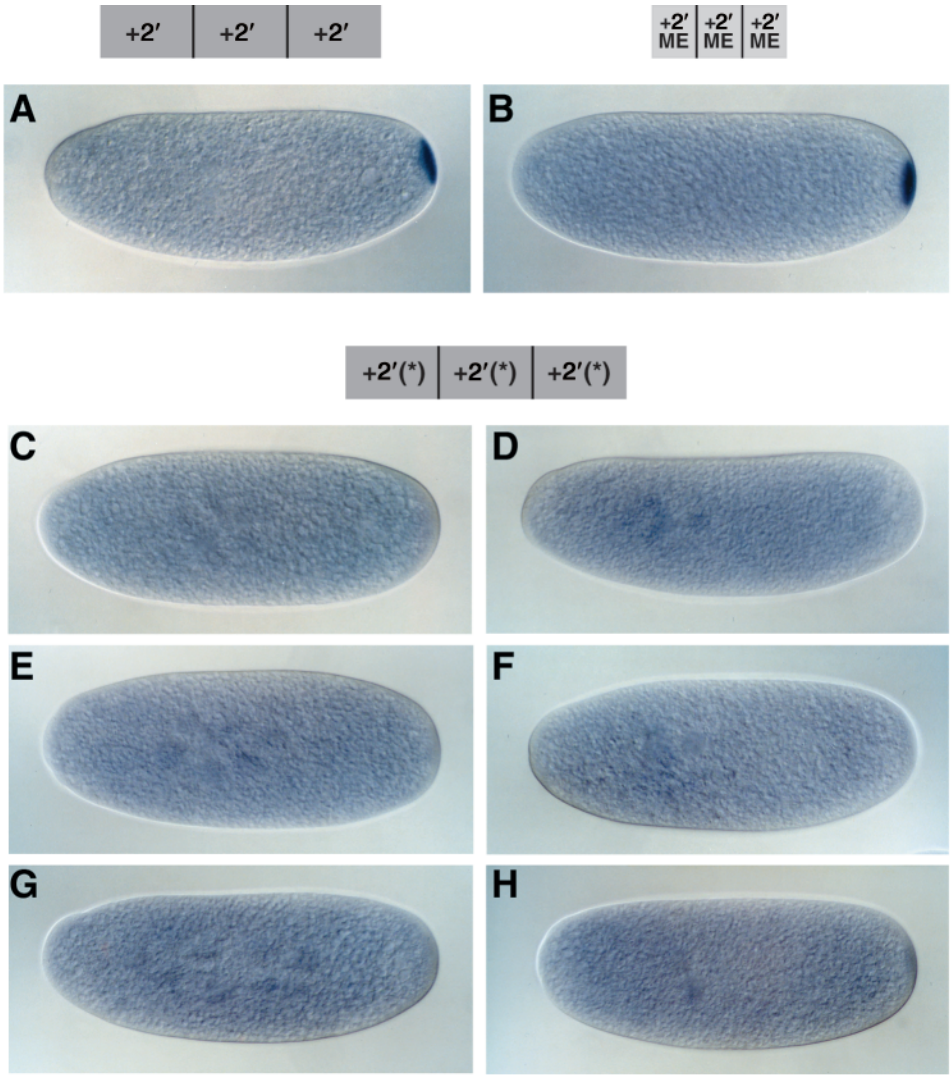

Fig. 3. Localization by the +2′ME and +2′ element mutants. (A,B) Whole-mount in situ hybridization to *nos* RNA in embryos from *nos*^{BN} homozygous females that carry the *nos-tub:nos+2′-3X* (A) and *nos-tub:nos+2′ME-3X* transgenes (B). The *nos* localization element sequences present in each transgene are indicated above. (C-H) Whole-mount in situ hybridization to *nos* RNA in embryos from *nos*^{BN} 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 *nos*^{BN} 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.

+2′ element mutations have long range effects on localization signal function

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-tub3′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.

Dependence of +2 element function on the organization of its sub-elements

The synergistic interaction between the +1 and +2′ elements and the long range effect of +2′ element mutations within the +2 element suggests that the spatial relationship between these elements may be important. To determine whether the +1 and +2′ elements must be adjacent to collaborate, we tested the effect of separating the +1 and +2′ elements using a 130 nucleotide spacer. Localization of *nos-tub:nos+2(^)* RNA is similar to that of *nos-tub:nos+2* RNA, which contains a wild-type +2 element (Fig. 6A,B). To determine whether the relative positions of the +1 and +2′ elements within the larger +2 element is critical to their ability to interact, we reversed their order and assayed localization of the resulting *nos-tub:nos+2(RO)* RNA. Localization of *nos-tub:nos+2(RO)* RNA is reduced relative to *nos-tub:nos+2* RNA, and is comparable with or slightly stronger than that of *nos-*

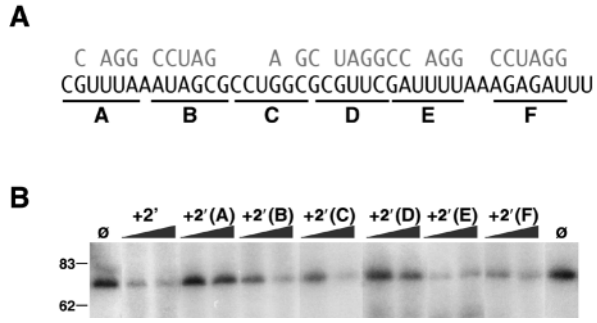

Fig. 4. Effect of +2′ element mutations on binding by p75. (A) +2′ element mutations. The sequence of +2′ME is shown with the nucleotides replaced by an *Avr*II restriction site in each mutant underlined. Nucleotide changes resulting from these replacements are indicated in gray above. (B) UV crosslinking competition assay similar to that shown in Fig. 2D. Each pair of lanes shows crosslinking of partially purified protein to the ^{32}P-labeled +2′ RNA probe in the presence of a 200- or 400-fold molar excess of unlabeled wild-type +2′ or +2′(*) RNA as indicated. Lanes designated as (ø) show crosslinking of p75 to the ^{32}P-labeled +2′ RNA probe in the absence of unlabeled competitor. Molecular mass standards are indicated.

tub:nos+1/TCE RNA (Fig. 6A,C, and data not shown). Taken together, these results show that the relative order of the +1 and +2′ sub-elements within the +2 element is critical for localization function, whereas the distance between them is not.

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 RNA, +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* mutants 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,

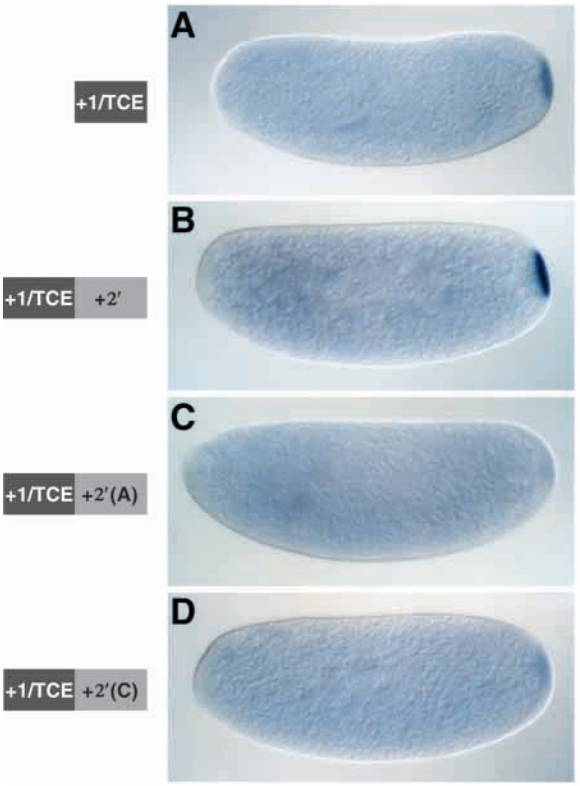

Fig. 5. Localization of the +2 element is disrupted by +2′ mutations. (A) Whole-mount in situ hybridization to *nos* RNA in embryos from *nos*^{BN} homozygous females that carry the following transgenes: (A) *nos-tub:nos+1/TCE*; (B) *nos-tub:nos+2*; (C) *nos-tub:nos+2(A)*; (D) *nos-tub:nos+2(C)*. The *nos* localization element sequences present in each transgene are indicated on the left. The *nos-tub:nos+2(E)* transgene (not shown) behaves identically to *nos-tub:nos+2(A)*.

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(A)*, *+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(^)* nor

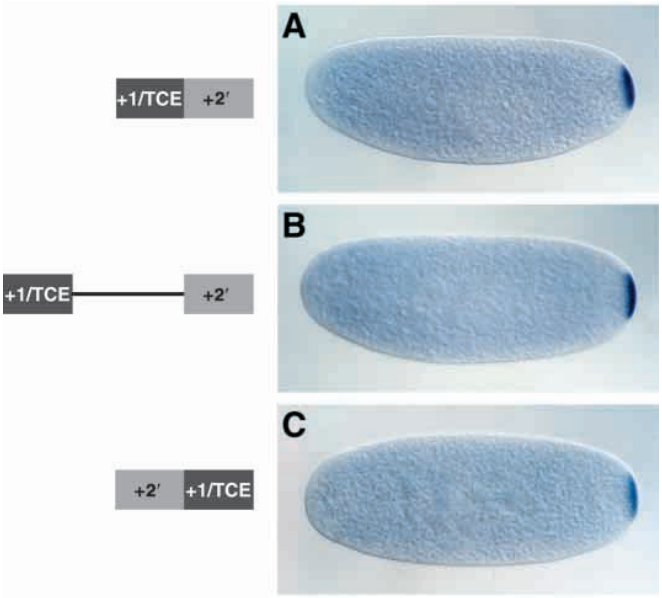

Fig. 6. Effect of +2 element organization on localization function. Localization of *nos-tub:nos+2* RNA (A) was compared with that of *nos-tub:nos+2(^)* RNA (B), in which the +1 and +2′ elements are separated by a 130 nucleotide spacer, or that of *nos-tub:nos+2(RO)* RNA (C), in which the relative positions of the +1 and +2′ elements are reversed. All embryos were derived from nos^{BN} females carrying the corresponding transgene and in situ hybridization was carried out using a nos probe.

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*$^-$	*vas*$^-$	*nos*$^-$	*vas*$^-$	*nos*$^-$	*vas*$^-$	*nos*$^-$	*nos*$^-$	*nos*$^-$
0	–	96	–	100	–	–	–	2	–	–	–
1-3	–	4	98	–	–	72		53	–	–	–
4-6	–	–	2	–	2	28	2	43	3	–	–
7-8	100	–	–	–	98	–	98	2	97	100	100
Anterior defects	100	–	–	–	–	–	2	–	–	2	100

	+2′(C)-3X	+2′(D)-3X	+2′(E)-3X	+2′(F)-3X	+2(A)	+2(C)	+2(E)
Abdominal segments	*nos*$^-$	*nos*$^-$	*nos*$^-$	*nos*$^-$	*nos*$^-$	*nos*$^-$	*nos*$^-$
0	–	–	–	2	92	21	56
1-3	–	–	28	64	8	79	44
4-6	–	–	67	34	–	–	–
7-8	100	100	5	–	–	–	–
Anterior defects	94	6	–	–	–	–	–

	+2(^)		+2(RO)	
Abdominal segments	*vas*$^-$	*nos*$^-$	*vas*$^-$	*nos*$^-$
0	100	–	100	–
1-3	–	–	–	94
4-6	–	6	–	6
7-8	–	94	–	–
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-tub:3′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^{BN} embryos, is comparable with that of other transgenes used in this analysis.

41 nucleotide domain each disrupt +2′ localization function when assayed in the context of the +2′-3X trimer and in the native context of the +2 element. These mutations define a recognition motif for at least one protein, p75. This protein is the first factor identified that interacts specifically with a *nos* localization signal element.

Binding of p75 to the +2′ element is disrupted by two mutations, A and D, which abolish +2′ element localization function. Although these mutations also disrupt translational repression, we believe that p75 is more likely to play a role in localization than in translational repression. First, p75 does not interact with the TCE. In addition, two other +2′ element mutants, B and C, which have more severe effects than A or D on translational repression, retain significant ability to bind p75. Mutation C alters one nucleotide of the Smg binding site (Smibert et al., 1996), whereas A, B and D lie outside the reiterated TCE loop motif. The potential for nucleotides altered in mutations A, B, and D to participate in formation of a Smg-binding loop (Dahanukar and Wharton, 1996; Smibert et al., 1995; our results) suggests that these mutations disrupt translational repression most likely by affecting Smg binding. Nonetheless, molecular identification and genetic analysis of p75 will be essential to determine its role in vivo.

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.

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 long range effects of +2′ element mutations are specific to the localization function of the +2 element. Translational repression by the TCE is not affected by +2′ mutations, indicating that they do not prevent formation of TCE structure in unlocalized *nos* RNA. Similarly, we have identified mutations in the +1 element that retain TCE function but disrupt the ability of the +1 and +2′ elements to interact (Crucs et al., 2000). Based on our analysis of +2′ element mutations, we suggest that localization factors recognize or promote formation of an alternate conformation of the *nos* 3′UTR than that recognized by translational repressors. Since p75 binding is independent of the germ plasm components, formation of this structural motif, aided by factors such as p75, may be required prior to association of *nos* RNA with the germ plasm anchor. Our previous studies suggest that binding by translational repressors and localization factors is mutually exclusive, and that RNA localization activates *nos* translation by preventing binding of translational repressors (Bergsten et al., 1999; Crucs et al., 2000). The ability to form alternative structures can explain the mutually exclusive relationship between translational repression and localization.

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