Cell, Vol. 80, 747–756, March 10, 1995, Copyright © 1995 by Cell Press

Binding of Pumilio to Maternal *hunchback* mRNA Is Required for Posterior Patterning in Drosophila Embryos

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Summary

Posterior patterning in Drosophila embryos is governed by nanos (nos), which acts by repressing the translation of maternal transcripts of the *hunchback* (*hb*) gene. Sites in *hb* mRNA that mediate this repression, named nanos response elements (NREs), have been identified. However, we know of no evidence of a direct interaction between nos, or any other protein, and the NRE. Here, we show that two proteins present in embryonic extracts, neither one nos, bind specifically to the NRE in vitro. Furthermore, we show that binding in vitro correlates with NRE function in vivo. One of the NRE-binding factors is encoded by *pumilio* (*pum*), a gene that, like *nos*, is essential for abdominal segmentation. These and other observations suggest that pum acts by recognizing the NRE and then recruiting nos. Presumably, the resulting complex inhibits some component of the translation machinery.

Introduction

During the syncytial development of Drosophila melanogaster embryos, two determinants localized to opposite poles of the egg organize most of the anteroposterior pattern (Frohnhöfer et al., 1986; Frohnhöfer and Nüsslein-Volhard, 1986; Lehmann and Nüsslein-Volhard, 1986). At the anterior, *bicoid* (*bcd*) mRNA is anchored to the cortical cytoplasm (Frigerio et al., 1986; St Johnston et al., 1989), while at the posterior, *nanos* (*nos*) mRNA is associated with the pole plasm, which contains the germline determinants (Wang and Lehmann, 1991; Ephrussi and Lehmann, 1992; Smith et al., 1992). Following fertilization, each of these transcripts gives rise to a gradient of protein emanating from the appropriate pole of the embryo (Driever and Nüsslein-Volhard, 1988; Ephrussi and Lehmann, 1992; Smith et al., 1992).

The mechanism by which the bcd gradient provides positional information is well established. In vivo, bcd regulates the zygotic transcription of *hunchback* (*hb*) (as well as other genes) (Tautz, 1988; Simpson-Brose et al., 1994; Cohen and Jürgens, 1990; Finkelstein and Perrimon, 1990). The bcd protein contains a homeodomain DNA recognition motif and a distinct transcriptional activation domain. In embryos, bcd binds to specific sites in the promoter region of target genes and stimulates their transcription (Driever and Nüsslein-Volhard, 1989; Struhl et al., 1989).

In contrast, the mechanism of nos action is less well understood. In the preblastoderm embryo, the nos gradient generates an opposing gradient of hb by blocking translation from maternal *hb* transcripts (Tautz, 1988; Wharton and Struhl, 1991). Genetic experiments have demonstrated that these transcripts are the only significant regulatory target of nos in the embryo (Hülskamp et al., 1989; Irish et al., 1989; Struhl, 1989). The sites in *hb* mRNA that mediate nos activity, called nanos response elements, or NREs, have been identified (Wharton and Struhl, 1989); however, we know of no evidence demonstrating a direct interaction of nos with these sites. Furthermore, the nos protein sequence bears no obvious homology to known RNA-binding proteins (Wang and Lehmann, 1991).

Recently, another factor that is required specifically for posterior patterning has been identified. In the absence of *pumilio* (*pum*) function (Lehmann and Nüsslein-Volhard, 1987), hb is expressed uniformly throughout the posterior of the embryo; in consequence, the embryo develops no abdominal segments (Barker et al., 1992). The *nos* and *pum* mutant phenotypes therefore are indistinguishable in this respect, and it has not been possible to determine the order of action of one with respect to the other. The sequence of pum suggests little about its function, and pum protein is distributed uniformly throughout the cytoplasm of the preblastoderm embryo (Macdonald, 1992). Pum generally has been considered to act as a cofactor in the repression of *hb* translation, with nos providing the spatial information as well as the molecular specificity to the reaction.

Translational regulation of maternal messages is thought to play a significant role during development in many organisms, including mice (Vassalli et al., 1989), Xenopus laevis (e.g., Klein and Melton, 1994), and Caenorhabditis elegans (Ahringer and Kimble, 1991; Goodwin et al., 1993; Lee et al., 1993; Wightman et al., 1993). However, in many of these cases, the *trans*-acting regulators have not been identified, or their interactions with *cis*-acting targets in the appropriate mRNA have not been well defined. In this report, we characterize factors in embryonic extracts that bind to NRE sequences, in order to understand better the mechanisms by which *hb* translation is regulated. We describe some properties of two factors present in embryonic extracts, neither one nos, that bind specifically to different regions of the NRE. Using mutant NREs, we show that the binding of these factors in vitro correlates with NRE function in vivo. Several lines of evidence lead to the conclusion that one of these factors is pum, and that both pum and a 55 kDa factor bind to *hb* mRNA in the absence of nos activity. These results suggest a model for NRE function in which the RNA is recognized by pum and perhaps by the 55 kDa factor. We further suggest that the NRE-bound proteins serve to recruit nos, which then interacts with the translation machinery.

Results

Embryonic Proteins That Bind Specifically to the *hb* NRE

To identify factors that interact with the regulatory target

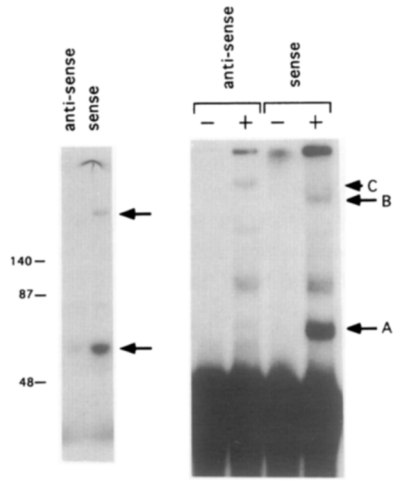

Figure 1. Two Proteins Bind Specifically to the NRE

Shown are the results of a UV cross-linking experiment on the left and of a gel mobility shift experiment on the right. Binding assays were performed in the presence of 100 mM KCl as described in the Experimental Procedures, using cytoplasmic extracts from wild-type embryos and either RNA molecules bearing a single copy of the wild-type NRE (sense), or molecules bearing sequences complementary to the NRE (antisense). The bold arrows labeled as A and B indicate the two protein–RNA complexes that we observe reproducibly in both assays, and the arrowhead labeled as C indicates an NRE-nonspecific complex that is barely detectable here (but is prominent in Figure 5, for example). Note that the 55 kDa NRE-binding protein also binds weakly to the antisense RNA and other control RNAs (see Figure 5, for example). For the UV cross-linking panel, the numbers indicate the sizes of protein molecular weight markers in kilodaltons. For the gel mobility shift panels, minus and plus indicate that reaction mixtures were incubated in the absence and presence of embryonic extract, respectively.

in *hb* mRNA, we prepared crude, unfractionated extracts containing cytoplasmic proteins from early Drosophila embryos. We also prepared small radiolabeled RNAs, each containing a single copy of an NRE that confers *nos*-dependent regulation in vivo. Protein and RNA solutions were incubated, and then protein–RNA interactions were detected by either of two methods: covalent transfer of ^{32}P to proteins following ultraviolet (UV) irradiation, or electrophoretic resolution of complexes in nondenaturing polyacrylamide gels.

As shown in Figure 1, two proteins with approximate molecular weights of 55 kDa and 165 kDa bind to the NRE, as assayed by UV cross-linking. In control experiments using similar RNA molecules that lack an NRE, binding of the 165 kDa protein is undetectable, and binding of the 55 kDa protein is dramatically reduced. Although we have not optimized the reaction conditions, two observations proved to be particularly useful for further characterization of the binding proteins. First, the 165 kDa activity appears to be relatively labile, as it is not detectable when extracts are prepared in the absence of protease inhibitors (data not shown). Second, the 55 kDa binding activity is observed in the presence of 100 mM KCl but not in its absence (data not shown).

The results of gel mobility shift assays are consistent with those obtained in UV cross-linking experiments. As shown in Figure 1, we have reproducibly resolved two NRE-specific protein–RNA complexes. Several lines of evidence support the idea that the complex identified as A in Figure 1 contains the 55 kDa protein, whereas complex B contains the 165 kDa protein. First, omission of KCl or protease inhibitors from the reaction mixture dramatically diminishes the quantity of complexes A and B, respectively. Second, isolation of complex A followed by UV irradiation results in the covalent transfer of ^{32}P to a protein that comigrates with the 55 kDa factor (data not shown; see Experimental Procedures). Finally, the results of experiments using mutant NREs (described below) are consistent with the idea that binding of the 55 kDa factor generates complex A and binding of the 165 kDa factor generates complex B.

We do not see evidence for the existence of a higher order complex consisting of both proteins bound to the same RNA molecule. Such a complex might either be unstable in our gel system or present at undetectably low levels. Alternatively, it might resolve poorly from either complex B or complex C; the latter, which we do not observe reproducibly, migrates more slowly than B and is not NRE specific (Figure 1; see Figure 5).

The 165 kDa Protein Is Pum

The pum protein in embryos has an approximate molecular weight of 160 kDa (Macdonald, 1992), which led us to consider whether the protein component of the 165 kDa covalent protein–RNA complex might be pum. This suggestion, that pum might bind directly to the NRE, was somewhat heterodox, since it is an ubiquitous rather than a spatially restricted protein; in addition, pum protein has none of the well-characterized RNA-binding motifs (Burd and Dreyfuss, 1994).

We first investigated the possibility that pum binds directly by examining the NRE-binding activities in extracts prepared from *pum* mutant embryos. As indicated in Figure 2A, the 165 kDa NRE-binding activity is not present in these *pum*$^-$ extracts, although the 55 kDa activity is present at levels comparable to those in wild-type extracts. Western blots probed with pum-specific antibodies demonstrated that the *pum*$^-$ embryo extracts contain no detectable full-length protein (data not shown). Thus, production of the 165 kDa factor is dependent on *pum* function in vivo.

We next asked whether the 165 kDa factor is recognized by pum-specific antibodies in vitro. As shown in Figure 2B, preincubation of wild-type embryo extracts with anti-pum antibodies, but not control antibodies, blocks the binding of the 165 kDa protein to the NRE; binding of the 55 kDa protein is unaffected by either type of antibody. The simplest interpretation of these observations is that the 165 kDa factor is pum.

To prove that pum binds directly to the NRE, we prepared extracts containing pum protein from two sources: COS cells transiently transfected with DNA that directs the expression of pum, and reticulocyte lysates programmed with synthetic *pum* message. As shown in Figure 2C, both extracts contain an activity that is indistinguishable from the activity of the 165 kDa factor present in embryonic extracts; in contrast, control extracts (prepared from

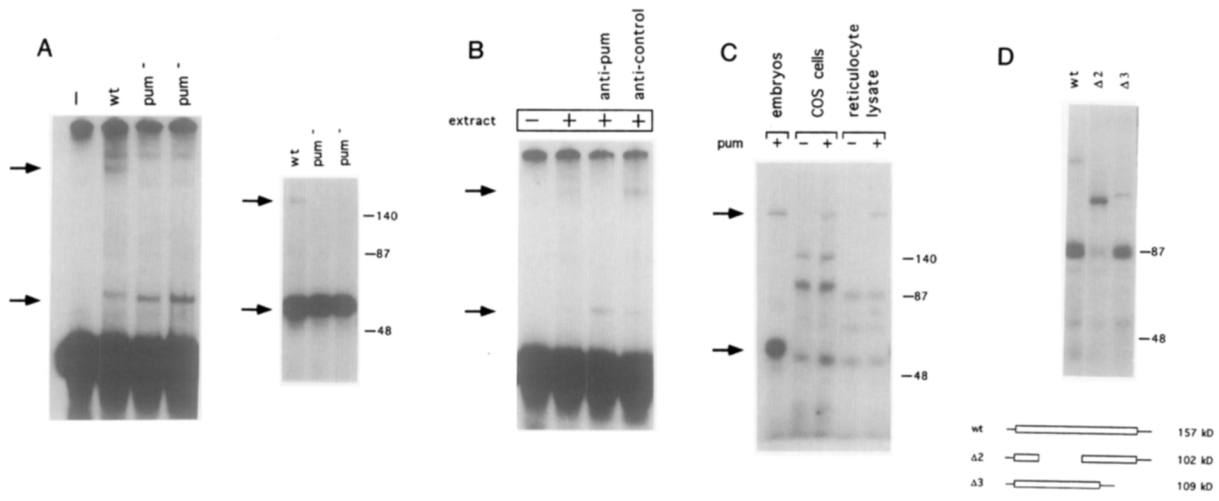

Figure 2. Pumilio Binds to the NRE

(A) *pum*⁻ embryos lack the 165 kDa NRE-binding activity. Shown are the results of gel mobility shift (left) and UV cross-linking (right) experiments in which RNA bearing a wild-type NRE was incubated with either no extract (minus), extract prepared from wild-type embryos (wt), or extracts prepared from embryos derived from two different types of *pum*⁻ females (pum⁻; see Experimental Procedures for details). The arrows point to the complexes formed by binding of pum (top) and the 55 kDa factor (bottom). The NRE-nonspecific complex C is clearly visible in lanes 2–4 of the gel shift experiment. Note that the gel shift experiment was performed in the absence of KCl.

(B) Pum-specific antibodies block binding of the 165 kDa protein. Wild-type embryo extracts were preincubated in the absence of KCl with serum containing pum-specific antibodies (anti-pum) or control serum (anti-control) containing antibodies that react weakly with an irrelevant protein, tailless. These pretreated extracts were then used in a gel mobility shift experiment, the results of which are shown. The arrows point to the complexes formed by binding of pum (top) and the 55 kDa factor (bottom). Note that the addition of serum slightly enhances binding of the 55 kDa factor to a variable extent; we assume that this is caused by the presence of salt(s) in the serum, as no similar effect is observed when the reaction mixture contains 100 mM KCl.

(C) Pum produced in COS cells or reticulocyte lysates binds to the NRE in the absence of other Drosophila proteins. Shown are the results of UV cross-linking experiments in which NRE-bearing RNA was incubated with extracts prepared from embryos (lane 1), extracts prepared from transiently transfected COS cells (lanes 2 and 3), or the products of in vitro protein synthesis in rabbit reticulocyte lysates (lanes 4 and 5). Above each lane, plus indicates the presence in embryos, COS cells, or lysate (as appropriate) of mRNA encoding pum, and minus indicates its absence. The arrows point to the complexes formed by binding of pum (top) and the 55 kDa factor (bottom), and the numbers at the right indicate the sizes of protein molecular weight markers in kilodaltons. Note that both COS cells and the reticulocyte lysate contain a protein that binds to the NRE-bearing RNA and is similar in size to the 55 kDa factor in embryonic extracts. We do not know whether this binding is NRE specific.

(D) Pum binds directly to the NRE. Rabbit reticulocyte lysates were programmed with RNA encoding either wild-type pum (wt) or two truncation derivatives (Δ2 and Δ3). The resulting lysates were then used in UV cross-linking experiments as described above. The proteins encoded by these derivatives, and their molecular weights, are indicated schematically below. PumΔ2 was constructed by deletion of a SmaI–SmaI fragment in *pum*, and PumΔ3 was constructed by deletion of sequences distal to a BglII site in *pum*.

mock-transfected COS cells or unprogrammed reticulocyte lysates) contain no such activity. Finally, we have shown that truncated derivatives of pum produced in reticulocyte lysates bind to the NRE in UV cross-linking assays (Figure 2D), demonstrating that the pum–NRE interaction is direct. We conclude that pum binds specifically to the NRE. Furthermore, it can do so in the absence of other Drosophila proteins, although we cannot exclude the possibility that both extracts contain auxillary factors that substitute for similar factors in the Drosophila embryo.

The 55 kDa Factor Is Not Nos

We next asked whether the 55 kDa factor is nos. The nos protein in embryos consists of a heterogeneous collection of molecules with a median molecular weight of approximately 47 kDa (Gavis and Lehmann, 1994). Thus, the protein component of the 55 kDa protein–RNA complex could be nos. However, three lines of evidence demonstrate that this is not the case.

First, the amount of 55 kDa activity we detect in embryonic extracts is insensitive to changes in the intracellular level of nos. We prepared extracts from mutant embryos that contain dramatically different levels of nos protein; relative to wild-type embryos, embryos from nos^{BN} females contain almost undetectable levels of nos, whereas embryos from females carrying an altered *nos* transgene (Gavis and Lehmann, 1992) substantially overproduce nos protein (Figure 3). Strikingly, the levels of 55 kDa binding activity in these two mutant extracts and in a wild-type extract are essentially indistinguishable.

Second, embryonic extracts prepared from *oskar* (*osk*)⁻ females contain levels of 55 kDa binding activity that are essentially indistinguishable from those in wild-type extracts (data not shown). Previous studies have shown that *osk* function is required for the localization of *nos* mRNA and the accumulation of nos protein in embryos (Smith et al., 1992; Ephrussi and Lehmann, 1992; Gavis and Lehmann, 1994).

Third, we prepared extracts from nos^{RC}, nos^{RD}, nos^{RW}, and nos^{L7} mutant flies. These alleles have different properties in vivo (Lehmann and Nüsslein-Volhard, 1991; Wang et al., 1994) and have different types of lesions (data not shown). Nevertheless, each mutant extract contains normal levels of a 55 kDa NRE-binding activity that is indistin-

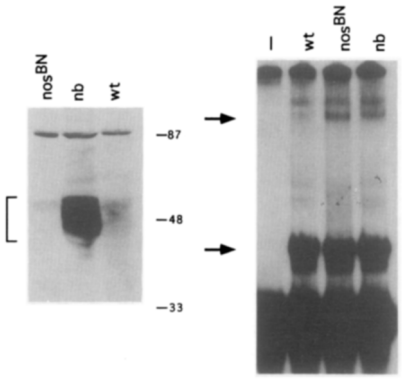

Figure 3. The 55 kDa Factor Is Not Nos

Shown on the left is a Western blot in which nos protein is detected with specific antibodies. Extracts were prepared from embryos derived from *nos*^{BN} mutant females (lane 1), females expressing an altered *nos* transgene (nb) that causes the ectopic expression of nos (lane 2; see Gavis and Lehmann, 1992), or wild-type embryos (lane 3). The bracket at the left indicates that the nos in wild-type embryos consists of a heterogeneous population of molecules, as seen by Gavis and Lehmann (1994). The prominent band near the top of the photograph is a cross-reacting protein. The sizes of protein molecular weight markers in kilodaltons are indicated at the right.

Shown on the right is the result of a gel mobility shift assay using aliquots of the same extracts. (In lane 1, minus indicates incubation in the absence of any extract.) The arrows point to the complexes formed by binding of pum (top) and the 55 kDa factor (bottom). Note that the relative amount of pum activity that we recover in different preparations of embryonic extract is somewhat variable, and we assume that this variability accounts for the small differences seen here.

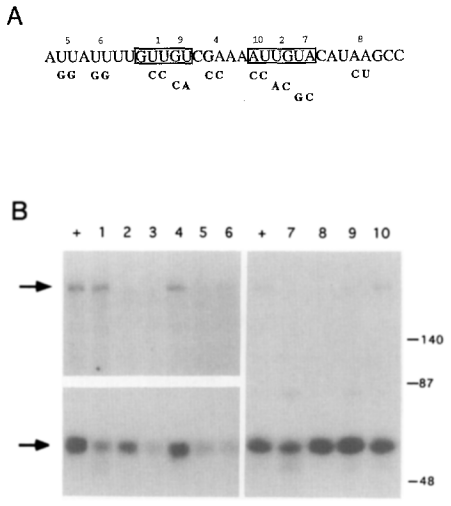

Figure 4. Binding of Pum and the 55 kDa Factor to Mutant NREs In Vitro

(A) Identities of the mutant NREs. Shown is the sequence of one of the wild-type NREs present in *hb* mRNA; when substituted for the endogenous sites, the resulting *hb* mRNA derivative is regulated in a manner indistinguishable from wild-type *hb* mRNA (Wharton and Struhl, 1991). Therefore, we have used this site as the basis for experiments in this report. Each mutant NRE (identified by number above) contains a tandem dinucleotide substitution (identified below). NRE3 is a compound mutant, containing the four substitutions present in NRE1 and NRE2.

(B) UV cross-linking assays. Shown are the results of UV cross-linking experiments in which aliquots of wild-type embryonic extract were incubated with RNA molecules bearing wild-type (plus) or various mutant NREs, as indicated at the top. Note that the figure is a composite of two experiments, with mutants 1–6 analyzed on one gel, and mutants 7–10 analyzed on another. Note also that the left part of the figure is derived from two different autoradiographic exposures of the same gel. Two additional points are as follows. First, the binding of pum to NRE9, which is weak in this experiment, is indistinguishable from binding to NRE^{+} in the presence of 0 mM KCl (data not shown). Second, NRE5 and NRE6 bind weakly to both proteins in this assay and in gel mobility shift assays (data not shown). The arrows point to the complexes formed by binding of pum (top) and the 55 kDa factor (bottom), and the sizes of protein molecular weight markers in kilodaltons are indicated at the right.

tinguishable from that in wild-type extracts (data not shown).

Taken together, these observations show that the 55 kDa factor is not nos. Furthermore, since the binding of the 55 kDa factor or pum occurs with equal efficiency in the presence and absence of nos function, it is unlikely that nos is required in order to stabilize the interaction between either of these factors and the NRE. Finally, we note that we have been unable to detect a specific interaction between nos and the NRE by using extracts containing high levels of nos protein prepared either from embryos or from transfected COS cells (data not shown).

Binding of Pum to the NRE Correlates with Its Function In Vivo

While the results described above show that pum and the 55 kDa factor bind specifically to the wild-type NRE in vitro, they do not demonstrate that these interactions are physiologically relevant. To address this issue, we tested the activities of a collection of mutant NREs in vitro and in vivo. Each of these sites bears a tandem dinucleotide substitution (Figure 4A). To assay NRE activity in vitro, we performed UV cross-linking and gel retardation experiments using wild-type embryonic extracts and synthetic NRE-bearing RNA (Figure 4B; see Figure 5). To assay activity in vivo, we determined whether each mutant NRE permits normal abdominal segmentation when substituted for the wild-type NREs in maternal *hb* mRNA (see Figure 6). Previous experiments have shown that the wild-type NREs mediate efficient translational repression by nos, resulting in the formation of a steep hb protein gradient in the posterior and the development of a normal complement of eight abdominal segments (Wharton and Struhl, 1991). Conversely, mutant NREs mediate inefficient repression, resulting in the formation of a shallow hb protein gradient and the development of fewer abdominal segments.

In summary, we observe (with a single exception) the following qualitative correlation: mutations that interfere specifically with the binding of either pum or the 55 kDa factor reduce NRE function in vivo, whereas mutations that have no specific effect on the binding of either factor have no detectable effect on NRE function (Table 1). The NRE mutations are divided into four classes, which are discussed in turn below: mutations that affect pum binding, a single mutation that affects binding of the 55 kDa

Table 1. Activity of Mutant NREs In Vitro and In Vivo

Mutant	Pum Binding In Vitro	55 kDa Binding In Vitro	Activity In Vivo
NRE1	+	−	−
NRE2	−	+	−
NRE3	−	−	ND
NRE4	+	+	+
NRE5	−[a]	−[a]	+
NRE6	−[a]	−[a]	+
NRE7	−	+	−
NRE8	−	+	−
NRE9	−[b]	+	−[b]
NRE10	+	+	−

For each mutant NRE, the table indicates the ability of the mutant to bind pum and the 55 kDa factor in vitro and to permit normal abdominal segmentation when substituted for the endogenous NREs in maternal *hb* mRNA. Binding activity, which was assessed by considering the results of both UV cross-linking and gel mobility shift experiments (Figures 4 and 5; data not shown), is scored relative to the binding activity of the wild-type NRE as either similar (plus) or significantly reduced (minus). Activity in vivo is scored as plus if maternal expression of the appropriate *hb*-NRE-containing mRNA leads to the development of a full complement of eight abdominal segments and is scored as minus if abdominal segmentation is disrupted (Figure 6). NRE3 (a compound mutant bearing the four substitutions present in both NRE1 and NRE2) was not tested in vivo. Thus, with the exception of NRE10, the activity of each site is correlated in vivo and in vitro.

[a] In these instances, NRE5 and NRE6 bind weakly to both proteins and thus exhibit no specific binding defect in vitro.

[b] In these instances, the defects associated with NRE9 are weak.

factor, mutations that nonspecifically affect the binding of both proteins, and mutations that affect the binding of neither protein.

Mutations That Affect Pum Binding

In vitro, pum does not bind detectably to NRE2, NRE7, or NRE8; it binds weakly to NRE9, and this binding is salt sensitive, unlike binding to the other mutants or the wild-type NRE (see Figures 1 and 4B). In contrast, all four of these sites bind the 55 kDa factor as well as does the wild-type NRE. Thus, the mutations in these four sites appear specifically to affect the binding of pum, suggesting that pum and the 55 kDa protein have at least partially distinguishable binding sites within the NRE.

As expected if pum binding is important in vivo, we find that none of these four sites permit normal abdominal development. The transgene directing expression of each of these *hb* mRNA derivatives causes dominant female sterility and embryonic lethality. The lethality is due to defects in abdominal segmentation: embryos containing the *hb*-NRE2 or *hb*-NRE7 mRNA typically develop no abdominal segments; embryos containing the *hb*-NRE8 mRNA typically develop four segments; and embryos containing the *hb*-NRE9 mRNA typically develop six or seven segments (see Figure 6). We have not measured the affinity of purified pum for these sites; thus, in general, we cannot correlate the extent of abdominal segmentation with the relative affinity of each site for pum. However, it is notable that among these mutants, NRE9 exhibits the weakest defects, both in vitro and in vivo. Taken together, the simplest interpretation of these results is that binding of pum to the NRE is essential for normal abdominal development.

A Mutation That Affects Binding of the 55 kDa Factor

In UV cross-linking experiments, the 55 kDa factor binds weakly to NRE1, whereas binding of pum to this site apparently is unaffected (Figure 4B). However, binding of the 55 kDa factor is reduced only slightly as measured in this assay, so we further investigated the properties of NRE1 in gel mobility shift experiments. As shown in Figure 5A, the 55 kDa factor binds very poorly to NRE1, whereas it binds normally to NRE2. Taken together, these observa-

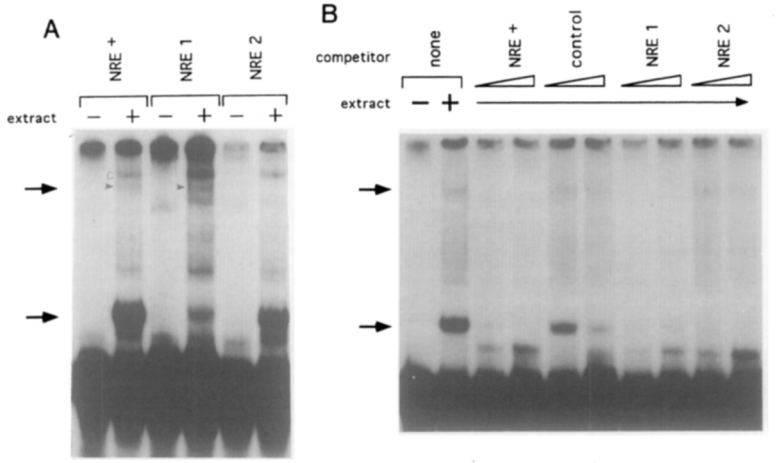

Figure 5. NRE1 Binds Weakly to the 55 kDa Factor and Binds Normally to Pum

(A) Shown are the results of a gel shift experiment using extract prepared from wild-type embryos and RNAs bearing various NREs (indicated at the top). In each pair of lanes, minus indicates that extract was omitted from the reaction mixture, and plus indicates that it was included. The arrows point to the complexes formed by binding of pum (top) and the 55 kDa factor (bottom). In addition, pum-dependent complexes are marked by arrowheads within the figure. The NRE-nonspecific complex C, which is described in the text, is identified only in the NRE$^+$ control lanes, although it is present in the other lanes also. Note that pum binding to NRE2 is undetectable and that pum binding to NRE1 is comparable to its binding to NRE$^+$.

(B) Shown are the results of a binding competition experiment in which various unlabeled RNA molecules (identified at the top) were used to compete the binding of proteins in embryonic extract to labeled NRE$^+$-bearing RNA. The control RNA was prepared by transcribing pSP73 polylinker sequences (see Experimental Procedures for other details). Where appropriate, the molar ratio of competitor to tester RNA was 250 in one reaction and 1000 in the other. In other experiments, lower concentrations of NRE$^+$- and NRE1-bearing RNA compete pum binding to the same extent (data not shown). Note that all RNA molecules we have tested compete binding of the 55 kDa factor, although we find that NRE-bearing RNA does so more effectively than does non-NRE-bearing RNA.

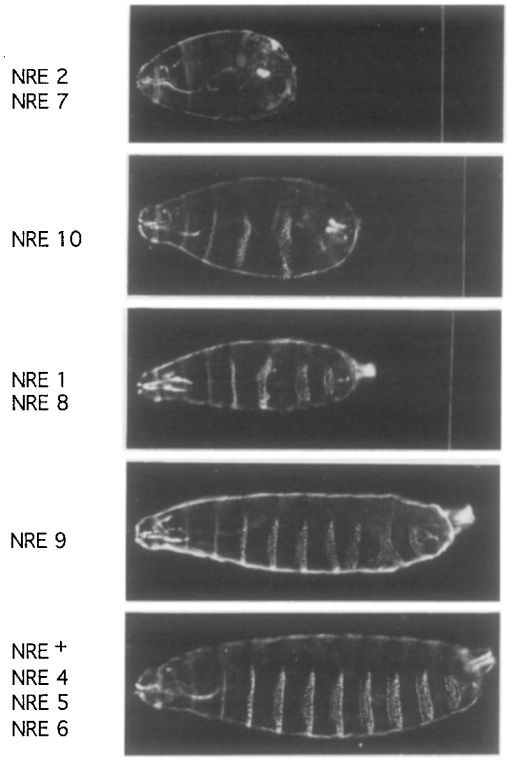

Figure 6. Activity of Mutant NREs In Vivo

Each photograph shows the segmentation pattern of a typical embryo that contained during its early development a particular maternal *hb* mRNA derivative. In each such derivative, two copies of the NRE identified at the left were substituted for the endogenous *hb* NREs (see Experimental Procedures). In the ventrolateral aspects shown, each abdominal segment is marked by a characteristic band of thick hairs that are readily visible in dark field. For each NRE, the median number of abdominal segments that develops is as follows: NRE2 and NRE7, 0 (although some embryos contain a disorganized patch of abdominal hairs); NRE10, 2.5; NRE1 and NRE8, 4.5; NRE9, 7; NRE$^+$, NRE4, NRE5, and NRE6, 7.5. Note that expression of the NRE2-*hb*, NRE7-*hb*, and NRE10-*hb* mRNA derivatives causes completely penetrant dominant female sterility, all of the progeny dying with fewer than the normal complement of eight abdominal segments. Note also that almost all embryos bearing NRE1-*hb* or NRE8-*hb* mRNA derivatives die with severe abdominal defects like those shown, but that the phenotype is not completely penetrant; a minority of these embryos develop more segments, and some lines have rare escapers that yield viable adults. Embryos containing NRE9-*hb* mRNAs are viable and develop only weak segmentation defects; most develop into adults. Head and thoracic development is normal in each case.

tions suggest that the NRE1 mutation specifically interferes with binding of the 55 kDa factor.

To test this idea further, we performed binding competition experiments, preincubating aliquots of wild-type extract with a vast molar excess of various unlabeled competitor RNAs prior to the addition of ^{32}P-labeled NRE$^+$-bearing RNA. We find that the NRE1 mutant site competes the binding of pum as effectively as does the wild-type NRE (Figure 5B). This result is consistent with the idea that NRE1 is specifically defective in binding the 55 kDa factor, an important point in considering its role in vivo (see below). Two additional observations from these experiments are as follows. First, while the 55 kDa factor binds preferentially to the wild-type NRE, it binds weakly to control RNA lacking an NRE (see Figure 1; Figure 5) as well as to NRE1. Thus, the binding specificity of the 55 kDa factor appears to be somewhat lower than that of pum under these conditions. Second, the affinity of pum for NRE2 is negligible, since this mutant site does not compete pum binding to NRE$^+$, even when present at a 1000-fold higher concentration (Figure 5B).

When in vivo tests are performed, we find that the NRE1 mutant site does not permit normal abdominal development. As is the case for embryos containing *hb* mRNA derivatives that do not bind pum, embryos containing the *hb*-NRE1 mRNA die, typically developing four abdominal segments (Figure 6). Thus, nucleotides within the NRE that are essential for binding the 55 kDa factor are essential for normal development. Proof that binding of the 55 kDa factor is essential in vivo awaits genetic evidence that it plays a role in regulating the translation of *hb* mRNA.

Mutations That Nonspecifically Affect the Binding of Both Proteins

In vitro, both pum and the 55 kDa factor bind very weakly to NRE5 and NRE6 (see Figure 4B). This finding suggests either that both proteins directly contact the bases altered in these mutants or that the substitutions affect the binding of both proteins indirectly, perhaps by altering the RNA structure. Consistent with the idea that binding in vitro is sensitive to changes in RNA structure, our observations have shown that the addition of flanking sequences at a remove from the NRE can eliminate NRE-specific binding (data not shown).

Embryos containing the *hb*-NRE5 and *hb*-NRE6 mRNAs develop a wild-type complement of eight abdominal segments (Figure 6). Thus, the NRE5 and NRE6 mutant sites function in vivo as well as does the wild-type site. As the NRE5 and NRE6 sites are not associated with any phenotype in vivo, we have not investigated their properties in vitro further.

Mutations That Bind Both Proteins Normally

The binding of both pum and the 55 kDa factor to NRE4 and to NRE10 is essentially indistinguishable from binding to the wild-type site (see Figure 4B). However, the activities of these mutant sites in vivo are dramatically different. Consistent with its properties in vitro, embryos containing *hb*-NRE4 mRNAs develop a wild-type complement of eight abdominal segments (Figure 6). In contrast, embryos containing *hb*-NRE10 mRNAs die, typically developing only three abdominal segments (Figure 6). The discrepancy between the activity of NRE10 in vitro and its activity in vivo suggests that our binding assays do not detect all of the interactions necessary for regulation of *hb* translation in vivo.

In summary, with the exception of NRE10, every mutation tested that disrupts NRE function in vivo disrupts specific binding of either pum or the 55 kDa protein in vitro (see Table 1). Moreover, in one case (NRE9), we observe a qualitative correlation between the extent to which NRE function is reduced in vivo and the extent to which pum binding is disrupted in vitro.

Discussion

In this report, we describe specific interactions between pum and the NRE, the sequence in *hb* mRNA that mediates its translational regulation. Furthermore, we observe that several mutations that block pum binding in vitro prevent normal abdominal segmentation. We conclude that recognition of the NRE by pum is essential in vivo. We also observe specific binding of a 55 kDa protein to the NRE and describe one mutation that disrupts this interaction in vitro and blocks normal NRE function in vivo. Finally, we see no evidence for a direct interaction between nos and the NRE; in addition, our mutational analysis of the NRE is consistent with the idea that most of the specific contacts it makes in vivo are with pum or the 55 kDa factor.

On the basis of these observations, we suggest the following speculative model for NRE function in vivo. We suggest that the NRE is a composite binding site for both pum and the 55 kDa factor. Both proteins are probably bound to NRE-bearing mRNA throughout the embryo, but this has no consequences for translation in the absence of nos activity. Then, in the posterior of the embryo, we suggest that nos is recruited to the NRE largely via weak protein–protein contacts with pum or the 55 kDa factor. Finally, nos acts (perhaps in conjunction with the other NRE-bound proteins) to block translation of the message. In this model, pum and the 55 kDa factor collaborate to form a landing pad for nos in the *hb* 3′ untranslated region.

Below, we discuss some of the evidence in support of these ideas, considering separately the roles of pum, the 55 kDa factor, and nos in the regulation of *hb* translation.

Role of Pum in Posterior Patterning

Our finding that pum binds specifically to the NRE was unanticipated, primarily because pum is distributed throughout the cytoplasm of the embryo during early development (Macdonald, 1992). In contrast, nos is distributed in a gradient emanating from the posterior pole; thus, by analogy with the anterior determinant system, it has generally been assumed that the spatially graded factor nos would recognize the NRE directly. These models left pum in an ill-defined role, in which it is required for efficient translational regulation of *hb* mRNA but does not necessarily contribute specificity to the repression reaction. Our results show that pum is a key specificity determinant, contributing most of the contacts involved in recognizing the NRE regulatory target. They also show that pum can bind efficiently to the NRE in the absence of other Drosophila proteins; therefore, we imagine that it is bound to *hb* mRNA via its NREs throughout the embryonic cytoplasm.

In addition, the sequence of pum protein (Macdonald, 1992; Barker et al., 1992) offered no intimation that it might bind to a specific RNA site. The protein contains none of the motifs associated with traditional RNA-binding proteins such as those in heterogeneous ribonucleoprotein particles or splicing factors, for example. Nor does it contain obvious homologies to less well-defined motifs that may mediate site recognition by other RNA-binding proteins (Burd and Dreyfuss, 1994). We have not yet localized the RNA-binding domain of pum; experiments such as that shown in Figure 2D suggest that noncontiguous regions of the protein may contribute to RNA binding (data not shown). As has been noted previously, the most prominent features of the 160 kDa pum protein are several homopolymeric amino acid patches in its amino terminus as well as eight tandem, imperfect repeats in its carboxyl terminus. Presumably only part of the protein is required in order to recognize the NRE, leaving the remainder free to interact with nos, the 55 kDa NRE-binding factor, or components of the translation machinery.

Role of the 55 kDa Factor in Posterior Patterning

Our results suggest that the 55 kDa factor may be involved in regulating the translation of *hb* mRNA and thereby governing abdominal segmentation. This idea derives from consideration of the properties of NRE1 in three different experiments. First, we find that expression of the *hb*-NRE1 mRNA is not normally regulated, leading to the development of only half the normal number of abdominal segments. Second, we find that the 55 kDa factor binds much more weakly to NRE1 than to NRE$^+$ in vitro. Third, we find that the binding of NRE1 to pum, the only other NRE-binding protein we have detected, is normal in binding competition experiments. As outlined above, we favor a model in which both pum and the 55 kDa factor bind the NRE in vivo. However, a critical test of the role of the 55 kDa factor awaits the isolation of mutations in the gene encoding this protein.

Role of Nos in Posterior Patterning

In the model we advance here, nos makes two main contributions to posterior patterning. First, as the only factor known to be distributed in a gradient in the posterior, we assume that it provides the positional information required for patterning. Second, as pum is incapable of repressing *hb* translation on its own, we assume that nos, perhaps in collaboration with the other NRE-bound proteins, makes inhibitory contacts with some component of the translation machinery. We believe that nos is recruited to the NRE largely or exclusively via protein–protein rather than specific protein–RNA contacts.

The argument that nos is unlikely to make extensive direct contacts with the NRE is based on two lines of evidence. The first is negative: we do not detect interactions between nos and the NRE even if the protein is overproduced in embryos or in COS cells. The second line of evidence involves consideration of the properties of mutant NREs in vivo and in vitro. Six of the mutants we have tested do not function as well as the wild-type NRE in vivo; of these, all but one are defective in binding to either pum or the 55 kDa factor in vitro (Table 1). Thus, with the exception of NRE10, the inactivity of the mutant sites in vivo can be accounted for by their inability to interact with one of the two NRE-binding proteins identified in this report. While our mutational analysis of the NRE is not exhaustive, we have altered 18 of its 32 bases, leaving no span of more than 3 nt unprobed by mutation. Thus, if nos does make contacts with the NRE that we cannot detect, they are probably either relatively nonspecific or limited to a few nucleotides, such as those altered in NRE10.

We suggest above that nos is recruited to the NRE indirectly, making contacts to the NRE-bound proteins. However, in the experiments reported here, we have been unable to detect interactions between nos and any protein–NRE complex. Indeed, in gel mobility shift experiments using crude embryonic extracts as a source of protein, we cannot even detect ternary complexes containing pum, the 55 kDa factor, and the NRE. Thus, if the addition of nos requires the binding of both pum and the 55 kDa factor to a single NRE, as we postulate, it is not surprising that we do not detect nos-dependent supercomplexes in our gel shift assays. Resolution of these issues awaits purification and further characterization of the NRE-binding proteins.

Although it is not clear why regulation of *hb* translation might require the unparsimonious assembly of two (or more) proteins on the NRE, there are other cases of RNA regulation in which the assembly of large multiprotein complexes apparently is required. For example, the sex-specific splicing of *doublesex* (*dsx*) RNA in Drosophila involves the binding of at least two general splicing factors as well as the specific factors tra and tra2 to a splicing enhancer (Amrein et al., 1994; Tian and Maniatis, 1993, 1994). Perhaps combinatorial, weak protein–protein and protein–RNA interactions are used to generate highly specific regulatory switches in each of these cases.

Nos and Pum as Translational Repressors

We do not yet understand the mechanisms by which nos and pum collaborate to block translation of *hb* mRNA. However, several recent observations suggest that translational control mediated by these proteins or their cognates may occur in a number of developmental systems. For example, sequences resembling NREs have been implicated in the temporal translational control of maternal *Cyclin B* (*CycB*) mRNA in Drosophila pole cells (Dalby and Glover, 1993). Also, genes with homology to *nos* and *pum* have been reported in Xenopus and Saccharomyces cerevisiae, respectively (Mosquera et al., 1993; Chen et al., 1991).

More intriguingly, NRE-like sequences are thought to confer spatial regulation on the C. elegans maternal *glp-1* mRNA, which encodes a member of the Notch/LIN-12 transmembrane receptor family (Evans et al., 1994). As is the case for *hb* in Drosophila embryos, maternal *glp-1* mRNA is uniformly distributed throughout the early C. elegans embryo; however, translation of the message is repressed in the posterior part of the embryo. An RNA element that includes NRE-like sequences is necessary and sufficient to confer this spatial regulation on microinjected reporter transcripts, which led Evans et al. to suggest that a conserved nos-like activity might be present in the worm embryo. The observations we report here suggest that worm homologs of pum or the 55 kDa factor might also play a role in regulating *glp-1* translation.

Experimental Procedures

Strains and Reagents

In the experiment shown in Figure 2A, the flies were heteroallelic combinations of pum^{ET3} (Bloomington Stock Center) with either *In(3R)Msc* or *T(3;1)FC8* (Barker et al., 1992). Elsewhere, the following mutant flies were used: nos^{BN} (Wang et al., 1994), *nb-5* (nb; Gavis and Lehmann, 1992), and $Df(3R)Dl^{K43}$ (Bloomington Stock Center). Wild-type flies were Oregon R. The rat anti-pum serum was a gift from P. Macdonald. For the control in the experiment shown in Figure 2B, we used serum from a rat immunized with a fragment of tailless. Rat anti-nos sera were generated by standard techniques (Harlow and Lane, 1988).

Construction of NRE-Bearing Plasmids

For testing the activity of each mutant NRE in vivo, two copies of a synthetic double-stranded fragment encoding the NRE were inserted in tandem into the unique SpeI site of p2343 essentially as described by Wharton and Struhl (1991).

For testing the binding activity of each mutant NRE in vitro, a single copy of each of the fragments described above was inserted separately into the unique SpeI site of p4684. p4684 was generated by insertion of a synthetic duplex containing the SpeI site of p2343 as well as flanking *hb* sequences into Bluescript KS(−) (Stratagene) between SacI and PstI, yielding a plasmid bearing the following sequence: GAGCTCACTATCATAAAGACTAGTCTGGAGAAACATCTGCAG.

RNA Binding Assays

Dechorionated embryos were resuspended in ice-cold extract buffer (25 mM HEPES [pH 7.5], 10% sucrose, 5% glycerol, 0.1 mM EDTA, 1 mM DTT, 0.2 mM AEBSF, 1 μg/ml aprotinin, 1 mM benzamidine, 10 μg/ml leupeptin, and 10 μg/ml pepstatin) and homogenized. Following centrifugation, the supernatant was incubated with RNA as described below. In some cases (particularly nos^{RC} and nos^{RD} mutants), similar extracts were prepared from adults. Synthetic ^{32}P-labeled RNA was prepared by standard procedures using EcoRI-linearized derivatives of p4684. Gel shift assays were performed essentially as described by Andino et al. (1990). Each reaction (total volume, 10 μl) contained the following: embryo extract (10–30 μg of total protein), binding buffer (10 mM HEPES [pH 7.9], 5 mg/ml heparin, 1 mM DTT, 10 μg/ml yeast tRNA), poly(rU) (0.1 mg/ml), and heat-denatured radiolabeled RNA (1×10^5 to 5×10^5 cpm, typically 1×10^{-14} to 5×10^{-14} mol). Following incubation at ambient temperature for 10 min, 2 μl of 50% glycerol was added, and the mixture was electrophoresed through a 5% native polyacrylamide gel (79:1 acrylamide:bisacrylamide) containing 5% glycerol and 0.5× TBE. Gels were electrophoresed at 210 V for 3 hr at 4°C, dried, and subjected to autoradiography. UV cross-linking experiments were carried out essentially as described by Gilmartin and Nevins (1989), except that the binding reactions (total volume, 10 μl) were prepared as described above, prior to UV irradiation. Following RNase A treatment, proteins covalently bound to RNA were electrophoresed through an 8% SDS–polyacrylamide gel and visualized by autoradiography.

To determine the identity of proteins in the gel shift complexes (A and B in Figure 1), we prepared photoreactive, radiolabeled RNA by using an equimolar mixture of 5-BrUTP and UTP (0.25 mM each). Following incubation with embryonic extract and electrophoresis as described above, the gel was irradiated with 300 nm UV light (Fotodyne) as described by Molitor et al. (1990), at room temperature for 30 min. Gel slices containing protein–RNA complexes were incubated at 37°C for 3 hr in 300 μl of buffer (6.7 mM Tris [pH 7.5], 6.7 mM $MgCl_2$, 50 mM NaCl, 0.5 mM DTT) containing 10 μg of RNase A. Reaction mixtures were then electrophoresed through a 10% SDS–polyacrylamide gel. For the lower complex (A in Figure 1), this procedure resulted in the covalent transfer of ^{32}P to a protein that comigrates with the 55 kDa complex seen in conventional UV cross-linking experiments. We did not recover detectable levels of labeled protein following irradiation of the upper complex (B).

Expression of Pum in COS Cells and Reticulocyte Lysates

Details of the construction of derivatives of pCMV4 (Andersson et al., 1989) that direct pum expression are available on request. COS cells were transfected by standard procedures (Cullen, 1987). Soluble extracts were prepared by harvesting cells 48 hr posttransfection, lysing them by sonication, and removing debris by centrifugation. Coupled in vitro transcription and translation using reticulocyte lysates was performed by using the TnT kit as described by the manufacturer (Promega).

Analysis of NRE Function In Vivo

Transformant lines bearing each modified *hb* gene were generated by microinjection into w^{1118} recipient embryos by use of standard techniques. For each mutant NRE, we analyzed the segmentation pattern in embryos from transformant females that were derived from at least three (and in most cases more) independent lines.

Acknowledgments

We thank Tammy Lee and Michelle Eagan for technical assistance; Jane Everson for secretarial help; Paul Macdonald for reagents and helpful discusssions; Liz Gavis, Doug Barker, and Ruth Lehmann for strains and plasmids; Rich Colvin, Masa Ikeda, Kim Mowry, Kiyoshi Ohtani, and Duen-Hwa Yan for helpful discussions; Gary Struhl for support during the initial phase of this work; and Anupama Dahanukar, Josh Dubnau, Mariano Garcia-Blanco, Joe Heitman, Tom Jessell, and Joe Nevins for advice and comments on the manuscript. This work was supported by the Howard Hughes Medical Institute. Y. M. was supported by a Medical Scientist Training Program fellowship.

Received October 21, 1994; revised December 15, 1994.

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