Pumilio is essential for function but not for distribution of the *Drosophila* abdominal determinant Nanos

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The *Drosophila* gene *pumilio* is expressed maternally, and its function is essential during early embryogenesis for the formation of abdominal segments. Our molecular analysis reveals that *pumilio* is a large gene that encodes a protein of 160 kD whose RNA is enriched at the posterior pole of the egg. As with *pumilio*, the maternal effect gene *nanos* is specifically required for abdomen formation. The Nanos protein is expressed in a posterior-to-anterior concentration gradient in the developing embryo. Previous experiments demonstrated a genetic interaction between *pumilio* and *nanos*, and led to the suggestion that *pumilio* is required for the proper spatial distribution of the Nanos protein. Here, we show that the expression and distribution of *nanos* RNA and protein in embryos derived from *pumilio* mutant females are indistinguishable from wild type. We conclude that abdomen formation depends both on Nanos activity, spreading from the localized posterior source, and on Pumilio activity, present throughout the embryo.

[Key Words: *pumilio*; *nanos*; pattern formation; abdomen formation; translational control; YGL023]

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Specification of cell fate along the anterior–posterior axis of the *Drosophila* embryo is initiated by the products of at least 30 maternal genes [Perrimon et al. 1986, 1989; Schüpbach and Wieschaus 1989; C. Nüsslein-Volhard, K. Anderson, G. Jürgens, and R. Lehmann, unpubl.]. These maternal genes establish three independently acting signaling systems that subdivide the early embryo into anterior, posterior, and terminal regions [Nüsslein-Volhard et al. 1987; Nüsslein-Volhard 1991; St. Johnston and Nüsslein-Volhard 1992]. The anterior and posterior systems act through gradients of morphogenetic proteins emanating from RNA molecules localized to the anterior and posterior poles of the oocyte (Frigerio et al. 1986; Berleth et al. 1988; St. Johnston et al. 1989; Wang and Lehmann 1991). Soon after fertilization, these morphogenetic proteins provide the initial spatial coordinates of the embryo by regulating the expression of the zygotic genome.

The posterior system is responsible for the formation of the segmented abdomen of the embryo [Boswell and Mahowald 1985; Lehmann and Nüsslein-Volhard 1986, 1987, 1991; Schüpbach and Wieschaus 1986; Mannesse and Schüpbach 1989, Boswell et al. 1991]. Of the 10 genes that constitute the posterior system, the gene *nanos* plays a central role (Lehmann and Nüsslein-Volhard 1991). The Nanos protein, the posterior determinant, is encoded by a maternal mRNA that is localized to the posterior pole of the oocyte [0% egg length] [Wang and Lehmann 1991]. However, the function of the Nanos protein is required in the presumptive abdomen of the embryo [20–50% egg length]. Nanos specifies abdominal development by repressing translation of maternally derived *hunchback* mRNA [Tautz et al. 1987; Tautz 1988; Schröder et al. 1988; Hülskamp et al. 1989, 1990; Irish et al. 1989; Struhl 1989]. The other known genes of the posterior system share the *nanos* phenotype as embryos derived from females mutant for any of these genes fail to form abdominal segments. Eight of these genes function before *nanos* and are required for the posterior localization of *nanos* RNA [Ephrussi et al. 1991; Lehmann and Nüsslein-Volhard 1991; Wang and Lehmann 1991; L.K. Dickinson, C. Wang, and R. Lehmann, unpubl.]. These eight genes also share a second phenotype; embryos derived from mutant females lack the specialized posterior pole plasm that is required for the formation of the pole cells, the germ-line precursors [Boswell and Mahowald 1985; Lehmann and Nüsslein-Volhard 1986; Schüpbach and Wieschaus 1986; Mannesse and Schüpbach 1989, Boswell et al. 1991]. In contrast, the remaining member of the posterior group, *pumilio*, has no pole plasm or germ cell defect and is specifically required for abdominal segmentation [this paper, Lehmann and Nüsslein-Volhard 1987].

Previous experiments suggested that *pumilio* is required to achieve the proper distribution of the Nanos protein within the embryo [Lehmann and Nüsslein-Volhard 1987]. Three independent lines of evidence supported this suggestion. First, cytoplasmic transplanta-
tion experiments implied that the posterior localization of nanos RNA is unaffected by pumilio mutations. The abdominal segmentation defect of most posterior group mutants can be fully rescued by the injection of nanos RNA or the transplantation of wild-type posterior pole plasm, which contains nanos RNA [Lehmann and Nüsslein-Volhard 1991; Wang and Lehmann 1991]. Posterior pole plasm from a pumilio mutant embryo contains wild-type levels of this rescuing activity, whereas the posterior pole plasm of nanos mutant embryos, as with all other posterior group mutants, contains no rescuing activity (Lehmann and Nüsslein-Volhard 1987, 1991). This suggests that nanos RNA is properly localized in pumilio mutant embryos and indicates that pumilio must function after the localization of nanos RNA. Second, the abdominal defect of a pumilio mutant embryo can be rescued simply by repositioning pumilio mutant pole plasm to the site of Nanos action, the presumptive abdomen (Lehmann and Nüsslein-Volhard 1987). This means that the functional Nanos protein can be expressed in a pumilio mutant embryo and led to the suggestion that Pumilio affects the transport of the Nanos protein from the pole plasm to the presumptive abdomen. Finally, when a pumilio mutant embryo is injected with pumilio pole plasm, wild-type pole plasm, or in vitro-synthesized nanos RNA, only partial rescue is observed, and this rescue is restricted to the site of injection (Lehmann and Nüsslein-Volhard 1987, 1991; Wang and Lehmann 1991). This observation supported a transport role for Pumilio, as the Nanos protein could not become widely distributed throughout the pumilio mutant embryo.

To understand the role of Pumilio in abdominal development, we have analyzed pumilio mutants and found that pumilio is absolutely required for abdomen formation. To investigate the relationship between pumilio and Nanos, we analyzed the expression of nanos mRNA and protein in pumilio mutant embryos, and demonstrated that pumilio has no effect on the posterior localization of nanos RNA or on the expression or distribution of the Nanos protein. These findings make a transport function for pumilio very unlikely and suggest that Pumilio and Nanos act in conjunction. To address the mechanism of Pumilio function we performed a molecular analysis of the pumilio gene. pumilio is a large gene that encodes an mRNA present in oocytes and early embryos. This RNA is preferentially enriched at the posterior pole of embryos and encodes a 160-kD protein. Near its carboxyl terminus the Pumilio protein contains eight tandemly repeated sequence motifs. A similar sequence of repeated motifs is found in the yeast gene YGL023.

Results

pumilio is essential for abdomen formation

Previous studies of pumilio suggested that its function was not absolutely required for abdomen formation. Abdominal segmentation could be restored by transplanta-

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tion of nanos RNA into pumilio mutant embryos or by allowing pumilio mutant embryos to develop at high temperature. This cold-sensitive pumilio phenotype, common to 16 ethylmethanesulfonate (EMS) induced alleles, has been described previously (Lehmann and Nüsslein-Volhard 1987, 1991): Embryos derived from mutant females kept at the restrictive temperature (18°C) develop very few or no abdominal segments, whereas those kept at the permissive temperature (29°C) can develop up to the normal number of abdominal segments. Because our molecular analysis [see below] indicated that two chromosomal rearrangements [In(3R)Msc and T(3;1)FC8, see Materials and methods] disrupt pumilio, embryos from females carrying these chromosomes would be unlikely to express any intact Pumilio protein. Therefore, we tested the cold sensitivity of these embryos and found them unable to develop abdominal segments at either temperature (see Materials and methods). Thus, the phenotype of this strong pumilio mutant is indistinguishable from that of nanos. In addition, we found that the injection of nanos RNA into embryos derived from In(3R)Msc/T(3;1)FC8 females was unable to restore abdominal segmentation (Table 1). This indicates that pumilio function is absolutely required for abdomen formation. Furthermore, it suggests that the EMS-induced pumilio alleles retain partial function and that nanos can only bypass a reduction but not the complete loss of pumilio function.

Pumilio and Nanos affect hunchback protein distribution

Because nanos and pumilio share the same phenotype, we wondered whether they exert the same effect on hunchback, the target of Nanos function. Nanos functions by repressing the translation of maternally derived hunchback mRNA in the presumptive abdomen [Tautz et al. 1987; Tautz 1988; Schröder et al. 1988; Hülskamp et al. 1989; Irish et al. 1989; Struhl 1989]. In doing so, Nanos indirectly regulates the expression of the zygotic gap genes required for abdomen formation, Krüppel, kniipps, and giant, which are under the direct control of the Hunchback protein [Hülskamp et al. 1990; Struhl et al. 1992]. To determine the effect of pumilio on Hunch-

| Maternal genotype of recipient | Embryos injected [number] | Embryos developed [number] | Number of abdominal segments formed [% total] |
|-------------------------------|--------------------------|----------------------------|--------------------------------------------|
| In(3R)Msc/Tp(3;1)FC8 nosT2    | 220                      | 60                         | 100                                        | 0–1 2–4 5–8 |
|                               |                           | 122                        | 3                                          | 97   |

*In vitro-synthesized nanos RNA was injected into the prospective abdominal region of embryos from females of the indicated genotype.
back, we compared the expression of the Hunchback protein in wild-type and pumilio mutant embryos. In cleavage-stage embryos, expression of the Hunchback protein is derived from a uniformly distributed maternal hunchback mRNA [Tautz et al. 1987; Tautz and Pfeifle 1989]. In wild-type embryos [Fig. 1] translation of the maternal Hunchback protein from this RNA is restricted to the anterior half of the embryo [Tautz 1988; Tautz and Pfeifle 1989]. In embryos from females mutant for EMS-induced pumilio alleles, the domain of Hunchback protein expression expands in the posterior direction [Fig. 1; see also Tautz 1988]; whereas in strong pumilio mutant embryos, the Hunchback protein is expressed at high levels throughout the embryo [Fig. 1]. Thus, Pumilio affects hunchback translation to the same extent as Nanos does [Tautz 1988, Wharton and Struhl 1991].

**Distribution of the Nanos protein is unaffected by pumilio**

The similarity between the nanos and pumilio phenotypes precludes the ordering of these two genes in a genetic pathway. To identify interactions between these two genes, we determined whether pumilio mutations interfere with the synthesis, distribution, or stability of nanos RNA and protein. In wild-type embryos nanos mRNA is localized to the posterior pole plasm [Wang and Lehmann 1991]. Transplantation of this cytoplasm into the presumptive abdomen of a nanos mutant embryo is sufficient to restore abdominal segmentation. Similarly, pole plasm derived from a pumilio mutant embryo is equally able to induce abdominal segmentation, suggesting that the localization of nanos mRNA is unaffected by the pumilio mutation [Table 2; Lehmann and Nüsslein-Volhard 1987, 1991]. To investigate this at the molecular level, we performed in situ hybridization to whole-mount embryos and confirmed that the localization of nanos RNA in pumilio mutant embryos appears normal [Fig. 2A,B]. Thus, Pumilio function is not required for nanos RNA synthesis or localization. This suggests that Pumilio interacts with the Nanos protein.

Pumilio might control Nanos protein activity in the presumptive abdomen and could do so by a number of different mechanisms. Because the abdomen is derived from a region of the embryo that is anterior of the pole plasm, Pumilio could be required to transport the Nanos protein from the posterior pole toward the prospective abdomen as suggested initially [Lehmann and Nüsslein-Volhard 1987]. Alternatively, Pumilio might control nanos translation or Nanos protein stability. Furthermore, Pumilio could be required for Nanos protein function without changing its concentration or distribution.

To distinguish among these possibilities, we compared the expression and distribution of the Nanos protein in wild-type and pumilio mutant embryos using an anti-Nanos antiserum. The Nanos protein forms a concentration gradient emanating from the posterior pole and extending into the presumptive abdomen. Our initial observations on large numbers of whole-mount embryos indicated that the expression of the Nanos protein is very similar in wild-type and pumilio embryos [Fig. 2C,D]. This indicates that Pumilio is not necessary for the synthesis or stability of the Nanos protein. To determine the relative distribution of the Nanos protein more objectively, we used a modification of the filtered fluorescence-imaging technique [Karr and Kornberg 1989],

**Table 2. Rescue of abdominal phenotype of nanos mutant embryos by posterior pole plasm**

| Genotype of donor* | Embryos injected (number) | Embryos developed (number) | Number of abdominal segments formed (% total) |
|-------------------|---------------------------|-----------------------------|------------------------------------------|
| In(3R)MSC/In(3R)MSC | 35                       | 17                         | 0–1                                       |
| Tp(3;1)FC8        | 35                       | 17                         | 0–1                                       |

*Posterior pole plasm of embryos derived from females of the indicated genotype was injected into the prospective abdominal region of embryos from nos2 females.
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Figure 2. 

The localization of nanos RNA in embryos derived from wild-type [A] and pumilio [B] females is indistinguishable. Similarly, the distribution of the Nanos protein in wild-type [C] and pumilio [D] embryos appears identical. Antibody staining was performed with a rabbit polyclonal anti-Nanos serum. Identical results were observed with embryos from females mutant for pumilio^68~ at the restrictive temperature 20°C and embryos from females transheterozygous for In(3R)Msc and T(3;1)FC8 [data not shown]. The embryos shown are in nuclear cycle seven or eight [Foe and Alberts 1983]. For all embryos, anterior is left and dorsal is up. Nomarski optics were used.

which combines the enhanced sensitivity of enzyme-linked immunohistochemistry with the high contrast of fluorescence microscopy. The data obtained using this technique demonstrate that the relative distribution of the Nanos protein in wild-type and pumilio embryos [Fig. 3A] is indistinguishable.

To confirm that this technique is capable of measuring differences in the relative distribution of the Nanos protein sufficient to account for the pumilio phenotype, we compared the distribution of the Nanos protein in embryos derived from females carrying either one copy or four copies of the nanos gene [Fig. 3B]. This difference in nanos gene dosage has no detectable effect in an otherwise wild-type background. However, in embryos from females mutant for an EMS-induced pumilio allele, which express partial pumilio function, this difference in nanos gene dosage is sufficient to induce the formation of three abdominal segments [Fig. 3C]. Embryos derived from females carrying one copy of nanos~ form ~0.6 abdominal segments, whereas embryos derived from females carrying two or four copies of nanos~ form ~1.7 and 3.4 abdominal segments, respectively. When the distribution of the Nanos protein was measured in these embryos, a small but significant difference was observed [Fig. 3B]. Thus, we are able to detect a difference in the relative distribution of the Nanos protein that is sufficient to produce a three-segment difference in phenotype. Therefore, our failure to detect a difference between wild-type and pumilio embryos, which differ by eight abdominal segments, means that the pumilio phenotype cannot be the result of an altered distribution of the Nanos protein. This result indicates that pumilio is not required for either the expression of the Nanos protein or its transport to the presumptive abdomen.

Molecular identification of pumilio

To address further the mechanism of Pumilio function we have isolated the pumilio gene and performed a molecular analysis. The cytological position of pumilio has been determined by deficiency mapping to position 85CD of the polytene chromosome [Lehmann 1985; Lehmann and Nüsslein-Volhard 1987]. To isolate pumilio sequences we took advantage of the observation that the inversion, In(3R)Msc, fails to complement pumilio [Lehmann 1985] and that the proximal breakpoint of In(3R)Msc at 84A lies within the Antennapedia complex [Scott et al. 1983; T. Kaufman, pers. comm.]. A bacteriophage clone from the Antp region [gift of M. Scott] was used to isolate fragments spanning the In(3R)Msc breakpoints. Sequences from the distal breakpoint at 85CD were used to begin a genomic walk in a wild-type cosmid library [Fig. 4A].

An embryonic cDNA library [gift of N. Brown; Brown
Figure 3. The distribution of the Nanos protein is not affected by pumilio. The relative distribution of the Nanos protein was measured in early embryos of the indicated genotypes using a modification of the filtered fluorescence imaging technique (Karr and Kornberg 1983). Embryos between nuclear cycles seven and nine (Foe and Alberts 1983) were photographed. These video images were then converted into digital form, and fluorescence intensity profiles were generated by computer. The profiles of eight embryos were averaged together and inverted to represent the level of the Nanos protein. A variable reduction of fluorescence was observed along the edge of the embryos independent of the Nanos antibody. (A) There is no significant difference in the distribution of the Nanos protein between wild-type (solid line) and pumilio (broken line) mutant embryos, whereas a statistically significant difference can be observed between embryos carrying one copy (Df(3R); Df(1F)X1/TM3; broken line) or four copies (P[gnos]/P[gnos]; solid line) of the nanos gene (B). (C) The effect of varying the nanos gene dosage on the phenotype of pumilio embryos mutant for EMS-induced alleles. The data were obtained by preparing cuticles and determining the average number of abdominal segments formed after embryogenesis at 20°C (restrictive temperature). These embryos were produced by females of the following genotypes: 1 x nos +, pumaT3/pum68~ nosLZ; 2 x nos, pumET3/pum68~ 4 x nos +, pumET3/pum68~ P[gnos]/P[gnos]. The mean numbers of abdominal segments, 1 x nos, 0.6; 2 x nos, 1.7; 4 x nos, 3.4, were calculated from sample sizes of 308, 160, and 235 embryos, respectively. The error bars indicate the standard deviation of these samples.

and Kafatos 1988] was screened with two genomic fragments from the distal breakpoint of the Msc inversion, and a group of related cDNAs was identified by hybridization to both probes. The largest of these, clone R7-1, was used to probe a blot containing mRNA isolated from different developmental stages (Fig. 4B). This clone hybridizes to an RNA molecule 6.5–7 kb in length that is present in oocytes and early embryos, a pattern consistent with the paternal requirement for pumilio function. In addition, a larger mRNA producing a weaker signal was also detected during the same stages. We determined that this cDNA clone was derived from pumilio by analyzing wild-type and mutant polytene chromosomes by in situ hybridization. cDNA R7-1 hybridizes to the position of pumilio (85CD), and this hybridization signal is interrupted by the two breakpoints known to disrupt pumilio function, In(3R)Msc and T(3;1)FC8 (data not shown; Lehmann 1985).

When fragments of this pumilio cDNA were hybridized to the cloned genomic DNA, it became apparent that the 3’ end of the pumilio transcription unit extends beyond the limits of the initial genomic walk (Fig. 4). Using R7-1 cDNA sequences as probes, cosmid clones spanning the entire pumilio gene were isolated, except for one region that is apparently not represented in the libraries we screened.

Structure of pumilio and its products

The nucleotide sequence of pumilio was determined by sequencing both strands of the cDNA R7-1 and portions of other cDNAs and has been confirmed by sequencing the corresponding genomic regions (Fig. 5). pumilio encodes two mRNAs that contain different 5’ exons and are both present in early embryos. Each mRNA contains 13 exons spanning >160 kb of the genome, including a single intron covering >120 kb (see Fig. 4A). We have obtained 6835 nucleotides of cDNA sequence corresponding to one RNA and 6674 nucleotides for the other, both encode an identical open reading frame. The 5’ untranslated regions contain multiple stop codons, indicating that this sequence contains the entire protein-coding
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Figure 4. Structure and expression of pumilio. (A) The intron–exon structure of the pumilio gene is indicated above an EcoRI restriction map of the region. Transcription proceeds from the right in a distal-to-proximal direction on chromosome 3. The exons are positioned above the restriction fragments from which they are derived but are not drawn to the same scale. The position of the In(3R)Msc breakpoint used to clone this region is indicated. The location of this breakpoint indicates that this mutant could express no more than a severely truncated protein. In addition, a breakpoint of the translocation T(3;1)FC8 falls within the pumilio gene; however, its location has not been identified. Also contained within these sequences is the gene for the chromosomal protein D1, which had been mapped previously to this region of the third chromosome (Ashley et al. 1989). Chromosomal protein D1 sequences have been identified in the distal (right) region of cosmid cB1.

Below the EcoRI restriction map, the position of genomic cosmid and bacteriophage λ clones are indicated. (B) The developmental expression of pumilio RNA was analyzed with an RNA blot. Polyadenylated RNA (2 μg) isolated from the developmental stages indicated were fractionated on a formaldehyde-agarose gel and transferred to a nylon membrane. This blot was then hybridized with a probe derived from the R7-1 cDNA that had been uniformly labeled with 32p. Hybridization with other probes, including bicoid and genomic clones that reveal chromosomal protein D1 transcripts, indicates that the apparent increase in pumilio expression at 5–8 hr is the result of overloading relative to other lanes.

region. Downstream of the open reading frame is a relatively large 3′-untranslated region of 1193 nucleotides. A portion of the pumilio sequence has also been reported by Macdonald (1992). The pumilio open reading frame encodes a protein with a predicted molecular mass of 160 kD. Its amino acid sequence indicates that it is a novel protein. One region of this protein, however, near the carboxyl terminus (amino acids 1111–1401) contains a repeated sequence motif [Macdonald 1992]. This sequence can be aligned to reveal the presence of eight repeated units [Fig. 5B]. Specific amino acids are conserved at a few positions; however, a more extensive conservation of the chemical nature of amino acids is observed throughout this region [Fig. 5B].

This region of the Pumilio protein is strikingly similar to a portion of the open reading frame encoded by the Saccharomyces cerevisiae gene YGL023 (Chen et al. 1991). This 888-amino-acid protein of unknown function contains a sequence near its carboxyl terminus that can also be aligned into eight repeated units that are similar to those of pumilio [Fig. 5B]. Comparison of specific Pumilio repeats to the corresponding Ygl023 repeats reveals additional similarities that indicate the presence of a further pattern appearing every two repeats. In addition, the positions of a number of other amino acids are conserved in specific repeats, which indicates that the different repeats may be functionally distinct and that the order of the repeats is conserved.

A pumilio minigene complements the pumilio mutant phenotype

The large size of pumilio makes it impossible to use P-element-mediated transformation to identify all of the genomic sequences required for pumilio function. Therefore, to demonstrate that the R7-1 cDNA encodes the functional Pumilio protein, we introduced a pumilio minigene containing the R7-1 open reading frame into the germ line of pumilio mutant flies and tested its ability to complement the pumilio mutant phenotype. To direct transcription of the cDNA, we used the oogenesis-specific nanos promoter (Gavis and Lehmann 1992), because we expected the temporal regulation of its expression to be appropriate for pumilio. The resulting nanos—

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Figure 5. Sequence of pumilio. [A] The composite cDNA nucleotide sequence was derived predominately from cDNA R7-1, which was isolated from a 4- to 8-hr embryonic library. In addition, the 5' sequences were obtained from clones isolated from a specifically primed library. Two different types of 5' clones were isolated that differ in their 5' exons, and the sequences of the longest clone are shown. Primer extension analysis (data not shown) indicates that the sequences shown correspond approximately to the 5' ends of these RNAs. The numbering of the nucleotide sequence is to the left. Sixteen nucleotide differences were observed between the cDNA sequence, and the genomic sequence, two of which resulted in an altered amino acid. Two of these changes are the result of the insertion of a TA between positions 5853 and 5854 which resulted in an altered amino acid. Therefore, the two changes are the result of the insertion of a TA between positions 5853 and 5854. The numbering of the nucleotide sequence is to the left.

[B] The predicted amino acid sequence of the Pumilio protein is indicated by the nucleotide sequence and is shown for the longest RNA. The numbering of the nucleotide sequence is to the left. Sixteen nucleotide differences were observed between the cDNA sequence, and the genomic sequence, two of which resulted in an altered amino acid. Two of these changes are the result of the insertion of a TA between positions 5853 and 5854. The numbering of the nucleotide sequence is to the left.
pumilio fusion gene (Fig. 6A) was inserted into the P-element vector pDM30, which contains the rosy+ marker gene, (Mismer and Rubin 1987) and was injected into embryos. Two of six transformant lines were analyzed in detail by examining the larval cuticles of the progeny of homozygous pumilio females that carry the transgene. Transformation with this nanos-pumilio fusion gene results in complete rescue of the pumilio phenotype (Fig. 6B). Essentially all of the embryos produced by these transgenic females developed into viable larvae. Thus, we have identified the protein sequences necessary for pumilio function during oogenesis and early embryogenesis.

pumilio RNA is localized to the posterior pole plasm

The similar phenotype of pumilio and nanos mutants suggests an interaction between their gene products. Because the nanos mRNA is localized to the posterior pole of early embryos [Wang and Lehmann 1991], we analyzed the distribution of the pumilio RNA by in situ hybridization with the R7-1 cDNA to whole-mount embryos. We found that pumilio RNA is enriched at the posterior pole of early embryos (Fig. 7). In addition, uniform staining was consistently observed, suggesting that unlocalized pumilio RNA is also present throughout the embryo. We cannot detect pumilio RNA localization in embryos from females with rearrangements in the pumilio gene, which disrupt the pumilio transcription unit (Fig. 7).

To determine the genetic requirements for pumilio RNA localization, we analyzed a number of posterior group mutants. Embryos from females mutant for a strong allele of nanos or one of the EMS-induced pumilio alleles show normal localization of pumilio RNA (Fig. 7; data not shown), whereas embryos from females mutant for posterior group genes that affect the formation of the pole plasm, such as vasa, oskar, and staufen, fail to localize pumilio RNA (Fig. 7, data not shown) (Lehmann and Nüsslein-Volhard 1986; Schüpbach and Wieschaus 1986; Hay et al. 1988a, b; Lasko and Ashburner 1988; Ephrussi et al. 1991; Kim'-Ha et al. 1991; St. Johnston et al. 1991). Thus, as with nanos RNA localization, pumilio RNA localization depends on the formation of the pole plasm.

Discussion

We have identified the pumilio gene by mapping two chromosomal rearrangements that disrupt the gene and by complementation of the pumilio mutant phenotype using germ-line transformation with a minigene encoding the Pumilio protein. Analysis of mutations that disrupt the pumilio transcript demonstrates that pumilio is absolutely required for abdomen formation. We have investigated Pumilio function by measuring the effect of pumilio mutations on the expression of the Nanos protein and found that pumilio has no effect on either the level of expression or the distribution of nanos RNA or protein. We conclude that pumilio is an essential factor for abdomen formation and that it acts in conjunction with the localized abdominal determinant Nanos.

pumilio mutations result in the uniform translation of the maternal Hunchback protein (Fig. 1; Tautz et al. 1991).
in the anterior of the embryo (Wharton and Struhl 1989; Lehmann and Nüsslein-Volhard 1991; L. Gavis and R. Lehmann, unpubl.). Second, the uniform distribution of Pumilio throughout the embryo (Macdonald 1992) is inconsistent with a direct role in establishing a concentration gradient of hunchback along the anterior–posterior axis. Finally, we cannot identify an independent activity for Pumilio. Although the localized nanos RNA present in pumilio mutant embryos is fully competent to rescue abdominal segmentation in posterior group mutants, no rescuing activity can be detected on transplantation of pumilio RNA present in nanos mutant embryos (Lehmann and Nüsslein-Volhard 1991) or in vitro-synthesized pumilio RNA (D. Barker and R. Lehmann, unpubl.). We find our data more consistent with a model in which Pumilio acts in conjunction with Nanos. Determining the role of Pumilio, therefore, will require an understanding of Nanos function.

How do Pumilio and Nanos cooperate and eventually control the repression of hunchback translation? Recent studies have identified a short RNA sequence present in the 3′-untranslated region of hunchback mRNA that is necessary and sufficient to confer Nanos-dependent translational repression (Wharton and Struhl 1991). However, it is presently unclear whether Nanos binds this sequence directly. A possible role for Pumilio could be to interact with hunchback mRNA, making the RNA more susceptible to translational repression. By interacting with hunchback mRNA, Pumilio might affect its secondary structure, a well-established mechanism of translational regulation (Altuvia et al. 1989). This might create an RNA structure or a Pumilio-RNA complex for which the Nanos protein has a higher affinity. Alternatively, the Pumilio protein may interact with the Nanos protein, enabling it to repress hunchback. Because we cannot order nanos and pumilio genetically, it is also formally possible that Nanos enables Pumilio to repress hunchback.

Little is known about mechanisms or molecules that control translation of specific mRNAs. Codependence of nanos and pumilio for translational repression could be achieved by a number of possible mechanisms, such as the introduction of a covalent protein modification or the formation of a protein–protein complex. Many of the pumilio alleles exhibit a cold-sensitive phenotype (Lehmann and Nüsslein-Volhard 1987). Cold sensitivity often indicates the assembly of large protein complexes, such as ribosomes (Guthrie et al. 1969; Tai et al. 1969; Moritz et al. 1991), bacteriophage capsids (Cox and Strack 1971; Jarvick and Botstein 1975), and microtubules (Schatz et al. 1988), owing to the inherent cold sensitivity of the hydrophobic interactions required for some of these associations (Correia and Williams 1983). Such a complex might involve an interaction between Pumilio and Nanos, between Pumilio and an unidentified protein, or among multiple Pumilio proteins. Finally, formation of a Nanos–Pumilio complex might be required for Nanos activity, possibly by increasing its affinity for hunchback mRNA.

Along with nanos and pumilio, at least eight other
genes make up the posterior group. In addition to their abdominal phenotype, these eight genes are also required for the posterior localization of nanos and pumilio RNAs. These genes specifically affect nanos function, as the mutant phenotype of these posterior group genes can be rescued by transplantation of nanos RNA alone. Therefore, it has been proposed that the abdominal phenotype of these genes is the result of the lack of localized nanos activity (Wang and Lehmann 1991; Gavis and Lehmann 1992). pumilio function, on the other hand, seems unaffected in these mutants, which indicates that pumilio function does not require RNA localization, pumilio function is not limited to the posterior pole, as Pumilio acts in conjunction with Nanos even when Nanos is localized to the anterior pole of the embryo (E. Gavis and R. Lehmann, unpubl.). Thus, pumilio provides a factor required for abdomen formation that is present throughout the embryo, whereas nanos controls embryonic polarity through its posteriorly localized source.

Only one pumilio allele was identified in the initial mutagenesis screen for maternal effect genes on the third chromosome involved in embryonic patterning (Lehmann and Nüsslein-Volhard 1987). We have isolated many additional alleles on the basis of their failure to complement the pumilio maternal or zygotic (see below) phenotypes (Lehmann and Nüsslein-Volhard 1987, 1991; D. Barker, C. Detweiler, and R. Lehmann, unpubl.). Some of these alleles show reduced viability and the duplication of thoracic bristles of mutant adults. Thus, Pumilio has additional zygotic functions. Because pumilio does not share these phenotypes with nanos (Lehmann and Nüsslein-Volhard 1991; C. Wang, L. Dickinson, and R. Lehmann, unpubl.), it is likely that Pumilio interacts with additional gene products during later stages of development. Sequence analysis of the existing pumilio alleles, as well as a functional analysis of derivatives generated from the pumilio minigene, should help to define regions in the Pumilio protein specifically required for interaction with Nanos and other factors.

Materials and methods

Nomenclature

The mutant phenotypes described here depend strictly on the maternal genotype. Throughout the text, we refer to embryos derived from females mutant for a maternal effect gene, for example, pumilio or nanos, as mutant embryos irrespective of the genotype of the embryo.

Mutant alleles and chromosomal rearrangements

Two chromosomal rearrangements disrupt the pumilio transcription unit. In(3R)Msc and T(3;1)FC8 in trans are subviable (<50% expected) and, in addition to the strong maternal phenotype, exhibit various morphological defects, such as small body size, aberrant wing posture, and additional bristles (most pronounced in the scutellar region). Because these defects were also observed in flies mutant for EMS-induced pumilio alleles, we consider them part of the pumilio phenotype (Lehmann and Nüsslein-Volhard 1987). The pumilio alleles used in this study are pumello and pumelt, the nanos allele is nos, and the vase allele is vas (as described in Lehmann and Nüsslein-Volhard 1987, 1991).

Preparation and analysis of nucleic acids

Plasmid, cosmid, and bacteriophage DNA were prepared by standard procedures (Sambrook et al. 1989). To prepare Drosophila genomic DNA, 50–100 adult flies were homogenized in 0.35 m sucrose, 0.1 m EDTA, and 0.05 m Tris-HCl (pH 8.0) and filtered through a Nitex screen. The nuclei were pelleted by centrifugation at 4000 rpm for 10 min at 4°C and resuspended in 2 ml of 0.1 m NaCl, 0.01 m EDTA, 0.02 m Tris-HCl (pH 8.0) and 10 μg/ml of RNase, and incubated at room temperature for 15 min. Proteinase K and SDS were added to concentrations of 100 μg/ml and 0.5%, respectively, and the solution was incubated for 1 hr at 65°C. The DNA was extracted twice with phenol-chloroform (1 : 1) and precipitated with ethanol. Southern blots were performed as described (Sambrook et al. 1989), except the DNA was transferred to Biotrans nylon membranes [ICN] with a Hoyer TransVac T80.

Total Drosophila RNA was prepared by homogenizing 50–100 adult flies in 3 ml of 8 m urea, 3 m LiCl, 0.005 m dithiothreitol, and 0.005 m EDTA for 1 min in a Brinkman polytron homogenizer at full speed. The homogenate was then centrifuged at 3000 rpm for 10 min at 4°C, and the clear supernatant transferred to a 15-ml Corex tube and incubated at 0°C overnight. The RNA was pelleted by centrifugation at 12000 rpm for 30 min in a Sorvall SS34 rotor at 4°C and resuspended in 0.01 m Tris-HCl (pH 7.0), 0.005 m EDTA, and 0.5% SDS. This solution was extracted once with phenol-chloroform (1 : 1), and the RNA was precipitated with ethanol. Selection of polyadenylated RNA and RNA blots was performed as described (Sambrook et al. 1989), using formaldehyde agarose gels.

Sequence analysis of the pumilio cDNA was performed using two nested sets of Tn9-induced deletions (Peng and Wu 1986), using Sequenase version 2.0 (U.S. Biochemical). Additional cDNA and genomic DNA were sequenced from double-stranded plasmid DNA using Sequenase version 2.0 or from cosmide DNA using the fmol sequencing system (Promega). Sequencing primers were synthesized with an Applied Biosystems 391 DNA synthesizer and deblocked oligonucleotides were precipitated with butanol (Sawadogo and Van Dyke 1991).

Compilation and initial analyses of DNA sequences were performed using the MacVector program [IBI]. A DNA homology search was performed with the FASTA program using the GenBank, EMBL, and NBRF nucleic acid data bases [Pearson and Lipman 1988]. Protein homology searches were performed with the TFASTA program using the above data bases and also with the BLAST network service of the National Center for Biotechnology Information using the PIR, SWISS-PROT, GenPept, and GUpdate data bases [Altschul et al. 1990]. The Pumilio, Ygu023 similarity was detected using a profile analysis (Gribskov et al. 1987).

Cloning of genomic and cDNA sequences

Two libraries were constructed from fractions of BamHI-di-
gested In(3R)Msc/+ DNA, purified by preparative agarose gel electrophoresis to include the two Msc breakpoint fragments (7.8 and 13.8 kb), in the vector λL47.1 and the Escherichia coli strain LE392 (Sambrook et al. 1989). Packaging was performed using the Packagene λ DNA packaging system (Promega). The proximal breakpoint of the Msc inversion maps within the Antennapedia complex (Scott et al. 1983). A bacteriophage clone spanning this region was obtained (gift of M. Scott), and the breakpoint was mapped further to a 5.2-kb BamHI restriction fragment within the Scr gene. This 5.2-kb Scr fragment was used to screen the Msc libraries. Fourteen positive clones (λMsc-S) were identified from a total of 15,000 clones of the library containing the smaller Msc breakpoint (7.8 kb), whereas four positive clones (λMsc-L) were identified from a total of 100,000 clones of the library containing the larger Msc breakpoint (13.8 kb). λMsc-S contained highly repetitive sequences in addition to the Scr sequences, whereas λMsc-L contained only unique sequences that hybridized, as expected, to the position of the Antennapedia complex (84A), and pumilio (85CD) of the polytene chromosome. Wild-type cosmids and bacteriophage libraries were obtained from J. Tamkun (Tamkun et al. 1992) and initially screened with the pumilio sequences contained within λMsc-L using standard techniques (Sambrook et al. 1989). Additional screens were performed with cosmid insert fragments and pumilio cDNA sequences.

A 4- to 8-hr Drosophila embryo cDNA library was provided by N. Brown (Brown and Kalatos 1988) in the form of purified plasmid DNA. This DNA was used to transform the E. coli strain DH5α (BRL) both directly as well as after selection of clones >5 kb by preparative agarose gel electrophoresis. Both platings of this library were screened with two genomic fragments from the pumilio region, a 7.3-kb EcoRI fragment covering the Msc breakpoint and a 6.8-kb PstI–BamHI fragment that maps ~20 kb distal to the former. The Msc breakpoint probe identified 26 positive clones from a total of ~400,000 screened. Most of these also hybridized to the distal probe. The distal probe identified several hundred positive clones. Sequence analysis indicates that some of these are derived from the gene encoding the chromosomal protein D1 that had been mapped previously to this region (Ashley et al. 1989).

**Construction of a nanos–pumilio fusion gene and germ-line transformation**

A SacI restriction site was added to the nanos sequence immediately 3' of the start codon by performing a polymerase chain reaction with a mutagenic oligonucleotide using the nanos genomic clone pCW1 as a template (C. Wang, unpubl.). The amplified fragment extended from an EcoRI site 213 nucleotides upstream of the nanos transcriptional start through the start codon and ended with the introduced SacI site. This fragment was subcloned and then fused to upstream nanos sequences extending 2.1 kb 5' from the EcoRI site to an EcoRI site. This EcoRI–SacI fragment, containing the nanos promoter, 5'-untranslated leader region, and start codon, was then fused to the pumilio cDNA at a naturally occurring SacI site located 213 nucleotides 5' of the pumilio start codon. This fusion gene, encoding the entire Pumilio protein with an additional 72 amino acids attached to its amino terminus, was inserted into the P-element vector pDM30 (Misser and Rubin 1987) to generate pDM30/nos-pum. Germ-line transformation was performed as described previously (Spradling 1986) by injecting a solution containing 500 μg/ml pDM30/nos-pum, 100 μg/ml pr25.7wAc2-3 (gift of D. Rio), 5 mM KCl, and 0.1 mM NaPO₄ [pH 6.8] into embryos derived from a st pum680 ry ss/TM3 stock [mutations as described in Lindsey and Zimm 1992]. Homozygous pum680 Go females were tested for fertility directly, whereas homozygous males and heterozygous animals were first backcrossed with the pum680 stock to generate pum680 homozygous females. Six transformant lines were identified and tested for complementation of the pumilio phenotype at 20°C, the restrictive temperature for the maternal phenotype produced by the pum680 allele.

**Injections into embryos**

Injections of cytoplasm and RNA were performed as described previously (Lehmann and Nüsslein-Volhard 1986, Wang and Lehmann 1991). Embryos were injected into the prospective abdominal domain, between 20% and 50% egg length (0% = posterior pole). The developed cuticles were prepared as described (Lehmann and Nüsslein-Volhard 1991; Wang and Lehmann 1991) and scored for the number of abdominal segments formed. nanos RNA was synthesized in vitro from the full-length nanos cDNA clone pN5 as described in Wang and Lehmann (1991). Data from two independent experiments are summarized in Table 1. In each experiment, freshly synthesized nanos transcript was injected in parallel into nanos and pumilio embryos. For transplantation of cytoplasm, posterior pole plasm was taken either from wild-type embryos or from embryos derived from females transheterozygous for In(3R)Msc and T(3;1)FC8 and injected into the prospective abdominal domain of embryos from females homozygous for nos27.

**Analysis of embryonic cuticles**

Cuticle preparations were performed essentially as described (Wieschaus and Nüsslein-Volhard 1986). To study the effect of temperature on mutant phenotypes, females mutant for a specific allele combination were kept at either 20°C or 29°C. Embryos were collected for several hours and left to develop into larva for 48 hr (20°C) or 12 hr (29°C), and their cuticles were prepared. Embryos from females transheterozygous for In(3R)Msc and T(3;1)FC8 were collected at 20°C and at 29°C. In each sample the cuticle patterns of 50 larvae were examined, and all larvae in both samples lacked abdominal segmentation. Embryos were dechoriuated with 50% bleach, dissected from vitelline membranes with a glass pipette, and fixed in 50% Foy- er’s mounting and 50% lactic acid overnight at 60°C. Cuticles were photographed using dark field optics on a Zeiss Axiopt microscope with Kodak Technical Pan film.

**In situ hybridization to whole-mount embryos**

In situ hybridizations were performed as described by Tautz and Pfeifle (1989). For pumilio, the insert of the cDNA clone R7-1 was used as a probe, whereas the insert of the cDNA clone pN5 was used as a nanos probe (Wang and Lehmann 1991). Embryos were collected from wild type and pumilio In(3R)Msc/T(3;1)FC8, nanos [nos27/nos27], and vasa [vas201/vas201] mutant females for 2 hr at 20°C. These embryos were fixed and processed for whole-mount in situ hybridization of RNA.

**Antibody staining of whole-mount embryos**

To analyze the distribution of Hunchback and Nanos proteins in wild-type and mutant embryos, embryos were collected for 2 hr at 20°C or at room temperature and fixed immediately. Embryos were dechoriuated, fixed, and devitellinized according to the method of Mitchison and Sedat (1983) and stored in methanol at ~20°C. Because In(3R)Msc/T(3;1)FC8 flies lay very few eggs, embryos were alternatively devitellinized using fine tung-
sten needles after fixation. Both devitellinization techniques resulted in identical antibody staining patterns. All incubations were performed in 1.5-ml polypropylene tubes with TBST [0.05 M Tris-HCl (pH 7.0) 0.15 M NaCl, and 0.01% Triton X-100] while agitating on an Adams nutator (Clay Adams). Embryos were rehydrated for 2 hr at room temperature, and blocked by incubation in 1 ml of 5% normal goat serum for 2 hr at room temperature. Incubation in primary antibody was performed in 2% normal goat serum overnight at 4°C. Embryos were washed with 1 ml, four times for 15 min, and incubated with a biotin-conjugated secondary antibody [goat] for 4 hr at room temperature. Embryos were washed as described above and incubated with a horseradish peroxidase (HRP)-biotin/avidin complex [ABC Elite, Vector Labs] for 1 hr at room temperature. Embryos were washed as described above and incubated with 1 ml of 5% normal goat serum for 2 hr at room temperature, washed as described above and incubated with a fluorescein isothiocyanate-conjugated secondary antibody for 2 hr at room temperature, washed again, and mounted in TBST, 70% glycerol. Embryos were visualized with epifluorescence optics on a Zeiss Axiophot microscope. Video images were collected with a Hamamatsu C2400 Newvicon camera and an Argus 10 image processor (Hamamatsu). These images were then digitized using a Bio-Rad MRC 600 confocal microscope workstation and quantitatively analyzed on a Macintosh with NIH Image [version 1.41]. Flourescence intensity profiles along the length of several wild-type and pumilio embryos were averaged and inverted to represent the distribution of the Nanos protein in graphic form (Fig. 3). Statistical comparisons were performed at several positions along the length of the embryos using the Wilcoxon rank sum test (Lindgren et al. 1978). No significant difference was found between wild-type and pumilio embryos, whereas there is >99.5% probability that the distribution of the Nanos protein is statistically different in embryos derived from females carrying one copy of the nanos gene [DF (3R);DF<sup>PKY</sup>/TM3] or four copies [P<nanos>/P<nanos>]. P<nanos> is a rosy <mark>*</mark>-marked P-element containing the entire nanos gene (Wang and Lehmann 1991).

Antibody production

The anti-Nanos antibody is a rabbit polyclonal serum raised against a Nanos fusion protein produced in bacteria. Briefly, a P<sub>BD1</sub>-BGJII fragment of the nanos cDNA p<i>NS</i> (Wang and Lehmann 1991) was subcloned into the expression vector pET-3c (Rosenberg et al. 1987) to produce a fusion protein encoding 11 amino acids of T7 gene 10 protein fused in-frame to amino acids 51–400 of the 400-amino-acid nanos open reading frame. The fusion protein was injected into rabbits as inclusion bodies using Freund’s adjuvant.

The specificity of the antibody is shown by staining embryos derived from females homozygous for the nanos<sup>SN</sup> allele [data not shown]; homozygous nanos<sup>SN</sup> females do not contain detectable levels of nanos RNA (C. Wang and R. Lehmann, unpubl.). Embryos derived from these females show a uniform level of staining comparable to the level at the anterior half of wild-type embryos stained in parallel. Therefore, we concluded that the staining seen in the posterior half of wild-type embryos is specific for Nanos. The background, nonspecific staining can be reduced, while preserving the specific staining, by repeatedly preabsorbing the serum against wild-type, gastrula embryos [data not shown].

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