A Selective Screen Reveals Discrete Functional Domains in Drosophila Nanos

Gustavo Arrizabalaga* and Ruth Lehmann†

**Biology Department, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139-4307 and †Howard Hughes Medical Institute, Developmental Genetics Program, Skirball Institute at New York University Medical School, New York 10016*

Manuscript received June 8, 1999
Accepted for publication September 3, 1999

ABSTRACT

The Drosophila protein Nanos encodes an evolutionarily conserved protein with two zinc finger motifs. In the embryo, Nanos protein function is required for establishment of the anterior-posterior body pattern and for the migration of primordial germ cells. During oogenesis, Nanos protein is involved in the establishment and maintenance of germ-line stem cells and the differentiation of oocyte precursor cells. To establish proper embryonic patterning, Nanos acts as a translational regulator of *hunchback* RNA. Nanos' targets for germ cell migration and development are not known. Here, we describe a selective genetic screen aimed at isolating new *nanos* alleles. The molecular and genetic analysis of 68 new alleles has allowed us to identify amino acids critical for *nanos* function. This analysis shows that the CCHC motifs, which coordinate two metal ions, are essential for all known functions of Nanos protein. Furthermore, a region C-terminal to the zinc fingers seems to constitute a novel functional domain within the Nanos protein. This "tail region" of Nanos is required for abdomen formation and germ cell migration, but not for oogenesis.

ESTABLISHMENT of polarity along the anterior-to-posterior body axis of the Drosophila embryo requires the function of the maternal effect gene *nanos* (*nos*; Wang and Lehmann 1991). *nanos* RNA is synthesized during oogenesis and becomes localized to the oocyte's posterior pole at the end of oogenesis (Wang *et al.* 1994). Upon fertilization, Nanos protein is translated from the posteriorly localized RNA to form a posterior-to-anterior protein gradient. *Cis*-acting sequences required for the localization of *nanos* RNA, as well as sequences that prevent translation of unlocalized RNA, have been mapped to its 3′ untranslated region (UTR) (Gavis and Lehmann 1992; Gavis *et al.* 1996a,b; Dahanukar and Wharton 1996; Smibert *et al.* 1996). During embryogenesis, Nanos acts together with Pumilio to repress translation of the maternally provided mRNA for the transcription factor *hunchback* (*hb*; Barker *et al.* 1992; Macdonald 1992). In wild-type embryos, this creates a Hunchback protein gradient reciprocal to that of Nanos protein. In the absence of either *nanos* or *pumilio* function, Hunchback protein is translated throughout the embryo. This ectopic translation of *hunchback* RNA in the posterior region leads to the transcriptional repression of genes normally required for abdomen formation. Thus, *nanos* or *pumilio* mutant females produce embryos that lack an abdomen.

Biochemical analysis has shown that an ~400-amino-acid C-terminal region of the Pumilio protein binds directly to a repeated sequence motif in the 3′ UTR of *hunchback* RNA, termed the nanos response elements (NREs; Murata and Wharton 1995; Zamore *et al.* 1997). The NREs are necessary and sufficient for the regulation of *hunchback* by Nanos and Pumilio (Wharton and Struhl 1991). A number of experiments suggest that Pumilio is bound to the NREs throughout the embryo (Murata and Wharton 1995; Zamore *et al.* 1999). *hunchback* translation, however, is only inhibited where Nanos is present. Thus, the distribution of Nanos somehow determines the spatial regulation of Hunchback. This conclusion is supported by experiments in which Nanos was expressed ectopically either at the anterior pole or throughout the embryo (Gavis and Lehmann 1992, 1994). In both cases, head structures were lost and replaced by more posterior structures. In addition to its effects on *hunchback*, ectopic expression of Nanos also affects translation of the anterior morphogen *bicoid* (*bcd*), whose function is required for the development of the head and thoracic structures. Like *hunchback*, *bicoid* contains NRE sequences in its 3′ UTR (Wharton and Struhl 1991). The specific role of Nanos in the translational regulation of *hunchback* is unclear.

In addition to early pattern formation, *nanos* function is also required for germ-line development. In the early embryo, *nanos* RNA and protein are taken up into the primordial germ cells as they form at the posterior pole (Wang *et al.* 1994). In the absence of *nanos* function, many aspects of normal primordial germ cell (PGC) behavior are affected (Kobayashi *et al.* 1996; Forbes and Lehmann 1998). Germ cells fail to migrate toward the mesodermal tissue that forms the somatic gonad.

Corresponding author: Ruth Lehmann, New York University Medical Center, Skirball Institute, Developmental Genetics Program, 540 First Ave., 4th Fl., New York, NY 10016.
E-mail: lehmann@saturn.med.nyu.edu

Germ cell morphology is aberrant, and certain transcripts that are only expressed at later stages of normal development are expressed early in *nanos* mutant germ cells. It has therefore been proposed that Nanos affects the translation of a number of maternally deposited RNAs that are required for early germ cell migration (Kobayashi *et al.* 1996; Forbes and Lehmann 1998). *Hunchback* RNA is not the target of Nanos in the PGCs, since the germ cell migration defect of *nanos* mutant PGCs is observed even in the absence of maternal *hunchback* product (Forbes and Lehmann 1998).

Transcription of *nanos* is initiated during the first larval instar and is restricted to the germ line throughout development. While *nanos* mutant males are fertile, several aspects of female germ-line development are affected in *nanos* mutant females (Forbes and Lehmann 1998; Bhat 1999). In the wild type, most PGCs that reach the embryonic gonad will become germ-line stem cells. During the life of the female, these germ-line stem cells maintain themselves and produce daughter cells that are termed cystoblasts. The cystoblast undergoes four rounds of division to give rise to 16 interconnected cells, one of which will become the oocyte. In *nanos* mutants, the total number of germ-line stem cells established in the ovary is reduced and the remaining ones do not maintain their stem cell character. Differentiation of the cyst into an egg chamber is also affected, leading to the premature degeneration of egg chambers (Forbes and Lehmann 1998; Bhat 1999). As a consequence, *nanos* mutant females produce very few eggs. Neither the mechanism of Nanos protein function nor the targets for Nanos during germ-line development have been identified. Interestingly, while *nanos* and *pumilio* share a role in the translational regulation of *hunchback* and in germ cell migration, the oogenesis phenotypes of *nanos* and *pumilio* mutants are different. This observation suggests that Nanos and Pumilio do not function together during oogenesis (Forbes and Lehmann 1998).

nanos encodes a protein of 402 amino acids with a C-terminal region that is highly conserved among metazoa (Curtis *et al.* 1995). The C terminus includes two zinc fingers of the CCHC type (Curtis *et al.* 1997). Zinc finger domains similar to those of Nanos have been identified in proteins from Xenopus, leech, and *Caenorhabditis elegans* (Mosquera *et al.* 1993; Curtis *et al.* 1997; Pilon and Weisblat 1997; *C. elegans* EST database; Kraemer *et al.* 1999; Subramaniam and Seydoux 1999). The function of these genes is not known. However, a role similar to that of Drosophila Nanos in pattern formation and germ-line development has been proposed on the basis of their expression patterns.

Only four alleles of *nanos* have been described (see materials and methods; Wang *et al.* 1994; Curtis *et al.* 1997). While one allele is a stop codon, three alleles map to the C terminus. One of these carries a mutation in a cysteine that is predicted to be involved in metal ion binding. This mutation leads to a strong phenotype similar to that of complete null mutations. The other two mutations are a deletion and a point mutation in a region C terminal to the zinc fingers, which is termed here the "tail domain." These two mutations have been shown to affect *hunchback* translational regulation and have no effect on female germ-line development (Lehmann and Nüsslein-Volhard 1991; Wang *et al.* 1994). The role of these tail domain mutations in germ cell migration has not been analyzed.

Injection experiments using wild-type and mutant *nanos* transcripts have been used to determine the regions of the Nanos protein that are required for *nanos* function (Curtis *et al.* 1997). These experiments also showed that the zinc finger region and tail domain are necessary for *nanos* function in *hunchback* regulation. However, the specific experimental design restricted the analysis to the role of *nanos* in *hunchback* regulation. Therefore, the isolation of new *nanos* mutants could identify domains in the protein that are important for *nanos* function. Such an analysis may also provide important information regarding the different aspects of the *nanos* mutant phenotype.

Here, we describe a selective genetic screen that allowed us to obtain 68 new *nanos* alleles. We have identified specific lesions in the protein-coding region of 60 alleles and have characterized the phenotype of each mutation. This analysis demonstrates the functional relevance of the zinc finger domain for all aspects of *nanos* function. Our experiments further suggest that the tail domain constitutes a separate domain required for *hunchback* regulation and PGC migration. Implications of our analysis on the functional, structural, and evolutionary aspects of *nanos* are discussed.

MATERIALS AND METHODS

Fly strains: For the screen, wg^{IL1l4} was utilized. *sax*[1] *bn sp*/ *CyO p*($ry^{+t7.2}$ = *en1*) wg^{en11} flies were a gift from Bill Gelbart. $nos^{RC}/Df(3R)Dl^{x43}$ was used when crossing the transgenes to a *nanos* null background (Curtis *et al.* 1997). nos^{RC} is a mutation in a splice donor (G734A), nos^{RD} (C354Y) is a missense mutation in the zinc finger, nos^{L7} carries a seven-amino-acid deletion (Δamino acids 378–385) and nos^{RW} (D380N) is a missense mutation in the tail of Nanos (Figure 3A; Curtis *et al.* 1997).

Transgene and *P*-element transformation: The *nos-tub3′UTR* hybrid gene has been described previously (Gavis and Lehmann 1994), and its structure is shown in Figure 1D. It contains genomic sequences of *nanos*, with its 3′ UTR replaced by that of *tubulin* (*tub*). It also contains a hemagglutinin epitope tag at the 5′ end of the coding region. A *Not*I fragment from pDM30*n(ha)-t* (Gavis *et al.* 1996a) was inserted into the w^+ *P*-element vector pCaSpeR3 (Pirrotta 1998).

Injection of CaSpeR*nos-tub3′UTR* [*P*(*nos-tub3′UTR*)] into *yw* flies was performed as described by Spradling (1986). Seven independently transformed lines were established. Those transgenes inserted into the second chromosome were kept for use in the screen. Females from all transformant lines were completely sterile, producing embryos with head defects and lack of thoracic segments (data not shown). Protein expression

was observed in all lines by staining embryos with anti-Nanos antibodies (data not shown).

To test whether female sterility of the P(*nos-tub3′UTR*) transgene was 100% penetrant, 200 *y w*; P(*nos-tub3′UTR*)/*CyO wg* females from line 198.1 were crossed to 100 Oregon-R males and allowed to lay eggs for 4 days. None of the eggs developed, assuring us that even in large scale, this transformant line does not yield viable progeny.

EMS mutagenesis and selection for *nanos* alleles: See Figure 2 for an outline of the screen. Males carrying the transgene P(*nos-tub3′UTR*) were balanced with a *CyO* chromosome containing a *P*-element-induced, homozygous lethal allele of *wingless* (*wg*). A total of 14,400 of such males were mutagenized with a 35-mm solution of EMS (Sigma, St. Louis) in 1% sucrose for 24 hr using standard procedures (Ashburner 1989). The males were starved for 6 hr before mutagenesis in a bottle with a Kimwipe saturated with water. After EMS treatment, males were crossed to 14,300 *wg*/*CyO* females. In the next generation, recessive lethality of *wg* and the balancer chromosome eliminate all flies except for those carrying the transgene. A total of 186,000 flies were divided into groups of ~200 animals and allowed to mass mate. Only groups with a female carrying a dominant suppressor mutation gave rise to crawling larvae. A total of 68 of ~900 groups had F_2 progeny that developed to adulthood.

The surviving flies have a 50% chance of having inherited the suppressor. To identify those that carry the mutation, w^+ virgin females were tested for production of viable offspring by crossing them to *y w*; *wg*/*CyO* males. In this manner, 68 balanced heterozygous mutant lines were established. To ensure that all mutants studied were independent events, only one isolate per group was used for analysis. In this screen, we obtained a mutagenesis rate of 1 in 1370 haploid genomes.

Females from the F_2 generation containing two transgenes were identified by their eye color and used to distinguish between a mutation in the transgene or a second site suppressor. If the mutation lies within the transgene, the second transgene should be a wild-type version of the fusion gene, thus rendering the flies sterile. A second site suppressor should affect both transgenes equally, thereby allowing the females to produce viable offspring.

Complementation analysis: To cross *P(nos-tub3′UTR)* transgenes into the background of a *nanos* null mutant, two stocks were established for each line, each carrying the mutated transgene and either the null allele nos^{RC} or $Df(3R)Dl^{X43}$, which deletes the *nanos* locus. A cross between the two resulting stocks gave rise to *nanos* null mutant flies carrying one, two, or no copies of the mutated transgene. The females and their progeny were assayed for the ability of the transgene to complement the effect of *nanos* lack-of-function mutations on *hunchback* regulation, germ cell migration, and oogenesis.

Sequencing: Genomic DNA was isolated from each of the mutant lines (Barker *et al.* 1992). PCR was used to obtain DNA fragments of the *P(nos-tub3′UTR)* transgene. To isolate only the mutant transgene and not the endogenous *nanos* gene, we specifically amplified two fragments using primers directed against sequences in the epitope tag and the *tubulin* 3′ UTR (Figure 1D). Two PCR fragments were amplified from all mutants: one fragment extending from the epitope tag to the first intron of *nanos* and a second fragment from the first intron to the 3′ UTR of *tubulin*. At least two independent amplifications were performed for each fragment, to distinguish mutations in the transgene from those induced by PCR errors. We sequenced directly from the PCR fragments using either the AmpliCycle or ABI Prism systems from Perkin-Elmer (Norwalk, CT).

RNA analysis: Ovaries from mutant lines were isolated according to Theurkauf and Hawley (1992). RNA was purified with Trizol Reagent (Life Technologies, Gaithersburg, MD), following the manufacturer's instructions. Northern blotting and hybridization was performed following standard methods (Sambrook *et al.* 1989). The blot was probed with a *nanos* cDNA probe. Since the 3′ UTR of the transgene is shorter than that of the endogenous gene, RNA from the transgene and the endogenous gene can be assayed simultaneously. Only five of the eight alleles that do not show any sequence changes in the coding region could be analyzed; the remaining three alleles were lost after phenotypic and sequence analysis.

Germ-line clones: *yw*; *FRT82* $hb^{FB}nos^{L7}$ females were crossed to *yw hsFLP*^{122}; *FRT82* ovo^{D} males (a gift from Claude Desplan), and their progeny were heat-shocked as second or third instar larvae to induce FLP expression (Chou and Perrimon 1996). Larvae were heat-shocked at 37° as described by Forbes and Lehmann (1998). *yw hsFLP*^{122}; *FRT82* $hb^{FB}nos^{L7}$/*FRT82* ovo^{D} females were crossed to nos^{RC}/$Df(3R)Dl^{x43}$. ovo^{D} females do not produce eggs; thus only hb^{FB} nos^{L7} homozygous germ-line clones will give rise to progeny.

Cuticle preparation and embryo staining: For cuticle preparations, embryos were dechorionated in 50% bleach, fixed in 1:4 glycerol and acetic acid, and mounted in Hoyer's medium (Lehmann and Nüsslein-Volhard 1986). Embryos derived from germ-line clones were stained with anti-Vasa antibody to visualize the germ cells as described previously (Forbes and Lehmann 1998).

RNA injections: To obtain nos^{WT} RNA, vector pN5 (Wang and Lehmann 1991) containing the full-length *nanos* cDNA was linearized with *Xho*I and transcribed with SP6 polymerase using the mMessage mMachine system (Ambion, Austin, TX). To isolate nos^{L7} RNA, we performed the same process with a derivative of pN5 carrying the small deletion in the carboxyl terminus (Curtis *et al.* 1997). The RNA was precipitated in ethanol and resuspended in water. Three concentrations were used in the injections: 250 ng/μl, 500 ng/μl, and 1.3 μg/μl.

Site-specific PCR mutagenesis was used to generate pN54CF1 and pN54CF2. pN54CF1 carries a mutation that changes the fourth Cys of the first finger to a Ser, while pN54CF2 carries a mutation in the fourth Cys of the second finger, changing it to a Ser. Both of these plasmids were linearized with *Xho*I and transcribed with SP6 polymerase. The two RNAs were injected at a concentration of 1.5 μg/μl.

A 0- to 45-min collection of embryos laid by nos^{L7}/nos^{L7} mothers was dechorionated, lined up for injection on a coverslip, and injected from the dorsal side. Embryos were allowed to develop for 2 days at 18°. Hatched larvae were collected, and unhatched embryos were hand devitellinized and mounted in 50% Hoyer's and 50% lactic acid for cuticle preparation.

RESULTS

Isolation of new *nanos* alleles: A large-scale analysis of the amino acids that are essential for Nanos protein function should provide useful information regarding structurally important domains within the Nanos protein. Only four of the known *nanos* alleles affect the protein coding region (Lehmann and Nüsslein-Volhard 1991; Curtis *et al.* 1997). We therefore designed a new scheme to identify important amino acids in Nanos that allowed specific recovery of a large number of new *nanos* alleles. This mutagenesis screen identifies mutations in *nanos* by suppressing the dominant female sterile phenotype that is caused by ectopic expression of Nanos. Replacing the *nanos* 3′ UTR with the *tubulin* 3′ UTR leads to stable expression of unlocalized *nanos*

RNA, which is translated throughout the embryo (Gavis and Lehmann 1994). The presence of Nanos protein in the anterior of the embryos inhibits the translation of *hunchback* and *bicoid* RNA (Gavis and Lehmann 1992). As a consequence, females that carry a transgene with the Nanos coding sequences fused to the 3′ UTR of *tubulin* [P(*nos-tub3′UTR*)] produce embryos that lack head and thoracic structures (Figure 1B). These embryos die at the end of embryogenesis, thereby rendering the females that carry this transgene dominant sterile. A mutation induced in the transgene that disrupts *nanos* function as a translational repressor of *bicoid* will revert the female sterile phenotype, thereby leading to viable offspring in the next generation (Figure 1C). The mutated transgene can be analyzed subsequently for mutations in the Nanos coding sequence.

While we are interested in identifying amino acids important for the biologically relevant functions of Nanos protein, the mutagenesis scheme selects for *nanos* mutations that suppress its ability to repress *bicoid* RNA translation. A number of observations suggest that the effects of Nanos on *hunchback* and *bicoid* translational regulation are quite similar and, therefore, that mutations affecting the regulation of one should also affect regulation of the other. First, *bicoid* regulation by Nanos is dependent on sequences in the *bicoid* 3′ UTR that are, with regard to base composition and function, similar to the NREs in the 3′ UTR of *hunchback* (Wharton and Struhl 1991). Second, Pumilio protein, which acts in conjunction with Nanos in the regulation of *hunchback*, is also required for translational repression of *bicoid* (Lehmann and Nüsslein-Volhard 1991). Third, we tested whether known alleles of *nanos*, which were identified on the basis of their effect on *hunchback* regulation, also abolish *bicoid* regulation. For this test, we took advantage of an *oskar* (*osk*) transgene *P*(*osk-bcd3′UTR*) that localizes *oskar* RNA and protein to the anterior pole of the egg (Ephrussi and Lehmann 1992). In the wild type, *nanos* RNA localization to the posterior is dependent on *oskar* activity (Ephrussi *et al.* 1991). Consequently, anterior localization of *oskar* by the transgene leads to ectopic localization of the endogenous *nanos* RNA and protein to the anterior. Ectopic Nanos protein causes anterior deletions and posterior duplications in embryos derived from females carrying the *P(osk-bcd3′UTR)* transgene (Ephrussi and Lehmann 1992). We placed the *P(osk-bcd3′UTR)* transgene into the background of two different, strong *nanos* missense mutations, nos^{RC} and nos^{RD}, and analyzed the phenotype of the progeny. Embryos derived from these females showed the *nanos* phenotype: head and thorax were formed normally, but there was no abdomen (data not shown). This result demonstrates that a mutant Nanos protein unable to repress *hunchback* regulation is also unable to repress *bicoid* translation. Thus, a genetic screen based on the suppression of Nanos-mediated regulation of *bicoid* should allow identification of mutations that are also deficient in *hunchback* regulation. Accordingly, we isolated 68 independent mutant lines through our suppression screen (see materials and methods).

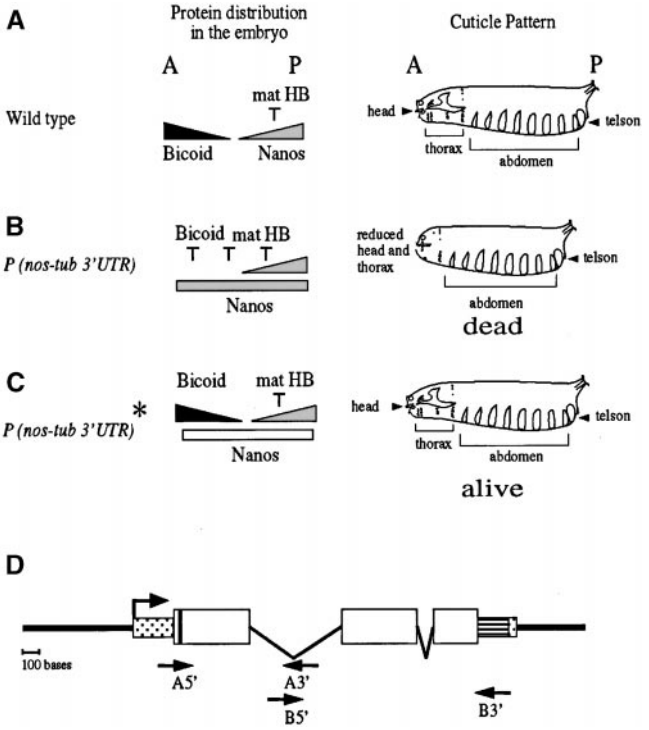

Figure 1.—Reversion of the dominant female sterile phenotype caused by ectopic Nanos. (A–C) The expression pattern of Nanos (gray), Hunchback (HB), Bicoid (black), and the resulting embryonic phenotype are depicted for different maternal genotypes: wild type, *P(nos-tub 3′UTR)*, and the suppressor mutants, respectively. Wild-type females (A) lay eggs with *nanos* RNA localized to the posterior pole. Nanos protein forms a posterior-to-anterior gradient that represses translation of maternal *hunchback* RNA in the posterior region (Wang and Lehmann 1991). The resulting larvae have eight abdominal segments, three thoracic segments, head structures in the anterior, and a telson and stretched filzkörper at the posterior. (B) Females carrying the *P(nos-tub 3′UTR)* transgene produce unlocalized Nanos (gray rectangle; Gavis and Lehmann 1994). The resulting larvae lack head structures and thoracic segments. (C) A mutation in the *P(nos-tub 3′UTR)* transgene abolishes ectopic Nanos function (white rectangle). Since endogenous *nanos* is unaffected in this mutant, the resulting larvae will be identical to the wild type. A, anterior; P, posterior. (D) Schematic of *P(nos-tub 3′UTR)* transgene (Gavis and Lehmann 1994). The genomic sequences of *nanos* were used. Plain thick lines, flanking genomic sequences from the *nanos* gene; open boxes, *nanos* exons; thin lines, introns; black bar, hemagglutinin epitope tag. Stippled boxes at the 5′ and 3′ ends depict *nanos* UTR sequences, and the box with vertical lines represents the *tubulin* 3′ UTR. The two pairs of primers used to specifically isolate the transgenic DNA are shown as arrowheads. Primers A5′ and A3′ produce a 559-bp fragment, while primers B5′ and B3′ amplify a 1102-bp fragment.

Two types of mutations can result in a reversion of the female sterile phenotype: intragenic and second site suppressors. A simple test allowed us to distinguish

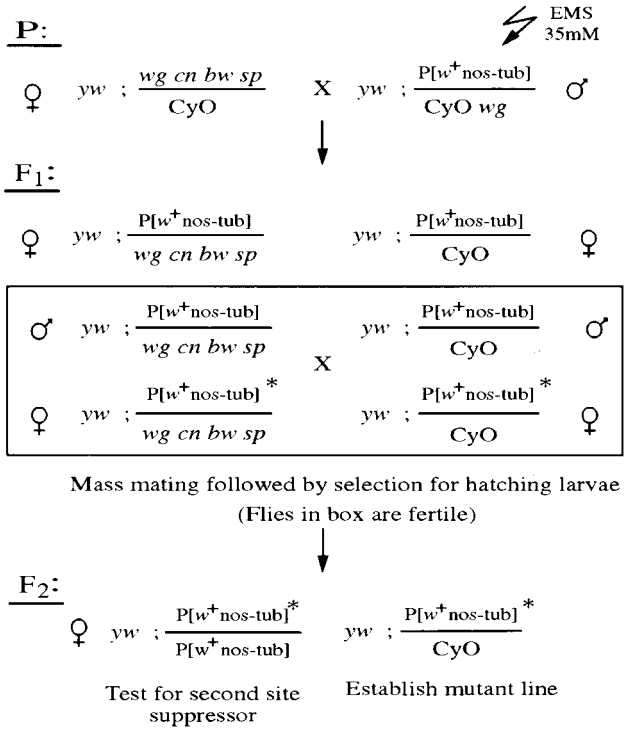

Figure 2.—Selection protocol and isolation of stable mutant lines. In the F_1 generation, all females are sterile except for those carrying a mutation (*) that affects the transgenic *nanos*. The box includes the genotype of the males and the fertile females in this generation. For details about the strains used and the screen itself, see materials and methods.

between these two possibilities (Figure 2). Since expression of the w^+ marker carried by the P(*nos-tub3′UTR*) transgene is dosage sensitive, F_2 female flies with two copies of the transgene could be easily distinguished from those carrying only one copy. Thus, to distinguish between intragenic and second site suppressors, we analyzed the phenotype of the progeny of females that carry two copies of the transgene. If the mutation lies within one of the transgenes, the second copy of the transgene should still render the flies sterile. On the other hand, a second site suppressor should affect both transgenes equally, therefore allowing females carrying two copies of the transgene to produce progeny that are normal or phenotypically less severe. All mutant lines were tested in this manner, and none proved to carry a second site suppressor (see below). We conclude that the 68 mutations identified are likely to affect directly the function of the *nanos* transgene.

Molecular analysis of *nanos* alleles: Once mutant lines were established, the transgenes were analyzed molecularly. Each line contains both a copy of the transgenic and endogenous *nanos* gene. Transgenic sequences were specifically isolated from the mutant lines by using primers unique to the transgene (see materials and methods). We have identified mutations in 60 alleles. All but two mutations affect a single base. EMS is an alkylating agent that can add an ethyl group to many positions in all four bases. In Drosophila, the prevalent mutation resulting from EMS mutagenesis is a GC → AT transition (Ashburner 1989). This type of change is seen in 50 of 58 mutants isolated (Tables 1–3). In addition, we identified six AT → TA and two TA → GC transversions. A change in the protein coding sequence was not detected in 8 of the 68 alleles. RNA blot analysis of five of these alleles shows that RNA from the transgene is either undetectable or significantly reduced compared to the endogenous RNA (data not shown). These alleles are therefore most likely to be defective in *nanos* RNA synthesis or stability.

We have divided the mutants into three categories: nonsense mutations (Table 1), rearrangements (Table 2), and missense mutations (Table 3). In the first category, we found 27 EMS-induced premature stop codons (Table 1). The stop codons are distributed throughout the coding sequence and show no specific pattern. The

TABLE 1

Nonsense mutations

nanos alleles	Base change	Amino acid change
286	C(565[a]) → T	Arg(102) → Stop[b]
635	C(568) → T	Gln(103) → Stop[b]
641	C(598) → T	Gln(113) → Stop[b]
554	C(625) → T	Gln(122) → Stop[b]
607	C(673) → T	Gln(138) → Stop[b]
517	C(1302) → T	Gln(165) → Stop[b]
539	C(1314) → T	Gln(169) → Stop[b]
524	T(1337) → A	Tyr(177) → Stop[c]
243	C(1390) → T	Gln(194) → Stop[c]
253	C(1390) → T	Gln(194) → Stop[c]
254	C(1390) → T	Gln(194) → Stop[c]
197	C(1401) → T	Gln(198) → Stop[b]
111	C(1404) → T	Gln(199) → Stop[b]
204	C(1404) → T	Gln(199) → Stop[b]
258	C(1404) → T	Gln(199) → Stop[b]
264	C(1410) → T	Gln(201) → Stop[b]
212	C(1461) → T	Gln(218) → Stop[b]
558	G(1549) → A	Trp(247) → Stop[b]
594	T(1594) → A	Leu(262) → Stop[b]
139	C(1596) → T	Gln(263) → Stop[b]
592	C(1671) → T	Gln(288) → Stop[b]
595	C(1671) → T	Gln(288) → Stop[b]
620	C(1671) → T	Gln(288) → Stop[b]
568	A(1719) → T	Lys(304) → Stop[b]
122	C(1912) → T	Gln(344) → Stop[d]
183	C(1912) → T	Gln(344) → Stop[d]
263	A(1999) → T	Lys(373) → Stop[b]

[a] The number of the base mutated corresponds to the numbering given by C. Wang for the genomic sequence of *nanos* (Wang and Lehmann 1991) with one correction. An extra three bases, AGA, were found between bases 1410 and 1411. Consequently, His (201) is replaced by Gln and Ile.
[b] Amber.
[c] Ochre.
[d] Opal.

TABLE 2

Rearrangements

Nanos alleles	Base change	Mutation
246	G(1827) → A	Last G of intron 2
526	G(1827) → A	Last G of intron 2
623	G(1827) → A	Last G of intron 2
272	Δ 1722-1775	In-frame deletion, amino acids 305–331
581	13-base insert	Between bases 1722 and 1723

mutants in the rearrangement category (Table 2) include a deletion (*nos*^{272}), an insertion (*nos*^{581}), and three alleles that affect the intron/exon structure of the transgene (*nos*$^{246/526/623}$). The deletion and the insertion mutation start at the same base. The deletion is in frame and eliminates 27 amino acids that affect only the first zinc finger, leaving the second finger and the end of the protein intact.

The 28 missense mutations have proven to be very informative. All of these mutations fall within the C-terminal region of the protein (Figure 3). This region spans the two zinc fingers and the last 37 amino acids, which include a 9-amino-acid stretch referred to as the "tail region." Most of the cysteines and histidines in the zinc finger motifs were mutated (Figure 3A), with the exception of the fourth Cys (C347 and C371) of each finger and the first Cys (C355) in the second finger. C355 is changed to a Tyr in the previously isolated allele *nos*RD (Curtis *et al.* 1997).

To determine whether the fourth Cys (C347 and C371) in each zinc finger is important for *nanos* function, we tested the effect of directly mutating the respective amino acids. For this assay, C347 and C371 were mutated to Ser to generate the mutated cDNAs 4CF1 and 4CF2. RNA was prepared from wild-type and each mutant cDNA and was injected into embryos from *nos*^{L7} females. While injection of wild-type *nanos* cDNA (pN5) rescues the abdominal phenotype of *nanos* mutant embryos at a concentration of 250 ng/μl, RNAs synthesized from the mutant cDNAs were unable to rescue, even at a concentration of 1.5 μg/ml (Table 4). We conclude that each amino acid of the CCHC motifs is important for *nanos* function.

The remaining missense alleles are distributed in four clusters in the C terminus of the protein (Figure 3A). The first group (*nos*^{512}, *nos*$^{505/520}$, and *nos*^{672}) falls between the His and last Cys of the first finger. A second group of alleles (*nos*$^{585/587}$, *nos*$^{152/154}$, and *nos*^{627}) affects the region between the two fingers. Two mutations (*nos*^{6} and *nos*$^{277/560/565/579/626}$) lie in the second finger between the second Cys and the His. One of these two mutations, A365T, was identified five times, more than any mutation in the screen. The last three mutations (*nos*^{295}, *nos*$^{3/108}$) fall into the "tail region" of Nanos. Previous screens had identified two other alleles (*nos*^{L7} and *nos*^{RW}) that map to this region of the protein (Figure 3A).

In summary, this screen identified 16 amino acids in the carboxyl terminal region of Nanos essential for the regulation of *bicoid* translation by Nanos. Eight mutations change histidines or cysteines in the CCHC motifs expected to have a function in the coordination of zinc, while the other 20 mutations identify other amino acids within the C terminus that are important for *nanos* function.

Phenotypic characterization of *nanos* alleles: The new *nanos* alleles were identified on the basis of their failure to repress *bicoid* translation. To determine whether the other known functions of *nanos* were also affected by the new mutations, we devised a complementation test. This genetic test rests on the observations that one copy of the wild-type *P(nos-tub3′UTR*) transgene is able to complement the oogenesis phenotype of *nanos* null mutant females and to rescue the abdominal phenotype of embryos produced by such females (this study; Gavis and Lehmann 1992). However, these embryos still show head structure defects and do not hatch due to translational repression of *bicoid* (Gavis *et al.* 1996b).

To determine the ability of the new alleles to regulate *hunchback* translation, we crossed all mutant *P(nos-tub3′UTR)* transgenes into the background of a *nanos* null mutant and analyzed the embryonic phenotype (see materials and methods). In the case of a mutation that affects both *bicoid* and *hunchback* regulation, the embryonic phenotype should be indistinguishable from that of a *nanos* null mutant. On the other hand, we would expect embryos to develop to adulthood if the mutant transgene was only defective in the regulation of *bicoid*. All new mutations affect *hunchback* translational regulation. The three rearrangement mutations and all but one missense mutation, when crossed to the null background in one or two copies, gave rise to embryos that were phenotypically indistinguishable from strong *nanos* mutant embryos (Table 3). These embryos have normal thoracic and head structures, but they lack all abdominal segments. The nonsense mutations behaved identically, with the exception of the amber mutants, which produced hatching larvae at low frequency and, thus, seem to allow occasional readthrough. Only one of the missense alleles, *nos*^{295}, partially complemented the abdominal phenotype caused by the *nanos* null background. Approximately 30% of the embryos laid by *nanos* null females carrying one copy of the *nos*^{295}

TABLE 3

Missense mutations

nanos alleles	Base change	Amino acid change	Abdominal phenotype	Oogenesis phenotype
273	G(1841) → A	Cys(320) → Tyr	Strong	Strong
516	G(1841) → A	Cys(320) → Tyr	Strong	Strong
599	G(1841) → A	Cys(320) → Tyr	Strong	Strong
18	A(1849) → T	Cys(323) → Ser	Strong	Strong
506	C(1888) → T	His(336) → Tyr	Strong	Strong
512	C(1892) → T	Ser(337) → Leu	Strong	Strong
505	T(1895) → A	Val(338) → Glu	Strong	Strong
520	T(1895) → A	Val(338) → Glu	Strong	Strong
672	G(1898) → A	Arg(339) → Gln	Strong	Strong
585	C(1924) → T	Pro(348) → Ser	Strong	Strong
587	C(1924) → T	Pro(348) → Ser	Strong	Strong
153	T(1931) → G	Leu(350) → Arg	Strong	Strong
154	T(1931) → G	Leu(350) → Arg	Strong	Strong
627	G(1934) → A	Arg(351) → Gln	Strong	Strong
538	G(1942) → A	Val(354) → Met	Strong	Strong
549	G(1942) → A	Val(354) → Met	Strong	Strong
165	G(1955) → A	Cys(358) → Tyr	Strong	Strong
614	G(1955) → A	Cys(358) → Tyr	Strong	Strong
6	G(1967) → A	Gly(362) → Glu	Strong	Strong
277	G(1975) → A	Ala(365) → Thr	Strong	Strong
560	G(1975) → A	Ala(365) → Thr	Strong	Strong
565	G(1975) → A	Ala(365) → Thr	Strong	Strong
579	G(1975) → A	Ala(365) → Thr	Strong	Strong
626	G(1975) → A	Ala(365) → Thr	Strong	Strong
19	C(1978) → T	His(366) → Tyr	Strong	Strong
295	C(2015) → T	Thr(378) → Ile	Weak	WT
3	T(2018) → A	Met(379) → Lys	Strong	WT
108	T(2018) → A	Met(379) → Lys	Strong	WT

Null mutant *nanos* females carrying a copy of the mutated *nanos* transgene were tested for complementation of abdominal and oogenesis *nanos* phenotype. "Strong" abdominal phenotype: embryos showed no abdominal segments. "Weak" abdominal phenotype: ~50% of embryos showed between two and eight abdominal segments. "Strong" oogenesis phenotype: females laid very few eggs, and their ovaries lacked mature eggs. WT oogenesis phenotype: females laid normal number of eggs, and their ovaries appeared to be wild type.

transgene form two to eight abdominal segments, while the remaining embryos formed no abdominal segments. With two copies of the transgene, the frequency of embryos showing a weak abdominal phenotype increased to ~50%. A previously isolated allele, nos^{RW}, shows a similarly weak phenotype (Lehmann 1988; Curtis *et al.* 1997). Like nos^{RW}, the mutation in nos^{295} changes an amino acid in the tail region of the protein (Curtis *et al.* 1997). Thus, all mutations that affect the ability of Nanos to regulate *bicoid* translation are also defective in *hunchback* regulation. Furthermore, the fact that we obtained an allele that only weakly affects abdomen but loses its ability to repress head formation indicates that the regulation of *bicoid* translation is more sensitive to a decrease in Nanos activity than *hunchback* regulation.

To examine whether the newly identified mutations also affect oogenesis, we tested the ability of the mutated transgenes to complement the oogenesis phenotype of females carrying a *nanos* null mutation *in trans* to a deletion of the gene (see materials and methods). Females that carry *nanos* null mutations produce very few eggs and show defects in early oogenesis (Forbes and Lehmann 1998). All missense alleles with mutations in the zinc finger region have a phenotype identical to that described for *nanos* null mutations (Figure 3A; Table 3). In contrast, the three alleles that are mutated in the tail region of *nanos* fully complement the oogenesis phenotype. *nanos* mutant females carrying one or two copies of these three transgenic alleles laid a normal number of eggs, and their ovaries looked indistinguishable from those of wild-type females. Alleles nos^{108} and nos^{3}, which both cause the same amino acid change, complement the oogenesis defect but completely fail to regulate *hunchback* translation. This phenotype is similar to that observed for the *nanos* mutation nos^{L7}, which deletes seven amino acids in the tail region. Two mutations, the allele nos^{RW} and the newly identified allele nos^{295}, weakly affect abdomen formation and fully complement the oogenesis phenotype. These results suggest that the tail domain of the Nanos protein may be re-

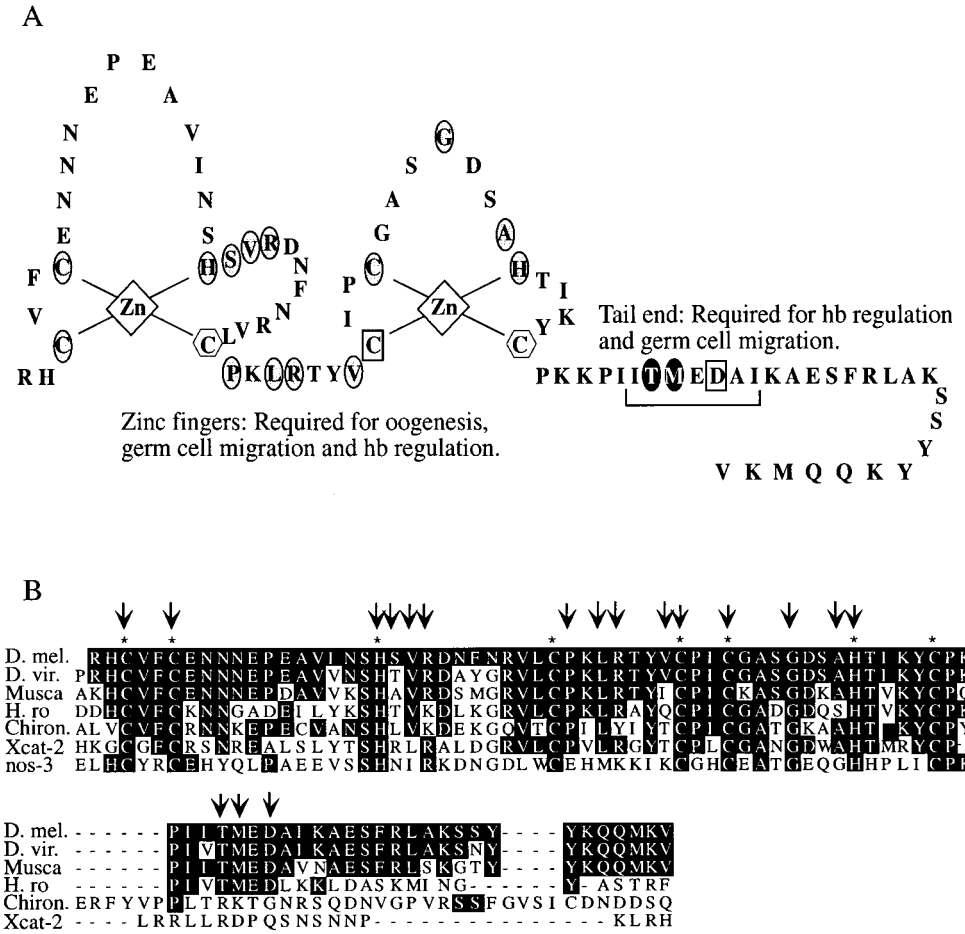

Figure 3.—Missense mutations in the carboxyl-terminal region of Nanos. (A) The last 87 amino acids of Nanos are depicted with the proposed configuration of the two zinc fingers (Curtis *et al.* 1997). Gray and black circles, amino acids mutated in the screen described here; hexagons, amino acids tested by *in vitro* injection assay; open boxes, amino acids mutated in previous screens. The bracket indicates the seven amino acids deleted in the previously described nos^{L7} allele (Curtis *et al.* 1997). (B) Alignment of the C terminus of Nanos from *Drosophila melanogaster* (*D. mel.*) and *virilis* (*D. vir.*), *Musca domestica* (Musca), *Chironomus samoensis* (Chiron.; Curtis *et al.* 1995), leech *Helobdella robusta* (*H. ro*; Pilon and Weisblat 1997), the *X. laevis* protein Xcat-2 (Mosquera *et al.* 1993), and the *C. elegans* protein Nos-3 (Wilson *et al.* 1994). For Nos-3, only the two zinc fingers are shown, although 118 amino acids follow this domain. Three *C. elegans* homologs have been identified (*C. elegans* EST database; Kraemer *et al.* 1999; Subramaniam and Seydoux 1999). Nos-3 is the only homologe that contains both zinc fingers with the exact spacing seen in Drosophila Nanos. Asterisks indicate the Cys and His involved in forming the two zinc fingers. Arrows point to amino acids that have been affected in this screen and previous screens.

quired specifically for the translational regulation of *hunchback* and *bicoid*, while the zinc finger domain seems to be necessary for all aspects of *nanos* function.

Nanos tail domain defines a functional domain: Five mutations have been identified that map to the tail region of the Nanos protein. These mutations specifically affect *hunchback* (and *bicoid*) regulation, but they do not interfere with normal oogenesis. One reason for this specificity could be that this region of the protein carries a function only required for the regulation of *hunchback* (and *bicoid*). Alternatively, these mutations may simply reduce *nanos* function. In this case, *hunchback* (and *bicoid*) regulation would be more sensitive to a reduction in *nanos* function than oogenesis. If this mutant form of Nanos simply has reduced activity, an increase in protein concentration should at least partially restore the ability of the mutant protein to regulate *hunchback* and, thus, rescue the abdominal phenotype. Such an increase of a Nanos protein lacking a specific, essential function should have no effect. Two different means were used to increase the level of Nanos protein with mutations in the tail domain. In the first test, one or two copies of the mutated transgene nos^{108} were crossed into a homozygous nos^{L7} background. This genetic combination approximately doubles the amount of Nanos "tail mutant" protein in the embryo (data not shown). Figure 4 shows the phenotype of an embryo from a mother homozygous for nos^{L7} and the transgene nos^{108}. All nos^{L7} embryos, which received either one or two copies of the nos^{108} transgene, completely lack abdomen and the filzkörper are not extended. Thus, increasing the amount of Nanos protein by adding two additional copies of the *nanos* mutant transgene is not sufficient to weaken the abdominal phenotype.

In the second test, we decided to increase the levels of mutant protein even further by injection of RNA that encodes a protein with a deletion of the tail region. We introduced the seven-amino-acid, in-frame deletion of the nos^{L7} mutation into the wild-type *nanos* cDNA (see materials and methods). RNA transcribed from this construct or a wild-type construct was injected into nos^{L7} mutant embryos. Wild-type *nanos* RNA restored the abdominal phenotype of 85% of *nanos* mutants when injected at a concentration of 250 ng/μl. The overall

TABLE 4

RNA injection into *nos*^{L7} embryos

RNA	No. abdominal segments 0	1–5	6–8	Overall rescue (%)	Strong rescue (%)	*N*
nos^{WT} 250 ng/μl	10	9	49	85	72	68
nos^{WT} 500 ng/μl	7	10	58	91	77	75
nos^{WT} 1.3 μg/μl	5	6	39	90	78	50
nos^{L7} 250 ng/μl	65	0	0	0	0	65
nos^{L7} 500 ng/μl	72	0	0	0	0	72
nos^{L7} 1.3 μg/μl	53	0	0	0	0	53
4CF1 1.5 μg/μl	113	0	0	0	0	113
4CF2 1.5 μg/μl	96	0	0	0	0	96
Uninjected	20	0	0	0	0	20

Overall rescue refers to embryos with any abdominal segments. Strong rescue refers to embryos with six to eight segments. 4CF1 has fourth Cys in first finger changed to a Ser. 4CF2 has fourth Cys in second finger mutated to a Ser. *N*, number of cuticles scored.

frequency of rescue as well as the average number of abdominal segments formed per embryo further increased with higher concentrations of wild-type RNA. In contrast, *nos*^{L7} RNA does not rescue the abdominal phenotype, even at a concentration as high as 1.3 μg/μl (Table 4). We conclude that mutations in the tail region of Nanos render the protein inactive for the regulation of *hunchback*. Taken together, these two experiments suggest that the region carboxyl-terminal to the Nanos Zn fingers defines a functional domain of the protein. This region is required for the translational regulation of *hunchback* and *bicoid* function, but not for *nanos* function, during oogenesis.

Nanos tail domain affects some aspects of germ cell migration: Primordial germ cells devoid of Nanos fail to migrate correctly through the embryo to reach the somatic portion of the embryonic gonad (Kobayashi *et al.* 1996; Forbes and Lehmann 1998). We therefore decided to test whether the tail region of Nanos is required for the migration of germ cells. Since embryos mutant for *nos*^{L7} lack abdomen, and the somatic part of the gonad forms from abdominal mesoderm, we could

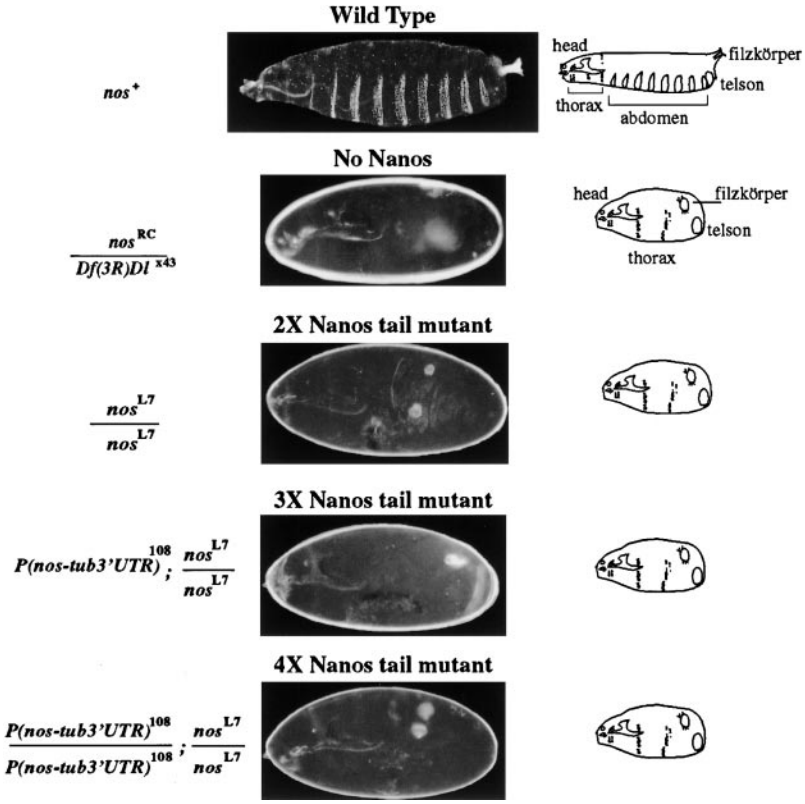

Figure 4.—Multiple copies of a *nanos* gene carrying a mutation in its tail region do not rescue the abdominal phenotype. Cuticle preparations and sketch of embryos derived from females with two, three, or four copies of *nanos* carrying a mutation in the tail region. These embryos look identical to those from *nanos* null females. All embryos display a strong *nanos* mutant phenotype: they lack abdominal segments and have a ventral scar and unstretched filzkörper. In all panels, anterior is left and dorsal is up.

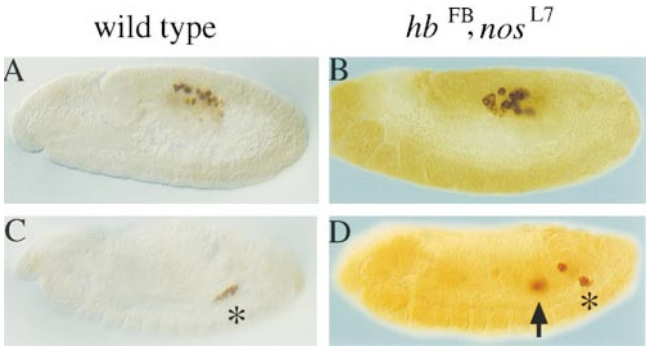

Figure 5.—Nanos tail region is required for germ cell migration. (A–D) Wild-type embryos (A and C) and mutant embryos from a hb^{FB} nos^{L7} germ-line clone (B and D) stained with anti-Vasa antibody to show germ cells in brown. (A and B) Stage 11 embryos. In the wild type (A), germ cells move from the midgut toward the lateral mesoderm. In the mutant embryo (B), the germ cells clump together on the midgut and do not migrate properly to the mesoderm. (C and D) Stage 13 embryos. In the wild type (C), germ cells have associated with the gonadal mesoderm to form the gonad, the germ band is retracted and the posterior midgut is approaching the anterior midgut rudiment. In the mutant embryo (D), primordial germ cells are organized in several clumps: one of these clumps is attached to the posterior midgut (arrow), and another clump (*) is in the same position as the gonad in the wild-type embryo (C). In all panels, anterior is left and dorsal is up.

not assay germ cell migration in this background. However, it was previously shown that embryos lacking maternally derived *hunchback* and *nanos* can develop a normal abdomen (Hülskamp *et al.* 1989; Irish *et al.* 1989; Struhl 1989). In these embryos, germ cell migration has been observed and it was shown that complete lack of Nanos dramatically affects this process (Forbes and Lehmann 1998). We tested the nos^{L7} mutant in a similar manner. Since *hunchback* is required for embryonic development, homozygous *hunchback* females do not survive. We therefore generated embryos mutant for both maternal *hunchback* and *nanos* by generating germ-line clones using the *FRT/FLP/Ovo*^{D} system (Chou and Perrimon 1996; see materials and methods).

Females with hb^{FB} nos^{L7} mutant germ-line clones were crossed to *nanos* null mutant males to produce embryos that are mutant in the maternal and zygotic contribution of *nanos.* The resulting embryos were stained with anti-Vasa antibodies to visualize the germ cells (Figure 5). Germ cells form normally in these embryos and first show defects after stage 10 of embryogenesis. At this stage, wild-type and mutant germ cells have traversed the posterior midgut and have moved on the gut toward the overlying mesoderm. While wild-type germ cells subsequently migrate toward the lateral mesoderm, many nos^{L7} mutant germ cells fail to leave the gut and tightly associate into clusters of cells (Figure 5, A and B). Not all mutant germ cells stay on the midgut; some germ cells follow a normal migratory pattern (Figure 5D). The morphology of the mutant cells seems normal, and the embryos go on to develop into fertile flies, confirming the observation that some germ cells associate correctly with gonadal tissues. The migration phenotype of germ cells that have the tail domain of Nanos mutated resembles that of germ cells completely lacking Nanos protein (nos^{BN} allele; Forbes and Lehmann 1998). However, the germ cell loss is more extensive in null mutants.

DISCUSSION

A selective screen was performed to identify amino acids in Nanos that are essential for its function. A total of 68 new alleles were identified and characterized molecularly. All 28 missense mutations affect the carboxyl-terminal region of the protein. This region harbors two CCHC-type zinc fingers that were both mutated in this screen. In addition, C terminal to the zinc fingers, a region of seven amino acids, the tail domain, has been identified as a separate domain required for Nanos activity during embryogenesis and germ cell migration, but not for oogenesis.

Given the design of the screen, it is not surprising that second site suppressors were not obtained. A mutation in another protein necessary for *bicoid* regulation by Nanos would likely affect *hunchback* regulation as well. While such a mutation would be expected to rescue the *bicoid* head defects, it may likely cause abdominal defects due to a failure in *hunchback* regulation. A second site mutation that would specifically affect *bicoid* regulation is one that would mutate the NRE in the *bicoid* 3′ UTR and thereby make *bicoid* nonresponsive to Nanos-mediated translational repression. Nevertheless, our screen does not seem to have isolated such mutations.

Genetic analysis of the mutant lines showed that all mutations, identified on the basis of their inability to repress *bicoid* translation, also affected the ability to regulate *hunchback* translation. The fact that we did not find any mutations that specifically affected *bicoid* regulation further supports the idea that both regulatory functions are performed via the same mechanisms. Indeed, it has been shown that both genes are regulated by similar *cis*-acting sequences, the NREs, and the same *trans*-acting factors, Pumilio and Nanos (Lehmann and Nüsslein-Volhard 1991; Wharton and Struhl 1991; Gavis and Lehmann 1992).

Sequence analysis of the mutated transgenes shows that all missense alleles identified map to the C terminus of Nanos. This region had already been shown to be important for *nanos* function in repressing *hunchback* (Curtis *et al.* 1997). The fact that mutations affecting amino acids outside of the C terminus were not found could suggest that the C terminus is the only region required for function of the protein. However, injection of an RNA, which encoded a truncated Nanos protein that lacked 285 amino acids from the N terminus but

had an intact C-terminal region, was unable to rescue the *nanos* abdominal phenotype (Curtis *et al.* 1997). Thus, although essential, the C terminus of Nanos may not be sufficient for normal function. One possibility is that a single-amino-acid change in the N-terminal half of Nanos may not have a detectable effect on *nanos* function. Indeed, homologs of Nanos from other insect species that are able to substitute for lack of Nanos show very little conservation at the amino acid level in the N-terminal region of the protein (Curtis *et al.* 1995). At this point, we also cannot exclude the possibility that we have missed essential amino acids in other regions of the protein. Our screen only assayed for the effect of Nanos on *bicoid* regulation and may not have identified mutations specifically affecting other aspects of *nanos* function, such as germ cell migration and oogenesis. Statistical analysis further suggests that our screen did not reach saturation. Each amino acid that can be mutated in our screen and can be identified by our selection criteria was hit on average 1.8 times, which corresponds to a saturation of ~80%, according to a Poisson distribution. Further evidence for the lack of saturation is the fact that three of the six cysteines, which were shown to be required for zinc coordination of the Nanos CCHC motif, were not identified in the mutagenesis screen, but are important for *nanos* function (Curtis *et al.* 1997; this study).

The zinc fingers: The last 87 amino acids of Nanos contain two metal-binding domains of the CCHC type. In addition to mutations that alter the CCHC motif and thereby affect the ability of the mutant protein to chelate Zn (Curtis *et al.* 1997), we identified mutations in 9 amino acids located within the Zn finger domain that completely abolish *nanos* function. While these mutations could affect the structure or stability of the protein, some of these mutations may identify amino acids important for the ability of Nanos protein to interact with RNA or protein targets.

Zinc fingers of the CCHC type are not commonly found. The spacing between the Cys and His residues in Nanos are unique to this protein and its homologs (Figure 3B). Other proteins, such as the HIV nucleocapsid protein (Dannull *et al.* 1994), Xenopus CNBP (Rajavashisth *et al.* 1989), and Drosophila Clipper (Bai and Tolias 1998), have multiple copies of CCHC zinc fingers, but the ligand spacing is different. All of these proteins have been implicated in binding to single-stranded RNA. For instance, Clipper (Clp) is a Drosophila endoribonuclease that cleaves RNA hairpins (Bai and Tolias 1998). This protein contains five CCCH fingers that confer the endonucleolytic function and two CCHC fingers implicated in specific RNA binding. In addition to the CCHC motif, the HIV-I nucleocapsid protein and Nanos share a seven-amino-acid spacing between the zinc fingers. Of particular interest is the fact that the fourth amino acid in this spacer is an Arg in both proteins. Our mutational analysis has identified this Arg351 as important for *nanos* function. This Arg is conserved among HIV nucleocapsid proteins (De Guzman *et al.* 1998) and has been shown to be required for viral genomic packaging (Ottmann *et al.* 1995). In addition, crystallography studies of the nucleocapsid protein bound to its RNA target showed that this Arg makes direct contact with nucleic acids (De Guzman *et al.* 1998). Thus, it is an intriguing possibility that this Arg plays a similar role in Nanos.

The specific function of the zinc fingers in Nanos is not known. However, it has been shown that Nanos can bind to RNA with high affinity and that the ability of Nanos to bind RNA resides in the C terminus (Curtis *et al.* 1997). While a specific, high-affinity interaction between Nanos and the NREs has not been established, Pumilio protein has been shown to bind with high affinity and specificity to the NREs (Murata and Wharton 1995; Zamore *et al.* 1997, 1999). Point mutations in the NREs that affect Pumilio binding do not affect the affinity of Nanos for the RNA (Curtis *et al.* 1997). Nevertheless, a small number of nucleotides outside the conserved NRE motif have been shown to affect translational regulation of *hunchback* binding, but not Pumilio binding (Wharton *et al.* 1998). Further experiments are required to determine whether Nanos indeed binds to the NREs or other RNA targets with sequence specificity and whether Arg351 plays a role in such an interaction.

The tail region: Mutations in a region C terminal to the zinc fingers of Nanos cause abdominal and germ cell migration defects without affecting the function of *nanos* in oogenesis. Since increasing the dosage of Nanos protein containing a mutation in the tail domain does not alter the abdominal phenotype of mutant embryos, we favor the hypothesis that the tail region of Nanos constitutes a separate functional domain.

Mutations in the tail domain affect germ cell migration differently from *nanos* null mutations. Null mutations or mutations in the zinc finger region of Nanos have been shown to have a dramatic effect on germ cell migration (Forbes and Lehmann 1998). Primordial germ cells devoid of Nanos have altered morphology, fail to leave the gut toward the mesoderm, and tightly associate with each other in clusters (Forbes and Lehmann 1998). Furthermore, Kobayashi and colleagues have shown that enhancer trap lines that are normally expressed in germ cells late during embryogenesis are expressed earlier in *nanos* mutant germ cells (Kobayashi *et al.* 1996; Asaoka *et al.* 1998). This has led to the hypothesis that some of the phenotypes displayed by *nanos* mutant germ cells may be caused by the precocious expression of genes normally expressed at a later stage (Kobayashi *et al.* 1996). Mutants in the tail domain affect germ cell migration to a lesser extent than null mutations. nos^{L7} germ cells, like germ cells lacking Nanos, form clusters and fail to leave the gut. However, aberrant germ cell clustering is not as extreme as that

seen for the null mutant, and germ cell morphology seems normal. Furthermore, premature gene expression has not been observed in *nos*^{L7} mutants (Heller and Steinmann-Zwicky 1998). Finally, many *nos*^{L7} germ cells reach the embryonic gonad and the embryos develop into fertile adults.

These differences in phenotypes might indicate that *nos*^{L7} is a weak allele with respect to germ cell migration. Contrary to what we observe in *hunchback* regulation, *nos*^{L7} may retain some function in germ cell migration. Alternatively, the tail domain may just affect a subset of the processes disrupted in the null mutants. Contrary to *nos*^{L7} germ cells, *nanos* null germ cells show aberrant morphology and even when some cells reach the gonad, the resulting adults are often sterile. Consequently, *nanos* might be required in the germ cells for two independent functions: migration, which requires the tail domain, and germ-line stem cell identity, which does not require the tail domain. Problems in germ cell identity might exacerbate the migration defect, hence the greater loss of germ cells in the null mutants. Clearly, the identification of Nanos germ-line targets is required to further address the function of its different domains in germ cell migration.

Evolutionary conservation: The Nanos CCHC motifs show significant homology with sequences from other insects, Xenopus, leech, and *C. elegans* (Figure 3B). The Xenopus *nanos* homolog *Xcat-2* and the leech homolog are expressed in the developing oocyte (Mosquera *et al.* 1993; Pilon and Weisblat 1997). *Xcat-2* RNA is localized to the vegetal pole of the oocyte during oogenesis. In the embryo, *Xcat-2* RNA is initially taken up into the vegetal blastomeres and its expression becomes restricted to the primordial germ cells later in embryogenesis. The function of the Nanos homolog in Xenopus is not known. However, the similarity in localization pattern suggests a role for the protein in establishing embryonic polarity or germ-line development (Mosquera *et al.* 1993). Most amino acids mutated in our screen are conserved in these divergent species. Only two mutations, alleles *nos*$^{538/549}$ and *nos*^{512}, lead to changes in nonconserved amino acids (V354M and S337L). Given that these amino acids are next to His and Cys residues of the CCHC motif, it is possible that these mutations may affect the metal coordination within the respective zinc finger. Despite the high degree of conservation between the two zinc finger domains of Drosophila Nanos and Xenopus Xcat-2, the two protein regions are functionally not interchangeable. An RNA in which the Nanos zinc fingers were replaced in frame by those of Xcat-2 was shown to be unable to rescue the *nanos* mutant abdominal phenotype (Wang 1995).

Three family members of Nanos have been identified in *C. elegans*, and a role for these three proteins in germ-line development has been suggested (Wilson *et al.* 1994; Kraemer *et al.* 1999; Subramaniam and Seydoux 1999). Among the three *C. elegans* Nanos homologs, the characteristic CCHC motif with the exact spacing within and between the two fingers is only conserved in Nos-3. Like Xcat-2, the homology between Drosophila Nanos and the *C. elegans* homolog includes only the zinc finger region, while there is no homology with the tail domain. Our results suggest that the tail domain of Nanos is specifically required for the regulation of *hunchback* and *bicoid* and certain aspects of germ cell migration. One possibility is that the tail domain carries out functions that are unique to insect development, while the zinc finger domain plays a conserved role in germ-line development. In support of a conserved role of Nanos, homologs of Pumilio, the functional partner of Nanos, have been found in many species, including *C. elegans*. There are at least eight *pumilio* homologs in *C. elegans* (*C. elegans* database), and the role of two functionally redundant homologs, FBF1 and FBF2, has been reported. Fem-3 binding factor (FBF) proteins, like their Drosophila counterpart, are specific RNA-binding proteins and act as translational repressors (Zhang *et al.* 1997). One of the identified FBF targets is the *fem-3* gene, which is involved in germ-line sex determination (Ahringer and Kimble 1991; Zhang *et al.* 1997). *Fem-3* directs spermatogenesis in the hermaphrodite, and its translation must be suppressed to allow the switch to oogenesis to occur (Ahringer *et al.* 1992). These observations suggest a role for FBF in translational regulation similar to that performed by Pumilio and Nanos in Drosophila. Furthermore, the fact that the FBFs like Pumilio and Nanos regulate germ-line cell fate may suggest a conserved role of Nanos and Pumilio in germ-line development.

In summary, our analysis of a large number of *nanos* mutants has led to the following model for Nanos protein function: The C terminus of Nanos plays a crucial role during three developmental stages of Drosophila development: embryogenesis, primordial germ cell migration, and oogenesis. Nanos' role during embryogenesis is to silence the translation of maternal *hunchback* RNA. This function requires the Nanos zinc finger and tail region as well as the RNA-binding protein Pumilio. During primordial germ cell development, Pumilio and Nanos are required for migratory behavior, the temporal control of gene expression, and the differentiation of germ cells into germ-line stem cells. These processes require Pumilio and the zinc finger region of Nanos. The function of Nanos' tail region seems to be restricted to certain aspects of germ cell migration. During oogenesis, only the zinc finger region is necessary for Nanos' function, which has aspects both overlapping with and separate from Pumilio. Nanos homologs in other organisms suggest a conserved role in germ cell development. In these organisms, the region of homology is restricted to the zinc finger motif and does not span the tail domain, suggesting that the tail domain may have been recruited later in evolution and may fulfill a more specialized role.

We are grateful to Laila Parker for her help during the genetic screen and to Zandy Forbes for her help in analyzing the migration phenotype. We want to thank past and present members of our laboratory for reagents and their helpful suggestions on the manuscript. We thank Bill Gelbart and Claude Desplan for supplying fly strains used in this study. G.A. is supported by a predoctoral fellowship from the National Institutes of Health, and R.L. is a Howard Hughes Medical Institute investigator.

LITERATURE CITED

Ahringer, J., and J. Kimble, 1991 Control of the sperm-oocyte switch in *Caenorhabditis elegans* hermaphrodites by the *fem-3* 3′ untranslated region. Nature **349:** 346–348.

Ahringer, J., T. A. Rosenquist, D. N. Lawson and J. Kimble, 1992 The *Caenorhabditis elegans* sex determining gene fem-3 is regulated post-transcriptionally. EMBO J. **11:** 2303–2310.

Asaoka, M., H. Sano, Y. Obara and S. Kobayashi, 1998 Maternal Nanos regulates zygotic gene expression in germline progenitors of Drosophila melanogaster. Mech. Dev. **78:** 153–158.

Ashburner, M., 1989 Mutation and mutagenesis, pp. 299–418 in *Drosophila: A Laboratory Handbook*, edited by M. Ashburner. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Bai, C., and P. P. Tolias, 1998 Drosophila clipper/CPSF 30K is a post-transcriptionally regulated nuclear protein that binds RNA containing GC clusters. Nucleic Acids Res. **26:** 1597–1604.

Barker, D. D., C. Wang, J. Moore, L. K. Dickinson and R. Lehmann, 1992 Pumilio is essential for function but not for distribution of the *Drosophila* abdominal determinant Nanos. Genes Dev. **6:** 2312–2326.

Bhat, K. M., 1999 The posterior determinant gene nanos is required for the maintenance of the adult germline stem cells during Drosophila oogenesis. Genetics **151:** 1479–1492.

Chou, T. B., and N. Perrimon, 1996 The autosomal FLP-DFS technique for generating germline mosaics in *Drosophila melanogaster*. Genetics **144:** 1673–1679.

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

Curtis, D., D. K. Treiber, F. Tao, P. D. Zamore, J. R. Williamson *et al.*, 1997 A CCHC metal-binding domain in Nanos is essential for translational regulation. EMBO J. **16:** 834–843.

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

Dannull, J., A. Surovoy, G. Jung and K. Moelling, 1994 Specific binding of HIV-1 nucleocapsid protein to PSI RNA in vitro requires N-terminal zinc finger and flanking basic amino acid residues. EMBO J. **13:** 1525–1533.

De Guzman, R. N., Z. R. Wu, C. C. Stalling, L. Pappalardo, P. N. Borer *et al.*, 1998 Structure of the HIV-1 nucleocapsid protein bound to the SL3 psi-RNA recognition element. Science **279:** 384–388.

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

Ephrussi, A., L. K. Dickinson and R. Lehmann, 1991 *Oskar* organizes the germ plasm and directs localization of the posterior determinant *nanos*. Cell **66:** 37–50.

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

Gavis, E. R., and R. Lehmann, 1992 Localization of *nanos* RNA controls embryonic polarity. Cell **71:** 301–313.

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

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

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

Heller, A., and M. Steinmann-Zwicky, 1998 No premature gene expression in germ cells of embryos deriving from *nos* females. Mech. Dev. **72:** 169–173.

Hülskamp, M., C. Schroder, C. Pfeifle, H. Jackle and D. Tautz, 1989 Posterior segmentation of the Drosophila embryo in the absence of a maternal posterior organizer gene. Nature **338:** 629–632.

Irish, V., R. Lehmann and M. Akam, 1989 The Drosophila posterior-group gene nanos functions by repressing hunchback activity. Nature **338:** 646–648.

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

Kraemer, B., S. Crittenden, M. Gallegos, G. Moulder, R. Barstead *et al.*, 1999 NANOS-3 and FBF proteins physically interact to control the sperm/oocyte switch in *Caenorhabditis elegans*. Curr. Biol. **9:** 1009–1018.

Lehmann, R., 1988 Phenotypic comparison between maternal and zygotic genes controlling the segmental pattern of the Drosophila embryo. Development **104**(Suppl.): 17–27.

Lehmann, R., and C. Nüsslein-Volhard, 1986 Abdominal segmentation, pole cell formation, and embryonic polarity require the localized activity of oskar, a maternal gene in Drosophila. Cell **47:** 141–152.

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

Macdonald, P. M., 1992 The Drosophila *pumilio* gene: an unusually long transcription unit and an unusual protein. Development **114:** 221–232.

Mosquera, L., C. Forristall, Y. Zhou and M. L. King, 1993 An mRNA localized to the vegetal cortex of *Xenopus* oocytes encodes a protein with a *nanos*-like zinc finger domain. Development **117:** 377–386.

Murata, Y., and R. P. Wharton, 1995 Binding of Pumilio to maternal *hunchback* mRNA is required for posterior patterning in Drosophila embryos. Cell **80:** 747–756.

Ottmann, M., C. Gabus and J. L. Darlix, 1995 The central globular domain of the nucleocapsid protein of human immunodeficiency virus type 1 is critical for virion structure and infectivity. J. Virol. **69:** 1778–1784.

Pilon, M., and D. A. Weisblat, 1997 A nanos homolog in leech. Development **124:** 1771–1780.

Pirrotta, V., 1998 Vectors for P-mediated transformations in *Drosophila*, pp. 437–456 in *Vectors: A Survey of Molecular Cloning Vectors and Their Uses*, edited by R. L. Rodriguez and D. T. Reinhardt. Butterworths Publishing, Woburn, MA.

Rajavashisth, T. B., A. K. Taylor, A. Andalibi, K. L. Svenson and A. J. Lusis, 1989 Identification of a zinc finger protein that binds to the sterol regulatory element. Science **245:** 640–643.

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

Smibert, C. A., J. E. Wilson, K. Kerr and P. M. Macdonald, 1996 Smaug protein represses translation of unlocalized nanos mRNA in the Drosophila embryo. Genes Dev. **10:** 2600–2609.

Spradling, A. C., 1986 P element-mediated transformation, pp. 175–197 in *Drosophila: A Practical Approach*, edited by D. B. Roberts. IRL Press, Oxford.

Struhl, G., 1989 Differing strategies for organizing anterior and posterior body pattern in Drosophila embryos. Nature **338:** 741–744.

Subramaniam, K., and G. Seydoux, 1999 *nos-1* and *nos-2*, two genes related to Drosophila *nanos*, regulate primordial germ cell migration and survival in *C. elegans*. Development **126:** 4861–4871.

Theurkauf, W. E., and R. S. Hawley, 1992 Meiotic spindle assembly in Drosophila females: behavior of nonexchange chromosomes and the effects of mutations in the nod kinesin-like protein. J. Cell Biol. **116:** 1167–1180.

Wang, C. I., 1995 The role of *nanos* in maternal specification of abdomen in Drosophila. Ph.D. Thesis. Massachusetts Institute of Technology, Cambridge, MA.

Wang, C., and R. Lehmann, 1991 *Nanos* is the localized posterior determinant in Drosophila. Cell **66:** 637–648.

Wang, C., L. K. Dickinson and R. Lehmann, 1994 The genetics of *nanos* localization in *Drosophila*. Dev. Dynam. **199:** 103–115.

Wharton, R. P., and G. Struhl, 1991 RNA regulatory elements

mediate control of Drosophila body pattern by the posterior morphogen *nanos.* Cell **67:** 955–967.

Wharton, R. P., J. Sonoda, T. Lee, M. Patterson and Y. Murata, 1998 The Pumilio RNA-binding domain is also a translational regulator. Mol. Cell **1:** 863–872.

Wilson, R., R. Ainscough, K. Anderson, C. Baynes, M. Berks *et al.*, 1994 2.2 Mb of contiguous nucleotide sequence from chromosome III of C. elegans. Nature **368:** 32–38.

Zamore, P. D., J. R. Williamson and R. Lehmann, 1997 The Pumilio protein binds RNA through a conserved domain that defines a new class of RNA-binding proteins. RNA **3:** 1421–1433.

Zamore, P. D., D. P. Bartel, R. Lehmann and J. R. Williamson, 1999 The PUMILIO-RNA interaction: a single RNA-binding domain monomer recognizes a bipartite target sequence. Biochemistry **38:** 596–604.

Zhang, B., M. Gallegos, A. Puoti, E. Durkin, S. Fields *et al.*, 1997 A conserved RNA-binding protein that regulates sexual fates in the *C. elegans* hermaphrodite germ line. Nature **390:** 477–484.

Communicating editor: T. Schüpbach