The role of *nanos* in maternal specification of abdomen in Drosophila

by

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ABSTRACT

This thesis describes the cloning and characterization of the *Drosophila* maternal-effect gene *nanos*. *nanos* encodes a posterior determinant which is localized to the posterior pole of the early embryo to specify abdomen. Localization of *nanos* mRNA to the posterior of the developing oocyte requires the function of eight other maternal-effect genes of the posterior group. The abdominal mutant phenotype of these eight genes can be rescued by injection of in vitro transcribed *nanos* RNA into the prospective abdomen. This shows that these genes are required for the localization or translation of the *nanos* mRNA, but are dispensible for the function of nanos protein. The abdominal phenotype of embryos derived from mothers mutant for another posterior group gene, *pumilio*, cannot be rescued by the injection of *nanos* RNA. *pumilio*, therefore, is absolutely required for the function of *nanos* in specifying abdomen. *nanos* mRNA is translated in the early embryo to form a protein gradient emanating from the posterior pole. This gradient specifies the formation of a complementary gradient of the abdominal repressor protein hunchback. Polysome fractionation analysis of wild-type and mutant embryo extracts suggests that *nanos* acts to suppress the initiation of translation of the *hunchback* mRNA. Preliminary structural analysis of the *nanos* open reading frame shows that the C-terminal of the *nanos* polypeptide is essential to its function. This thesis also describes the transient expression of nanos protein early in oogenesis. This expression may account for the oogenesis defect associated with some *nanos* alleles.

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Chapter 1

Introduction

A primary event in the development of an embryo is specification of the body axes (reviewed in Gurdon, 1992)). In the fruitfly *Drosophila melanogaster*, embryonic axis specification depends largely on maternally provided factors. The prominent role of maternal factors in *Drosophila* is probably related to the relatively short period in which a fertilized egg develops into a free-living 1st instar larvae - just 24 hours at 25 °C. A large scale screen for maternal-effect mutations affecting pattern formation identified a large number of genes, which fall into four classes, affecting either dorsal/ventral, anterior, posterior, or terminal structures. These gene classes act largely independently of one another, that is, mutations in one gene do not affect structures specified by another gene class. Extensive genetic and molecular studies have led to a detailed understanding of the function of each of the genes within each group (reviewed in St. Johnston and Nüsslein-Volhard, 1992). These studies have shown that within each group, one gene acts as a localized signal to specify axis formation. For the dorsal/ventral group the gene *dorsal* is active only on the ventral side of the embryo. Nuclear transport of the dorsal protein is regulated such that it is found in a nuclear gradient with highest levels on the ventral side (Roth et al., 1989; Rushlow et al., 1989; Steward, 1989). For the anterior class, *bicoid* encodes an RNA which is tightly localized the the anterior pole of embryos (Berleth et al., 1988; Frigerio et al., 1986). Bicoid protein acts as a morphogen transcription factor to direct the formation of head and thoracic structures (Driever and Nüsslein-Volhard, 1988; Driever and Nüsslein-Volhard, 1989; Struhl et al., 1989). For the terminal class, the *torso* transmembrane receptor tyrosine kinase is selectively activated at the embryonic termini to specify formation of the acron and telson (Sprenger and Nüsslein-Volhard, 1992; Sprenger et al., 1989). For the posterior group, the gene *nanos* encodes the localized signal, and is the topic of this thesis.

The posterior class comprises 12 genes, *cappuccino, spire, staufen, oskar, vasa, valois, tudor, mago nashi, pipsqueak, orb, nanos,* and *pumilio* (Boswell and Mahowald, 1985; Boswell et al., 1991; Lehmann and Nüsslein-Volhard, 1986; Lehmann and Nüsslein-Volhard, 1987a; Lehmann and Nüsslein-Volhard, 1991; Manseau and Schüpbach, 1989; Schüpbach and

Wieschaus, 1986a; Siegel et al., 1993; Lantz et al., 1994; Christerson and McKearin, 1994; for a review see St. Johnston, 1993). Mothers mutant for any of these genes produce embryos which lack abdominal segments. For ten of these genes (*cappuccino, spire, staufen, oskar, vasa, valois, tudor, mago nashi, pipsqueak, orb*), these embryos have a second phenotype - they lack poleplasm, a specialized cytoplasm from which the germ cell precursors (called pole cells in *Drosophila*) originate. The poleplasm is assembled during oogenesis at the posterior pole of the growing oocyte. Four of the cloned "poleplasm" genes encode gene products which are indeed localized to or enriched in the poleplasm (Bardsley et al., 1993; Ephrussi et al., 1991; Hay et al., 1988; Kim-Ha et al., 1991; Lasko and Ashburner, 1988; St. Johnston et al., 1991). In particular, *oskar* appears to encode a germ cell determinant, since localization of *oskar* to the anterior is sufficient to induce pole cell formation (Ephrussi and Lehmann, 1992), and overexpression results in the apparent formation of germ cells ectopically in the embryo (Smith et al., 1992).

Unlike the other posterior group genes, *nanos* and *pumilio* are specific to the process of abdominal segmentation, and mutant embryos form normal pole cells (Lehmann and Nüsslein-Volhard, 1987a; Lehmann and Nüsslein-Volhard, 1991). However, whereas the pumilio protein is distributed throughout the early embryo (Barker et al., 1992; Macdonald, 1992), *nanos* RNA is strictly localized to the posterior pole (Wang and Lehmann, 1991), and nanos protein forms a posterior concentration gradient (Barker et al., 1992; Smith et al., 1992). In addition, misexpression of *nanos* at the anterior of the embryo induces the formation of a second abdomen in mirror image to the first (Gavis and Lehmann, 1992). These results imply that *nanos* acts as a localized determinant of abdomen. *nanos* and *pumilio* act jointly to specify abdomen by repressing the translation of the maternal *hunchback* mRNA (Barker et al., 1992). *Hunchback* acts as a transcriptional repressor of the zygotic abdominal gap genes *knirps* and *giant* (Kraut and Levine, 1991a; Rothe et al., 1989). In the presence of the *nanos/pumilio* system, *hunchback* expression is repressed in the prospective abdomen, allowing expression of abdominal gap genes, and subsequent specification of abdominal segments.

The specification of abdomen by *nanos* includes aspects of RNA localization and translational control, which form recurring themes in the

maternal control of pattern formation. The remainder of this chapter will therefore focus on these two processes and their role in specifying developmental fate.

A. Localized RNAs and pattern formation

Axis specification in a growing oocyte requires some mechanism for regional specification. In many instances, this mechanism is the localization of mRNAs encoding developmental or cell fate regulators. Recent studies in both *Drosophila* and *Xenopus* have led to the identification and analysis of several localized mRNA species.

1. Localized RNAs in *Drosophila*

A large number of transcripts are now known to be localized in the *Drosophila* oocyte or early embryo (reviewed in Ding and Lipshitz, 1993). Many of these transcripts have been identified in the course of genetic screens for pattern formation genes. The known localized transcripts may be subdivided into classes according to the timing of their translation. The first class, which includes *oskar*, *gurken*, and *orb*, comprises transcripts that are localized and then translated during oogenesis (for a description of oogenesis, see Chapter 4). The *oskar* RNA is localized to the oocyte posterior pole at stage 9 of oogenesis (Ephrussi et al., 1991; Kim-Ha et al., 1991) (stages according to King, 1970). Oskar protein is translated at stage 9 of oogenesis (C. Rongo, E. Gavis, R. Lehmann, submitted), and acts as a poleplasm organizer which is both necessary and sufficient for the specification of the poleplasm (Ephrussi and Lehmann, 1992; Lehmann and Nüsslein-Volhard, 1986). The *gurken* RNA is localized to the dorsal anterior corner of the developing oocyte, also at stage 9 (Neuman-Silberberg and Schüpbach, 1993). *Gurken* encodes a protein of the TGF-alpha family which signals to adjacent follicle cells to specify dorsal fate and establish the dorsal-ventral axis. Localization of the *orb* gene product follows a more complex pattern - at stage 7 both the RNA and the protein are found in the cortical region at the posterior end of the oocyte, but at stages 8-10 the gene products are found at the anterior margin of the oocyte, enriched at the dorsal and anterior cortical regions (Lantz et al., 1992;

Lantz et al., 1994; Christerson and McKearin, 1994). The *orb* open reading frame contains two copies of the RNA recognition motif sequence (RRM) (Lantz et al., 1992) , and orb protein is postulated to play a role in localizing RNAs to both the posterior and anterior margins of the growing oocyte.

The second class of transcripts are localized during oogenesis, but not translated until egg activation. This class includes the positional determinant genes *nanos* and *bicoid*. *nanos* RNA is localized to the posterior pole of the oocyte at stage 10 (Wang and Lehmann, 1991), whereas *bicoid* RNA is localized at the anterior margin of the growing oocyte (Berleth et al., 1988), but the respective proteins are not expressed until after egg activation (Wang et al., 1994; Driever and Nüsslein-Volhard, 1988). The RNA product of the gene *germ cell-less* (*gcl*) is also localized to the posterior poleplasm of embryos, and the gcl protein is necessary but not sufficient for germ cell formation (Jongens et al., 1994; Jongens et al., 1992). The final class of localized transcripts includes the cyclin B RNA, which is localized late in oogenesis to the posterior poleplasm (Dalby and Glover, 1992; Whitfield et al., 1989). Cyclin B protein is not translated until 9 hours after pole cell formation, when its function is presumably required to drive the mitotic divisions of the proliferating pole cells (Dalby and Glover, 1993; Raff et al., 1990).

2. Localized RNAs in *Xenopus*

The *Xenopus* oocyte is another attractive system for studying localized transcripts, due to its large size and the ease in obtaining large numbers of oocytes for analysis. Successful experimental approaches to identifying localized transcripts include the manual dissection of oocytes into vegetal and animal halves, as well as the isolation of cytoskeletal-associated mRNAs (Mosquera et al., 1993; Regabliatti et al., 1985). These localized transcripts are found in one of three compartments of the *Xenopus* oocyte. The transcripts An1, An2, and An3 are enriched in the cytoplasm of the animal hemisphere (Gururajan et al., 1991; Regabliatti et al., 1985; Weeks and Melton, 1987). The transcripts Xcat-2, Xcat-3, and Xlsirt are cortically localized with the germ plasm to the vegetal pole (Elinson et al., 1993; Kloc et al., 1993; Mosquera et al., 1993). Finally, the Vg1 and Xwnt11 transcripts also localized to the vegetal cortex, but in a somewhat broader region (Ku and Melton, 1993; Melton, 1987). However, in contrast to the situation in *Drosophila*, the function of these

localized RNAs in pattern formation remains unclear. Fortunately, in several cases sequence homologies can provide a basis for speculation as to function. Vg1 and Xwnt11 are related to growth factors of the TGF-ß and Wnt families, respectively (Ku and Melton, 1993; Weeks and Melton, 1987). Forced expression of a secreted form of Vg1 which contains the TGF-ß sequences is able to induce mesoderm, as well as rescue dorsal-ventral axis formation in UV-irradiated embryos (Dale et al., 1993; Thomsen and Melton, 1993), suggesting that localized processing of Vg1 may play a role in mesoderm induction. Xcat-2 and Xcat-3 are both related to Drosophila genes which are localized as proteins to the poleplasm. Xcat-2 has homology to the C-terminal region of *nanos* (Mosquera et al., 1993). Xcat-3 encodes a DEAD box protein related to *vasa* (M.L. King, personal communication, cited in St Johnston, 1995). Combined with the recent finding that Xcat-2 and Xcat-3 RNAs segregate with the germplasm into a subset of vegetal blastomeres that eventually give rise to the primordial germ cells (Forristall et al., 1995), these results suggest that these RNAs may encode *Xenopus* germ line determinants.

3. Localization signals and trans-acting factors

In all cases studied thus far, sequences specifying RNA localization map to the 3' untranslated region (3'UTR) of the transcript. More detailed studies have invariably shown that the "localization signal" entails a relatively large sequence, on the order of a few hundred nucleotides or so. This suggests that localization signals may be complex secondary structures, containing multiple protein binding sites. One of the best studied examples is the *bicoid* RNA, for which much is known about the genes and cis-acting sequences required for localization. Localization of *bicoid* to the anterior pole of the early embryo is a stepwise process requiring the functions of the anterior group genes *exuperantia* (*exu*), *swallow*, and *staufen* (St Johnston et al., 1989). Early in oogenesis, *bicoid* RNA is transiently localized to the apical region of the nurse cells, then transported to the anterior margin of the oocyte in an *exu*-dependent process (Marcey et al., 1991). Anchoring of the RNA in the cortex depends on *swallow* activity (Stephenson et al., 1988). The RNA is released from the cortex at egg activation, and anchoring of the RNA in the anterior cytoplasm requires the function of the double-stranded RNA binding

protein staufen (St. Johnston et al., 1992). The 625 nucleotide (nt) bicoid localization signal contains a 53 nt element required for the early, *exu*-dependent steps in localization (Macdonald et al., 1993; Macdonald and Struhl, 1988). Two copies of this BLE1 element inserted into a heterologous transcript direct early localization in the oocyte, but not later anchoring of the RNA to the cortex. Finally, RNA injection experiments have defined a 400 nt region of the *bicoid* 3'UTR distinct from the localization signal, but including BLE1, which associates with staufen protein in embryos (Ferrandon et al., 1994).

Similarly, genetic and molecular analyses have shown that localization of *oskar* RNA to the posterior pole of the oocyte and early embryo occurs via a stepwise process. Early enrichment in the oocyte requires the functions of *Bicaudal-D* and *egalitarian* (Ephrussi et al., 1991; Kim-Ha et al., 1991; Ran et al., 1994) . At stage 7, the RNA is transiently localized to the anterior of the oocyte in a *cappuccino* and *spire*-dependent process. Transport of *oskar* RNA to the posterior pole requires the functions of *staufen* and *mago nashi* (Newmark and Boswell, 1994). Maintainance of *oskar* localization at the posterior requires the function of oskar protein itself (Ephrussi et al., 1991; Kim-Ha et al., 1991; C. Rongo, E. Gavis, R. Lehmann, submitted). Oskar protein directs the localization of the *nanos* RNA, whose localization also requires the downstream function of *vasa* and *tudor* (Wang et al., 1994). Mapping of sequence elements for *oskar* and *nanos* RNA localization again suggest redundant elements distributed throughout the 3'UTR (Kim-Ha et al., 1993; E. Gavis, personal communication). Similarly, the 3'UTR of the *orb* RNA contains redundant localization elements, although a 280 nt region of the 3'UTR is sufficient for proper localization (Lantz and Schedl, 1994).

4. Conclusions

A few localized transcripts have been studied in detail to date, and features of the RNA elements required for their localization are beginning to emerge. Namely, these localization elements are often redundant and encompass hundreds of nucleotides, suggesting that a complex secondary structure and/or multiple RNA binding proteins are involved. Genetic analyses in *Drosophila* have identified numerous genes required for localization of particular RNAs, and two in particular, *staufen* and *orb*, may

encode RNA binding proteins. Elucidation of the molecular mechanisms behind the function of the remaining localization genes, as well as the link between these genes and the cytoskeleton, should provide many additional years of fruitful study.

An interesting additional note is that localized RNAs are likely to be translationally regulated. In order to restrict a gene product to a particular location, it seems logical that protein synthesis would occur only when the transcript is properly localized. In fact, the *nanos* RNA is under such translational control, and the RNA sequences required for this control have been mapped to the 3'UTR (Gavis and Lehmann, 1994). The *oskar* 3'UTR also contains sequence elements that repress translation of the unlocalized RNA in the developing oocyte, and recently a protein has been identified in oocyte extracts which binds to this sequence element (Kim-Ha et al., 1995). Clearly, RNA localization is linked to a second issue in the maternal control of development, translational control.

B. Translational regulation in development

The very first stages of development after fertilization take place in the absence of transcription - the early embryo executes its developmental program using only maternally provided gene products. The translational control of maternal mRNAs is the primary mode of gene regulation during these stages. The remainder of this review will focus on the role of translational regulation in developmental control.

1. Generalized translational control

a. Translational masking

A classically studied form of translational control is masking (Spirin, 1966). The term masking describes the phenomenon whereby more than 80% of the maternal mRNA synthesized in vivo is translationally silent when tested in an in vitro translation system (Davidson, 1986), yet after phenol extraction, these same RNAs are translationally active in oocytes (Richter, 1988; Richter and Smith, 1984). Purified mRNAs can be partially

translationally suppressed by the addition of proteins isolated from storage ribonucleoprotein particles (Kick et al., 1987; Richter and Smith, 1984). In *Xenopus*, the two major protein components of these particles are the closely related proteins mRNP3 and FRGY2 (Darnborough and Ford, 1981), which belong to the family of nucleic-acid binding proteins which bind the Y box, a sequence element found in the promoters of genes which are specifically active in oocytes (Murray et al., 1992; Tafuri and Wolffe, 1990; Tafuri and Wolffe, 1993). Misexpression of FRGY2 in somatic cells results in an increase in mRNA accumulation from Y-box containing promoters, as well as a general decrease in translation of all transcripts examined (Ranjan et al., 1993). A recent paper (Bouvet and Wolffe, 1994) has shown that in vivo synthesized transcript is translated much less efficiently than in vitro synthesized transcript when tested by injection into *Xenopus* oocytes. In addition, the translational efficiency of in vivo synthesized RNA was further decreased by overexpression of FRGY2 in oocytes, but the translation of in vitro synthesized RNA was unaffected. These results suggest that FGRY2 acts to suppress translation of a wide variety of messages by a mechanism which is closely coupled to transcription. FGRY2 and associated proteins may therefore mediate transcription-dependent translational repression in a fashion analagous to the DNA-replication dependent transcriptional repression mediated by histone proteins.

b. Polyadenylation control

The translation of a group of maternal mRNAs is regulated by cytoplasmic poly(A) elongation. These transcripts exist in a deadenylated (or under-adenylated) state in the cytoplasm and as such are translationally dormant. Upon oocyte maturation, the transcripts are readenylated, and become actively translated. One of the first demonstrations that polyadenylation state regulates translation and that this regulation requires specific sequences in the 3'UTR came from studies of the tissue plasminogen activator (tPA) RNA in the mouse oocyte (Strickland et al., 1988; Vassalli, 1989). Translation of tPA RNA is activated during meiotic maturation, and this activation occurs via a polyadenylation which is specified by two sequence determinants in the 3'UTR - an AU-rich adenylation control element (ACE), and the hexanucleotide AAUAAA polyadenylation signal

(Huarte et al., 1992). In addition, the ACE specifies the initial cytoplasmic deadenylation event which inactivates the tPA transcript, indicating that adenylation control is a reversible event.

A more extensive understanding of the sequence requirements, dynamics, and transacting factors involved in regulated polyadenylation has come from studies in the *Xenopus* oocyte model system. Polyadenylation control in the *Xenopus* oocyte requires a uridine-rich sequence similar to ACE known as the cytoplasmic polyadenylation element (CPE), as well as the AAUAAA signal (Fox et al., 1989; McGrew et al., 1989). The spacing between the CPE and the polyadenylation signal determines the timing and extent of polyadenylation after oocyte maturation of both the C12 and C11 transcripts of *Xenopus* (Simon and Richter, 1994; Simon et al., 1992). A recent comparison of polyadenylation control elements in the 3'UTRs of cell cycle regulators confirms that element spacing contributes to timing and extent of adenylation (Sheets et al., 1994). The differential translational regulation of the cyclin and *c-mos* mRNAs conferred by the regulatory sequences in their 3'UTRs may represent an important mechanism for cell cycle control in the maturing oocyte. In summary, these studies show that for a given transcript, translation state is generally correlated with the degree of polyadenylation. However, a given length of poly(A) will result in different degrees of translation for different transcripts, indicating that other, less well characterized sequence elements play a role in specifying translational state.

Several transacting factors which interact specifically with the CPE have been identified. Two proteins from mature eggs have been shown to interact specifically with the CPEs of the *Xenopus* C12 and C11 RNAs (Simon and Richter, 1994), although the function of these proteins remains to be defined. Another CPE-binding protein, this one isolated by virtue of its interaction with the B4 RNA, was shown to be phosphorylated by p34/cdc2 kinase, indicating a possible link between reactivation of the cell cycle at egg maturation and polyadenylation control (Paris et al., 1991). Recently, RNA affinity chromatography has been used to isolate this CPE-binding protein (CPEB), which is required for polyadenylation in egg extracts (Hake and Richter, 1994). Interestingly enough, this protein contains significant homology (62% identity) to the *Drosophila* orb protein, which has been

shown to play a role in RNA localization during oogenesis (Christerson and McKearin, 1994; Lantz et al., 1994). This homology in the RNA recognition motif region suggests that orb may itself bind to CPE sequences. The diversity of identified CPE-binding proteins is perhaps not surprising, since different CPE elements were used for each study, and the timing and extent of adenylation is known to vary among different messages, suggesting message-specific elements (see above). In vitro studies of CPE-dependent cytoplasmic polyadenylation should yield a better understanding of the specific factors required. Preliminary results suggest that the enzymes which catalyze nuclear polyadenylation can participate in CPE-dependent polyadenylation in vitro (Bilger et al., 1994; Fox et al., 1992)

Adenylation and translational activation at fertilization have recently been examined in *Drosophila* (Sallés et al., 1994). The *bicoid*, *Toll*, *torso*, and *hunchback* mRNAs are all adenylated between oogenesis and embryogenesis, and this addition of poly(A) correlates with translational activation of these messages. In the case of *bicoid* mRNA, it was shown that this adenylation is required for efficient translation. By contrast, the *nanos* RNA was not found to change its adenylation state between oocytes and embryos, suggesting that translational activation of the *nanos* transcript in the early embryo may occur via a different mechanism. This hypothesized second pathway may be necessary to ensure early and/or efficient translation of *nanos*, which is itself a translational regulator (see below).

In contrast to the above studies of transcripts which are adenylated at egg maturation, sequence elements directing deadenylation in embryos have been described. The Eg maternal RNAs in *Xenopus* are deadenylated and released from polysomes after fertilization, despite the presence of CPE sequences in their 3'UTRs (Paris et al., 1988; Paris and Philippe, 1990). The Eg2 mRNA contains a 17 nt deadenylation control element which when deleted results in adenylation in the embryo (Bouvet et al., 1994). Similarly, sequences distinct from the CPE and AAUAAA regions of the 3'UTR of Eg1 (cdk2) regulate its deadenylation, and preliminary evidence shows that a maternal mRNA product controls this deadenylated state (Stebbins-Bouz and Richter, 1994). Two proteins, p53 and p55, have been identified which bind to the deadenylation control region of the Eg2 mRNA, and whose binding is

correlated with the rate of deadenylation (Bouvet et al., 1994; Legagneux et al., 1992). The function of these proteins remains unknown, however it seems that regulation by the deadenylation element can override adenylation specified by the CPE.

2. Specific translational control

Genetic studies of *C. elegans* and *Drosophila* have identified a number of instances in which translational regulation of specific RNAs has distinct consequences for development. These consequences may include determination of cell fate, or regional specification in the early embryo. One emerging theme is that in each case the sequence elements specifying translational regulation map to the 3'UTR.

a. Translational regulation and cell fate specification

Translational control has recently been shown to play a role in the sex determination pathway of *C. elegans*. The *tra-2* gene is repressed in hermaphrodites to allow spermatogenesis (Doniach, 1986; Schedl and Kimble, 1988). Translational regulation of *tra-2* was revealed by gain-of-function (gof) mutations which overexpress *tra-2*, resulting in hermaphrodites which are unable to produce sperm. These gof alleles are due to changes within a pair of direct repeat sequences in the *tra-2* 3'UTR (Goodwin et al., 1993), suggesting post-transcriptional control. Analysis of transcript abundance in polysome-containing fractions shows that the *tra-2* gof RNA is translationally activated. The *C. elegans* gene *fem-3* is also negatively regulated in hermaphrodites, to allow the switch from spermatogenesis to oogenesis (Barton et al., 1987; Hodgkin, 1986). Gain of function mutations in *fem-3* map to a 6 nt region of the 3'UTR (Ahringer and Kimble, 1991) . The finding that steady-state levels of wild-type and gof *fem-3* RNA in hermaphrodites are equivalent suggests that this regulation is translational. In addition, changes in the poly(A) tail length which correlate with translation have been shown for *fem-3* (Ahringer et al., 1992). Translational repression of these RNAs may be mediated by repressor proteins which bind the regulatory sequences and promote a stable but untranslatable deadenylated state (Goodwin et al., 1993).

The regulation of the *lin-14* gene by *lin-4* in C. elegans reveals an apparently novel mechanism of translational control. *lin-14* and *lin-4* are required for the proper temporal expression of cell lineages during larval development (Ambros and Horvitz, 1987; Chalfie et al., 1981). Loss of *lin-14* function results in precocious larval development, manifested by the appearance of cell lineages characteristic of one or two larval stages later. *Lin-14* gain of function mutations result in a retarded phenotype - later stages of development reiterate cell fate specifications characteristic of earlier stages. The *lin-14* gain-of-function alleles map to the 3'UTR and result in abnormally high levels of LIN-14 protein, suggesting that *lin-14* is subject to translational repression (Wightman et al., 1991). *lin-4* is a negative regulator of *lin-14* (Ambros, 1989; Arasu et al., 1991), and a *lin-4* null mutation results in a phenotype identical to *lin-14* gain-of-function alleles. Surprisingly, the *lin-4* gene does not encode a protein, but produces two small RNA transcripts which include a sequences with complementarity to a seven-fold repeated sequence motif in the 3'UTR of the *lin-14* RNA (Lee et al., 1993; Wightman et al., 1993). These results suggest that the *lin-4* may suppress translation of *lin-14* via an RNA/RNA pairing mechanism, with seven *lin-4* molecules per *lin-14* transcript. This resulting complex may itself constitute an untranslatable RNA, or may nucleate formation of a ribonucleoprotein particle resistant to translational initiation.

b. Translational regulation and regional specification

Another characterized function of translational control in development is as a mechanism for defining specialized regions within a single cell. In these cases, regional specialization is usually facilitated by a localized RNA. These principles are exemplified by the maternal system of abdomen specification in *Drosophila*. *nanos* is translated from its posteriorly localized RNA to form a posterior protein gradient. *nanos* acts in conjunction with the pumilio protein, which is distributed throughout the embryo, to repress the translation of the maternal *hunchback* RNA. This regulation is mediated by a pair of bipartite sequence motifs, the *nanos* response elements (NREs), in the *hunchback* 3'UTR which direct *nanos*-mediated repression (Wharton and Struhl, 1991). Deletion of the NRE sequences from *hunchback* results in the same phenotype (absence of

abdominal segmentation) as loss of either *nanos* or *pumilio* function, suggesting that *nanos* and *pumilio* act exclusively through these sequences. Recently it has been shown that pumilio protein and a 55kD protein from embryo extract bind to NRE sequences in vitro (Murata and Wharton, 1995). In addition, the sequence requirements for NRE function in vivo correlate with the requirements for binding of either pumilio or the 55kD factor, indicating that both these proteins play an important role in regulation in vivo. The NRE binding activity of these proteins is not dependent on *nanos*. These results suggest that *hunchback* RNA is complexed to one or both of these proteins throughout the embryo, independent of its translational state. Repression of translation would then require the additional presence of nanos protein. pumilio and the 55kD protein may act to provide a "landing pad" on the *hunchback* RNA for nanos and possibly other proteins involved in translational repression (Murata and Wharton, 1995).

A related translational regulatory mechanism may govern the expression of *glp-1* in the early *C. elegans* embryo. *glp-1* RNA is present throughout the early embryo, but is only translated in the anterior cells during the first two embryonic divisions (Evans et al., 1994). This translational regulation is conferred by sequences in the *glp-1* 3'UTR. Most strikingly, a 61 nt region required for repression of translation in the posterior cells contains one and a half copies of a bipartite sequence motif very similar to the *hunchback* NRE. These results suggest that 3'UTR-mediated translational repression of *glp-1* may occur via repressor proteins localized in the posterior half of the embryo.

Studies of the translation of the *nanos* RNA itself have revealed another link between localized RNAs, translational regulation, and regional specification. Unlocalized *nanos* RNA is not translated, and this translational suppression is mediated by sequences in the 3'UTR (Gavis and Lehmann, 1994). Levels of nanos protein at the posterior pole depend on levels of oskar protein, suggesting that *oskar* may act as a translational activator of localized *nanos* RNA (Ephrussi and Lehmann, 1992; Smith et al., 1992). Another protein localized to the posterior poleplasm, vasa, has sequence similarity to the translation initiation factor eIF-4A and has RNA helicase activity in vitro (Hay et al., 1988; Lasko and Ashburner, 1988; Liang et al., 1994). These results

suggest that oskar and vasa proteins may interact with *nanos* RNA at the posterior pole to facilitate its translation. However, no direct interaction between these species has yet been shown.

Translation of the Drosophila *oskar* RNA is also regulated by its localization. *oskar* is localized to the posterior pole of the growing oocyte, and translation does not occur until after the RNA is localized (Ephrussi et al., 1991; Kim-Ha et al., 1991; C. Rongo, E. Gavis, and R. Lehmann, submitted). Localized oskar protein specifies formation of the posterior poleplasm (Ephrussi and Lehmann, 1992). Recently it has been shown that the translational repression of unlocalized *oskar* RNA is dependent on a short sequence motif present in two copies in the 3'UTR (Kim-Ha et al., 1995). In addition, a protein in ovary extracts, bruno, binds to RNA containing this sequence motif (the bruno response element, or BRE) in vitro. BRE sequences inserted into a heterologous RNA confer partial translational suppression in the oocyte. It will be interesting to see if translational suppression of unlocalized forms of localized RNAs by bruno and the BREs bears mechanistic parallels to the translational suppression of CPE-containing transcripts in *Xenopus* oocytes.

3. Conclusions

It is clear that translational control plays an important role in the maternal control of development. For the majority of transcripts in the oocyte, repression of translation is likely to involve packaging into translationally inert ribonucleoprotein particles. For a subset of transcripts, a cytoplasmic adenylation element (CPE) sequence in the 3'UTR acts in conjunction with the hexanucleotide AAUAAA polyadenylation signal to direct the adenylation of these transcripts in the maturing oocyte, activating their translation. The spacing of the CPE and hexanucleotide sequences controls the timing and extent of post-maturation adenylation. Control of polyadenylation is also implicated in the translational regulation of the *tra-2*, *fem-3*, and possibly the *hunchback* RNAs. These results, combined with the observation that the 3'UTR seems invariably to be central to developmentally regulated translational control, highlight the importance of understanding the relationship between adenylation and translational initiation. Studies in yeast imply a link between the poly(A) metabolism and the initiation of

translation (Sachs and Davis, 1989; Sachs and Deardorff, 1992), although the significance of these studies to the strict dependence of translation on poly(A) tail length observed in *Xenopus* and mouse oocytes, as well as in *Drosophila* and *C. elegans*, remains unclear. The link between 3'UTR regulatory sequences and translational initiation suggests that the initiating mRNA may have a complex secondary structure, perhaps even a circular or looped configuration. Clarification of this and other issues awaits the reconstitution of regulated translation in vitro.

The aims of this thesis

The focus of the work presented in this thesis is the structure and function of the Drosophila maternal-effect gene *nanos*. Chapter 2, which has been previously published as an independent paper (Wang and Lehmann, 1991), describes the cloning of *nanos*, its localization as an RNA to the posterior pole of wild-type embryos, and introduces an RNA injection assay which was used to show that *nanos* RNA is sufficient to rescue the abdominal phenotype of all posterior group mutants tested. Chapter 3 describes use of this RNA injection assay to perform a preliminary analysis of possible functional domains in the *nanos* open reading frame. Also included in this chapter is a functional comparison between *nanos* and Xcat-2, a *Xenopus* gene encoding an RNA localized in oocytes. Chapter 4 (previously published as (Wang et al., 1994)) describes the localization of *nanos* RNA and protein in a number of mutant backgrounds, and includes a brief discussion of the *nanos* oogenesis phenotype. Chapter 5 describes polysome fractionation studies designed to elucidate the mechanism of *nanos*-dependent translational suppression of the maternal *hunchback* mRNA. The single appendix describes another test using the RNA injection assay, this time to determine whether *pumilio*, another posterior group gene, is absolutely required for *nanos* function.

REFERENCES

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

Ahringer, J., Rosenquist, T. A., Lawson, D. N., and Kimble, J. (1992). The *Caenorhabditis elegans* sex determining gene fem-3 is regulated post-transcriptionally. The European Molecular Biology Organization Journal *11*, 2303-2310.

Ambros, V. (1989). A hierarchy of regulatory genes controls a larva-to-adult developmental switch in C. elegans. Cell *57*, 49-57.

Ambros, V., and Horvitz, R. (1987). The *lin-14* locus of *Caenorhabditis elegans* controls the time of expression of specific postembryonic developmental events. Genes and Development *1*, 398-414.

Arasu, P., Wightman, B., and Ruvkun, G. (1991). Temporal regulation of *lin-14* by the antagonistic action of two other heterochronic genes, *lin-4* and *lin-28*. Genes & Development *5*, 1825-1833.

Bardsley, A., Macdonald, K., and Boswell, R. E. (1993). Distribution of tudor protein in the Drosophila embryo suggests separation of function based on site of localization. Development *119*, 207-219.

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

Barton, M. K., Schedl, T. B., and Kimble, J. (1987). Gain-of-function mutations of *fem-3*, a sex-determination gene in *Caenorhabditis elegans*. Genetics *115*, 107-119.

Berleth, T., Burri, M., Thoma, G., Bopp, D., Richstein, S., Frigerio, G., Noll, M., and Nüsslein-Volhard, C. (1988). The role of localization of bicoid RNA in organizing the anterior pattern of the Drosophila embryo. The European Molecular Biology Organization Journal *7*, 1749-1756.

Bilger, A., Fox, C. A., Wahle, E., and Wickens, M. (1994). Nuclear polyadenylation factors recognize cytoplasmic polyadenylation elements. Genes & Development *8*, 1106-1116.

Boswell, R. E., and Mahowald, A. P. (1985). *tudor*, a gene required for assembly of the germ plasm in *Drosophila melanogaster*. Cell *43*, 97-104.

Boswell, R. E., Prout, M. E., and Steichen, J. C. (1991). Mutations in a newly identified *Drosophila melanogaster* gene, *mago nashi*, disrupt germ cell formation of mirror-image symmetrical double abdomen embryos. Development *113*, 373-384.

Bouvet, P., Omilli, F., Arlot-Bonnemains, Y., Legagneux, V., Roghi, C., Bassezx, T., and Osborne, H. B. (1994). The deadenylation conferred by the 3'UTR of a developmentally controlled mRNA in Xenopus embryos is switched to polyadenylation by deletion of a short sequence element. Molecular and Cellular Biology *14*, 1893-1900.

Bouvet, P., and Wolffe, A. P. (1994). A role for transcription and FRGY2 in masking maternal mRNA within Xenopus oocytes. Cell *77*, 931-941.

Chalfie, M., Horvitz, R., and Sulston, J. (1981). Mutations that lead to reiterations in the cell lineages of C. elegans. Cell *24*, 59-69.

Christerson, L. B., and McKearin, D. M. (1994). *orb* is required for anteroposterior and dorsoventral patterning during Drosophila oogenesis. Genes & Development *8*, 614-628.

Dalby, B., and Glover, D. M. (1992). 3' non-translated sequences in *Drosophila* cyclin B transcripts direct posterior pole accumulation late in oogenesis and peri-nuclear association in syncytial embryos. Development *115*, 989-997.

Dalby, B., and Glover, D. M. (1993). Discrete sequence elements control posterior pole accumulation and translational repression of maternal cyclin B RNA in *Drosophila*. The EMBO Journal *12*, 1219-1227.

Dale, L., Matthews, G., and Colman, A. (1993). Secretion and mesoderm-inducing activity of the TGF-beta related domain of *Xenopus* Vg1. The EMBO Journal *12*, 4471-4480.

Darnborough, D. H., and Ford, P. J. (1981). Identification in *Xenopus laevis* of a class of oocyte specific proteins bound to messenger RNA. European Jounal of Biochemistry *113*, 415-424.

Davidson, E. H. (1986). Gene activity in early development. (Orlando: Academic Press, Inc.).

Ding, D., and Lipshitz, H. D. (1993). Localized RNAs and their functions. BioEssays *15*, 651-658.

Doniach, T. (1986). Activity of the sex-determining gene *tra-2* is modulated to allow spermatogenesis in the *C. elegans* hermaphrodite. Genetics *114*, 53-76.

Driever, W., and Nüsslein-Volhard, C. (1988). The *bicoid* protein determines position in the Drosophila embryo in a concentration-dependent manner. Cell *54*, 95-104.

Driever, W., and Nüsslein-Volhard, C. (1988). A gradient of bicoid protein in Drosophila embryos. Cell *54*, 83-93.

Driever, W., and Nüsslein-Volhard, C. (1989). The bicoid protein is a positive regulator of hunchback transcription in the early Drosophila embryo. Nature *337*, 138-143.

Elinson, R. P., King, M. L., and Forristall, C. (1993). Isolated vegetal cortex from *Xenopus* oocytes selectively retains localized mRNAs. Developmental Biology *160*, 554-562.

Ephrussi, A., Dickinson, L. K., and Lehmann, R. (1991). *oskar* organizes the germ plasm and directs localization of the posterior determinant *nanos*. Cell *66*, 37-50.

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

Evans, T. C., Crittenden, S. L., Kodoyianni, V., and Kimble, J. (1994). Translational control of maternal *glp-1* mRNA establishes an asymmetry in the C.elegans embryo. Cell *77*, 183-194.

Ferrandon, D., Elphick, L., Nüsslein-Volhard, C., and St Johnston, D. (1994). Staufen protein associates with the 3'UTR of bicoid mRNA to form particles that move in a microtubule-dependent manner. Cell *79*, 1221-1232.

Forristall, C., Pondel, M., Chen, L., and King, M. L. (1995). Patterns of localization and cytoskeletal association of two vegetally localized RNAs, *Vg1* and *Xcat-2*. Development *121*, 201-208.

Fox, C. A., Sheets, M. D., Wahle, E., and Wickens, M. (1992). Polyadenylation of maternal mRNA during oocyte maturation: poly(A) addition in vitro requires a regulated RNA binding activity and a a poly(A) polymerase. The European Molecular Biology Organization Journal *11*, 5021-5032.

Fox, C. A., Sheets, M. D., and Wickens, M. P. (1989). Poly(A) addition during maturation of frog oocytes: distinct nuclear and cytoplasmic activities and regulation by the sequence UUUUUAU. Genes & Development *3*, 2152-2162.

Frigerio, D., Burri, M., Bopp, D., Baumgartner, S., and Noll, M. (1986). Structure of the segmentation gene paired and the Drosophila PRD gene set as part of a gene network. Cell *47*, 479-746.

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

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

Goodwin, E. B., Okkema, P. G., Evans, T. C., and Kimble, J. (1993). Translational regulation of *tra-2* by its 3' untranslated region controls sexual identity in C. elegans. Cell *75*, 329-339.

Gurdon, J. B. (1992). The generation of diversity and pattern in animal development. Cell *68*, 185-199.

Gururajan, R., Perry-O'Keefe, H., Melton, D. A., and Weeks, D. L. (1991). The Xenopus localized messenger RNA An3 may encode an ATP-dependent RNA helicase. Nature *349*, 717-719.

Hake, L. E., and Richter, J. D. (1994). CPEB is a specificity factor that mediates cytoplasmic polyadenylation during Xenopus oocyte maturation. Cell *79*, 617-627.

Hay, B., Jan, L. Y., and Jan, Y. N. (1988). A protein component of Drosophila polar granules is encoded by vasa and has extensive sequence similarity to ATP-dependent helicases. Cell *55*, 577-587.

Hodgkin, J. (1986). Sex determination in the nematode *C. elegans*: analysis of *tra-3* suppressors and characterization of fem genes. Genetics *114*, 15-52.

Huarte, J., Stutz, A., O'Connell, M. L., Gubler, P., Belin, D., Darrow, A. L., Strickland, S., and Vassalli, J.-D. (1992). Transient translational silencing by reversible mRNA deadenylation. Cell *69*, 1021-1030.

Jongens, T. A., Ackerman, L. D., Swedlow, J. R., Jan, L. Y., and Jan, Y. N. (1994). *Germ cell-less* encodes a cell type-specific nuclear pore-associated protein and functions early in the germ-cell specification pathway of *Drosophila*. Genes & Development *8*, 2123-2136.

Jongens, T. A., Hay, B., Jan, L. Y., and Jan, Y. N. (1992). The *germ cell-less* gene product: a posteriorly localized component necessary for germ cell development in Drosophila. Cell *70*, 569-584.

Kick, D., Barrett, P., Cummings, A., and Sommerville, J. (1987). Phosphorylation of a 60 kDa polypeptide from Xenopus oocytes blocks messenger RNA translation. Nucleic Acids Research *15*, 4099-4109.

Kim-Ha, J., Kerr, K., and Macdonald, P. M. (1995). Translational regulation of *oskar* mRNA by bruno, a newly-identified RNA binding protein, is essential. Cell, in press.

Kim-Ha, J., Smith, J. L., and Macdonald, P. M. (1991). oskar mRNA is localized to the posterior pole of the Drosophila oocyte. Cell *66*, 23-35.

Kim-Ha, J., Webster, P. J., Smith, J. L., and Macdonald, P. M. (1993). Multiple RNA regulatory elements mediate distinct steps in localization of *oskar* mRNA. Development *119*, 169-178.

King, R. C. (1970). Ovarian Development in *Drosophila melanogaster*. (New York: Academic Press).

Kloc, M., Spohr, G., and Etkin, L. D. (1993). Translocation of repetitive RNA sequences with the germ plasm in *Xenopus* oocytes. Science *262*, 1712-1714.

Kraut, R., and Levine, M. (1991a). Spatial regulation of the gap gene giant during development. Development *111*, 601-609.

Ku, M., and Melton, D. A. (1993). *Xwnt-11*: a maternally expressed *Xenopus wnt* gene. Development *119*, 1161-1173.

Lantz, V., Ambrosio, L., and Schedl, P. (1992). The Drosophila orb gene is predicted to encode sex-specific germline RNA-binding proteins and has localized transcripts in ovaries and early embryos. Development *115*, 75-88.

Lantz, V., Chang, J. S., Horabin, J. I., Bopp, D., and Schedl, P. (1994). The *Drosophila orb* RNA-binding protein is required for the formation of the egg chamber and establishment of polarity. Genes & Development *8*, 598-613.

Lantz, V., and Schedl, P. (1994). Multiple *cis*-acting targeting sequences are required for *orb* mRNA localization during *Drosophila* oogenesis. Molecular and Cellular Biology *14*, 2235-2242.

Lasko, P. F., and Ashburner, M. (1988). The product of the Drosophila gene *vasa* is very similar to eukaryotic initiation factor-4A. Nature *335*, 611-617.

Lee, R. C., Feinbaum, R. L., and Ambros, V. (1993). The C.elegans heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. Cell *75*, 843-854.

Legagneux, V., Bouvet, P., Omilli, F., Chevalier, S., and Osborne, H. B. (1992). Identification of RNA-binding proteins specific to *Xenopus* Eg maternal mRNAs: association with the portion of Eg2 mRNA that promotes deadenylation in embryos. Development *116*, 1193-1202.

Lehmann, R., and Nüsslein-Volhard, C. (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 Nüsslein-Volhard, C. (1987a). Involvement of the pumilio gene in the transport of an abdominal signal in the Drosophila embryo. Nature *329*, 167-170.

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

Liang, L, Diehl-Jones, W., and Lasko, P. (1994). Localization of vasa protein to the *Drosophila* pole plasm is independent of its RNA-binding and helicase activities. Development *120*, 1201-1211.

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

Macdonald, P. M., Kerr, K., Smith, J. L., and Leask, A. (1993). RNA regulatory element BLE1 directs the early steps of *bicoid* mRNA localization. Development *118*, 1233-1243.

Macdonald, P. M., and Struhl, G. (1988). Cis-acting sequences responsible for anterior localization of bicoid mRNA in Drosophila embryos. Nature *336*, 595-598.

Manseau, L. J., and Schüpbach, T. (1989). cappuccino and spire: two unique maternal-effect loci required for both the anteroposterior and dorsoventral patterns of the Drosophila embryo. Genes & Development *3*, 1437-1452.

Marcey, D., Watkins, W. S., and Hazelrigg, T. (1991). The temporal and spatial distribution pattern of maternal exuperantia protein —evidence for a role in establishment but not maintenance of bicoid messenger RNA localization. The European Molecular Biology Organization Journal *10*, 4259-4266.

McGrew, L. L., Dworkin-Rastl, E., Dworkin, M. B., and Richter, J. D. (1989). Poly(A) elongation during Xenopus oocyte maturation is required for translational recruitment and is mediated by a short sequence element. Genes & Development *3*, 803-815.

Melton, D. A. (1987). Translocation of a localized maternal mRNA to the vegetal pole of *Xenopus* oocytes. Nature *328*, 80-82.

Mosquera, L., Forristall, C., Zhou, Y., and King, M. L. (1993). A 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 Wharton, R. P. (1995). Binding of Pumilio to maternal *hunchback* mRNA is required for posterior patterning in Drosophila embryos. Cell *80*, 747-756.

Murray, M. T., Schiller, D. L., and Franke, W. W. (1992). Sequence analysis of cytoplasmic mRNA-binding proteins of *Xenopus* oocytes identifies a family of RNA-binding proteins. Proceedings of the National Academy of Science USA *89*, 11-15.

Neuman-Silberberg, F. S., and Schüpbach, T. (1993). The Drosophila dorsoventral patterning gene *gurken* produces a dorsally localized RNA and encodes a TGF α-like protein. Cell *75*, 165-174.

Newmark, P. A., and Boswell, R. E. (1994). The *mago nashi* locus encodes an essential product required for germ plasm assembly in *Drosophila*. Development *120*, 1303-1313.

Paris, J., Osborne, H. B., Couturier, A., LeGuellec, R., and Philippe, M. (1988). Cloning by differential screening of a *Xenopus* cDNA coding for a protein highly homolgous to cdc2. Proceedings of the National Academy of Sciences USA *88*, 1039-1043.

Paris, J., and Philippe, M. (1990). Poly(A) metabolism and polysomal recruitment of maternal mRNAs during early *Xenopus* development. Developmental Biology *140*, 221-224.

Paris, J., Swenson, K., Piwnica-Worms, H., and Richter, J. D. (1991). Maturation-specific polyadenylation: in vitro activation by p34-cdc2 and phosphorylation of a 58-kD CPE-binding protein. Genes and Development *5*, 1697-1708.

Raff, J. W., Whitfield, W. G. F., and Glover, D. M. (1990). Two distinct mechanisms localise cyclin B transcripts in synctial *Drosophila* embryos. Development *110*, 1-13.

Ran, B., Bopp, R., and Suter, B. (1994). Null alleles reveal novel requirements for *Bic-D* during *Drosophila* oogenesis and zygotic development. Development *120*, 1233-1242.

Ranjan, M., Tafuri, S. R., and Wolffe, A. P. (1993). Masking mRNA from translation in somatic cells. Genes & Development *7*, 1725-1736.

Regabliatti, M. R., Weeks, D. L., Harvey, R. P., and Melton, D. A. (1985). Identification and cloning of maternal mRNAs in *Xenopus* eggs. Cell *42*, 769-777.

Richter, J. D. (1988). Information relay from gene to protein: the mRNP connection. Trends in Biochemical Sciences *13*, 483-486.

Richter, J. D., and Smith, L. D. (1984). Reversible inhibition of translation by *Xenopus* oocyte-specific proteins. Nature *309*, 378-380.

Roth, S., Stein, D., and Nüsslein-Volhard, C. (1989). A gradient of nuclear localization of the dorsal protein determines dorsoventral pattern in the Drosophila embryo. Cell *59*, 1189-1202.

Rothe, M., Nauber, U., and Jäckle, H. (1989). Three hormone receptor-like *Drosophila* genes encode an identical DNA-binding finger. The European Molecular Biology Organization Journal *8*, 3087-3094.

Rushlow, C. A., Han, K., Manley, J. L., and Levine, M. (1989). The graded distribution of the dorsal morphogen is initiated by selective nuclear transport in Drosophila. Cell *59*, 1165-1177.

Sachs, A. B., and Davis, R. W. (1989). The Poly(A) binding protein is required for poly(A) shortening and GOS ribosomal subunit-dependent translational initiation. Cell *58*, 857-867.

Sachs, A. B., and Deardorff, J. A. (1992). Translation initiation requires the PAB-dependent Poly(A) ribonuclease in yeast. Cell *70*, 961-973.

Sallés, F. J., Lieberfarb, M. E., Wreden, C., Gergen, J. P., and Strickland, S. (1994). Coordinate initiation of *Drosophila* development by regulated polyadenylation of maternal messenger RNAs. Science *266*, 1996-1999.

Schedl, T., and Kimble, J. (1988). *fog-2*, a germ-line-specific sex determination gene required for hermaphrodite spermatogenesis in *Caenorhabditis elegans*. Genetics *119*, 43-61.

Schüpbach, T., and Wieschaus, E. (1986a). Maternal-effect mutations altering the anterior-posterior pattern of the Drosophila embryo. Wilhelm Roux's Archives of Developmental Biology *195*, 302-317.

Sheets, M. D., Fox, C. A., Hunt, T., Vande Woude, G., and Wickens, M. (1994). The 3'-untranslated regions of *c-mos* and cyclin mRNA stimulate translation by regulating cytoplasmic polyadenylation. Genes and Development *8*, 926-938.

Siegel, V., Jongens, T. A., Jan, L. Y., and Jan, Y. N. (1993). *pipsqueak*, an early acting member of the posterior group of genes, affects *vasa* level and germ cell-somatic cell interaction in the developing egg chamber. Development *119*, 1187-1202.

Simon, R., and Richter, J. D. (1994). Further analysis of cytoplasmic polyadenylation in Xenopus embryos and identification of embryonic cytoplasmic polyadenylation element-binding proteins. Molecular and Cellular Biology *14*, 7867-7875.

Simon, R., Tassan, J.-P., and Richter, J. D. (1992). Translational control by poly(A) elongation during *Xenopus* development; differential repression and enhancement by a novel cytoplasmic polyadenylation element. Genes & Development *6*, 2580-2591.

Smith, J. L., Wilson, J. E., and Macdonald, P. M. (1992). Overexpression of *oskar* directs ectopic activation of *nanos* and presumptive pole cell formation in Drosophila embryos. Cell *70*, 849-859.

Spirin, A. S. (1966). On "masked" forms of messenger RNA in early embryogenesis and in other differentiating systems. Current Topics in Developmental Biology *1*, 1-63.

Sprenger, F., and Nüsslein-Volhard, C. (1992). Torso receptor activity is regulated by a diffusible ligand produced at the extracellular terminal regions of the Drosophila egg. Cell *71*, 987-1001.

Sprenger, F., Stevens, L., and Nüsslein-Volhard, C. (1989). The Drosophila gene torso encodes a putative receptor tyrosine kinase. Nature *338*, 478-483.

St Johnston, D., Driever, W., Berleth, T., Richstein, S., and Nüsslein-Volhard, C. (1989). Multiple steps in the localization of *bicoid* RNA to the anterior pole of the Drosophila oocyte. Development *Suppl. 107*, 13-19.

St Johnston, D. (1995). The intracellullar localisation of messenger RNAs. Cell *in press*.

St. Johnston, D. (1993). Poleplasm and the posterior group genes. In The development of *Drosophila melanogaster*, M. Bate, and Martinez Arias, A., eds. (Cold Spring Harbor: Cold Spring Harbor Press).

St. Johnston, D., Beuchle, D., and Nüsslein-Volhard, C. (1991). *staufen*, a gene required to localize maternal RNAs in the Drosophila egg. Cell *66*, 51-63.

St. Johnston, D., Brown, N. H., Gall, J. G., and Jantsch, M. (1992). A conserved double-stranded RNA-binding domain. Proceedings of the National Academy of Science USA *89*, 10979-10983.

St. Johnston, D., and Nüsslein-Volhard, C. (1992). The origin of pattern and polarity in the Drosophila embryo. Cell *68*, 201-219.

Stebbins-Bouz, B., and Richter, J. D. (1994). Multiple sequence elements and a maternal mRNA product control cdk2 RNA polyadenylation and translation during early *Xenopus* development. Molecular and Cellular Biology *14*, 5870-5880.

Stephenson, E. C., Chao, Y. C., and Fackenthal, J. D. (1988). Molecular analysis of the swallow gene of Drosophila melanogaster. Genes & Development *2*, 1655-1665.

Steward, R. (1989). Relocalization of the dorsal protein from the cytoplasm to the nucleus correlates with its function. Cell *59*, 1179-1188.

Strickland, S., Huarte, J., Belin, D., Vassalli, A., Rickles, R. J., and Vassalli, J.-D. (1988). Antisense RNA directed against the 3' noncoding region prevents dormant mRNA activation in mouse oocytes. Science *241*, 680-684.

Struhl, G., Struhl, K., and Macdonald, P. M. (1989). The gradient morphogen *bicoid* is a concentration-dependent transcriptional activator. Cell *57*, 1259-1273.

Tafuri, S. R., and Wolffe, A. P. (1990). Xenopus Y-box transcription factors: molecular cloning, functional analysis and developmental regulation. Proceedings of the National Academy of Sciences USA *87*, 9028-9032.

Tafuri, S. R., and Wolffe, A. P. (1993). Selective recruitmen of masked maternal mRNA from messenger ribonucleoprotein particles containing FRGY2 (mRNP4). Journal of Biological Chemistry *268*, 24255-24261.

Thomsen, G. H., and Melton, D. A. (1993). Processed Vg1 protein is an axial mesoderm inducer in Xenopus. Cell *74*, 433-441.

Vassalli, J.-D., Huarte, J., Belin, D., Gubler, P., Vassalli, A., O'Connell, M.L., Parton, L.A., Rickles, R.J., Strickland, S. (1989). Regulated polyadenylation controls mRNA translation during meiotic maturation of mouse oocytes. Genes & Development *3*, 2163-2171.

Wang, C., Dickinson, L. K., and Lehmann, R. (1994). Genetics of *nanos* localization in *Drosophila*. Developmental Dynamics *199*, 103-115.

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

Weeks, D. L., and Melton, D. A. (1987). An mRNA localized to the animal pole of *Xenopus* eggs encodes a subunit of a mitochondrial ATPase. Proceedings of the National Academy of Science USA *84*, 2798-2802.

Wharton, R. P., and Struhl, G. (1991). RNA regulatory elements mediate control of Drosophila body pattern by the posterior morphogen *nanos*. Cell *67*, 955-967.

Whitfield, W. G. F., Gonzáliz, C., Sánchez-Herrero, E., and Glover, D. M. (1989). Transcripts of one of two Drosophila cyclin genes become localized in pole cells during embryogenesis. Nature *338*, 337-340.

Wightman, B., Bürglin, T. R., Gatto, J., Arasu, P., and Ruvkun, G. (1991). Negative regulatory sequences in the *lin-14* 3'-untranslated region are necessary to generate a temporal switch during *Caenorhabditis elegans* development. Genes & Development *5*, 1813-1824.

Wightman, B., Ha, I., and Ruvkun, G. (1993). Posttranscriptional regulation of the heterochronic gene *lin-14* by *lin-4* mediates temporal pattern formation in C.elegans. Cell *75*, 855-862.

Chapter 2

Cloning and characterization of *nanos*

AUTHOR'S NOTE

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ABSTRACT

Segmental pattern in the *Drosophila* embryo is established by two maternal factors localized to the anterior and posterior poles of the egg cell. Here we provide molecular evidence that the localized posterior factor is the RNA of the *nanos* (*nos*) gene. *nos* RNA is localized to the posterior pole of early embryos, and *nos* protein acts at a distance to direct abdomen formation. Synthetic *nos* RNA has biological activity identical to that of the posterior poleplasm. Injection of *nos* RNA rescues the segmentation defect of embryos derived from females mutant for all nine known posterior group genes. Injection of *nos* RNA into the anterior is able to direct formation of ectopic posterior structures. Our results demonstrate that a localized source of *nos* RNA is sufficient to specify abdominal segmentation, and imply that other posterior group genes are required for localization, stabilization or distribution of the *nos* gene product.

INTRODUCTION

Establishment of polarity in the *Drosophila* embryo requires maternal information that is provided to the egg cell during its maturation. Information for the establishment of the anterior-posterior axis consists of three independent morphogenetic systems which control the spatially restricted expression of target genes in the embryo (Nüsslein-Volhard et al., 1987). The anterior system controls the development of head and thorax, the posterior system, the abdominal region, and the terminal system, the most anterior and posterior larval structures (Frohnhöfer and Nüsslein-Volhard, 1986; Lehmann and Nüsslein-Volhard, 1991; Klingler et al., 1988). The anterior and posterior systems act through factors localized to the respective poles (Frohnhöfer and Nüsslein-Volhard, 1986; Lehmann and Nüsslein-Volhard, 1991). At the anterior end, the determining factor is the product of the *bicoid* (*bcd*) gene (Frohnhöfer and Nüsslein-Volhard, 1986). Localization

of *bcd* to the anterior pole requires other anterior group genes (Frohnhöfer and Nüsslein-Volhard, 1987). *bcd* RNA localization establishes a concentration gradient of *bcd* protein over the anterior half of the embryo (St. Johnston et al., 1989; Driever and Nüsslein-Volhard, 1988a) that elicits proper expression of zygotic target genes such as the gap gene *hunchback* (*hb*) (Driever and Nüsslein-Volhard, 1988b; Driever and Nüsslein-Volhard, 1989; Struhl et al., 1989).

For the posterior system, at least nine genes (*nanos (nos), pumilio (pum), oskar (osk), vasa (vas), tudor (tud), staufen (stau), valois (vls), cappuccino (capu),* and *spire (spir)*) are required for abdomen formation (Boswell and Mahowald, 1985; Schüpbach and Wieschaus, 1986; Lehmann and Nüsslein-Volhard, 1986; Lehmann and Nüsslein-Volhard, 1987; Manseau and Schüpbach, 1989; Lehmann and Nüsslein-Volhard, 1991). Embryos derived from females mutant for any of these genes lack abdominal segments. This defect can be rescued by transplantation of cytoplasm from the posterior pole of wild-type embryos into the mutant abdomen (Lehmann and Nüsslein-Volhard, 1986; Lehmann and Nüsslein-Volhard, 1987; Lehmann and Nüsslein-Volhard, 1991). This result led to the hypothesis that the posterior poleplasm serves as the source of a signal whose function is required at a distance, in the prospective abdominal region. Synthesis of this rescuing activity during oogenesis is normal in all mutants, with the notable exception of *nos* mutant females (Lehmann and Nüsslein-Volhard, 1991). However, all posterior group mutant embryos, except *pum*, lack this activity at the posterior pole (Lehmann and Nüsslein-Volhard, 1986; Lehmann and Nüsslein-Volhard, 1987; Lehmann and Nüsslein-Volhard, 1991). These results suggested that the posterior group mutants affect a common, *nos*-dependent activity, synthesized during oogenesis, and localized to the posterior pole of the embryo.

Although the pathway for the establishment of posterior pattern predicted the existence of a maternally provided "determinant" localized to the posterior pole, the molecular nature of such a determinant remained unclear. Here, we describe the isolation and molecular characterization of the *nos* gene and present evidence that *nos* RNA is functionally equivalent to the

morphogenetic activity found in the posterior poleplasm, and that *nos* protein acts at a distance, to direct abdomen development.

RESULTS

The *nanos* gene

Cytological mapping places *nos* at band 91F13 on the right arm of the third chromosome (Lehmann and Nüsslein-Volhard, 1991). The *nos* genomic region was cloned by chromosomal walking (Figure 2.1A). Two deficiencies, *Df(3R)Dl-A143,* which uncovers the *nos* gene, and *Df(3R)Dl-HD28,* which complements all *nos* mutants (Vässin and Campos-Ortega, 1987; Alton et al., 1988; Lehmann and Nüsslein-Volhard, 1991), define a 20 kilobase (kb) region containing an essential part of *nos* gene function within a 120 kb chromosomal walk. Since genetic evidence indicated that *nos* function is only required maternally, the 20 kb region was analyzed for maternally expressed transcripts. This analysis revealed a 2.5 kb SalI-XhoI genomic DNA fragment which hybridizes strongly to a single RNA species of 2.4 kb present predominantly in ovaries and 0-2 hour old embryos, and weakly in 2-8 hour old embryos. No transcript is detected in older embryos, larvae, or pupae (Figure 2.1B). The developmental profile of this transcript is consistent with the maternal mode of *nos* action and parallels that of another maternal-effect gene, *bicoid* (Berleth et al., 1988; Figure 2.1B). Low levels of transcript are also detected in males. At present there is no genetic evidence for a role of *nos* in males. P-element mediated transformation of *nos* mutants with a 7.5 kb genomic DNA fragment (Figure 2.1A) that includes the 2.5 kb SalI-XhoI fragment rescues the abdominal phenotype of *nos* mutants (Gavis and Lehmann, 1992). We will thus refer to the 2.4 kb transcript as *nos* RNA.

The 2.5 kb SalI-XhoI genomic fragment was used as a probe to screen cDNA libraries (Brown and Kafatos, 1988; Frigerio et al., 1986). The nearly full length (2.3 kb) cDNA clone N5, as well as 3 kb of genomic DNA encompassing this cDNA, were sequenced (Figure 2.2). Comparison of cDNA and genomic sequences indicates that the *nos* primary transcript contains 2 introns which are excised to produce a single 2.4 kb mRNA. The single large open reading

frame encodes a protein of 400 amino acids with a predicted molecular weight of 43 kilodaltons (Figure 2.2). The *nos* polypeptide is somewhat basic (predicted pI=9.1), and contains several stretches of polyglutamine (encoded by OPA-sequences or M-repeats) and polyasparagine which are commonly found in *Drosophila* proteins (Wharton et al., 1985). Database comparisons (March 1995) show two sequences with some degree of homology to the nanos polypeptide (Altschul et al., 1990). The first is the *Xenopus* gene Xcat-2, which is localized as an mRNA to the vegetal cortex of *Xenopus* oocytes (see Chapter 3 for details). The second is a *C. elegans* gene of unknown function discovered in the genome sequencing project (Wilson et al., 1994), which is 70% identical and 88% similar to nanos in a 26 amino acid region at the C-terminus of nanos. The functional significance of this similarity is unknown.

Distribution of *nanos* mRNA

A central prediction of the model for the establishment of posterior pattern is that *nos* should encode the posterior rescuing activity and that the *nos* gene product should be localized to the posterior pole of early embryos (Lehmann and Nüsslein-Volhard, 1991). Using a digoxygenin-labeled *nos* cDNA as a probe for *in situ* hybridization, we find that the *nos* transcript is concentrated at the posterior pole of freshly laid eggs and cleavage stage embryos (stage 2; Figure 2.3A). At the pole bud stage (stage 3), the transcript segregates into the nascent pole cells (Figure 2.3B). The transcript remains concentrated in the pole cells during blastoderm stages (stages 4 and 5; Figure 2.3C). During gastrulation and germband extension when the pole cells are carried dorsally and internalized into the embryo, staining for *nos* RNA is visible in the pole cells, although reduced in intensity as compared to earlier stages (Figure 2.4D, stages 7 and 8). While the transcript is still visible in the pole cells at the fully extended germ band stage (stage 10, Figure 2.3E) localized staining is no longer detected once the pole cells have left the pocket formed by the posterior midgut invagination (stage 11, Figure 2.3F). In summary, the developmental profile of *nos* RNA detected by *in situ* hybridization to embryos and by RNA blot analysis suggests that maternally provided *nos* transcript is present during oogenesis and early stages of embryogenesis, and that transcript localization is confined to the poleplasm and pole cells.

Synthetic *nanos* RNA is biologically active, and depletes maternal *hunchback* in embryos

The localization of *nos* RNA at the posterior pole of early embryos and the presence of *nos* RNA in the pole cells suggests that *nos* RNA is responsible for the rescuing activity assayed in transplantation experiments (Lehmann and Nüsslein-Volhard, 1986). If *nos* RNA is the rescuing activity found in the poleplasm, synthetic *nos* transcript should provide rescuing activity in an embryo injection assay. Sense strand RNA was synthesized *in vitro* from the *nos* cDNA, N5. This RNA was injected into the prospective abdominal region of cleavage stage (stage 2) embryos derived from nos^{L7} homozygous mutant females. Uninjected *nos* mutant embryos lack all abdominal segments, as seen by the lack of ventral abdominal denticle belts (Figure 2.4A). By contrast, sibling embryos injected with *nos* RNA show rescue of the abdominal segmentation defect (Figures 2.4B, 2.4C). Completely rescued embryos are indistinguishable from wild type, and hatch and develop into normal, fertile adult flies.

The role of *nos* in abdominal segmentation is mediated by the maternally provided product of the *hunchback* (*hb*) gene (Hülskamp et al., 1989; Irish et al., 1989; Struhl, 1989). In wild-type embryos, *nos* is required to exclude *hb* protein from the posterior half of the embryo (Tautz, 1988). The resulting gradient of maternal *hb* allows proper expression of abdominal gap genes such as *knirps* and *giant* (Hülskamp et al., 1990; Eldon et al., 1990; Kraut and Levine, 1990). To see if the phenotypic rescue observed correlated with a change in the expression pattern of the maternal *hb* protein, we stained injected embryos with an antibody directed against *hb* (Figures 2.4D, 2.4E). RNA synthesized from the N5 *nos* cDNA template was injected into embryos derived from homozygous nos^{L7} females under the same conditions as described for phenotypic rescue (see above). After injection the embryos were incubated for one hour and assayed for *hb* protein. These embryos were compared to embryos treated identically but injected with a *nos* RNA containing a frameshift mutation at amino acid 51 (Table 1). Uninjected or frameshift injected (Figure 2.4D) embryos show a uniform pattern of *hb* protein staining at pole bud stage. By contrast, embryos injected with wild-

type *nos* transcript lack detectable *hb* protein in the posterior region (Figure 2.4E). In most of these embryos *hb* protein can be seen at the anterior, furthest from the site of injection (25-50% egg length, where 0%=posterior pole). Of 22 embryos injected with wild-type *nos* RNA and stained for *hb* protein, 5% showed homogeneous staining, 90% showed only anterior staining, and 5% gave no detectable staining. For comparison, of 19 embryos injected with *nos* frameshift RNA, 84% showed homogeneous staining, and 16% showed anterior staining. These results demonstrate that *nos* RNA depletes *hb* protein from the vicinity of the site of injection, and strongly suggests that the phenotypic rescue of embryos by *nos* RNA injection is mediated by this interaction.

nanos RNA is equivalent to the posterior rescuing activity

The degree of rescue by *nos* RNA depends on the concentration of the injected RNA. Rescue was tested over a 4-fold range in concentration (Table 2.1). At the lowest concentration tested, most embryos show only partial rescue (Figure 2.4B). At the highest concentration tested, all embryos are completely rescued and form all eight abdominal segments (Figure 2.4C). RNA transcribed from a template containing a frame shift mutation fails to rescue the abdominal defect when assayed by injection (Table 2.1). These results indicate that the N5 cDNA contains a functional *nos* open reading frame and that translation of *nos* RNA after egg deposition is sufficient to fully restore the mutant defect.

The degree of rescue by *nos* RNA depends on the age of the mutant embryo and the position of injection. Injection of *nos* RNA into *nos* mutant embryos results in optimal rescue when embryos are injected before pole cell formation (Figure 2.5A). We conclude that *nos* function is required early in embryogenesis, at the time when the concentration gradient of maternal *hb* product is established (Tautz, 1988). Further, optimal rescue is achieved if *nos* RNA is injected into the prospective abdominal region (25-50% egg length) (Figure 2.5B), indicating that the injected RNA is translated at the site of injection and does not require localization to the posterior pole for translation. In summary, the activity of synthetic *nos* RNA is equivalent to

the rescuing activity found in posterior poleplasm with respect to the spatial and temporal parameters of rescue (Lehmann and Nüsslein-Volhard, 1986; Lehmann and Nüsslein-Volhard, 1991).

Synthetic *nanos* RNA rescues abdominal phenotype of all posterior group mutants

Embryos derived from females defective for seven of the nine posterior group genes lack the specialized posterior poleplasm, including the characteristic polar granules, and subsequently fail to form pole cells, the germ line precursors (Lehmann and Nüsslein-Volhard, 1986; Schüpbach and Wieschaus, 1986; Boswell and Mahowald, 1985; Manseau and Schüpbach, 1989). Cytoplasmic transplantation experiments have shown that these embryos lack posterior rescuing activity (Lehmann and Nüsslein-Volhard, 1986; Lehmann and Nüsslein-Volhard, 1991). To demonstrate the exclusive role of *nos* as a localized posterior determinant, we tested the ability of *nos* RNA to rescue the abdominal phenotype of all posterior group mutants. Injection of *nos* RNA completely rescues the abdominal phenotype of the genes *osk, stau, tud, vas, vls, capu,* and *spir* (Table 2.2). The germ-cell defect, however, remains. These results indicate that *nos* acts as a posterior determinant, and suggest that these posterior group genes are dispensable for translation and/or activity of *nos* protein. Since embryos from mothers mutant for these genes lack the specialized posterior poleplasm, we propose that localization of *nos* mRNA to the posterior pole depends on the presence of an intact poleplasm. The abdominal segmentation defect of these mutants may therefore be a secondary effect of failure to localize or stabilize poleplasm components, including the *nos* mRNA.

Embryos derived from *pum* mutant mothers have intact poleplasm and form germ cells. Cytoplasmic transplantation experiments have shown that the posterior poleplasm of *pum* embryos contains normal levels of rescuing activity for all posterior group mutants (Lehmann and Nüsslein-Volhard, 1987; Lehmann and Nüsslein-Volhard, 1991). *pum* mutant embryos injected with *nos* RNA show rescue of the abdominal segmentation phenotype. However, rescue is limited to six abdominal segments or less

(Table 2.2), and segmentation is restored only locally, near the site of injection (data not shown). We conclude that *pum* affects the ability of *nos* to act at a distance on abdomen formation and propose that *pum* affects the distribution of *nos* protein, rather than the localization of *nos* RNA. Further experiments are required to determine whether *pum* function involves intracellular transport, stabilization, abundance or activity of *nos* protein.

Presence of *nanos* at the anterior suppresses *bicoid*

Previous studies have shown that transplantation of posterior poleplasm to an ectopic anterior position suppresses the formation of head and thoracic structures and directs the formation of a second abdomen at the anterior end of the embryo (Nüsslein-Volhard et al., 1987; Frohnhöfer et al., 1986). If this respecification is due to *nos*, injection of *nos* RNA into the anterior of embryos should suppress anterior development and cause development of ectopic posterior structures. We injected synthetic *nos* RNA into the prospective head region of *nos* mutant embryos and followed their development (Figure 2.6).

The cephalic furrow which normally forms during gastrulation (stages 6-7) at 65% egg length serves as a marker for the anterior region of the embryonic fate map. In many cases, injected embryos show an anterior shift in the position of the cephalic furrow, and cuticle preparations of first instar larvae reveal a reduction of head skeletal structures. In more extreme instances, injected embryos lack a cephalic furrow altogether, and an ectopic posterior midgut invagination is observed at the anterior end (Figures 2.6A, 2.6E). The result of this extreme fate map shift is seen more clearly in cuticle preparations. In the case shown, head structures are completely absent and structures normally found at the posterior, including a telson and several abdominal denticle belts, are duplicated in mirror image at the anterior ("bicaudal" phenotype, Figure 2.6F). Of 57 embryos injected anteriorly and scored for cuticle phenotype, 32% had normal head structures, 30% showed reduction of head structures, 33% lacked head structures altogether, and 5% gave a bicaudal phenotype which included reversal of abdominal segments. These results demonstrate that anteriorly introduced *nos* RNA is sufficient to

suppress formation of head and thoracic structures, and is able to induce the formation of posterior structures.

The suppression of head structures observed in *nos* injected embryos suggests that *nos* affects genes specifying head and thoracic structures. These anterior defects resemble those of *bicoid* and *bicaudal* mutants (Frohnhöfer and Nüsslein-Volhard, 1986; Nüsslein-Volhard, 1977). In no case were phenotypes characteristic of zygotic *hb* mutants, such as thoracic segment deletions, produced. To further distinguish between a direct effect of *nos* on *bcd*, as opposed to an effect of *nos* on the zygotic *hb* product, which is under the control of *bcd* (Driever and Nüsslein-Volhard, 1989; Struhl et al., 1989), we stained embryos with antibodies against *bcd* protein (Driever and Nüsslein-Volhard, 1988a). At the syncytial blastoderm stage (stages 3-4), levels of *bcd* protein are reduced in embryos injected with *nos* RNA, whereas normal levels of *bcd* protein are found in embryos injected with a frame shifted *nos* RNA (Figures 2.6D, 2.6H, Table 2.3). In addition, suppression of anterior structures is only observed after *nos* RNA injection in regions where *bcd* RNA is normally localized (data not shown). *In situ* hybridization analysis of injected embryos indicates that the stability and distribution of *bcd* mRNA is not significantly affected by the ectopic introduction of *nos* (Figures 2.6C, 2.6G, Table 2.3). These results taken together suggest that *nos* can affect the synthesis and/or stability of *bcd* protein.

DISCUSSION

Previous genetic studies led to identification of nine genes which are required for normal abdomen formation in Drosophila (Boswell and Mahowald, 1985; Schüpbach and Wieschaus, 1986; Lehmann and Nüsslein-Volhard, 1986; Lehmann and Nüsslein-Volhard, 1987; Manseau and Schüpbach, 1989; Lehmann and Nüsslein-Volhard, 1991). These studies revealed a factor concentrated in the poleplasm of wild-type embryos that rescues the abdominal defect of posterior group mutants (Lehmann and Nüsslein-Volhard, 1991). It was suggested that *nanos* (*nos*) is involved in the synthesis of this factor (Lehmann and Nüsslein-Volhard, 1991). Here, we have demonstrated that the *nos* gene product is the rescuing factor. *nos*

mRNA is localized at the posterior pole and serves as a source of the *nos* protein, which acts as a signal in the prospective abdomen. Synthetic *nos* RNA is biologically active and rescues the abdominal phenotype of *nos* and all other posterior group mutant embryos. This result shows that the other posterior group genes are not required for *nos* function, but rather play a role in localization or distribution of *nos* gene products. Finally, transplantation of *nos* RNA into the anterior pole suppresses head and thoracic development and promotes the formation of posterior structures in reversed orientation at the anterior end. These results indicate that a localized source of *nos* product is sufficient to specify abdominal segmentation.

How does the *nos* product determine posterior pattern? The anterior, posterior and terminal systems of maternal genes regulate the spatial domains of gap gene transcription in the embryo (Nüsslein-Volhard et al.,1987; Ingham, 1988). For the posterior system, *nos* activity is required for the correct embryonic transcription of at least three gap genes: *Krüppel* (*Kr*), *knirps* (*kni*) and *giant* (*gt*) (Gaul and Jäckle, 1987; Nauber et al., 1988; Eldon and Pirrotta, 1991; Kraut and Levine, 1991). However, *nos* does not regulate embryonic transcription directly. *nos* negatively regulates the abundance of the maternally provided *hunchback* (*hb*) gene products such that a concentration gradient of *hb* RNA and protein is established, with highest concentrations in the anterior (Tautz, 1988). Maternally provided *hb* itself is believed to act as a transcriptional repressor, such that low levels of *hb* in the abdomen allow proper expression of the gap genes *Kr*, *kni* and *gt* (Hülskamp et al., 1989; Irish et al., 1989; Struhl, 1989; Hülskamp et al., 1990).

Changes in the levels of *nos* along the anterior-posterior axis shift the embryonic fate map (Lehmann, 1988) and can alter the polarity of the segmental pattern (Lehmann and Nüsslein-Volhard, 1991), presumably by affecting the maternal *hb* concentration gradient (Tautz, 1988; Wharton, and Struhl, 1989). However, recent experiments indicate that, under some conditions, the maternal *hb* gradient is dispensable for the formation of a normal segmental pattern (Hülskamp et al., 1989; Irish et al., 1989; Struhl, 1989). Embryos that lack *nos* and maternal *hb* products, thereby completely lacking maternally provided posterior information, develop with a normal segmentation pattern (Hülskamp et al., 1989; Irish et al., 1989; Struhl, 1989),

which probably reflects normal expression of gap genes. Segmentation in such embryos must operate by a second, "*nanos*-independent" pathway. Gap gene expression in the abdomen of these embryos is set by neighboring gap genes, whose spatial domains of expression depend in turn on the anterior and terminal maternal systems (Hülskamp et al., 1990). Thus, in the abdomen, two redundant systems can establish the spatial expression of gap genes, which control segmentation and polarity. The *nos* system acts via a concentration gradient of the transcriptional repressor *hb* and is independent of other maternal information (Nüsslein-Volhard et al., 1987). By contrast the zygotic system acts via gap gene interactions established by positional cues provided by the anterior and terminal maternal genes (Lehmann and Frohnhöfer, 1989; Hülskamp et al., 1990).

This feature of the abdominal pattern forming system may account for the observed lack of correlation between the local concentration of *nos* and the abdominal structures formed. For example, injection of *nos* RNA into *nos* mutant embryos at various positions results in the formation of abdominal segments of normal polarity (Figure 2.4, Lehmann and Frohnhöfer, 1989). According to the model, *nos* removes the repressor *hb* , such that *kni* and *gt* can be expressed in the abdominal region. The relative position of *kni* and *gt* expression, however, is determined by interaction with neighboring gap genes (Lehmann and Frohnhöfer, 1989). This model for posterior pattern formation can also explain how injection of *nos* RNA into the anterior of a *nos* mutant embryo can result in the formation of two abdomens in mirror image (Figure 2.6): First, *nos* suppresses *bicoid* function in the anterior (see below); then, ectopically introduced *nos* eliminates maternal *hb* throughout the embryo. This allows expression of abdomen-specific gap genes in both the anterior and posterior halves of the embryo. The polarity of the resulting abdomens is most likely set by the terminal genes (Lehmann and Frohnhöfer, 1989), which are active at either end (Pignoni et al., 1990).

Transplantation of *nos* RNA to the anterior not only specifies the formation of abdominal segments, but also suppresses formation of head and thoracic structures. This suggests that *nos* can negatively regulate *bcd*, the anterior determinant. Indeed, antibody staining of injected embryos shows

that *bcd* protein levels are decreased after injection of *nos* RNA in the anterior (Figure 2.6). In addition, *in situ* hybridization analysis of injected embryos shows that the stability and localization of *bcd* RNA is not significantly affected (Figure 2.6). This suggests that *nos* acts on *bcd* at the level of translation or protein stability.

We favor the idea that *nos* regulates the translation of *hb* and *bcd*, since the presence of *nos* has a pronounced effect on levels of the *hb* and *bcd* proteins, whereas the effect of *nos* on *hb* and *bcd* RNA levels is more subtle, and appears to occur later (Driever and Nüsslein-Volhard, 1988b; Tautz, 1988; Wharton, and Struhl, 1989; Table 3). One possibility is that *nos* protein binds to *hb* and *bcd* RNA, decreases their rate of translation, and ultimately these transcripts are degraded. Evidence from experiments involving replacement of *hb* 3' region with the *lacZ* gene (Hülskamp et al., 1989), and examination of the *bcd* RNA in genetically bicaudal embryos (Wharton and Struhl, 1989) suggests that targets of *nos* action reside in the 3' regions of the *bcd* and *hb* RNAs. Analysis of the *nos* protein sequence, however, has not revealed homology to sequences known to be involved in RNA binding (Bandziulis et al., 1989). Further experiments are necessary to determine whether *nos* protein directly binds *bcd* and *hb* RNA, and to identify sequences within the *nos* protein required for such an interaction.

MATERIALS AND METHODS

Cloning of *nanos*

The *nos* genomic region is defined by the chromosomal deficiencies *Df[3R]Dl-HD28* and *Df[3R]Dl-KX12* which complement all *nos* mutants, and *Df[3R]Dl-A143*, which uncovers the *nos* gene (Vässin and Campos-Ortega, 1987; Alton et al., 1988; Lehmann and Nüsslein-Volhard, 1991). The region defined by the *Df[3R]Dl-A143* and *Df[3R]Dl-KX12* interval contains in addition to *nos* three lethal complementation groups: *l(3)91Fb* (= *l(DlX43)c3*), *l(3)91Fe* (= *l(DlX43)c4*), and *l(3)91Fc* (Vässin and Campos-Ortega, 1987; Alton et al., 1988). Representative alleles of each of these complementation groups complement *nos* mutants. Two lambda phage genomic libraries (in Charon 4 and EMBL 4

vectors) were used for the chromosomal walk, which was carried out according to standard procedures (Sambrook et al., 1989). The direction and progress of the walk was monitored by *in situ* hybridization of biotinylated phage DNA to salivary gland chromosomes of larvae heterozygous for the deficiencies *Df[3R] Dl-A143*, *Df[3R] Dl-KX12*, and *Df[3R] Dl-HD28* (Vässin and Campos-Ortega, 1987; Alton et al., 1988). The *nos* region was further mapped by blot hybridization of DNA prepared from flies heterozygous for the deficiencies *Df[3R] Dl-A143* and *Df[3R] Dl-HD28* with representative clones from the walk (F. Pelegri and R.L., unpublished data). To identify the genomic region encoding *nos*, radiolabeled cDNA from specific stages was hybridized to a blot of genomic DNA from the walk. By this analysis, the 2.5 kb fragment described hybridizes to the most strongly expressed maternal transcript in this region (data not shown).

Northern blot analysis

Poly A^+ RNA prepared from the indicated stages (1.5 µg per lane) was fractionated on a 1.2% agarose/formaldehyde gel. After transfer to nylon membranes, the filter was ultraviolet-crosslinked and probed with the appropriate ^{32}P-labeled DNA fragment. The 2.5 kb SalI-XhoI DNA fragment indicated in Figure 1A was used as the *nanos* probe. Blots were exposed to X-ray film with an intensifying screen for 48, 24, or 6 hours, for *nos*, *bcd*, or actin, respectively. Preparation of poly A^+ RNA as well as hybridization and washing of blots were done by standard methods (Sambrook et al., 1989).

cDNA cloning and DNA sequencing

The 2.5 kb SalI-XhoI genomic DNA fragment described in Figure 1A was labelled with [alpha-^{32}P] dCTP by random primer oligolabelling and used to screen both lambda and plasmid cDNA libraries. Three independently isolated cDNA clones were subcloned into Bluescript vectors (Stratagene), nested deletions were made using exonuclease III and mung bean nuclease according to manufacturers' protocols (Stratagene), and sequenced using Sequenase (United States Biochemical). The genomic DNA was sequenced using sequence-specific oligonucleotide primers, or, for some regions, by subcloning small fragments. In all cases, sequence was confirmed on the opposite strand. For comparison of the *nanos* sequence to those in the latest

version of the sequence databases (March 1995), the computation was performed at the NCBI using the BLAST network service (Altschul et al., 1990).

In situ hybridization

In situ hybridization was performed as described by Tautz and Pfeifle, 1989.

RNA injections

RNA for injection assays was synthesized *in vitro* from the full-length cDNA clone N5, which includes a 43 nucleotide ß-globin 5' untranslated leader sequence fused to the complete *nos* open reading frame (Brown and Kafatos, 1988). In vitro transcript lacking globin sequences has equivalent rescuing activity (data not shown). Methods for RNA synthesis were as described by Krieg and Melton, 1984. The *in vitro* transcription products were precipitated and resuspended in DEPC-treated water. Concentrations were determined by optical density measurement at 260 and 280 nm and are corrected for the presence of residual template DNA. Since the level of active transcription products obtained varies, the product of a single transcription reaction was used for any given experiment. "No template" embryos were injected with product of an *in vitro* transcription reaction from which template DNA was omitted. The frameshift template was constructed by filling in a PstI site at nt 406 of the insert, followed by addition of a BamHI linker and religation, creating a frameshift mutation after amino acid 50 of the open reading frame.

Injections and cuticle preparations were carried out as previously described (Lehmann and Nüsslein-Volhard, 1986; Lehmann and Nüsslein-Volhard, 1991). All embryos for injection were derived from nos^{L7} mutant females and injected at early cleavage stage (stage 2) at 25-50% egg length unless otherwise indicated. Final concentration of donor RNA was 2 µg/µl in DEPC-treated water. Assuming an embryo volume of 2 nanoliters, and an injection volume of 1% egg volume (20 picoliters) (Ashburner, 1989), each embryo receives approximately $3x10^7$ molecules of injected RNA. In addition, quantitative hybridization comparisons of embryonic RNA and prepared in vitro transcript indicate that a single wild-type embryo contains approximately 800,000 molecules of *nanos* mRNA (data not shown).

In general, 50-80% of injected embryos developed a cuticle and could be scored for segmentation phenotype. For the injections into other posterior group mutants (Table 2), all allele combinations used were the strongest available for the abdominal segmentation phenotype (Boswell and Mahowald, 1985; Schüpbach and Wieschaus, 1986; Lehmann and Nüsslein-Volhard, 1986; Lehmann and Nüsslein-Volhard, 1987; Manseau and Schüpbach, 1989; Lehmann and Nüsslein-Volhard, 1991). In experiments where rescued embryos were allowed to develop through adulthood, both heterozygous and homozygous progeny were obtained. For the experiment in Figure 6, embryos were injected at 75% egg length, observed through gastrulation by phase microscopy and photographed, then allowed to develop through the end of embryogenesis and mounted for scoring of cuticle phenotype. Staging of embryos was as described by Campos-Ortega and Hartenstein, 1985.

hb and *bcd* antibody staining of injected embryos

Embryos derived from nos^{L7} mutant females were injected with the appropriate RNA at 25-50% egg length for the *hb* stainings, and at 75% egg length for the *bcd* stainings. Injected embryos were aged at 18°C for 1 to 1.5 hours after injection, then fixed at pole bud and syncytial blastoderm stages (stages 3 and 4) and devitellinized by hand. Antibody distribution was detected using a biotinylated anti-mouse secondary antibody and a commercially available horseradish peroxidase detection kit (Vector labs).

in situ hybridization of injected embryos

Injected embryos were prepared, fixed, and devitellinized as described above for the antibody staining, then hybridized with a *bicoid* cDNA probe for whole mount *in situ* hybridization as described by Tautz and Pfeifle, 1989.

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REFERENCES

Alton, A. K., Fechtel, K. , Terry, A. L., Meikle, S. B., and Muskavitch, M. A. T. (1988). Cytogenetic definition and morphogenetic analysis of *Delta*, a gene affecting neurogenesis in *Drosophila melanogaster*. Genetics *118*, 235-245.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). Basic local alignment search tool. J. molec. Biol. *215*, 403-410.

Ashburner, M. (1989). Drosophila, a laboratory handbook (Cold Spring Harbor: Cold Spring Harbor Laboratory Press), p. 216.

Bandziulis, R. J., Swanson, M. S., and Dreyfuss, G. (1989). RNA-binding proteins as developmental regulators. Genes & Dev. *3*, 431-437.

Berleth, T., Burri, M., Thoma, G., Bopp, D., Richstein, S., Frigerio, G., Noll, M., and Nüsslein-Volhard, C. (1988). The role of localization of *bicoid* RNA in organizing the anterior pattern of the *Drosophila* embryo. EMBO J. *7*, 1749-1756.

Boswell, R. E., and Mahowald, A. P. (1985). *Tudor*, a gene required for assembly of the germ plasm in *Drosophila melanogaster*. Cell. *43*, 97-104.

Brown, N. H., and Kafatos, F. C. (1988). Functional cDNA libraries from *Drosophila* embryos. J. molec. Biol. *203*, 425-437.

Campos-Ortega, J. A., and Hartenstein, V. (1985). The Embryonic Development of *Drosophila* melanogaster (Heidelberg: Springer-Verlag).

Cavener, D. R. (1987). Comparison of the consensus sequences flanking translational start sites in *Drosophila* and vertebrates. Nucleic Acids Res. *15*, 1353-1361.

Driever, W., and Nüsslein-Volhard, C. (1988a). A gradient of bicoid protein in *Drosophila* embryos. Cell *54*, 83-93.

Driever, W. and Nüsslein-Volhard, C. (1988b). The bicoid protein determines position in the *Drosophila* embryo in a concentration-dependent manner. Cell *54*, 95-104.

Driever, W., and Nüsslein-Volhard, C. (1989). The bicoid protein is a positive regulator of *hunchback* transcription in the early *Drosophila* embryo. Nature *337*, 138-143.

Eldon, E. D., and Pirrotta, V. (1991). Interactions of the *Drosophila* gap gene *giant* with maternal and zygotic pattern-forming genes. Development *111*, 367-378.

Frigerio, D., Burri, M., Bopp, D., Baumgartner, S., and Noll, M. (1986). Structure of the segmentation gene *paired* and the *Drosophila* PRD gene set as part of a gene network. Cell *47*, 479-746.

Frohnhöfer, H. G., and Nüsslein-Volhard, C. (1986). Organization of the anterior pattern in the *Drosophila* embryo by the maternal gene *bicoid*. Nature *324*, 120-125.

Frohnhöfer, H. G., and Nüsslein-Volhard, C. (1987). Maternal genes required for the anterior localization of *bicoid* activity in the embryo of *Drosophila*. Genes & Dev. *1*, 880-890.

Frohnhöfer, H. G., Lehmann, R., and Nüsslein-Volhard, C. (1986). Manipulating the anteroposterior pattern of the *Drosophila* embryo. J. Embryol. Exp. Morphol. (supplement) *97*, 169-179.

Fyrberg, E. A., Mahaffey, J. W., Bond, B. J., and Davidson, N. (1983). Transcripts of the six *Drosophila* actin genes accumulate in a stage- and tissue-specific manner. Cell *33*, 115-123.

Gaul, U., and Jäckle, H. (1987). Pole region-dependent repression of the *Drosophila* gap gene *Krüppel* by maternal gene products. Cell *51*, 549-555.

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

Hülskamp, M., Pfeifle, C., and Tautz, D. (1990). A morphogenetic gradient of *hunchback* protein organizes the expression of the gap genes *Krüppel* and *knirps* in the early *Drosophila* embryo. Nature *346*, 577-580.

Hülskamp, M., Schröder, C., Pfeifle, C., Jäckle, H., and Tautz, D. (1989). Posterior segmentation of the *Drosophila* embryo in the absence of a maternal posterior organizer gene. Nature *338*, 629-632.

Ingham, P. W. (1988). The molecular genetics of embryonic pattern formation in *Drosophila*. Nature *335*, 25-34.

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

Klingler, M., Erdeleyi, M., Szabad, J., and Nüsslein-Volhard,C. (1988). Function of *torso* in determining the terminal anlagen of the *Drosophila* embryo. Nature *335*, 275-277.

Kraut, R. and Levine, M. (1991). Spatial regulation of the gap gene *giant* during *Drosophila* development. Development *111*, 601-609.

Krieg, P. A., and Melton, D. A. (1984). Functional messenger RNAs are produced by SP6 in vitro transcription of cloned cDNAs. Nucleic Acids Res. *12*, 7057-7070.

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

Lehmann, R., and Frohnhöfer, H. G. (1989). Segmental polarity and identity in the abdomen of *Drosophila* is controlled by relative position of gap gene expression. Development (supplement) *107*, 21-29.

Lehmann, R., and Nüsslein-Volhard, C. (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 Nüsslein-Volhard, C. (1987). Involvement of the *pumilio* gene in the transport of an abdominal signal in the *Drosophila* embryo. Nature *329*, 167-170.

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

Manseau, L. J., and Schüpbach, T. (1989). *cappuccino* and *spire*: Two unique maternal-effect loci required for both the anteroposterior and dorsoventral patterns of the *Drosophila* embryo. Genes & Dev. *3*, 1437-1452.

Nauber, U., Pankratz, M., Kienlin, A., Seifert, E., Klemm, U., and Jäckle, H. (1988). Abdominal segmentation of the *Drosophila* embryo requires a hormone receptor-like protein encoded by the gap gene *knirps*. Nature *336*, 489-492.

Nüsslein-Volhard, C. (1977). Genetic analysis of pattern-formation in the embryo of *Drosophila melanogaster*. Wilhelm Roux's Arch. *183*, 249-268.

Nüsslein-Volhard, C., Frohnhöfer, H. G., and Lehmann, R. (1987). Determination of anteroposterior polarity in *Drosophila*. Science *238*, 1675-1681.

Pignoni, F., Baldarelli, R. M., Steingrimsson, E., Diaz, R. J., Patapoutian, A., Merriam, J. R., and Lengyel, J. A. (1990). The *Drosophila* gene *tailless* is expressed at the embryonic termini and is a member of the steroid receptor superfamily. Cell *62*, 151-163.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular Cloning, A Laboratory Manual (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory).

Schüpbach, T., and Wieschaus, E. F. (1986). Maternal-effect mutations altering the anterior-posterior pattern of the *Drosophila* embryo. Wilhelm Roux's Arch. *195*, 302-317.

St. Johnston, D., Driever, W. , Berleth, T., Richstein, S., and Nüsslein-Volhard, C. (1989). Multiple steps in the localization of *bicoid* RNA to the anterior pole of the *Drosophila* oocyte. Development (supplement) *107*, 13-19.

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

Struhl, G., Struhl, K., and MacDonald, P. (1989). The gradient morphogen *bicoid* is a concentration-dependent transcriptional activator. Cell *57*, 1259-1273.

Tautz, D. (1988). Regulation of the *Drosophila* segmentation gene *hunchback* by two maternal morphogenetic centers. Nature *332*, 281-284.

Tautz, D., and Pfeifle, C. (1989). A non-radioactive in situ hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene *hunchback*. Chromosoma *98*, 81-85.

Vässin, H., and Campos-Ortega, J. A. (1987). Genetic analysis of *Delta*, a neurogenic gene of *Drosophila melanogaster*. Genetics *116*, 433-445.

Vässin, H., Bremer, K. A. , Knust, E., and Campos-Ortega, J. A. (1987). The neurogenic gene *Delta* of *Drosophila melanogaster* is expressed in neurogenic territories and encodes a putative transmembrane protein with EGF-like repeats. EMBO J. *6*, 3431-3440.

Wharton, K. A., Johansen, K. M., Xu, T., and Artavanis-Tsakonas, S. (1985). Nucleotide sequence from the neurogenic locus *Notch* implies a gene product that shares homology with proteins containing EGF-like repeats. Cell *43*, 567-581.

Wharton, R. P., and Struhl, G. (1989). Structure of the *Drosophila* Bicaudal-D protein and its role in localizing the posterior determinant *nanos*. Cell *59*, 881-892.

Wieschaus, E. F., and Nüsslein-Volhard, C. (1986). Looking at embryos. In *Drosophila*: a Practical Approach, D.B. Roberts, ed. (Washington, D.C.: IRL Press), pp. 199-227.

Wilson, R., Ainscough, R., Anderson, K., Baynes, C., Berks, M., Bonfield, J., Burton, J., Connell, M., Copsey, T., Cooper, J., Coulson, A., Craxton, M., Dear, S., Du, Z., Durbin, R., Favellow, A., Fulton, L., Gardner, A., Green, P., Hawkins, T., Hillier, L., Jier, M., Johston, L., Jones, M. Kershaw, J., Kirstern, J., Laister, N., Latreille, P., Lightning, J., Lloyd, C., McMurray, A., Mortimore, B.,

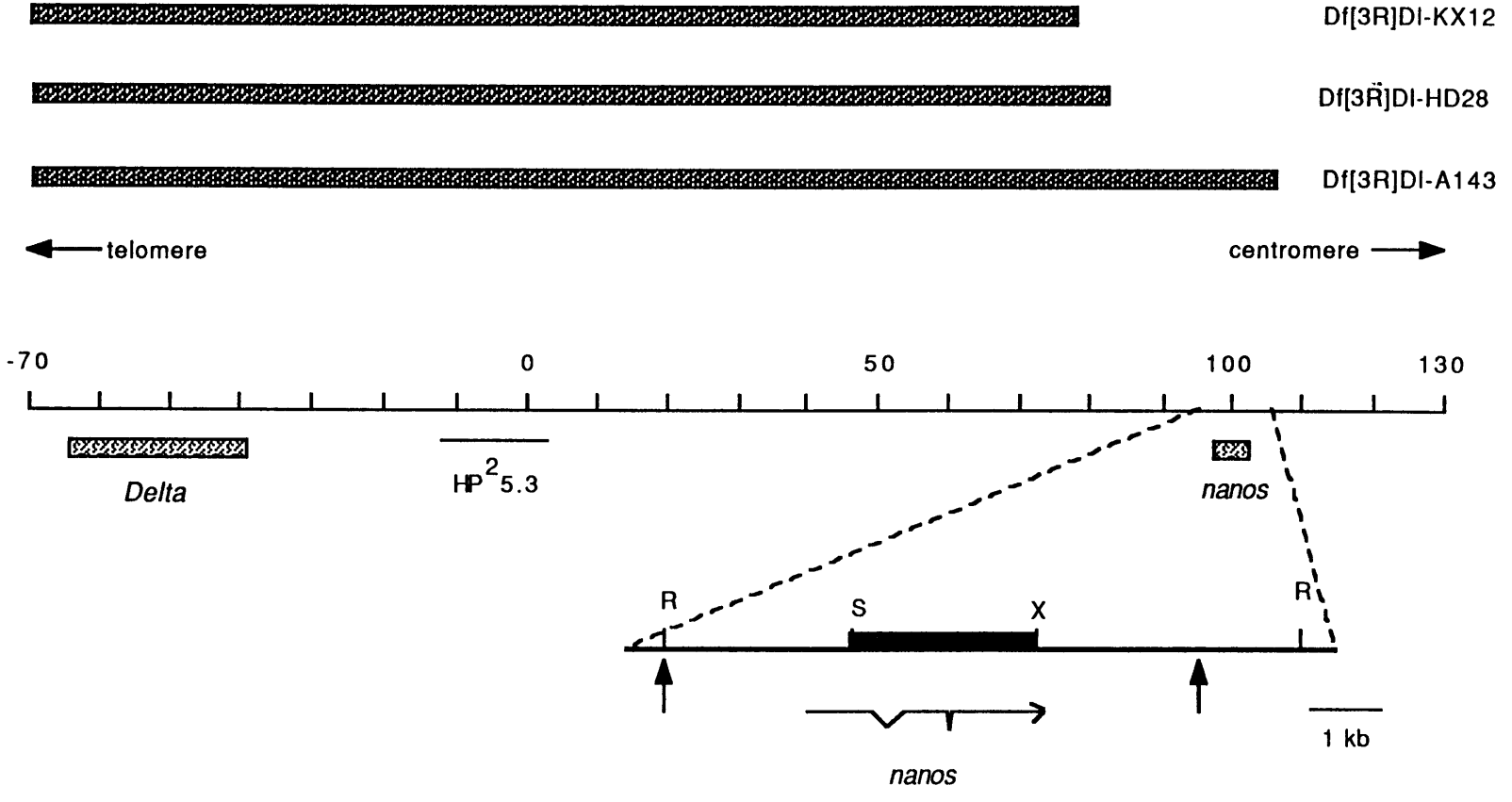
O'Callaghan, M., Parsons, J., Percy, C. Rifken., L., Roopra, A., Saunders, D., Shownkeen, R., Smaldon, N., Smith, A., Sonnhammer, E., Staden, R., Sulston, J., Thierry,-Mieg, J., Thomas, K., Vaudin, M., Vaughan, K., Waterston, R., Watson, A., Weinstock, LO., Wildinson-Sproat, J. and Wohldman, P. (1994). 2.2 Mb of contiguous nucleotide sequence from chromosome II of *C. elegans*. Nature *368*, 32-38.

Figure 2.1. Cloning of *nanos*

A) Physical map of the *nos* region.

Shaded bars at top indicate DNA which is absent in the indicated deficiency chromosomes; the *Delta-nos* genomic region is represented by the central line; the location of the starting clone ($HP^{2}5.3$) (Vässin et al., 1987) of the chromosomal walk, as well as the locations of the *Delta* and *nos* transcribed regions are shown. Distances are given in kb with numbering starting at the beginning of the walk. The *nos* region is shown in greater detail below. Heavy line indicates 2.5 kb genomic fragment which hybridizes to *nos* transcript. The 7.5 kb DNA fragment used for P-element mediated transformation spans from an EcoRI to an SphI site as indicated by the vertical arrows. Arrow at bottom indicates structure and 5' to 3' orientation of *nos* primary transcript (restriction sites for the following enzymes are shown: R = EcoRI, S = SalI, X = XhoI; scale as indicated).

A


Df[3R]DI-KX12
Df[3R]DI-HD28
Df[3R]DI-A143
telomere
centromere
-70
0
50
100
130
Delta
HP2 5.3
nanos
R
S
X
R
1 kb
nanos

B) Northern blot analysis.

Main panel shows *nos* transcript. *nos* is a single species present predominantly in ovaries, early embryos, and females. In this exposure, a weak signal is also detectable in males. For comparison, the same blot was reprobed for *bcd*, a maternal transcript which serves as a comparison for the profile of a maternal transcript (Frigerio et al., 1986), and actin 5C, a constitutively expressed transcript (Fyrberg et al., 1983) to show equal amounts loaded in each lane.

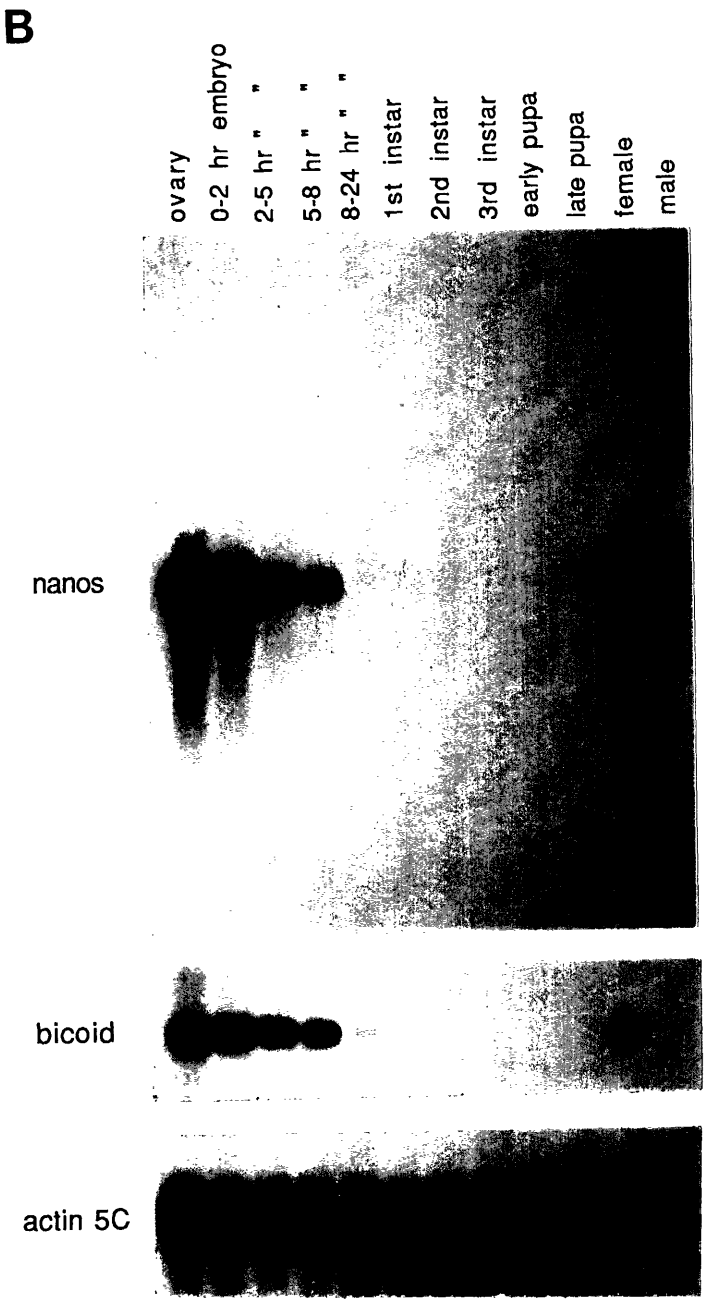
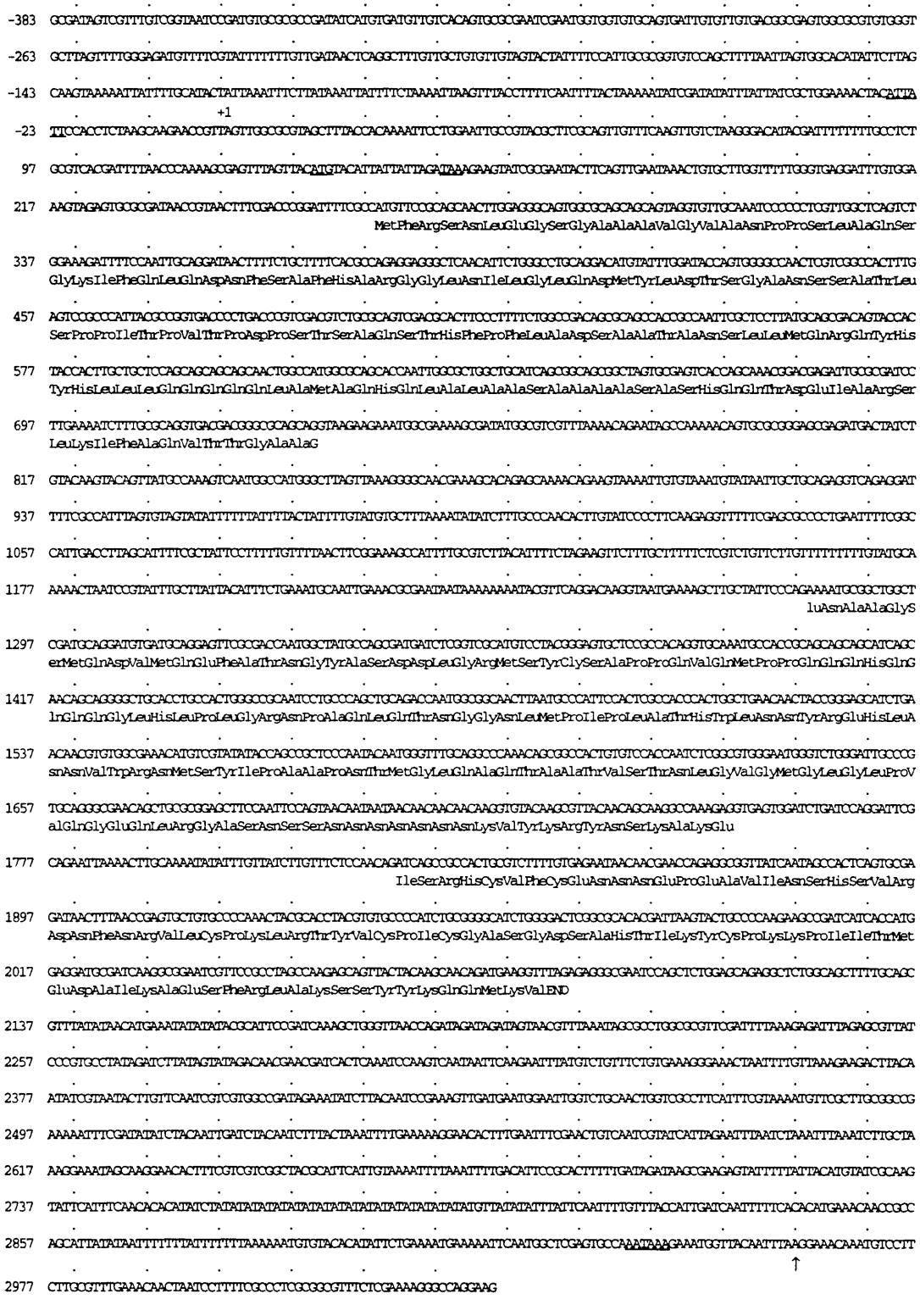

B
ovary
0-2 hr embryo
2-5 hr " "
5-8 hr " "
8-24 hr " "
1st instar
2nd instar
3rd instar
early pupa
late pupa
female
male
nanos
bicoid
actin 5C

Figure 2.2. Sequence of the predicted *nos* polypeptide.

This is a translation of the single large open reading frame encoded by the *nanos* gene. The transcriptional start site is designated as residue 1. The position of the transcriptional start was verified by primer extension (data not shown), and lies 20 nucleotides upstream of the 5' end of the cDNA N5. A potential TATA box sequence, ATTATT (underlined), occurs 25 nucleotides upstream of the transcriptional start. The first AUG (underlined) begins a short ORF which is followed by several stop codons (the first in-frame stop is underlined). The second AUG, at residue 263, begins the single large open reading frame. This translational start site shows a 2 out of 4 match with the Drosophila translation initiation consensus as determined by Cavener (Cavener, 1987). The *nos* polypeptide sequence is shown as a translation of the large open reading frame which is interrupted by two introns, spanning nucleotides 734-1281 and 1752-1824. The ATATAA polyadenylation signal is underlined, and the polyadenylation site is marked by an arrow.



65

Figure 2.3. Whole mount *in situ* hybridization.

The cDNA N5 (see Figure 2A legend) was used to prime synthesis of a uniformly labeled digoxygenin-UTP probe which was hybridized to wild-type embryos. A) Early cleavage stage embryo (stage 2, 0:25-1:20 hours). The probe recognizes a transcript which is localized to the posterior pole. B) Embryo at pole bud stage (stage 3, 1:20-1:30 hours). The transcript is concentrated in the pole cells. C) Embryo at syncytial blastoderm stage (stage 4, 1:30-2:30 hours). Transcript is restricted to pole cells and lacking from the blastoderm which will give rise to the somatic tissues. D) Embryo during early germband extension (stage 8, 3:45-4:30 hours). Pole cells are carried inside the embryo and are clustered inside a pocket of cells which will give rise to the posterior midgut. Reduced levels of *nos* RNA are detected in the pole cells. E) Embryo with fully extended germband (stage 9, 4:30-5:10 hours). By this stage most pole cells have migrated through the midgut epithelium. Only a few pole cells are found within the midgut pocket, and only those show residual staining. F) Embryo just prior to germband retraction (stage 10, 5:10-6:15 hours). No specific staining for *nos* RNA can be detected. In all cases anterior is left, dorsal up. The final staining reaction was carried out longer in embryos shown in D-F than in A-C. Developmental stages are as described in Campos-Ortega and Hartenstein, 1985, duration of stages at room temperature (22°C) are according to Wieschaus and Nüsslein-Volhard, 1986.

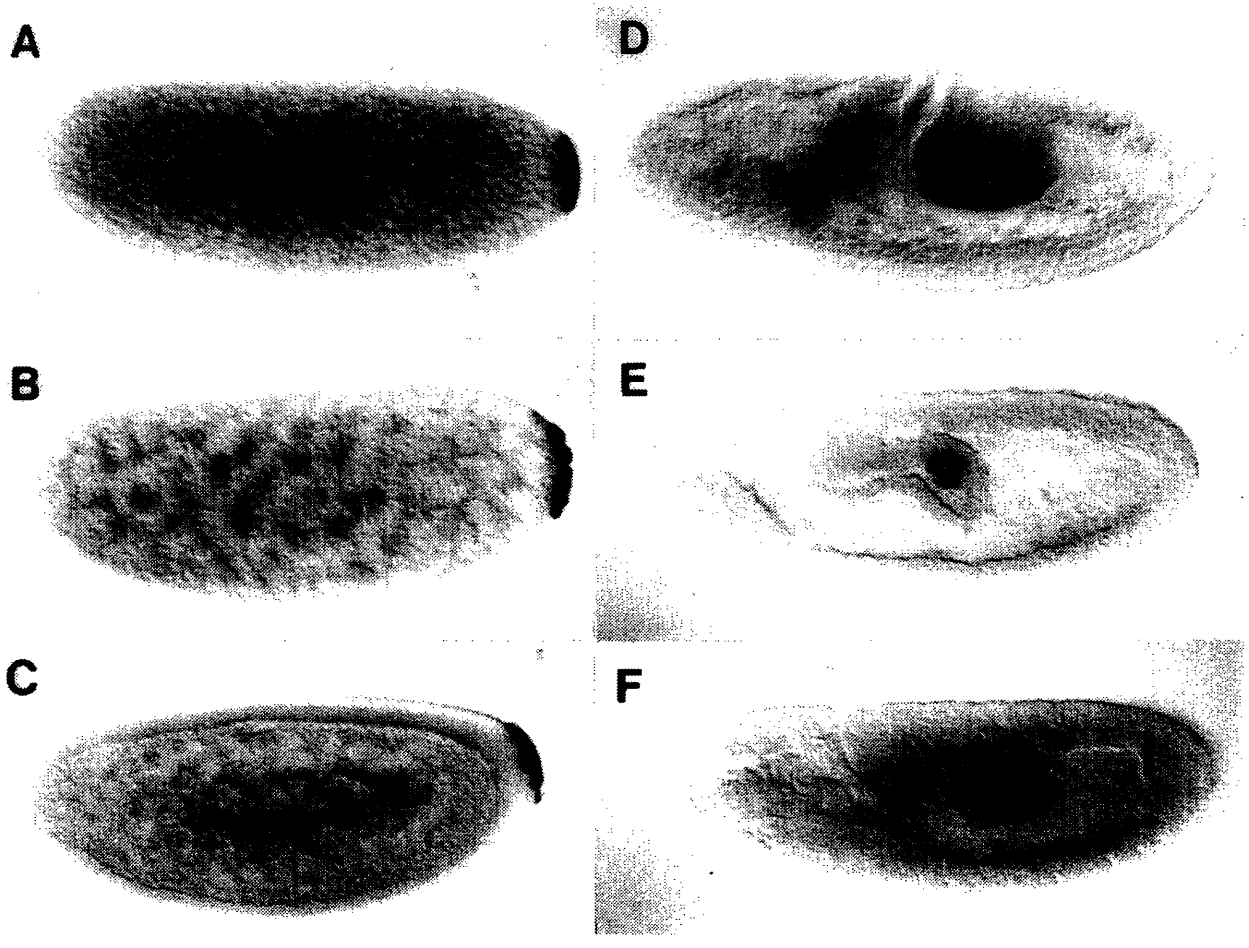
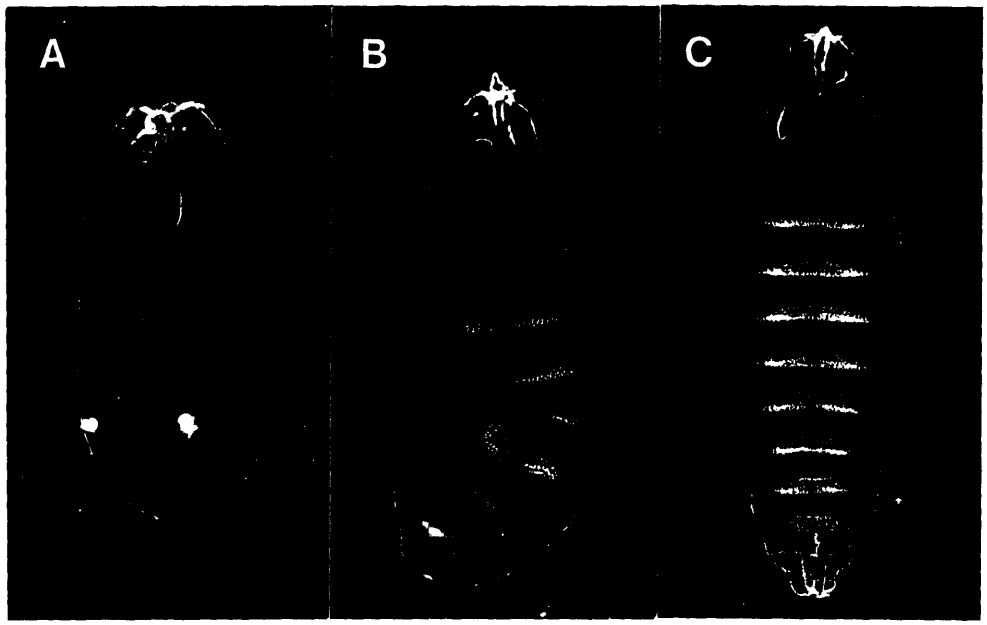

A
B
C
D
E
F

Figure 2.4. Rescue of the *nos* mutant phenotype by *in vitro* synthesized *nos* transcript.

A-C) Dark field photographs of cuticle preparations of first instar larvae derived from females homozygous for nos^{L7}. A) Uninjected embryo, anterior up, dorsal aspect. Note lack of abdominal segments. Head and thoracic segments are normal. B) Partially rescued embryo, anterior up, ventral aspect. This embryo shows bilaterally asymmetric rescue and has developed 6 partial abdominal segments. C) Fully rescued embryo, anterior up, ventral aspect. This pattern is indistinguishable from wild type. D, E) Embryos injected with *nos* RNA and stained for *hb* protein using an anti-*hb* antibody. Embryos shown are at late cleavage stage (stage 2), anterior left, dorsal up. D) Embryo injected with RNA synthesized from a template containing a frameshift mutation. E) Embryo injected with RNA from a template containing wild-type *nos* RNA sequences. The control embryo (D) shows uniform levels of *hb* protein while the embryo injected with functional *nos* RNA (E) shows reduced levels of *hb* protein in the posterior but normal levels in the anterior third. In some of the control embryos we detected slightly reduced levels of *hb* protein at the posterior pole which may indicate that the nos^{L7} mutation is not a complete lack of function mutation.


A
B
C

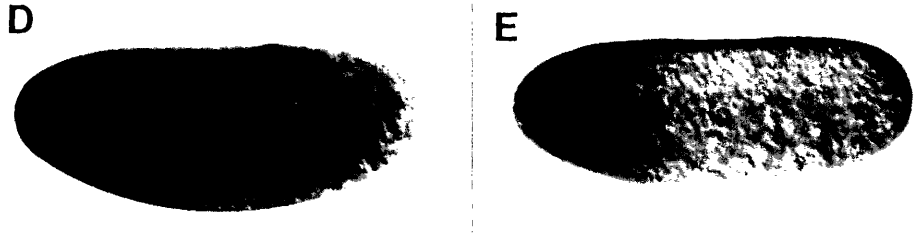

D
E

TABLE 1. RESCUE OF *NANOS* ABDOMINAL PHENOTYPE BY *NANOS* TRANSCRIPT

	embryos scored (N)	abdominal segments formed (% total)		
DONOR FRACTION		0-1	2-5	6-8
uninjected	29	100	-	-
no template	49	100	-	-
frameshift template (1.7µg/µl)*	79	96	4	-
pN5-RNA (0.03µg/µl)	50	66	28	6
pN5-RNA (0.07µg/µl)	45	15	49	36
pN5-RNA (0.13µg/µl)	46	-	-	100

*Of the 79 cuticles scored, 3 developed more than one segment. Of these, 2 had 2 segments, and one cuticle had 3 segments. Note that the concentration of frameshift transcript injected was about 10-fold higher than the concentration of wild-type transcript used. We interpret the low degree of rescue observed as a reflection of some low level of readthrough translation, or a residual activity of the 50 aa amino terminal polypeptide.

Figure 2.5. Developmental stage and position dependence of rescue.

"Strong rescue" (open squares) indicates more than five abdominal segments, and "rescue" (solid squares) indicates two or more abdominal segments, as judged by presence of abdominal ventral denticle belts. Fifteen to eighty cuticles were scored for each data point. A) Developmental stage dependence. Staging of recipient embryos as in Campos-Ortega and Hartenstein, 1985 (stages 1 and 2, cleavage stage; stage 3, pole bud formation; stage 4, syncytial blastoderm; stage 5, cellular blastoderm; stage 6, beginning of gastrulation). Developmental events are indicated along horizontal axis. B) Position dependence. Cleavage stage embryos were injected at the positions shown along the horizontal axis.

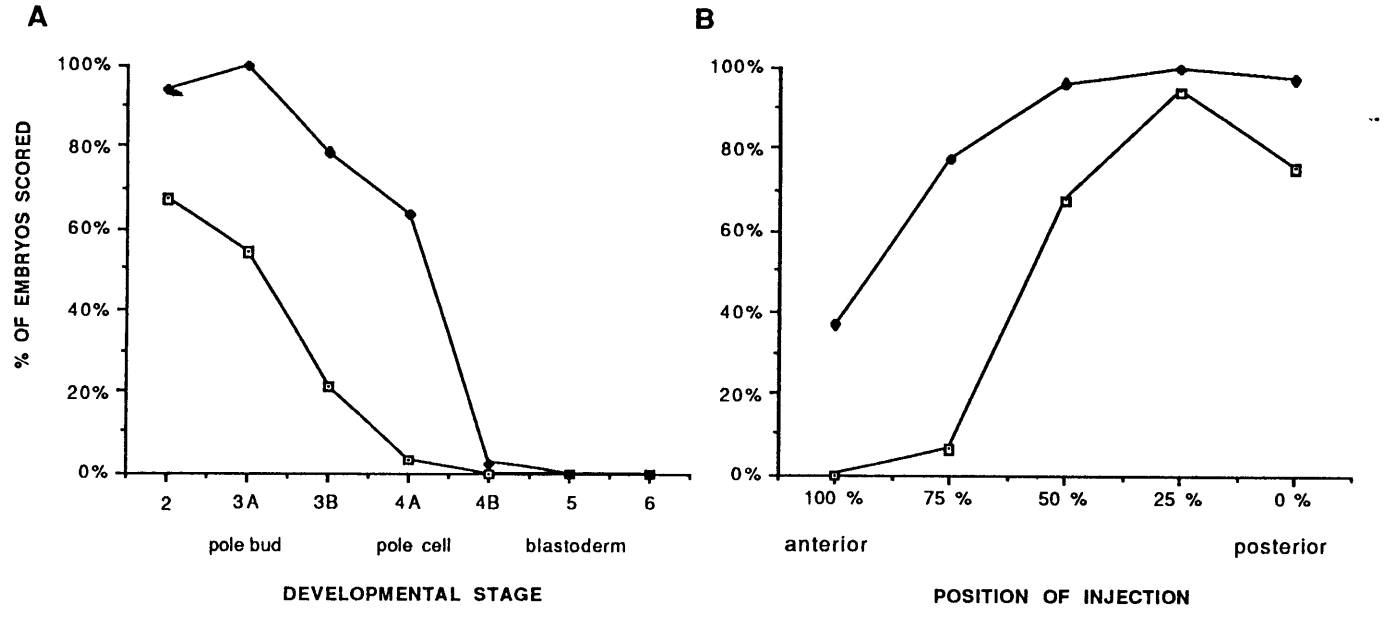

A
100%
80%
60%
40%
20%
0%
% OF EMBRYOS SCORED
2
3A
3B
4A
4B
5
6
pole bud
pole cell
blastoderm
DEVELOPMENTAL STAGE
B
100%
80%
60%
40%
20%
0%
100 %
75 %
50 %
25 %
0 %
anterior
posterior
POSITION OF INJECTION

TABLE 2 : RESCUE OF POSTERIOR GROUP ABDOMINAL PHENOTYPE BY *NANOS* TRANSCRIPT

MATERNAL GENOTYPE OF RECIPIENTS		EMBRYOS ANALYZED (N)	EMBRYOS DEVELOPED (N)	NUMBER OF ABDOMINAL SEGMENTS FORMED (% TOTAL) 0-1	2-5	6-8
nanos^{L7}						
	injected	72	58	5	5	90
	control	41	29	100	-	-
oskar^{166}						
	injected	37	29	-	10	90
	control	ND	35	100	-	-
vasa^{PD}/*vasa*^{D1}						
	injected	95	50	8	36	56
	control	52	38	100	-	-
staufen^{D3}						
	injected	108	56	29	16	56
	control	ND	39	100	-	-
pumilio^{680}						
	injected	140	75	33	65	2
	control	24	19	84	16	-
valois^{PE}/*valois*^{PG}						
	injected	180	9	22	22	55
	control	167	25	92	8	
tudor^{WC8}/Df[2R]*Pu*^{rP133}*						
	injected	115	62	2	5	93
	control	193	67	6	24	70
cappuccino^{G7}/Df[2L]*ed*$^{S71.2}$						
	injected	41	14	21	21	58
	control	24	11	91	9	-
spire^{RP}						
	injected	164	42	12	12	76
	control	53	28	86	14	-

"Control" indicates phenotype of uninjected embryos. ND = not determined.
*No *tudor* alleles with complete penetrance for the abdominal phenotype have been identified. For the *tudor* embryos, the phenotypic scores for control and injected populations were compared by the Wilcoxon-Mann-Whitney ranks test and shown to be significantly different with $P \leq 0.004$.

Figure 2.6. Ectopically introduced *nos* alters embryonic pattern.

A-D) Control embryos derived from nos^{L7} mutant females (A, B, uninjected; C, D, injected with *in vitro* synthesized *nos* RNA containing a frameshift mutation) show normal head development (A, B), and wild-type pattern of *bcd* RNA (C) and *bcd* protein (D) distribution. E-H) Embryos derived from nos^{L7} mutant females injected with RNA synthesized from a wild-type *nos* cDNA template develop posterior structures at the expense of head structures (E, F), and show normal levels and distribution of *bcd* RNA (G), while levels of *bcd* protein are reduced (H). Top panel (A, E): living embryos during gastrulation (stage 7); the position of the cephalic furrow is indicated in (C) by an arrow. Star in (E) indicates the position of an ectopic posterior midgut invagination at the anterior. Second panel (B, F): dark-field photographs of cuticle preparations from first instar larvae developed from embryos similar to those shown above. Embryo in F shows a bicaudal phenotype such that the telson and posterior abdomen is duplicated in mirror image at the expense of head and thoracic structures. Third panel (C, G): syncytial blastoderm (stage 4) embryos stained for *bcd* RNA. Bottom panel (D, H): syncytial blastoderm (stage 4) embryos stained for *bcd* protein. In all cases anterior is to the left, dorsal up. All embryos were injected at 75% egg length.

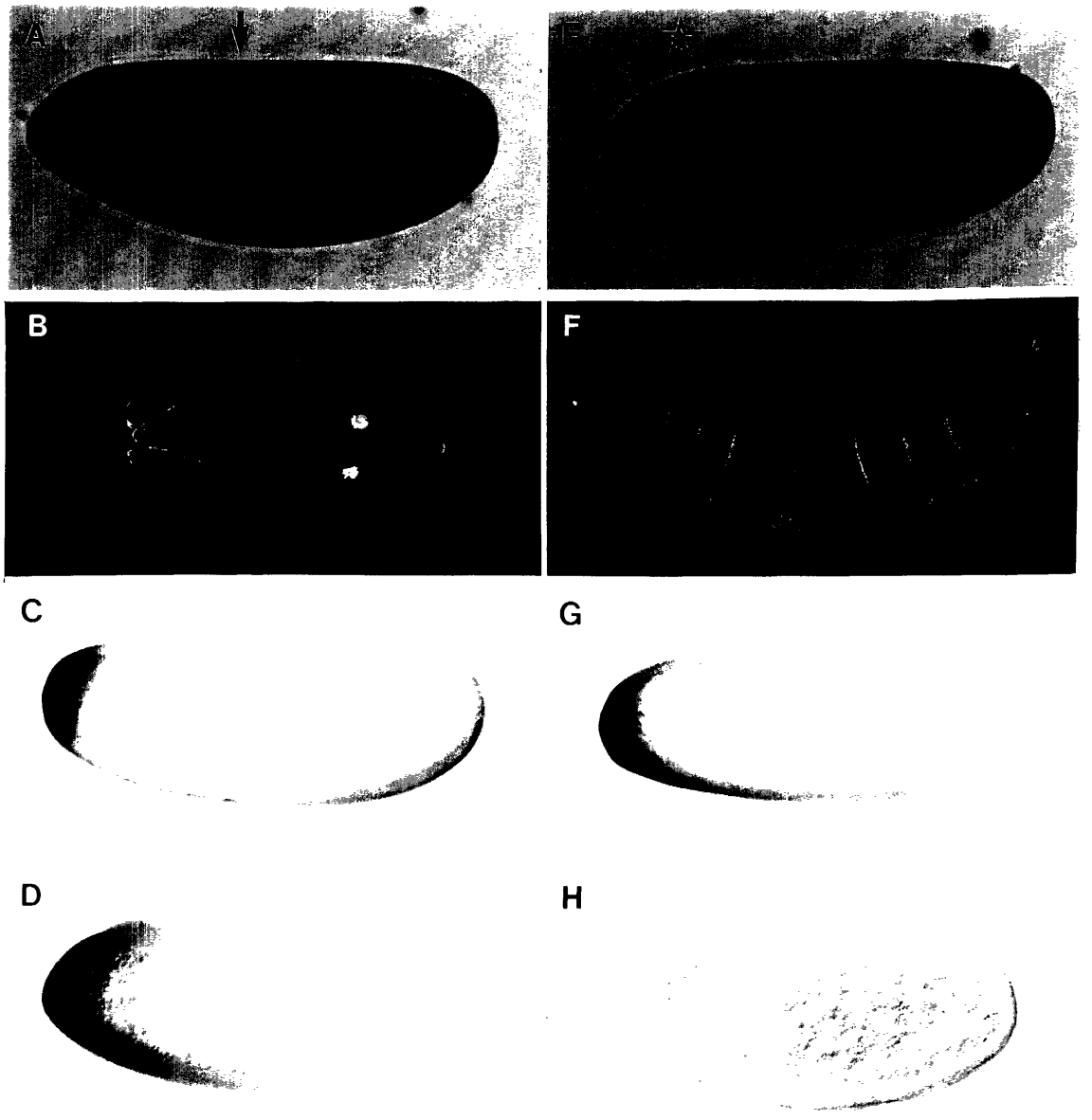

A
E
B
F
C
G
D
H

TABLE 3: EFFECT OF *NANOS* ON *BICOID*

species	*nanos* transcript injected	N	Staining pattern (%)			
			wildtype	weak	none	ectopic
bicoid RNA	frameshift	30	47	30	10	13
	wildtype	29	31	38	27	4
bicoid protein	frameshift	49	61	25	14	
	wildtype	45	20	13	67	

All embryos were derived from homozygous nos^{L7} mothers, and injected at 75% egg length with the indicated RNA. N= number of embryos analyzed.

Chapter 3

Preliminary structural analysis of the *nanos* open reading frame

INTRODUCTION

The maternal-effect gene *nanos* (*nos*) encodes a posterior determinant which is localized to the posterior pole of early embryos. Embryos derived from females homozygous mutant for *nos* develop into first instar larvae which completely lack abdominal segments. The mutant phenotype of these embryos (henceforth referred to as "mutant embryos") can be rescued by injection of in vitro transcribed *nos* RNA into the prospective abdominal region of early-stage embryos. The extent of this phenotypic rescue can be assessed by examining the cuticles of the resulting larvae and counting the number of abdominal segments formed. Injections of serially diluted samples of in vitro transcribed *nos* RNA have shown that the RNA injection assay is sensitive over a ten-fold range of RNA concentration (i.e., the minimal RNA concentration giving full rescue is ten times that required for minimally detectable rescue) (Curtis et al., 1995; Wang and Lehmann, 1991) . Thus the RNA injection assay constitutes a rapid and sensitive method to assess the potential function of any constructs containing *nanos* sequences.

Nanos encodes a novel 400 amino acid protein, which bears no homology to proteins of characterized function. Therefore the sequence itself provides limited clues as to the biochemical function of the protein. One strategy for defining functional domains of the *nanos* polypeptide is to make derivatives of the *nanos* cDNA which encode truncated versions of the protein. These cDNA constructs can then be easily transcribed and injected into *nos* mutant embryos to assay for the ability to rescue the abdominal phenotype. These experiments should eventually define the minimal *nanos* sequences required for protein function, and these regions of the polypeptide can then be singled out for further, more detailed studies.

Another approach to defining residues critical to the function of the *nos* polypeptide is to isolate sequence homologues, which can then be tested for function by the RNA injection assay. The *Xenopus* gene Xcat-2 was originally identified as a transcript associated with the insoluble cytoskeletal fraction, is localized as an mRNA to the vegetal cortex of late stage oocytes (Forristall et al., 1995; Mosquera et al., 1993) The putative Xcat-2 polypeptide

contains a 53 amino acid region with strong sequence homology to a C-terminal region of *nos* (55% identity, 64% conservation). These striking similarities between *nanos* and Xcat-2 in both RNA localization pattern and peptide sequence suggest that the parallels may extend to protein function. Therefore we used the RNA injection assay to test whether Xcat-2 sequences provide *nos* rescuing activity, either on their own or in the context of *nos* sequences.

RESULTS AND DISCUSSION

In-frame deletion derivatives of the nanos open reading frame

As a first step towards characterizing functional domains of the nanos polypeptide, we constructed a number of in-frame deletion derivatives of the *nos* open reading frame in the context of a full length *nos* cDNA. The deletion sites were chosen to take advantage of naturally occurring restriction endonuclease sites within the *nos* coding sequence (see Materials and Methods). The smallest deletion tested shortened the 400 amino acid *nos* open reading frame by 70 amino acids; the largest deletion omitted 259 amino acids (Table 3.1, Materials and Methods). After transciption in vitro, the RNAs were injected into embryos derived from nos^{L7} mutant females. The resulting larval cuticles were scored for the rescue of abdominal segments. Of the four constructs tested, only one, deleting amino acids 218-288, showed any rescuing activity (Table 3.1). The 218/288 construct gives rescuing activity essentially equivalent to wild-type RNA. None of the other deletion constructs showed any detectable rescuing activity.

These results suggest that the 70 amino acids between residues 218 and 288 are dispensible for *nanos* function. In addition, these results suggest that areas flanking this region are essential for function. Recently published evidence is consistent with this hypothesis (Curtis et al., 1995). Specifically, sequence comparison between nanos homologues from other insect species (homologues which have been shown to be functional in *D. melanogaster*) reveals several regions of significant homology, including residues 61-97 and

317-400 (high degree of conservation), and residues 170-180 and 290-415 (enrichments of particular amino acids). This pattern of conserved sequences is entirely consistent with the results obtained from the deletion injection assays. In sum, these results suggest that the C-terminus of nanos is almost certainly key to its function, and that other important regions lie N-terminal to amino acid 218 (see Figure 1). It should be noted that definitive interpretation of the deletion analysis described here requires proof that the protein derivatives which fail to rescue are stably expressed in embryos. However, the in vitro transcribed RNAs which fail to rescue when injected are competent for translation in vitro in rabbit reticulocyte extract (data not shown).

Xcat-2 - a functional homolog?

The Xenopus gene Xcat-2 encodes an RNA which is localized to the vegetal cortex of late stage oocytes. In addition, a portion of the Xcat-2 open reading frame bears significant homology to the C-terminal region (Forristall et al., 1995; Mosquera et al., 1993) of the nanos open reading frame. Given these interesting parallels to *nanos*, we decided to assay Xcat-2 sequences for *nanos* activity by RNA injection. Initially, we tested an intact Xcat-2 cDNA by this assay in nos^{L7} mutant embryos. Out of 125 embryos injected, no rescue was observed (Table 3.2). We conclude that Xcat-2 sequences alone do not provide functional *nanos* activity. This result might have been expected, however, since the Xcat-2 protein is only 128 amino acids long, and the homology to nanos is only over a 53 amino acid region of the 400 amino acid nanos open reading frame. Also, independent evidence suggests that regions besides the C-terminus of nos are important for its function (see above). In addition, a C-terminal 87 amino acid fragment of nos lacks rescuing activity (D. Curtis, personal communication).

In an effort to provide a sequence "context" which might be required for the Xcat-2 domain to provide *nanos* function in embryos, we next constructed a chimeric cDNA. Xcat-2 sequence was substituted for *nanos* sequence within the 53 amino acid region of homology, in the background of the wild-type *nanos* cDNA (Fig 3.1). This chimeric cDNA fails to rescue when assayed by RNA injection (Table 3.2). In order to test whether the chimeric fusion protein was properly synthesized and stable in embryos, the injections

were repeated, into *nos*^{BN} mutant embryos, which lack endogenous nanos protein and therefore provide a negative background for antibody staining. As a control, embryos were injected with RNA transcribed from an intact *nanos* cDNA. Just as with the *nos*^{L7} embryos, the abdominal phenotype of *nos*^{BN} embryos is fully rescued by the wild-type, but not the chimeric transcript (data not shown). Antibody staining of the injected embryos shows that both the wild-type and the chimeric fusion proteins are stably synthesized to roughly equal degrees (Figure 3.2). We therefore conclude that despite the high degree of sequence conversation, Xcat-2 is not a functional homologue of *nanos*. In addition, we may conclude that the 53 amino acid region of homology is absolutely essential to *nanos* function.

The nanos C-terminus includes a pair of cysteine containing motifs similar to that found in the retroviral nucleocapsid class of zinc finger proteins (Curtis et al., 1995). Most interestingly, these paired cysteine motifs occur in the region of Xcat-2 homology, and are also present in the Xcat-2 sequence (Mosquera et al., 1993). In addition, preliminary evidence suggests that bacterially produced nanos protein binds zinc (A. Hannaford and D. Curtis, personal communication). However, while the retroviral nucleocapsid proteins contain the invariant motif C-X$_2$-C-X$_4$-H-X$_4$-C (Schwabe and Klug, 1994), the *nanos*/Xcat-2 sequences differ in spacing both between the members of the pairs, and from the nucleocapsid consensus. These findings suggest that Xcat-2 and *nanos* may constitute a novel family of zinc-finger domain proteins. In this context, the failure of Xcat-2 to rescue in the RNA injection assay could be interpreted as a difference in substrate specificity of the two proteins.

MATERIALS AND METHODS

Cloning

The *nanos* deletion clones were derived from the plasmid pN5, which contains a full-length *nanos* cDNA (Wang and Lehmann, 1991). DNA manipulations were carried out using standard protocols (Sambrook et al., 1989). Briefly, for the 51/220 deletion construct, the plasmid was digested to

completion with restriction endonuclease PstI, then religated to generate the in-frame deletion of nucleotides 391-898 (numbering from the beginning of the full length cDNA (Wang and Lehmann, 1991)). For the 119/378 deletion, the same procedure was carried out with restriction endonuclease NcoI, generating an in-frame deletion of nucleotides 592-1369. For the 218/288 deletion, the plasmid was partially digested with restriction endonuclease PvuII and the partial digestion product representing cleavage at nucleotides 893 and 1103 of the cDNA was gel purified and religated to generate the in -frame deletion. For the 258/400 deletion, the pN5 plasmid was digested to completion with restriction endonuclease BstXI, then treated with T4 polymerase and calf intestinal phosphatase to generate a linear blunt-ended fragment. The linearized plasmid was religated in the presence of polynucleotide kinase-treated NheI linker (New England Biolabs, #1060) to generate stop codons in all reading frames at nucleotide 1016.

For the Xcat-2 cDNA injection construct, the Xcat-2 coding sequence was placed behind a *Xenopus* ß-globin 5' untranslated leader sequence (in order to match the *nanos* cDNA plasmid). Briefly, a pSPORT (GibcoBRL) clone containing Xcat-2 (Mosquera et al., 1993) was digested with SalI, treated with Klenow fragment, then digested with NotI to excise an intact 780 base pair cDNA. As a vector, an insert-containing plasmid from the Nick Brown 4-8 hour cDNA library was digested to completion with NheI and NotI to generate a 2150 base pair truncated version of the cDNA vector pNB40 (Brown and Kafatos, 1988). To generate a fragment containing the ß-globin linker which could be ligated to the Xcat-2 sequence, the polymerase chain reaction (PCR) was employed using the the primers CW50 and CW53 and a template plasmid containing pNB40. CW50 overlaps the unique NheI site in pNB40 and adds a KpnI site; CW53 overlaps the 3'end of the ß-globin 5' untranslated leader and adds a BamHI site.

Primer CW50: 5'-CGATCGGTACCGATCTGGCTAGCGATGAC-3'

Primer CW53: 5'-GCGGATCCCCCAAAGTTGAGCGTTTATTC-3',

The PCR product was filled in with Klenow fragment and digested with NheI to generate a 300 base pair NheI/blunt fragment. The three fragments were directionally ligated to generate the plasmid pNBXCAT2.

For the Xcat-2/*nanos* "swap" construct, PCR was used to generate two fragments. The first, a 936 base pair fragment containing most of the *nanos* coding region, was generated from a pN5 template using the primers Nde-R and CW56. Nde-R overlaps with and creates an NdeI site at the start codon of the *nanos* open reading frame; CW56 hybridizes perfectly to the *nanos* open reading frame just upstream of the homology region, and contains a NaeI half-site.

Nde-R: 5' TTCCATATGTTCCGCAGC-3'

CW56: 5'-GGCTGATCTCTTTGGCCTTG-3'

After PCR and Klenow fill-in, this fragment was cut at a unique BstEII site to generate a 741 base pair product. The second PCR-generated product of 190 base pairs, containing the homology bearing region of Xcat-2 was generated from the Xcat-2 clone using the primers CW55 and CW54. CW55 contains 5 residues at the 5'end which introduce a NaeI half site while preserving the amino acid coding; CW54 is a 'bridge' primer containing both Xcat-2 and *nanos* sequences, including an NcoI site from the *nanos* coding region.

CW55: 5'-GGCACTGCGGGTTCTGCAGGAGC-3'

CW54:5'-ATCCTCCATGGTGATGATCGGCTTCTTGGGGCAGTACCGCATG-3'

After PCR and Klenow fill-in, this second fragment was cut with NcoI to generate a 185 base pair product. The 3.9 kilobase vector backbone fragment which includes the *nanos* 5' and 3' untranslated regions was generated by digesting pN5 to completion with BstEII and NcoI. The three fragments were directionally ligated to generate the plasmid pN5/X1.

All DNA constructs were checked by DNA sequencing of junction and PCR-generated regions.

RNA injection and subsequent analysis

RNA injections were carried out as previously described (Wang and Lehmann, 1991). Recipient embryos were derived from females homozygous for either the nos^{L7} or nos^{BN} allele(Lehmann and Nüsslein-Volhard, 1991; Wang et al., 1994). The nos^{L7} allele is a hypomorph, producing a small amount of *nanos* activity, whereas nos^{BN} is a null in embryos (Wang and Lehmann, 1991; Wang et al., 1994). For staining experiments, the injected embryos were fixed after injection as previously described (Wang and Lehmann, 1991), and stainings were carried out as previously described (Gavis

and Lehmann, 1992) using an anti-nanos antibody which was raised against a nanos C-terminal peptide (Wang et al., 1994). This peptide is preserved intact in the chimeric fusion protein.

REFERENCES

Brown, N. H., and Kafatos, F. C. (1988). Functional cDNA libraries from Drosophila embryos. Journal of Molecular Biology *203*, 425-437.

Curtis, D., Apfeld, J., and Lehmann, R. (1995). *nanos* is an evolutionarily conserved organizer of anterior-posterior polarity. Development *in press*,

Forristall, C., Pondel, M., Chen, L., and King, M. L. (1995). Patterns of localization and cytoskeletal association of two vegetally localized RNAs, *Vg1* and *Xcat-2*. Development *121*, 201-208.

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

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

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

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular cloning: a laboratory manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Schwabe, J. W. R., and Klug, A. (1994). Zinc mining for protein domains. Nature Structural Biol *1*, 345-349.

Wang, C., Dickinson, L. K., and Lehmann, R. (1994). Genetics of *nanos* localization in *Drosophila*. Developmental Dynamics *199*, 103-115.

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

Table 1. RNA injection rescue assay of *nanos* deletion constructs

Construct	Percent of developed embryos				
	N	0-1	2-5	6-7	8 or hatch
wild-type *nanos*	70	11	4	6	79
*nos*Δ51/220	84	100	-	-	-
*nos*Δ119/378	60	100	-	-	-
*nos*Δ218/288	57	17	16	4	63
*nos*Δ258/400	78	100	-	-	-

Recipient embryos for the injection assay were derived from homozygous nos^{L7} mutant females. Uninjected embryos develop into larvae which completely lack abdominal segments. "Construct" denotes the cDNA template used for in vitro transcription of RNA for injection. "N" is the number of scored larval cuticles. The table entries represent the percent of the total number of larval cuticles scored showing a given number of abdominal segments.

Figure 3.1. Structural analysis of the *nanos* polypeptide

This figure depicts a schematic of the *nanos* open reading frame. The top half of the figure shows the in-frame deletion clones which were tested for function by the RNA injection assay. The column on the right shows whether a given deletion was able to rescue the *nanos* mutant phenotype. The lower half of the figure shows the 53 amino acid region of homology between *nanos* and the Xenopus gene Xcat-2. Residues which are identical between the two polypeptide sequences are denoted with a dot, and a connecting line denotes conservative amino acid changes. The percentages to the right are based on 29/53 identities, plus 3 conservative changes. Both the *nanos* and Xcat-2 open reading frames are depicted as open boxes, with lines drawn to indicate the location of the 53 amino acid homology region for each protein. N and C, the N- and C- termini of the respective open reading frames; aa, amino acids. Drawing is made to scale.

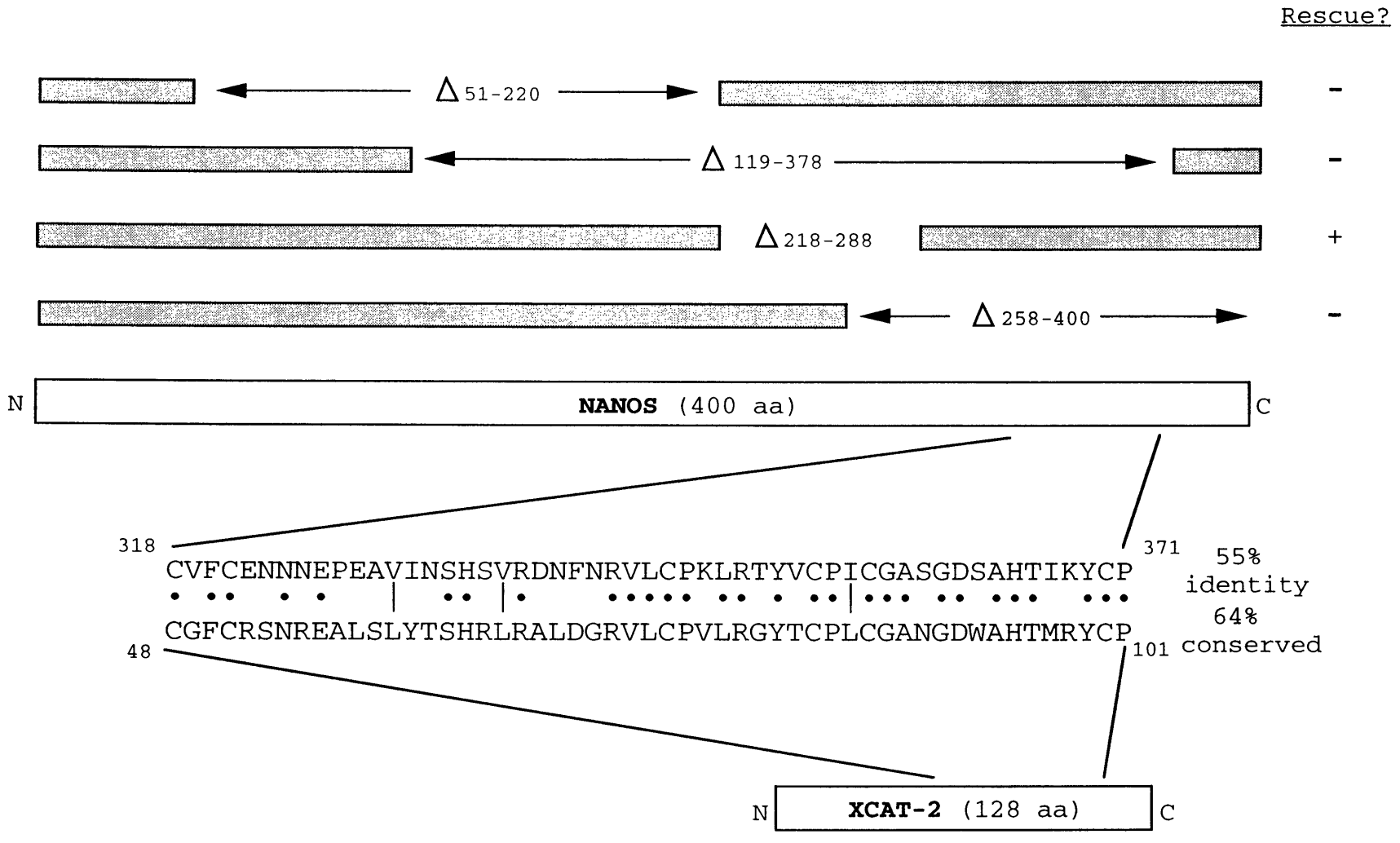

Rescue?
Δ 51-220
-
Δ 119-378
-
Δ 218-288
+
Δ 258-400
-
N
NANOS (400 aa)
C
318
371
CVFCENNNEPEAVINSHSVRDNFNRVLCPKLRTYVCPICGASGDSAHTIKYCP
CGFCRSNREALSLYTSHRLRALDGRVLCPVLRGYTCPLCGANGDWAHTMRYCP
48
101
55% identity
64% conserved
N
XCAT-2 (128 aa)
C

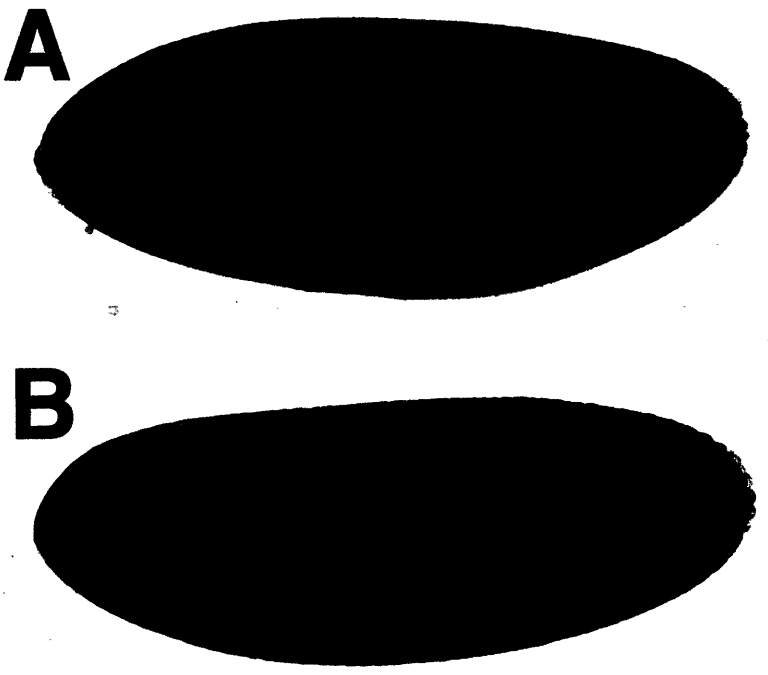
Table 2. RNA injection rescue assay of Xcat-2 sequences

		# of abdominal segments (%)		
RNA injected	N	0-1	2-5	6-8
pN5/X1	132	99	1	0
(swap)				
pNBXCAT2	125	100	0	0
(intact Xcat-2)				
pN5	84	0	2	98
(intact *nanos*)				

Recipient embryos for the injection assay were derived from homozygous nos^{L7} mutant females. Uninjected embryos develop into larvae which completely lack abdominal segments. "RNA injected" denotes the cDNA template used for in vitro transcription of RNA for injection. "N" is the number of scored larval cuticles. The table entries represent the percent of the total number of larval cuticles scored showing a given number of abdominal segments.

Figure 3.2. Expression of injected RNAs detected by whole-mount antibody staining

Embryos were injected with RNA, then fixed and stained in whole-mount to detect expression of *nanos*-derived protein sequences. Recipient embryos were obtained from nos^{BN} homozygous mutant mothers. These embryos lack endogenous nanos protein. A. Embryo injected with pN5 (full length *nanos* cDNA) in vitro transcript. B. Embryo injected with pN5/X1 (chimeric nanos/Xcat-2 protein) in vitro transcript. In both cases, high levels of expression of protein derived from injected RNA are observed. Embryos are oriented with the anterior on the left, dorsal side towards the top of the figure.


A
B

Chapter 4

The genetics of *nanos* localization in Drosophila

AUTHOR'S NOTE

This chapter has been previously published as Wang, C., Dickinson, L.K., and Lehmann, R. (1994) "Genetics of *nanos* localization in *Drosophila*" Developmental Dynamics **199**, 103-115. Laura Dickinson participated in the whole-mount in situ hybridization and antibody staining analyses of ovaries and embryos.

ABSTRACT

The Drosophila gene *nanos* is required for two processes. During oogenesis, *nanos* function is required for the continued production of egg chambers, and *nanos* is expressed in the early germarium. During embryogenesis, *nanos* is required maternally to specify abdominal segmentation. *Nanos* shares this latter function with nine other genes, collectively known as the posterior group. Of this group, *nanos* encodes a determinant, and is localized as an RNA to the posterior pole of early embryos. This RNA is translated to form a gradient of nanos protein with highest concentrations at the posterior. Analysis of the distribution of *nanos* gene products in embryos mutant for posterior group genes shows that eight of these genes are required for localization, but not stability, of the *nanos* RNA. Embryos mutant for posterior group alleles which produce weak abdominal phenotypes show reduced amounts of localized *nanos* RNA. This correlation between *nanos* RNA localization and abdominal phenotype suggests that *nanos* acts as a localization-dependent posterior determinant. Localization of *nanos* is not affected by mutations in *bicoid* or *torso*, confirming that the three maternal systems of anterior-posterior determination initially act independently.

INTRODUCTION

In Drosophila, three classes of maternal-effect genes, the anterior, posterior, and terminal classes, control embryonic pattern formation along the anterior-posterior axis (Nüsslein-Volhard et al., 1987; Nüsslein-Volhard, 1991; St. Johnston and Nüsslein-Volhard, 1992). The posterior class comprises ten known genes (*cappuccino* (*capu*), *spire* (*spir*), *mago nashi* (*mago*), *oskar* (*osk*), *staufen* (*stau*), *vasa* (*vas*), *tudor* (*tud*), *valois* (*vls*), *nanos* (*nos*), and *pumilio* (*pum*)) which are required for formation of the abdominal segments (Boswell and Mahowald, 1985; Schüpbach and Wieschaus, 1986; Lehmann and Nüsslein-Volhard, 1986; Lehmann and Nüsslein-Volhard, 1987b; Schüpbach and Wieschaus, 1989; Manseau and Schüpbach, 1989; Lehmann

and Nüsslein-Volhard, 1991; Boswell et al., 1991). Eight of these genes (*capu, spir, mago , stau, osk, vas, tud,* and *vls*) display a second mutant phenotype, the grandchildless phenotype. Grandchildless mutant mothers produce embryos which lack poleplasm, the specialized cytoplasm at the posterior pole. The poleplasm contains large ribonucleoprotein particles known as polar granules, and becomes incorporated into the germline precursor cells, the pole cells. Several of the grandchildless genes have been cloned, and the gene products shown to be localized to the poleplasm as RNAs and/or proteins (Hay et al., 1988; Lasko and Ashburner, 1988; Ephrussi et al., 1991; Kim-Ha et al., 1991; St. Johnston et al., 1991). Genetic analysis of the localization of these poleplasm components has resulted in a model in which the grandchildless genes act in a stepwise pathway to assemble poleplasm (Hay et al., 1990; Lasko and Ashburner, 1990; Kim-Ha et al., 1991; Ephrussi et al., 1991; St. Johnston et al., 1991). Genes early in the pathway nucleate or otherwise achieve the assembly of downstream gene products into the poleplasm. In this pathway, *capu, spir, stau,* and *mago* act upstream of *osk,* which in turn acts upstream of *vas,* followed by *tud* and *vls.*

Cytoplasmic transplantation experiments have shown that wild-type poleplasm contains an activity which rescues the abdominal segmentation phenotype of posterior group mutant embryos. This activity is absent at the posterior pole of posterior group mutant embryos (Lehmann and Nüsslein-Volhard, 1986; Lehmann and Nüsslein-Volhard, 1987b; Lehmann and Nüsslein-Volhard, 1991; Boswell et al., 1991). Synthesis of this rescuing activity during oogenesis depends strictly on the gene *nos* (Lehmann and Nüsslein-Volhard, 1991). Subsequent cloning of *nos* revealed that the *nos* messenger RNA is localized to the posterior pole of embryos, and that *nos* RNA is functionally equivalent to the posterior rescuing activity (Wang and Lehmann, 1991). *nos* therefore encodes a posterior determinant which is localized to the posterior pole of wild-type embryos, and posterior group mutant embryos lack localized *nos* activity. In wild-type embryos, localized *nos* RNA is a source for a gradient of nos protein with highest levels at the posterior (this report; Ephrussi and Lehmann, 1992; Smith et al., 1992; Barker et al., 1992). nos protein prevents expression of hunchback (hb) protein from the maternal *hunchback* RNA (Hülskamp et al., 1989; Irish et al., 1989a; Struhl, 1989). *Hb,* in turn, acts as a morphogen to repress expression of

abdominal gap genes (Hülskamp et al., 1990; Struhl et al., 1992). In the posterior half of the embryo, reduced levels of hb protein allow abdomen development.

Analysis of strong *nos* alleles reveals that, in addition to its role in specifying abdomen, *nos* also plays a role in oogenesis. Females mutant for either of two *nos* alleles lay very few eggs. Examination of the mutant ovarioles shows that while oogenesis continues normally once it has been initiated, stem cell proliferation appears to be defective such that few cysts are produced (Lehmann and Nüsslein-Volhard, 1991). The target of *nos* function in oogenesis remains unknown.

In this report, we examine the distribution of *nos* gene products during development. Furthermore, we show that the genes of the grandchildless class are required to localize *nos* RNA to the posterior pole of early embryos. Mutations in these genes do not affect the stability of the *nos* RNA. Embryos mutant for posterior group alleles giving weak abdominal segmentation phenotypes show abnormal or reduced localized RNA. Finally, we show that other maternally-acting segmentation genes do not play a role in *nos* localization.

RESULTS

Genetic analysis has shown that *nos* is required at two stages of development, oogenesis and early embryogenesis. RNA analysis confirms that *nos* transcript is detectable at significant levels only in females and early embryos (Wang and Lehmann, 1991). Moreover, genetic and cytoplasmic transplantation analyses suggest that *nos* function depends strictly on the correct spatial distribution of its gene product(s). We therefore examined the distribution of *nos* gene products in both ovaries and embryos. In summary, we find that patterns of *nos* expression correspond to the sites defined by genetic analysis as critical for *nos* function.

Distribution of nanos gene products in embryos

nos transcript is tightly localized to the posterior pole of early embryos, and appears to be restricted to the poleplasm (Figure 4.1A). *nos* RNA is included in the forming pole buds (Figure 4.1B), and is visible afterwards in the pole cells (Figures 4.1C,D). The transcript remains detectable in pole cells until the extended germ band stage, where it is faintly visible within the pocket of the posterior midgut invagination (Figure 4.1E). After germ band retraction, no RNA is detectable within the early embryonic gonad (Figure 4.1F). To test whether *nos* is zygotically transcribed in embryos, we examined embryos produced by nos^{BN} mutant females which had been crossed with wild-type males (data not shown). Females mutant for the nos^{BN} allele deposit no *nos* RNA into embryos (see below). In situ hybridization analysis of these embryos failed to detect any *nos* RNA, indicating that *nos* is not newly transcribed in either the pole cells or the embryonic somatic tissue.

Translation of nos protein from its localized RNA begins soon after egg deposition. At the earliest embryonic stages examined, faint staining is visible at the posterior pole (Figure 1G). As the cleavage stages progress, the level of staining increases, and a gradient of nos protein is generated which is highest at the posterior pole. This gradient reaches its most anterior extent, about 30% egg length (0%= posterior end), at the pole bud stage (Figure 4.1H). More sensitive staining methods have been employed to show that nos protein can be detected as anteriorly as 50% egg length (Barker et al., 1992). Both the nanos and hunchback protein gradients are detected in unfertilized eggs (data not shown). nos protein is incorporated into the pole cells, and staining outside the pole cells is largely undetectable by blastoderm stage (Figures 4.1I, J). The pole cells continue to stain strongly for nos protein through gastrulation and germ band extension (Figures 4.1K, L). nos protein can be detected in the primordial germ cells as late as embryonic stage 15 (stages according to Campos-Ortega and Hartenstein, 1985), when the cells have reached the bilaterally symmetric embryonic gonad. The persistence of the protein in pole cells until late embryogenesis contrasts with the *nos* RNA, which is undetectable in pole cells after germ band extension (see above).

Distribution of *nanos* gene products in oogenesis

The observation that some *nos* alleles exhibit oogenesis defects implies that *nos* is expressed very early in oogenesis. Detection of *nos* transcript in the pre-vitellogenic germarium is made difficult by very low amounts of *nos* RNA present in these stages (Figure 4.2) (see figure legend for a description of oogenesis). More sensitive in situ hybridization methods occasionally detect low concentrations of *nos* RNA in regions 2 and 3 of the germarium. *nos* transcript is clearly detected by stage 5 of oogenesis, when both the nurse cells and the oocyte contain *nos* RNA, which appears to be enriched in the oocyte (Figure 4.2A). During stages 7/8, *nos* RNA appears to be transiently localized to the anterior margin of the growing oocyte. Several other localized transcripts in Drosophila show a similar pattern of transient anterior localization in these stages (Suter et al., 1989; Ephrussi et al., 1991; Kim-Ha et al., 1991; Cheung et al., 1992; Lantz et al., 1992; see also Theurkauf et al., 1992). At stage 10, the nurse cells contain high levels of *nos* RNA. Shortly afterwards, the nurse cells contract and deposit their contents, including *nos* RNA, into the oocyte. *Nanos* RNA localization occurs during the final stages of oogenesis (stages 13/14), and small amounts of RNA can be observed accumulating at the posterior pole of the oocyte as early as stage 12 (Figure 4.2A).

nos protein is first detected in regions 1/2a of the germarium (Figures 4.2A, B). The 4 and 8 cell cysts (region 1) stain most strongly, and lower levels of staining are observed in the 16 cell cyst (region 2a). In the subsequent vitellogenic stages, nos protein is present at low levels in nurse cell/oocyte clusters in stages 3 through 6. High levels of expression are observed in nurse cells at stage 10. At no point in oogenesis do we observe nos protein in the growing oocyte.

Nanos mutant alleles

To further investigate the dual role of *nos* in development, we examined the distribution of mutant *nos* gene products (Table 4.1) (note: for maternal effect genes, we will hereafter refer to embryos derived from

homozygous mutant females as "mutant embryos"). The five existing *nos* alleles fall into three phenotypic classes. The first class, which includes nos^{L7} and nos^{RW}, results in abdominal, but not oogenesis, defects. nos^{L7} produces stronger abdominal defects than nos^{RW} (Lehmann, 1988; Lehmann and Nüsslein-Volhard, 1991). Embryos mutant for the nos^{L7} or nos^{RW} alleles show normal distribution patterns of both *nos* RNA and protein. These data suggest that these alleles encode nos proteins of either reduced function, or with defects specific for abdomen formation. The nos^{BN} allele, the result of a P element insertion in the *nos* promoter region (see Materials and Methods), comprises the second class of *nos* mutations. nos^{BN} produces strong abdominal defects, but oogenesis is unaffected. nos^{BN} embryos lack *nos* RNA (see Figure 4.4) and do not contain detectable nos protein (Table 4.1). nos^{BN} mutant ovaries contain greatly reduced amounts of nanos protein (Table 4.1). We conclude that the nos^{BN} allele reduces *nos* transcription below a level critical for abdomen formation, but still sufficient to complete oogenesis. The third class of *nos* alleles includes the two strongest alleles, nos^{RC} and nos^{RD}. Females homozygous for these alleles produce only a few embryos, all of which show strong abdominal defects. Both the oogenesis and abdominal segmentation phenotypes are fully complemented by a transgene containing 5 kb of genomic DNA capable of encoding only the *nanos* transcript (E. Gavis, personal communication). nos^{RD} encodes a stable RNA (data not shown), which is properly localized to the posterior pole (Table 4.1). However, nos^{RD} protein appears to be poorly synthesized or unstable, as protein levels are severely reduced in both ovaries and embryos (Table 4.1). nos^{RC} encodes an unstable transcript (data not shown), and we detect no protein in either ovaries or embryos (Table 4.1). We conclude that the nos^{RC} phenotype represents the *nos* null phenotype.

Posterior group mutants

Embryos derived from females mutant for any posterior group gene lack abdominal segments. Eight of the ten known posterior group genes display a second, grandchildless phenotype. Cytoplasmic transplantation and RNA injection experiments both suggest that grandchildless mutant embryos lack localized posterior rescuing activity (i.e. *nos* activity) at the posterior pole

(Lehmann and Nüsslein-Volhard, 1986; Lehmann and Nüsslein-Volhard, 1987b; Lehmann and Nüsslein-Volhard, 1991; Wang and Lehmann, 1991). We were therefore interested to see how the grandchildless mutations affect the distribution of *nos* gene products.

strong mutants

This group comprises strong alleles of *capu*, *spir*, *stau*, *osk*, and *vas*. These genes function early in the genetically defined poleplasm assembly pathway, and mutant embryos develop strong abdominal defects (Manseau and Schüpbach, 1989; Lehmann and Nüsslein-Volhard, 1986; Schüpbach and Wieschaus, 1986). The distribution of *nos* gene products in embryos derived from the respective mutant females (see Materials and Methods for a description of the alleles examined) was analyzed by in situ hybridization and antibody staining. No localized RNA or protein can be detected in embryos mutant for *vas*, *capu*, *spir*, *stau*, or *osk* (data shown for *vas*, Figure 4.3). We therefore conclude that *capu*, *spir*, *stau*, *osk*, and *vas* play an essential role in *nos* RNA localization.

weak mutants

We next examined the localization of *nos* in mutant embryos that fail to form poleplasm or pole cells, but give weak abdominal phenotypes (Figure 4.3). The mutation osk^{301} is temperature sensitive for abdomen formation. At the permissive temperature (20°C), normal, complete abdomens form, whereas at the restrictive temperature (29°C), strong abdominal defects occur (Lehmann and Nüsslein-Volhard, 1986). At the permissive temperature, *nos* RNA is most often observed as a small, strongly staining area at the posterior pole (Figure 4.3D). This localization pattern is distinct from the posterior "crescent" of staining seen in wild-type embryos (Figure 4.3A). At the restrictive temperature, no localized *nos* RNA or nos protein is observed (Figures 4.3C, J). The distribution of nos protein in osk^{301} at the permissive temperature parallels the altered RNA distribution, with a reduction in both the amount of protein as well as the anterior extent of the protein gradient (Figure 4.3K). Alleles of *capu* and *stau* which produce weak abdominal phenotypes show patterns of *nos* distribution similar to osk^{301} at the permissive temperature (data not shown).

Of the known grandchildless genes, *tud* and *vls* are the farthest downstream in the genetic pathway of poleplasm assembly. Both mutations partly affect *nos* RNA localization. *Tud* mutant embryos develop a variable number of abdominal segments, with the majority forming 6 to 8 of the normal 8 segments (Boswell and Mahowald, 1985; Wang and Lehmann, 1991). About half of *tud* embryos contain localized RNA, but the signal appears to be weaker than in wild type, while the other half have very little or no localized RNA (Figure 4.3E). *Vls* mutations have strong effects on abdomen formation, with greater than 90% of the developed embryos completely lacking abdominal segments (Schüpbach and Wieschaus, 1986; Wang and Lehmann, 1991). Correspondingly, the majority of embryos produced by *vls* mutant females fail to localize *nos* RNA, while approximately 10% show localized RNA which appears to occupy a smaller area than in wild type (Figure 4.3F). As might be expected from their patterns of RNA localization, *tud* and *vls* embryos differ in their patterns of nos protein distribution. Most *tud* mutant embryos show a fairly normal or slightly reduced nos protein gradient (Figure 4.3L). Most *vls* mutant embryos lack detectable nos protein. However, a small number of *vls* mutant embryos (about 10%) show a weak concentration of nos protein at the posterior (Figure 4.3M).

Mago is unique among the posterior group genes in that a variety of mutant phenotypes have been reported, ranging from weak to strong abdominal defects, as well as bicaudal (double abdomen) embryos at a low frequency (Boswell et al., 1991). The role of *mago* in poleplasm assembly remains unclear, but genetic analysis suggests that *mago* acts upstream of *osk* in the pathway (Ephrussi and Lehmann, 1992). In our observations, *mago* embryos frequently lacked abdominal segments. Bicaudal embryos were observed much less commonly than previously reported. Most *mago* mutant embryos lack localized *nos* RNA, although some embryos are observed with a "dot" of localized RNA at the posterior (Figure 4.3G). Correspondingly, many *mago* embryos have no detectable nanos protein, although some weak gradients are seen (Figure 4.3N). We did not detect at a significant frequency any *nos* RNA or protein at the anterior of *mago* mutant embryos.

In summary, we conclude that these mutations which give weak abdominal phenotypes partly disrupt posterior localization of *nos* RNA. These results suggest that the extent of *nos* function depends on the degree of *nos* RNA localization.

RNA analysis of posterior group mutants

As shown above, grandchildless mutant embryos are impaired for *nos* RNA localization. It remains possible, however, that instead of or in addition to their role in localizing *nos* RNA, the grandchildless genes might affect RNA stability. We therefore prepared RNA from grandchildless mutant and wild-type embryos for analysis by Northern blot hybridization. Similar amounts of intact *nos* transcript were detected in both wild-type embryos and mutant embryos lacking localized *nos* RNA (Figure 4.4). In particular, grandchildless mutations do not appear to affect the stability of the RNA.

Mutants in other maternal-effect systems

Genetic analysis has shown that each of the anterior, posterior, and terminal classes of maternal effect genes affects a subset of the complete embryonic pattern (Nüsslein-Volhard et al., 1987; Nüsslein-Volhard, 1991; St. Johnston and Nüsslein-Volhard, 1992). Each of these classes defines a system for specifying pattern. These three systems are thought to act largely independently of one another (Nüsslein-Volhard et al., 1987; Nüsslein-Volhard, 1991; St. Johnston and Nüsslein-Volhard, 1992). To confirm the independent action of the three maternal systems, we examined the localization of *nanos* gene products in embryos mutant for *torso*, *bicoid*, *exuperantia*, or *swallow*.

The terminal class gene *torso* (*tor*) encodes a tyrosine kinase receptor which plays a key role in specification of the specialized terminal structures, including the acron and telson (Klingler et al., 1988; Sprenger et al., 1989). Mutant embryos lacking *tor* activity show normal distribution patterns of *nos*

gene products (Figures 4.5A,E). Terminal class activity is therefore independent of, and not required for, *nos* RNA localization.

Bicoid (*bcd*), *exuperantia* (*exu*), and *swallow* (*swa*) are anterior class genes required for proper formation of the head and thoracic structures (Frohnhöfer and Nüsslein-Volhard, 1986; Frohnhöfer and Nüsslein-Volhard, 1987). *Bcd* encodes a homeodomain-containing transcription factor which acts as an anterior morphogen, and its RNA is localized to the anterior end of the embryo (Berleth et al., 1988; Driever and Nüsslein-Volhard, 1988). *Exu* and *swa* are required for proper localization of the *bcd* RNA (Berleth et al., 1988). *Bcd* mutations do not affect *nos* RNA localization or protein distribution (Figures 4.5B, F). The posterior localization machinery therefore functions independently of the activity of the anterior morphogen. Similarly, mutations in *exu* do not affect localization of *nos* RNA (Figure 4.5C). In *swa* mutant embryos, *nos* RNA is localized to the posterior, but the staining appears patchy or ragged (Figure 4.5D). nos protein distribution in *exu* or *swa* mutants is consistent with their respective RNA patterns (Figures 4.5G, H). We conclude that while *swa* is not absolutely required for localization of RNAs to the posterior, some components of the RNA localization machinery may be shared between the anterior and posterior systems.

DISCUSSION

Nanos during oogenesis

We have detected nos protein expression during two distinct periods of oogenesis. The early phase of nos expression is primarily limited to the early stages of the germarium and correlates with the genetic requirement for *nos* in oogenesis. Females mutant for the alleles nos^{RC} or nos^{RD} lay very few eggs. Examination of the mutant ovarioles shows that although oogenesis proceeds normally once initiated, stem cell proliferation appears to be defective, and few cysts are produced (Lehmann and Nüsslein-Volhard, 1991). Combined with the observed expression pattern, this phenotype suggests that *nos* may act to promote stem cell or cystoblast divisions. How would *nos* carry out this function? By analogy to its later role in embryonic

development, *nos* could repress *hb* translation. Absence of hb protein would allow oogenesis to continue normally. In the absence of *nos*, *hb* would repress stem cell or cystoblast divisions. This scenario appears unlikely, however, since there is no evidence that *hb* is expressed in early oogenesis (F. Pelegri, unpublished data). Moreover, oogenesis occurs normally in females which lack *hb* in the germline (Lehmann and Nüsslein-Volhard, 1987a). Finally, expression of a *hb* transgene lacking the RNA *nos* response elements (NREs), which have been shown to be necessary and sufficient for regulation by *nos* in embryogenesis, has no dominant phenotype in oogenesis (Wharton and Struhl, 1991). Thus it seems likely that, during oogenesis, *nos* acts not through *hb*, but rather through a different target. *nos* function in oogenesis does not require its 3' untranslated region (UTR), as a *nos* transgene containing a different 3'UTR fully complements the oogenesis defect (Gavis and Lehmann, 1992).

nos protein can be detected at a second time in oogenesis, in the nurse cells at stage 10 (Figure 4.2A). The function of this expression remains unclear. However, the presence of high levels of nos protein in stage 10 nurse cell cytoplasm is consistent with the earlier finding that this cytoplasm is able to rescue the abdominal phenotype of posterior group mutant embryos when assayed by cytoplasmic transplantation (Sander and Lehmann, 1988). Although staining for nos protein is no longer detectable after stage 10, it should be noted that formation of the chorion and vitelline membrane during the later stages of oogenesis probably renders the growing oocyte impermeable to antibody. In any case, it is critical that nos protein be excluded from the oocyte, because *nos* activity can interfere with the expression of the anterior morphogen *bcd* (Wharton and Struhl, 1989; Wang and Lehmann, 1991; Gavis and Lehmann, 1992).

Other posteriorly localized elements

Past work has identified several genes whose products are localized to the posterior pole of embryos as RNAs or as proteins. In some cases, these components have been shown to be part of the poleplasm, the specialized cytoplasm at the embryonic posterior pole. Products localized to the posterior

pole include *osk* RNA and protein (Ephrussi et al., 1991; Kim-Ha et al., 1991; Ephrussi and Lehmann, 1992), stau protein (St. Johnston et al., 1991), vas protein (Hay et al., 1988; Lasko and Ashburner, 1988), cyclin B RNA (Whitfield et al., 1989), and the *germ cell-less* (*gcl*) RNA (Jongens et al., 1992). Localization of these components depends on the grandchildless genes. Analysis of the distribution of localized components in grandchildless mutants defines a genetic hierarchy, and therefore a pathway for poleplasm assembly. Localization of *nos* RNA occupies the same position in the assembly pathway as localization of the cyclin B and *gcl* RNAs (this work, Raff et al., 1990; Jongens et al., 1992). That is, localization of these three RNAs requires the function of all known grandchildless genes.

However, the *Bicaudal-D* (*Bic-D*) mutation reveals a difference in the localization of these three RNAs. Embryos produced by *Bic-D* mutant females develop two abdomens in mirror image (Mohler and Wieschaus, 1986). Poleplasm and pole cells are observed at the posterior, but the anterior end of these mutant embryos contains neither poleplasm nor pole cells (Mohler and Wieschaus, 1985). *Osk* RNA, *nos* RNA, and cyclin B RNA are ectopically localized to the anterior in *Bic-D* mutant embryos, but stau protein, vas protein, and *gcl* RNA are not detectably enriched at the anterior (Ephrussi et al., 1991; Raff et al., 1990; St. Johnston et al., 1991; Hay et al., 1990; Lasko and Ashburner, 1990; Jongens et al., 1992). These results suggest that at the anterior, localization of *stau*, *vas*, and *gcl* is not necessary for *nos* RNA localization.

RNA localization and abdominal phenotype

Grandchildless mutant embryos exhibit a series of *nos* RNA localization patterns. How do these patterns relate to the abdominal phenotypes observed? For strong grandchildless mutations (i.e. strong alleles of *capu*, *spir*, *stau*, *osk*, and *vas*), no localized *nos* RNA is seen, and no nos protein is detected. These embryos lack *nos* activity, and develop strong abdominal defects. Most *vls* mutant embryos lack localized *nos* RNA, but a few embryos appear to contain reduced amounts of localized *nos* RNA. Accordingly, a large majority of *vls* mutant embryos lack localized protein,

and develop with strong abdominal defects (Schüpbach and Wieschaus, 1986; Wang and Lehmann, 1991). *Tud* and *mago* mutant embryos show a broad range of *nos* distribution patterns, resulting in variable and weak abdominal defects (Boswell and Mahowald, 1985; Schüpbach and Wieschaus, 1986; Boswell et al., 1991; Wang and Lehmann, 1991). Osk^{301} embryos at the permissive temperature show reduced amounts of localized *nos* RNA and protein. However, this reduced amount of nos protein is sufficient for the formation of a complete abdomen. These results demonstrate a strong correlation between the degree to which *nos* RNA is localized, the amount of nos protein observed, and the degree of abdominal segmentation achieved. In addition, an increase in maternal *osk* gene dosage results in increased amounts of localized *nos* RNA, and increased *nos* activity (Ephrussi and Lehmann, 1992; Smith et al., 1992). These correlations suggest that *nos*' activity as an abdominal determinant is localization-dependent.

Grandchildless mutant embryos which lack localized *nos* RNA nevertheless contain normal levels of stable, but unlocalized, *nos* transcript (Figure 4.4). These embryos lack posterior rescuing activity as assayed by cytoplasmic injection (Lehmann and Nüsslein-Volhard, 1986; Lehmann and Nüsslein-Volhard, 1991) and develop severe abdominal defects. This result suggests that the unlocalized *nos* RNA in these embryos is inactive. Additionally, comparison of the levels of *nos* RNA detected by whole-mount in situ hybridization to wild-type versus nos^{BN} embryos (nos^{BN} is an RNA null allele in embryos) shows that wild-type embryos contain detectable levels of unlocalized *nos* RNA (data not shown). If this unlocalized RNA were active, ectopic *nos* activity at the anterior might interfere with the function of the anterior morphogen *bcd*. However, no difference in head development is observed between wild-type and nos^{BN} embryos (unpublished data). These observations suggest that the unlocalized *nos* RNA in wild-type embryos, like the unlocalized *nos* RNA in grandchildless mutant embryos, is inactive. One possible explanation for this result is that the levels of nos protein produced by the unlocalized *nos* RNA are insufficient to prevent translation of *hb* or *bcd* RNAs. An alternative hypothesis is that expression of *nos* in the early embryo is regulated at the level of translation such that only localized RNA is active. We favor this latter hypothesis, since experimental evidence indicates that localization of *nos* RNA to the posterior pole is specified by its 3'UTR

(Gavis and Lehmann, 1992), and that substitution of an unregulated 3'UTR for the *nos* 3'UTR allows unrestricted nos activity (Gavis and Lehmann, 1994). This 3'UTR-mediated control mechanism apparently ensures that only *nos* transcript which is properly localized to the posterior pole can produce active nos protein, which acts as a determinant to specify abdomen.

MATERIALS AND METHODS

Fly stocks

The nos^{BN} allele, a generous gift of T. Schüpbach, was isolated in a P element mutagenesis screen using a *white*-marked P element (Bier et al., 1989). nos^{BN} homozygous mutant females produce embryos which completely lack abdominal segments (unpublished data). These mutant females do not display the oogenesis defects associated with some *nos* alleles (Lehmann and Nüsslein-Volhard, 1991). Genomic Southern blot analysis was used to map the insertion site of the P element to a 220 bp ClaI-NruI restriction fragment which spans the putative *nos* transcription start site (L.K.D., unpublished data). The P element was mobilized by the introduction of transposase, and resulting stocks were screened for the loss of the *white* marker. *White*$^-$ revertants were then tested for the maternal-effect abdominal defect. This screen produced both wild-type revertants as well as new *nos* alleles (L.K.D., unpublished data). Genomic Southern analysis of revertant lines shows that the wild-type revertants are the result of precise P element excision events. Revertants which give *nos* mutant phenotypes are associated with the partial loss of P element DNA (imprecise excisions) (L.K.D., unpublished data). These results indicate that the nos^{BN} allele is caused by a P element insertion in the *nos* gene.

For all other mutants, the alleles used are the strongest available combination, unless otherwise noted. Specifically : *nanos* - nos^{L7}/nos^{L7}, nos^{BN}/nos^{BN}, nos^{RC}/nos^{BN}, nos^{RD}/nos^{BN}, or nos^{RW}/nos^{BN} (weak) (Lehmann and Nüsslein-Volhard, 1991; this work). *oskar* - osk^{54}/osk^{54}, osk^{166}/osk^{166}, or osk^{301}/osk^{54} (weak) (Lehmann and Nüsslein-Volhard,

1986; Lehmann and Nüsslein-Volhard, 1991). *cappuccino/spire* - *capu*^{RK}/*capu*^{RK}, *capu*^{HK}/*capu*^{HK} (weak), *spir*^{RP}/*spir*^{RP}, or *capu*^{RK}*spir*^{035}/*capu*^{RK}*spir*^{035} (Manseau and Schüpbach, 1989). *staufen* - *stau*^{D3}/*stau*^{D3}, or *stau*^{C8}/*stau*^{D3} (weak) (Lehmann and Nüsslein-Volhard, 1991). *vasa* - *vasa*^{D1}/*vasa*^{PD} (Schüpbach and Wieschaus, 1986; Lehmann and Nüsslein-Volhard, 1991). *tudor* - *tud*^{WC8}/Df[2R]*Pu*^{rP133}, or *tud*^{WC8}/*tud*^{WC8} (Boswell and Mahowald, 1985; Schüpbach and Wieschaus, 1986). *valois* - *vls*^{PE}/Df[2R]TW2, or *vls*^{PE}/*vls*^{PG} (Schüpbach and Wieschaus, 1986; Schüpbach and Wieschaus, 1989). *mago nashi* - *mago*^{1}/*mago*^{3}, or *mago*^{1}/Df[2R]*Pu*^{F36} (Boswell et al., 1991). *pumilio* - *pum*^{680}/*pum*^{680} (Lehmann and Nüsslein-Volhard, 1987b). Note that for technical reasons, the strongest available *pum*$^{-}$ combination, *In(3R)Msc/T(3,1)FC8* was not used (Barker et al., 1992). *torso* - *tor*^{XR1}/*tor*^{XR1} (Sprenger et al., 1989). *bicoid* - *bcd*^{E1}/*bcd*^{E1} (Frohnhöfer and Nüsslein-Volhard, 1986). *exuperantia* - *exu*^{PJ}/*exu*^{PJ} (Schüpbach and Wieschaus, 1986). *swallow* - *swa*^{14}/*swa*^{14} (Frohnhöfer and Nüsslein-Volhard, 1987). All laying cages for embryo collections were kept at 22°C, except for *mago* females, which were kept at 18°C, the restrictive temperature for abdomen formation (Boswell et al., 1991).

Whole-mount in situ hybridization

Whole-mount in situ hybridizations to embryos and ovaries were performed as described previously (Tautz and Pfeifle, 1989), with modifications as described (Ephrussi et al., 1991; Gavis and Lehmann, 1992). Antisense RNA probes were synthesized by in vitro transcription of a plasmid containing an intact *nos* cDNA (pN5; Wang and Lehmann, 1991) with T7 RNA polymerase.

Antibodies

The anti-nos antibody is directed against a 14 amino acid peptide which includes the C-terminal 13 residues of the predicted nos polypeptide (RLAKSSYYKQQMKV) (Wang and Lehmann, 1991) and an additional N-terminal cysteine to facilitate chemical coupling. The peptide was coupled to keyhole limpet hemocyanin (KLH) using the Imject kit according to the manufacturer's directions (Pierce). This KLH-peptide conjugate was injected into rabbits, using Freund's adjuvant. The resulting antiserum was used for

whole-mount staining of embryos or ovaries directly after preabsorption to 0-2 hour old wild-type embryos. Collection, preparation, and staining of embryos and ovaries were carried out as described previously (Gavis and Lehmann, 1992).

It should be noted here that *vls* mutant embryos are inefficiently devitellinized by the standard methanol protocol referred to above. This defect may be related to the cellularization defects seen in a large percentage of *vls* mutant embryos (Schüpbach and Wieschaus, 1986; Schüpbach and Wieschaus, 1989; Lehmann and Nüsslein-Volhard, 1991). Only a small percentage of *vls* mutant embryos are therefore recovered for staining. This poor recovery could possibly result in a bias in the population of stained embryos observed.

RNA analysis

Poly A^+ RNA was prepared from 0-2.5 hour oldembryos and analyzed by Northern blot hybridization as described previously (Wang and Lehmann, 1991). The blot was probed with random-primed labeled *nos* cDNA (a 1.7 kb SalI-EcoRI fragment) (Wang and Lehmann, 1991), or with an actin 5C probe as a control for loading (Fyrberg et al., 1983).

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REFERENCES

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

Berleth, T., Burri, M., Thoma, G., Bopp, D., Richstein, S., Frigerio, G., Noll, M. and Nüsslein-Volhard, C. (1988) The role of localization of *bicoid* RNA in organizing the anterior pattern of the *Drosophila* embryo. EMBO J. 7: 1749-1756.

Bier, E., Vaessin, H., Shepherd, S., Lee, K., McCall, K., Barbel, S., Ackerman, L., Carretto, R., Uemura, T., Grell, E., Jan, L.Y. and Jan, Y.N. (1989) Searching for pattern and mutation in the *Drosophila* genome with a P-*lacZ* vector. Genes Dev. 3: 1273-1287.

Boswell, R.E. and Mahowald, A.P. (1985) *tudor*, a gene required for assembly of the germ plasm in *Drosophila melanogaster*. Cell 43: 97-104.

Boswell, R.E., Prout, M.E. and Steichen, J.C. (1991) Mutations in a newly identified *Drosophila melanogaster* gene, *mago nashi*, disrupt germ cell formation of mirror-image symmetrical double abdomen embryos. Development 113: 373-384.

Campos-Ortega, J.A. and Hartenstein, V. (1985) "The embryonic development of Drosophila melanogaster". Heidelberg: Springer-Verlag.

Cheung, H.-K., Serano, T.L. and Cohen, R.S. (1992) Evidence for a highly selective RNA transport system and its role in establishing the dorsoventral axis of the *Drosophila* egg. Development 114: 653-661.

Driever, W. and Nüsslein-Volhard, C. (1988) The *bicoid* protein determines position in the Drosophila embryo in a concentration-dependent manner. Cell 54: 95-104.

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

Ephrussi, A.E., Dickinson, L.K. and Lehmann, R. (1991) *oskar* Organizes the Germ Plasm and Directs Localization of the Posterior Determinant *Nanos*. Cell 66: 37-50.

Frohnhöfer, H.G. and Nüsslein-Volhard, C. (1986) Organization of the anterior pattern in the *Drosophila* embryo by the maternal gene *bicoid*. Nature 324: 120-125.

Frohnhöfer, H.G. and Nüsslein-Volhard, C. (1987) Maternal genes required for the anterior localization of *bicoid* activity in the embryo of *Drosophila*. Genes Dev. 1: 880-890.

Fyrberg, E.A., Mahaffey, J.W., Bond, B.J. and Davidson, N. (1983) Transcripts of the six Drosophila actin genes accumulate in a stage-and tissue-specific manner. Cell 33: 115-123.

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

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

Hay, B., Jan, L.Y. and Jan, Y.N. (1988) A protein component of Drosophila polar granules is encoded by vasa and has extensive sequence similarity to ATP-dependent helicases. Cell 55: 577-587.

Hay, B., Jan, L.Y. and Jan, Y.N. (1990) Localization of *vasa*, a component of Drosophila polar granules, in maternal-effect mutants that alter embryonic anteroposterior polarity. Development 109: 425-433.

Hülskamp, M., Pfeifle, C. and Tautz, D. (1990) A morphogenic gradient of hunchback protein organizes the expression of the gap genes Krüppel and knirps in the early Drosophila embryo. Nature 346: 577-580.

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

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

Jongens, T.A., Hay, B., Jan, L.Y. and Jan, Y.N. (1992) The *germ cell-less* Gene Product: A Posteriorly Localized Component Necessary for Germ Cell Development in Drosophila. Cell 70: 569-584.

Kim-Ha, J., Smith, J.L. and Macdonald, P.M. (1991) oskar mRNA is localized to the posterior pole of the Drosophila oocyte. Cell 66: 23-35.

King, R.C. (1970) "Ovarian development in *Drosophila melanogaster*". New York: Academic Press.

Klingler, M., Erdelyi, M., Szabad, J. and Nüsslein-Volhard, C. (1988) Function of torso in determining the terminal anlagen of the Drosophila embryo. Nature 335: 275-277.

Lantz, V., Ambrosio, L. and Schedl, P. (1992) The *Drosophila orb* gene is predicted to encode sex-specific germline RNA-binding proteins and has localized transcripts in ovaries and early embryos. Development 115: 75-88.

Lasko, P.F. and Ashburner, M. (1988) The product of the Drosophila gene *vasa* is very similar to eukaryotic initiation factor-4A. Nature 335: 611-617.

Lasko, P.F. and Ashburner, M. (1990) Posterior localization of vasa protein correlates with, but is not sufficient for, pole cell development. Genes Dev. 4: 905-921.

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

Lehmann, R. and Nüsslein-Volhard, C. (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 Nüsslein-Volhard, C. (1987a) hunchback, a gene required for segmentation of an anterior and posterior region of the Drosophila embryo. Dev. Biol. 119: 402-417.

Lehmann, R. and Nüsslein-Volhard, C. (1987b) Involvement of the pumilio gene in the transport of an abdominal signal in the Drosophila embryo. Nature 329: 167-170.

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

Mahowald, A.P. and Kambysellis, M.P. (1980). Oogenesis. /In: "The Genetics and Biology of Drosophila,"/ Ashburner, M. and Wright, T.R.F. (ed). London: Academic Press, 141-224.

Manseau, L.J. and Schüpbach, T. (1989) cappuccino and spire: Two unique maternal-effect loci required for both the anteroposterior and dorsoventral patterns of the Drosophila embryo. Genes Dev. 3: 1437-1452.

Mohler, J. and Wieschaus, E.F. (1985) Bicaudal mutations of Drosophila melanogaster: alteration of blastoderm cell fate. Cold Spring Harbor Symp. Quant. Biol. 50: 105-111.

Mohler, J. and Wieschaus, E.F. (1986) Dominant maternal effect mutations of Drosophila melanogaster causing the production of double-abdomen embryos. Genetics 112: 808-822.

Nüsslein-Volhard, C. (1991) Determination of the embryonic axis of Drosophila. Development Supplmt 1: 1-10.

Nüsslein-Volhard, C., Frohnhöfer, H.G. and Lehmann, R. (1987) Determination of anteroposterior polarity in Drosophila. Science 238: 1675-1681.

Raff, J.W., Whitfield, W.G.F. and Glover, D.M. (1990) Two distinct mechanisms localise cyclin B transcripts in synctial *Drosophila* embryos. Development 110: 1-13.

Sander, K. and Lehmann, R. (1988) Drosophila nurse cells produce a posterior signal required for embryonic segmentation and polarity. Nature 335: 68-70.

Schüpbach, T. and Wieschaus, E. (1986) Maternal-effect mutations altering the anterior-posterior pattern of the *Drosophila* embryo. Roux's Arch. Dev. Biol. 195: 302-317.

Schüpbach, T. and Wieschaus, E. (1989) Female Sterile Mutations on the Second Chromosome of *Drosophila melanogaster*. I. Maternal Effect Mutations. Genetics 121: 101-117.

Smith, J.L., Wilson, J.E. and Macdonald, P.M. (1992) Overexpression of *oskar* Directs Ectopic Activation of *nanos* and Presumptive Pole Cell Formation in Drosophila Embryos. Cell 70: 849-859.

Sprenger, F., Stevens, L. and Nüsslein-Volhard, C. (1989) The Drosophila gene torso encodes a putative receptor tyrosine kinase. Nature 338: 478-483.

St. Johnston, D., Beuchle, D. and Nüsslein-Volhard, C. (1991) *staufen*, a gene required to localize maternal RNAs in the Drosophila egg. Cell 66: 51-63.

St. Johnston, D. and Nüsslein-Volhard, C. (1992) The Origin of Pattern and Polarity in the Drosophila Embryo. Cell 68: 201-219.

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

Struhl, G., Johnston, P. and Lawrence, P.A. (1992) Control of Drosophila Body Pattern by the hunchback Morphogen Gradient. Cell 69: 237-249.

Suter, B., Romberg, L.M. and Steward, R. (1989) *Bicaudal-D*, a *Drosophila* gene involved in developmental asymmetry: localized transcript accumulation in ovaries and sequence similarity to myosin heavy chain tail domains. Genes Dev. 3: 1957-1968.

Tautz, D. and Pfeifle, C. (1989) A non-radioactive in situ hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene *hunchback*. Chromosoma 98: 81-85.

Theurkauf, W.E., Smiley, S., Wong, M.L. and Alberts, B.M. (1992) Reorganization of the cytoskeleton during *Drosophila* oogenesis: implications for axis specification and intercellular transport. Development 115: 923-936.

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

Wharton, R.P. and Struhl, G. (1989) Structure of the Drosophila BicaudalD protein and its role in localizing the posterior determinant nanos. Cell 59: 881-892.

Wharton, R.P. and Struhl, G. (1991) RNA regulatory elements mediate control of Drosophila body pattern by the posterior morphogen *nanos*. Cell 67: 955-967.

Whitfield, W.G.F., Gonzalez, C., Sanchez-Herrero, E. and Glover, D.M. (1989) Transcripts of one of two *Drosophila* cyclin genes become localized in pole cells during embryogenesis. Nature 338: 337-340.

Figure 4.1. *Nanos* RNA and protein distribution in wild-type embryos

Fixed embryos were analyzed by whole-mount in situ hybridization to detect *nos* RNA (A-F) or stained with antibodies directed against nos protein (G-L) as described in Materials and Methods. A, G) cleavage stage (stage 1); B, H) pole bud formation (stage 3a); C, I) pole cell formation (syncytial blastoderm) (stage 3b); D, J) blastoderm (stage 4); E, K) late gastrula (stage 8); F, L) post-germ band retraction (stage 13). Note that after pole bud formation, nos protein is rapidly (1-2 nuclear cycles) degraded outside of the pole cells, implying that the nos protein has a relatively short half life. This short half life suggests a mechanism for the formation of the nos protein gradient. Stages are as described in Campos-Ortega and Hartenstein, 1985. Photos were taken at 200X magnification. Anterior to the left, dorsal up.

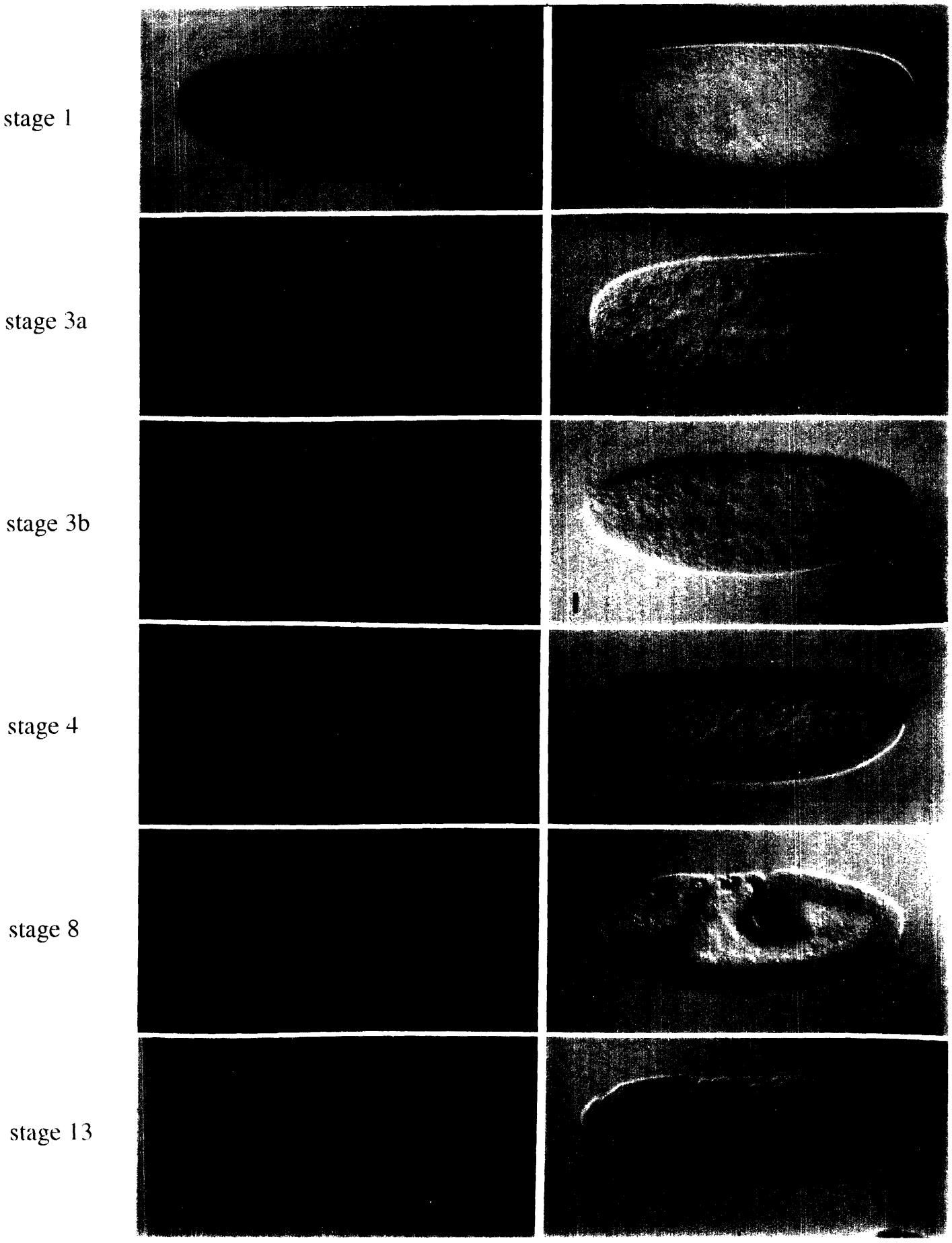
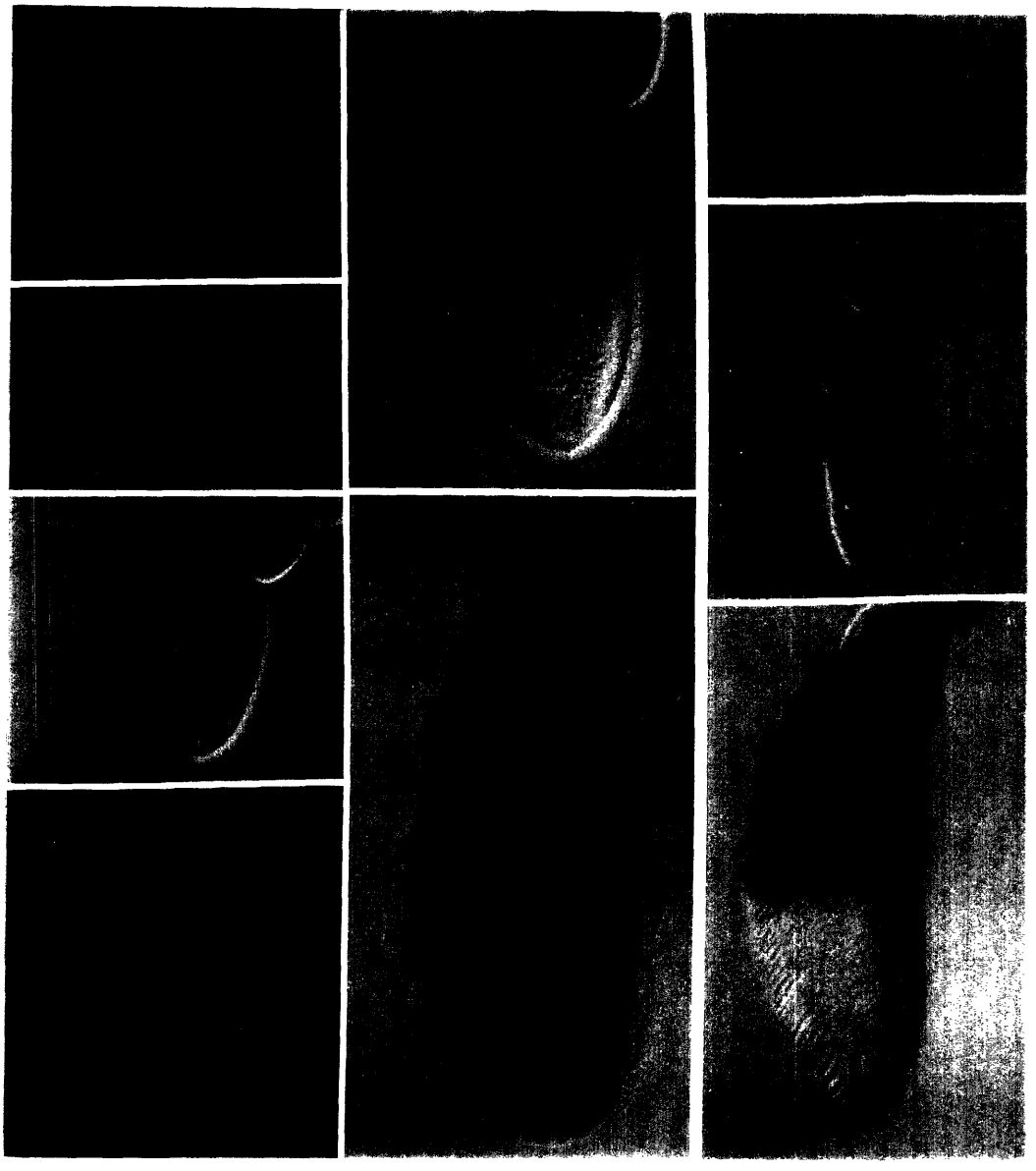

RNA
protein
stage 1
stage 3a
stage 3b
stage 4
stage 8
stage 13

Figure 4.2. *Nanos* RNA and protein distribution in wild-type ovaries

Ovaries dissected from wild-type females were analyzed by whole-mount in situ hybridization to detect *nos* RNA, or stained with antibodies directed against nos protein. Drosophila oogenesis is divided into previtellogenic and vitellogenic stages. In the three regions of the germarium (the previtellarium), stem cells divide 4 times without cytokinesis to form 16 cell clusters. One of these sixteen cells becomes the oocyte, while the other cells become polyploid nurse cells. The oocyte is always situated at one end (the posterior end) of the cluster. The entire cluster is surrounded by somatic follicle cells, which secrete the chorion and vitelline membrane. Vitellogenesis is divided into 14 stages. By stage 6, the oocyte is visibly larger than any of the other 15 cells. By stage 10, the oocyte and the nurse cell cluster are approximately equal in size. During stages 11-14, the chorion and vitelline membrane are secreted by the follicle cells, and the oocyte grows to its final size as the nurse cells degenerate. (See also King, 1970 and Mahowald and Kambysellis, 1980 for descriptions of oogenesis.) A) 200X magnification view; oogenetic stages are indicated for each panel. Anterior up, dorsal right. B) 640X magnification view; whole-mount germarium stained for nos protein. Diagram to right of panel shows a schematic presentation of the germarium, with the regions of the germarium labelled.

RNA protein



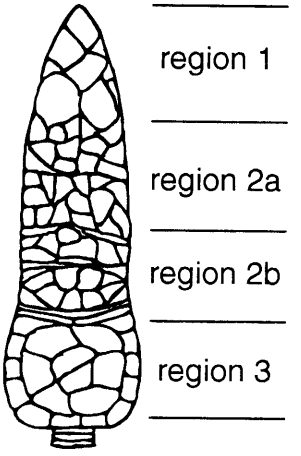

region 1
region 2a
region 2b
region 3

Table 1. Nanos localization in *nanos* mutants

Localization pattern in :

	Ovary (germarium)	Ovary (st. 10 nurse cells)	Embryos	
Allele	Protein	Protein	RNA	Protein
L7	normal	normal	normal	normal
R W	N. D.	N. D.	normal	normal
RC	none	none	none	none
RD	reduced	normal	normal	weak
B N	reduced	reduced	none	none

Ovaries derived from females mutant for various *nos* alleles were stained with antibodies against nos protein, and scored for *nos* expression both in the germarium and in stage 10 nurse cells (see Figure 2 for wild-type staining pattern). Cleavage stage embryos derived from females mutant for various alleles of *nos* were analyzed by whole-mount in situ hybridization to detect *nos* RNA, or stained with antibodies directed against nos protein. Entries in the table reflect the degree to which the various *nos* species are localized in the relevant genotypes (see Materials and Methods for genotype descriptions). For the ovary antibody stainings, "reduced" indicates that protein was detectable, but at lower amounts than in wild type. "Weak" indicates that levels of protein were not much above background staining. For the embryo antibody stainings, "weak" indicates that localized signal is readily detectable, but the localization is not as tight as in wild type. N. D., not determined.

Figure 4.3. *Nanos* localization in posterior group mutants

Cleavage stage embryos derived from females mutant for posterior group genes were analyzed by whole-mount in situ hybridization to detect *nos* RNA (left) or stained with antibodies directed against nos protein (right). A, H) wild type; B, I) $vasa^{PD}/vasa^{D1}$; C, J) $oskar^{301}/oskar^{54}$, 29°C; D, K) $oskar^{301}/oskar^{54}$, 20°C; E, L) $tudor^{WC8}$/Df[2R]Pu^{rP133}; F, M) $valois^{PE}$/Df[2R]TW2; G,N) *mago nashi*^{1}/*mago nashi*^{3}. In the cases of *tud*, *vls*, and *mago*, the localization patterns observed were variable, and the embryos shown in the figure do not necessarily represent the majority pattern (see text). The distributions of observed localization patterns were: *tudor* - 50% have reduced amounts of localized RNA as shown in the figure, 20% have even less localized RNA, and 30% show no visibly localized RNA (N= 112), 50% show gradients of nanos protein as in the figure, 30% show fainter gradients, and 20% show no detectable protein gradient (N= 94); *valois* - >90% of embryos show no localized RNA, <10% show reduced amounts of localized RNA as shown in the figure (N= 61), >90% show no protein gradient, <10% show a reduced gradient as shown in the figure (N= 58); *mago nashi* - 75% show no localized RNA, 25% show reduced amounts of localized RNA, or "dot" localization as shown in the figure (N= 42), 70% of embryos show no detectably localized protein, 30% of embryos show very faint traces of posteriorly localized protein as shown in the figure (N=101). Photos were taken at 200X magnification. Anterior to the left, dorsal up.

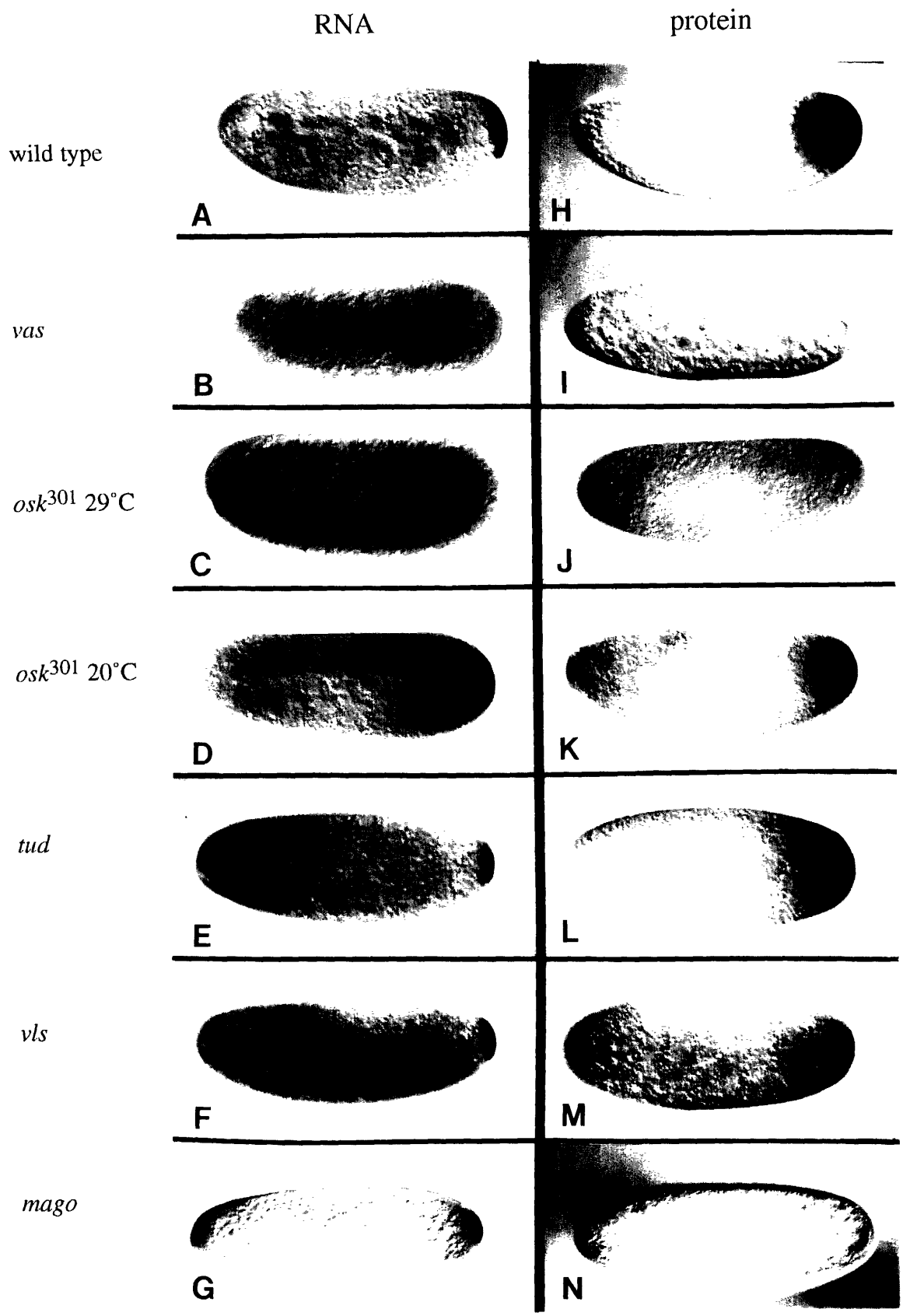

RNA
protein
wild type
A
H
vas
B
I
osk301 29°C
C
J
osk301 20°C
D
K
tud
E
L
vls
F
M
mago
G
N

Figure 4.4. *Nanos* RNA in posterior group mutants

Poly-A$^+$ RNA was prepared from cleavage-stage embryos derived from posterior group mutant females. Main panel shows *nos* transcript. Normal levels of *nos* RNA are present in all genotypes examined, except *nos*^{BN}. The lower panel shows the same blot reprobed for actin 5C, a constitutively expressed transcript (Fyrberg et al., 1983), as a loading control. Mutant genotypes analyzed were as follows: *mago*^{1}/Df[2R]F36; *capu*^{RK}*spir*^{035}/*capu*^{RK}*spir*^{035}; *stau*^{D3}/*stau*^{D3}; *osk*^{166}/*osk*^{166}; *vasa*^{D1}/*vasa*^{PD}; *tud*^{WC8}/*tud*^{WC8}; *val*^{PE}/Df[2R]TW2; *nos*^{L7}/*nos*^{L7}; *nos*^{BN}/*nos*^{BN}; *pum*^{680}/*pum*^{680}. Note that the *vas* mutant embryo lane is underloaded. Also note that the *pumilio* allele used was *pum*^{680} instead of the strongest available combination, *In(3R)Msc/T(3,1)FC8* (Barker et al., 1992). *In(3R)Msc/T(3,1)FC8* embryos contain localized nanos RNA and protein in patterns indistinguishable from wild-type embryos (Barker et al., 1992). We therefore conclude that *pum* function in abdominal segmentation lies downstream of *nos* translation, and anticipate that *In(3R)Msc/T(3,1)FC8* embryos contain normal levels of intact *nos* RNA.

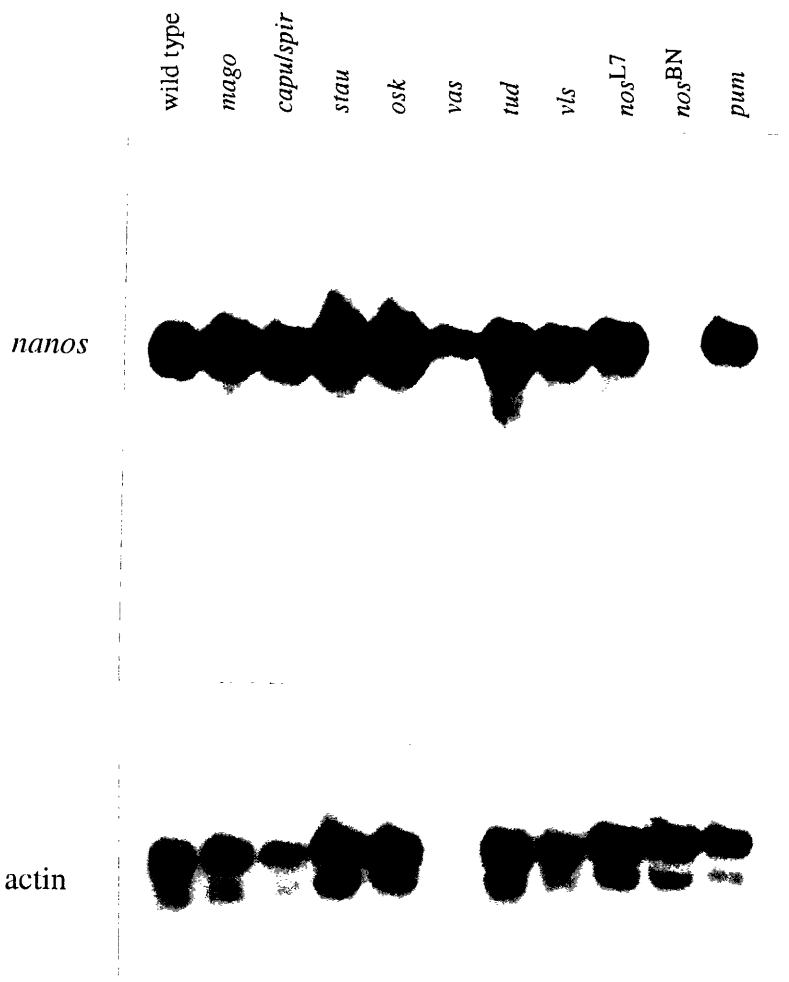
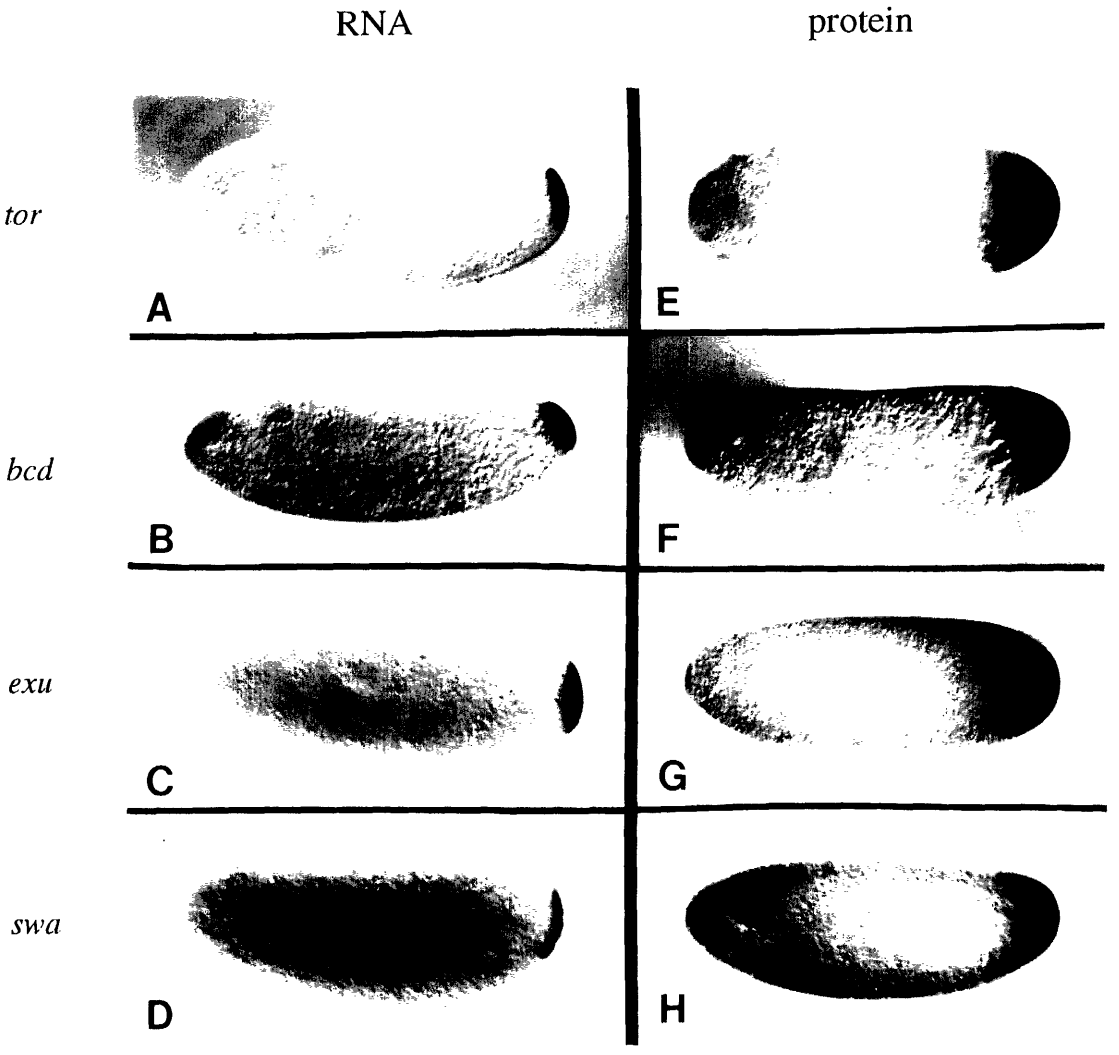

wild type
mago
capu/spir
stau
osk
vas
tud
vls
nosL7
nosBN
pum
nanos
actin

Figure 4.5. *Nanos* localization in other maternal-effect mutations

Cleavage stage embryos derived from females mutant for other maternal systems were fixed and analyzed by whole-mount in situ hybridization to detect *nos* RNA (left panels), or stained with antibodies directed against nanos protein (right panels). A, E) tor^{XR1}; B, F) bcd^{E1}; C, G) exu^{PJ}; D,H) swa^{4}. Photos taken at 200x magnification. Anterior to the left, dorsal up.


RNA
protein
tor
bcd
exu
swa
A
B
C
D
E
F
G
H

Chapter 5

nanos and translational control of maternal *hunchback*

INTRODUCTION

The Drosophila gene *nanos* (*nos*) acts as a posterior determinant whose RNA is localized to the posterior pole of early embryos (Wang and Lehmann, 1991). This localized RNA is the source for a nanos protein gradient which extends to about 50% egg length (0%=posterior pole) (Barker et al., 1992; Ephrussi and Lehmann, 1992; Smith et al., 1992). *nanos* mutant embryos completely lack abdominal segments, showing that *nos* is essential for abdomen formation(Lehmann and Nüsslein-Volhard, 1991). In terms of zygotic gene expression, abdominal segmentation requires the properly restricted expression of abdominal gap genes including *knirps* and *giant*. In the absence of *nos*, *knirps* and *giant* are not expressed in the prospective abdomen (Kraut and Levine, 1991a; Rothe et al., 1989). However, it has been shown that activation of gap gene expression by *nos* occurs by an indirect, double negative mechanism (Hülskamp et al., 1989; Irish et al., 1989; Struhl, 1989). *nanos* suppresses the function of an intermediary gene product, the maternal *hunchback* (*hb*) RNA. Hunchback protein represses transcription of the abdominal gap genes. In the posterior half of the embryo, therefore, nanos protein is present, hunchback protein is not translated from the maternal RNA, and *knirps* and *giant* are transcribed. In the absence of *nos* (for instance in the anterior of wild-type embryos, or throughout *nos* mutant embryos), maternal *hunchback* RNA is freely translated, and the hunchback protein suppresses abdominal gap gene expression.

Several lines of evidence indicate that this regulation of maternal *hunchback* is at the level of RNA translation. First, whole mount in situ hybridization analysis of wild-type embryos shows that *hb* RNA is initially distributed evenly throughout the embryo, whereas hb protein is expressed only in the anterior half of the embryo (Tautz and Pfeifle, 1989). Secondly, the *hb* RNA has been shown to contain sequence elements in its 3' untranslated region (3'UTR) which are both necessary and sufficient for *nos*-dependent translational regulation (Wharton and Struhl, 1991). These elements have been named NREs, for *nos* regulatory elements. This latter result indicates

that *nos*-dependent regulation of *hunchback* occurs at the RNA level, and not at the level of protein stability.

Overall this evidence suggests that *nos* acts as a translational repressor of the maternal hunchback RNA, and that this regulation is mediated by the NRE sequences in the mRNA. The *nos* open reading frame contains conserved C-terminal sequences related to sequences in retroviral nucleocapsid proteins which have been shown to bind zinc and package single stranded nucleic acid (Curtis et al., 1995; Schwabe and Klug, 1994). Indeed, preliminary evidence suggests that bacterially synthesized nanos protein is able to bind RNA in vitro (D. Curtis and P. Zamore, personal communication). Another approach to investigating the mechanism of *nos*-mediated regulation of *hb* translation is to recapitulate this regulation in an in vitro or a cell culture assay system.

None of these described experiments, however, address the more basic question of the manner in which *hunchback* translation is regulated. A direct way to examine translational control is to fractionate polysomes. Polysomes represent messages which are bound to multiple ribosomes, so a polysome-enriched fraction would be expected to contain mRNAs which are being actively translated. Conversely, mRNAs that are found predominantly in non-polysome (i.e. monosome or smaller) fractions are not being actively translated. Such a correlation between translational state and residence in a polysomal fraction would indicate that the transcript in question is regulated at the level of translational initiation. With these considerations in mind, we set out to examine the polysome distribution of maternal *hb* RNA in embryos derived from wild-type mothers, or from mothers that either lack or misexpress *nos*. In an adjunct experiment, we also examined the effect of *nos* on the polysome distribution of another maternal RNA which is also subject to *nos*-dependent translational control, the *bicoid* (*bcd*) mRNA. Finally, we outline a transgenic strategy designed to clarify the role of *nos* in mediating NRE-dependent translational regulation in embryos.

RESULTS AND DISCUSSION

Polysome fractionation of maternal *hunchback* RNA

The interpretation of polysome gradients derived from wild-type embryos can be somewhat unclear due to the presence of a mixed population of maternal *hunchback* RNA - in the anterior of the embryo there is no nanos protein and *hb* RNA is freely translated, whereas in the posterior half nanos protein suppresses the translation of *hb*. Fortunately *nos* mutant backgrounds are available which provide embryos constituting homogeneous sources of regulated maternal *hb* RNA. Specifically, embryos derived from homozygous nos^{BN} mothers completely lack detectable nos protein (Wang et al., 1994), and maternal *hb* RNA is translated throughout the embryo. To achieve expression of nanos protein throughout the embryo, transgenic females carrying the *nanos-bicoid* 3'UTR fusion transgene (hereafter referred to as *nos-bcd*) were used. This transgene contains a fusion of the nos open reading frame to the *bicoid* RNA localization sequence, resulting in anterior localization and expression of *nanos* (Gavis and Lehmann, 1992). Embryos derived from otherwise wild-type mothers carrying a single copy of the *nos-bcd* transgene contain high levels of nanos protein, due to synthesis of nos from the anteriorly localized transgenic *nanos/bicoid* 3'UTR RNA. In this latter case, maternal *hb* RNA translation is suppressed throughout the embryo.

Embryos aged 0.5-2.5 hours were chosen for the fractionation protocol, since this time window includes the period when the hunchback protein gradient is most distinct as shown by whole mount antibody staining (Tautz, 1988). A post-mitochondrial supernatant was prepared (see Materials and Methods), and sedimented over a 20-45% sucrose gradient. The gradient was collected and analyzed using a gradient analyzer in conjunction with a UV detector. Figure 5.1 shows a typical profile in which peaks corresponding to free ribosomal subunits, monosomes, and polysomes numbering from 2 to about 11 are readily detected. Fractions corresponding to free subunits, monosomes, low and high numbered polysomes were pooled into 5 fractions covering the entire gradient as indicated in the figure, and RNA was extracted for Northern blot hybridization analysis. The blots were probed for maternal

hb and actin 5C transcripts. The actin signal serves as a control, since actin translation (and translation in general) are not expected to be regulated by *nos*. Data was collected on a phosphorimager , and the signal of each band was quantitated and is displayed in Figure 5.2 as the percentage of the summed signal for all the fractions.

The results show that there is a small but detectable shift in the distribution of maternal *hb* RNA on polysomes. Specifically, a comparison of fraction 2 shows that about 20% of the maternal *hb* RNA of wild-type (Figure 5.2A) and *nos-bcd* (Figure 5.2C) embryos is found in this monosome-containing fraction (with *nos-bcd* embryos containing a slightly higher percentage), whereas only 12% of the *hunchback* in nos^{BN} embryos is found in this fraction (Figure 5.2B). This result suggests that in the absence of *nos*, a smaller portion of *hb* RNA is found in the monosomes, which are relatively inactive for translation. Conversely, examination of fraction 4 (the polysome-containing fraction) shows that in both wild-type and nos^{BN} embryos, about 40% of the *hunchback* RNA is in this fraction, but in *nos-bcd* embryos that figure is reduced to approximately 30%. This result suggests that when *nanos* is overexpressed, the proportion of maternal *hunchback* RNA present on polysomes is reduced. As an internal control, these blots were reprobed for the actin 5C transcript. Examination of the actin signals in the various backgrouinds studied shows that the distribution of actin transcripts on polysomes is unaffected. This indicates that *nos* does not affect translation at a global, or housekeeping, level. As an additional control, preparation of RNA from unfractionated post-mitochondrial extract shows that equivalent amounts of *hunchback*, actin, *bicoid* and *nanos* mRNAs are recovered in the various *nanos* backgrounds (data not shown). This latter control shows that changing *nanos* dosage does not result in a large-scale sequestration of transcripts into fractions that pellet at low speed (i.e. transcript association with the cytoskeleton).

In sum, these results are consistent with the hypothesis that *nos* regulates maternal *hb* at the level of translational initiation. In the absence of *nos*, less *hb* transcript is found associated with monosomes, implying that the RNA is enriched in larger complexes. Accordingly, more hb protein is present in this circumstance. When *nos* is overexpressed, less *hb* mRNA is

found associated with polysomes, implying that the RNA is shifted to smaller complexes. In this case, less hb protein is present. Since in this case enrichment of mRNA in monosome fractions corresponds to low levels of protein synthesis, and enrichment of mRNA in polysomes corresponds to high levels of protein synthesis, we may tentatively conclude nos interferes with the initiation of maternal hunchback translation. Regulation of the rate of translational elongation may be ruled out, since if this was the case enrichment of transcript in the polysome fraction should lead to decreased protein synthesis.

Polysome fractionation of *bicoid* RNA

The translation of *bicoid* RNA was examined by reprobing the same Northern blots with a *bicoid* probe. Although the *bcd* mRNA and nanos protein are normally found at opposite ends of the wild-type embryo, *bcd* RNA is subject to translational repression by *nos*. In circumstances where *nos* is present at the anterior of embryos, translation of the *bcd* mRNA is undetectable (Gavis and Lehmann, 1992). Accordingly, we examined the polysome distribution of *bcd* mRNA in wild-type and nos^{BN} embryos, where *bcd* mRNA is translated normally, as well as in *nos-bcd* embryos, where *bcd* mRNA translation is repressed. Figure 5.3 shows that *nos* does not detectably affect the distribution of *bcd* mRNA on polysomes. Specifically, the profile of *bcd* mRNA in *nos-bcd* embryos (Figure 5.3C) is not significantly different from the profile in wild-type (Figure 5.3A) or nos^{BN} (Figure 5.3B) embryos. This result constitutes a difference between *nos*-mediated regulation of the *hb* and *bcd* RNAs, and suggests that translational repression of the two transcripts might occur by differing mechanisms.

Independent evidence has also shown differences in the regulation of these transcripts. For example, the translationally repressed *bcd* mRNA has a significantly shorter poly-A tail than the actively translated form of the mRNA (Wharton and Struhl, 1991). However no such difference has been found for the regulated maternal *hb* RNA (Wharton and Struhl, 1991), although this latter result cannot be definitive due to technical limitations (e.g. the higher abundance of zygotic *hb* mRNA interferes with detection of

the maternal transcript). In addition, the *bcd* 3'UTR contains NRE-like sequences which are necessary for regulation by *nanos*, but do not fully functionally substitute for *hb* NREs (Wharton and Struhl, 1991). Also, although *nos* regulation of *hb* is strictly dependent on the NRE sequences (Wharton and Struhl, 1991), preliminary evidence suggests that *bcd* sequences outside of the NREs can confer *nos*-mediated translational regulation (E. Gavis, personal communication). Specifically, when *nos* is mislocalized to the anterior (*nos-bcd*), the resulting head defect cannot be suppressed by one copy of a transgene encoding a *bcd* transcript which lacks the NRE sequences. However, two copies of this *bcd* ΔNRE transgene do suppress the head defects of *nos-bcd* (Simpson-Brose et al., 1994). This suggests that some aspect of *nos*-mediated suppression of *bcd* translation may be directed by sequences outside of the *bcd* NRE, but this effect is weak. Thus it is plausible that translational repression by *nos* of the *hb* and *bcd* maternal RNAs may occur by differing mechanisms, and that these differences account for the failure to detect altered polysome distribution of the *bcd* mRNA. An alternative postulate is that *nos* regulation affects the sequestration or packaging of mRNAs into ribonucleoprotein particles. In the case of maternal *hb*, the sucrose gradient conditions employed resolve the *nos*-repressed versus the translatable forms of the mRNA. However, these gradient or buffer conditions do not resolve the analogous forms of the *bcd* mRNA. Further investigation using varying buffer and or fractionation conditions should clarify this issue.

Transgenic analysis

While early results with detection of the endogenous maternal *hb* transcript on polysomes were encouraging, the polysome assay in this form was somewhat problematic. First, unambiguous detection of the relatively unabundant maternal *hb* transcript is made difficult by the complicated transcriptional control of the *hb* gene. *Hb* has two promoters, one (termed P1) is primarily maternal, while the other (P2) is zygotically active and provides the gap gene function of *hb* (Schröder et al., 1988). Although specific probes can distinguish P1 versus P2 transcripts, the P1 promoter undergoes a second, zygotic mode of expression in a posterior stripe at about 3-3.5 hours of development (division cycle 14, cellular blastoderm stage). Thus in the

previous experiments a P1-specific probe will detect not only the maternal transcript, but also this "posterior stripe" expression in incorrectly staged embryos, confusing the interpretation. Another problem with the previous experimental design is the inability to accurately quantitate levels of synthesized hunchback protein. Whole mount staining of embryos does not give quantitative data, and interpretation of Western blots is severely complicated by the expression of zygotic hunchback protein which is indistinguishable from the maternal. Accurate quantitation of levels of the translationally regulated protein is especially important in light of the relatively low magnitude of the mRNA polysome shifts that were observed.

In order to simplify the interpretation of the polysome fractionation experiments, a transgenic reporter construct was designed which should offer unambiguous identification of the regulated RNA. This reporter construct contains the entire *hb* 3'UTR sequence, including the two NREs which have been shown to confer *nos*-dependent translational regulation to a heterologous transcript (Wharton and Struhl, 1991). Regulation of this transgenic message should thus authentically reflect *nos*-mediated translational control in vivo. The transgene itself consists of the 5' end of *nos* including a functional promoter and a short (nonfunctional) region of the open reading frame fused in-frame to the *Aequoria victoria* green fluorescent protein (GFP) coding sequence, attached to the *hb* 3'UTR and downstream region (see Figure 5.4). The *nos* promoter should provide strictly maternal expression, and the GFP sequence can be unambiguously identified as an RNA in Drosophila extracts. In addition, the intrinsic fluorescent properties of GFP allow for easy detection and quantification (Chalfie et al., 1994; Inouye and Tsuji, 1994). Transformant lines can be easily checked quite easily for proper regulation of the reporter by confocal microscopic examination of living embryos.

At present, these experiments are in progress and multiple stable transformant lines have been obtained which apparently express the transgene. After confocal microscopy has established that the chimeric transcript is properly translationally regulated (see above), the transgene will be crossed into *nos* loss- and gain-of-function backgrounds. Polysome analysis will be carried out on the resulting mutant embryos to test if the

reporter RNA varies in its abundance among the gradient fractions in a fashion similar to the endogenous maternal *hunchback* transcript. Concomitantly, the levels of the green fluorescent reporter protein, which presumably reflect the efficiency of translation of the reporter RNA, will be accurately quantitated in crude extracts by simple fluorimetry. In addition, a version of the construct which is identical in all respects, but lacks the NREs, will be examined using polysome gradients. This RNA should be translated in a *nos*-independent fashion, and its polysome profile will presumably represent a transgenic RNA which is being actively translated in embryos. It is hoped that these transgenic experiments will provide quantitative proof that *nos* suppresses the initiation of *hb* mRNA translation via an NRE-dependent mechanism.

MATERIALS AND METHODS

Polysome fractionation

Embryos staged 0.5-2.5 hour after egg deposition were collected, washed, and dechorionated as previously described (Lehmann and Nüsslein-Volhard, 1986). For mutant genotypes, embryos were collected in batches, dechorionated, then frozen and stored at -80 °C. A crude extract was prepared by homogenization using a hand pestle in 2 volumes of the following lysis buffer (0.5 M NaCl, 25 mM MgOAc, 50 mM Tris HCl pH 7.5, 0.2% Triton X-100, 2 mg/mL heparin, 1 µg/mL cycloheximide). This extract was spun in an Eppendorf microfuge for 10 minutes at 10,000 rpm (12,000 g). The resulting post-mitochondrial supernatant was collected carefully and loaded onto a 20-45% sucrose gradient prepared in gradient buffer (0.25 M NaCl, 25 mM MgOAc, 50 mM TrisHCl pH 7.5). For 12 mL gradients, approximately 35 A_{260} units were loaded. The gradients were spun for 1.5 hours at 41,000 rpm in a Beckman SW41Ti rotor at 4 °C. Fractions were collected using an ISCO gradient analyzer, and elution was monitored via an ISCO UA-5 absorbance detector at 254 nm. All biochemical reagents were obtained from Sigma.

Preparation and analysis of RNA

Appropriate fractions were pooled, and RNA was prepared by the following method. SDS, EDTA and proteinase K (Boehringer Mannheim) stock solutions were added to each pool to bring the final concentrations to 1%, 10 mM, and 150 μg/mL, respectively. Digestions were carried out for 30 minutes at 25 °C after which the RNA was precipitated by addition of 3 volumes of EtOH. After resuspension in DEPC-treated ddH2O, the RNA was analyzed by Northern blot hybridization as described previously (Wang and Lehmann, 1991).

In order to obtain a hybridization probe specific for the maternal *hunchback* transcript, a full length cDNA (hbm1) corresponding to transcript from the *hunchback* P1 promoter was isolated from the Nick Brown 4-8 hour plasmid cDNA library (Brown and Kafatos, 1988) by standard methods (Sambrook et al., 1989). A 550 bp HindIII/XbaI fragment from this cDNA was labelled as the probe for maternal *hunchback*. The other probes (*bicoid*, actin, and *nanos*) have been previously described (Wang and Lehmann, 1991). The resulting hybridization signals were analyzed and quantitated by phosphorimager analysis (Fuji). Each signal was expressed as the percentage of the sum of the signals from all fractions. Data was pooled from two independent trials.

Mutant strains

The wild-type strain used was Canton-S. The nos^{BN} and *nanos-bicoid* 3-UTR (i.e. *nos-bcd*) strains have been described previously (Gavis and Lehmann, 1992; Wang et al., 1994).

P-element construction and transformation

In order to construct the plasmids pDM30/nostGFP+NRE and pDM30/nostGFPΔNRE, first a version of the 5' end of the *nanos* gene was constructed which contains a hemagglutinin epitope tag cloned in-frame just upstream of the *nanos* open reading frame. This "epitope tag" should allow detection of the transgenic protein (Kolodziej and Young, 1991). The tagged nanos fragment was excised from the transformation plasmid pDM30-5'tag

(D. Curtis, personal communication) as a 2.0 kilobase (kb) HindIII fragment, which was subcloned into Bluescript (Stratagene) to yield the intermediate plasmid pCW9tag. The in-frame *nanos*-GFP fusion was achieved by digesting pCW9tag to completion with HindIII and NlaIV, which cuts early in the *nanos* open reading frame. The insert fragment,derived from the GFP cDNA-containing plasmid TU#65 (Chalfie et al., 1994), was prepared as a 0.75 kb blunt/EcoRI GFP-containing fragment by digestion with AgeI, treatment with Klenow fragment, then secondary digestion with EcoRI. The two fragments were ligated directionally into a HindIII/EcoRI digested Bluescript vector to create another intermediate clone, pBSnostag/GFP. The resulting fusion contains the first 58 amino acids of the *nanos* open reading frame, and introduces 4 extra amino acids (proline-valine-glutamatic acid-lysine) upstream of the GFP starting methionine. The junction region was checked by DNA sequencing. The *hunchback* 3'UTR fragment was reconstructed by ligating the DdeI/XbaI 150 base pair NRE-containing fragment (Wharton and Struhl, 1991) which had been isolated as the subclone pSP72DX (D. Curtis, personal communication) back to the 1.6 kb XbaI fragment from the downstream genomic region of *hunchback* (Tautz et al., 1987). In the process of cloning a portion of polylinker sequence from the plasmid pSP72 (EcoRV to XbaI) (Promega) was inadvertently retained at the internal XbaI junction. For the ΔNRE version, the 1.6 kilobase XbaI fragment itself was used. In both cases, the *hunchback* fragments were cloned between the BglII and XbaI sites of pSP72. The pBSnostag/GFP plasmid was then prepared by digestion with XbaI, Klenow treatment, then secondary digestion with BamHI. The *hunchback* sequences were then subcloned in as BglII/PvuII or BamHI/PvuII fragments for the NRE and ΔNRE versions, respectively to create the plasmids pBS nostag/GFP+NRE and pBS nostag/GFPΔNRE. The inserts were finally subcloned as unique SalI fragments into the ry^+ marked P-element transformation vector pDM30 (Mismer and Rubin, 1987). Injections were performed as previously described (Gavis and Lehmann, 1992).

REFERENCES

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

Brown, N. H., and Kafatos, F. C. (1988). Functional cDNA libraries from Drosophila embryos. Journal of Molecular Biology *203*, 425-437.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W., and Prasher, D. C. (1994). Green fluorescent protein as a marker for gene expression. Science *263*, 802-805.

Curtis, D., Apfeld, J., and Lehmann, R. (1995). *nanos* is an evolutionarily conserved organizer of anterior-posterior polarity. Development *in press*,

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

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

Hülskamp, M., Schröder, C., Pfeifle, C., Jäckle, H., and Tautz, D. (1989). Posterior segmentation of the *Drosophila* embryo in the absence of a maternal posterior organizer gene. Nature *338*, 629-632.

Inouye, S., and Tsuji, F. I. (1994). Aequorea green fluorescent protein, expression of the gene and fluorescence characteristics of the recombinant protein. FEBS Letters *341*, 277-280.

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

Kolodziej, P. A., and Young, R. A. (1991). Epitope tagging and protein surveillance. Meth. Enzymol. *194*, 508-519.

Kraut, R., and Levine, M. (1991a). Spatial regulation of the gap gene giant during development. Development *111*, 601-609.

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

Mismer, D., and Rubin, G. M. (1987). Analysis of the promoter of the *nina E* opsin gene in *Drosophila melanogaster*. Genetics *116*, 565-578.

Rothe, M., Nauber, U., and Jäckle, H. (1989). Three hormone receptor-like *Drosophila* genes encode an identical DNA-binding finger. The European Molecular Biology Organization Journal *8*, 3087-3094.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular cloning: a laboratory manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Schröder, C., Tautz, D., Seifert, E., and Jäckle, H. (1988). Differential regulation of the two transcripts from the Drosophila gap segmentation gene hunchback. The European Molecular Biology Organization Journal *7*, 2881-2887.

Schwabe, J. W. R., and Klug, A. (1994). Zinc mining for protein domains. Nature Structural Biol *1*, 345-349.

Simpson-Brose, M., Treisman, J., and Desplan, C. (1994). Synergy between the hunchback and bicoid morphogens is required for anterior patterning in Drosophila. Cell *78*, 855-865.

Smith, J. L., Wilson, J. E., and Macdonald, P. M. (1992). Overexpression of *oskar* directs ectopic activation of *nanos* and presumptive pole cell formation in Drosophila embryos. Cell *70*, 849-859.

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

Tautz, D. (1988). Regulation of the *Drosophila* segmentation gene *hunchback* by two maternal morphogenetic centres. Nature *332*, 281-284.

Tautz, D., Lehmann, R., Schnurch, H., Schuh, R., Seifert, E., Kienlin, A., Jones, K., and Jäckle, H. (1987). Finger protein of novel structure encoded by *hunchback*, a second member of the gap class of Drosophila segmentation genes. Nature *327*, 383-389.

Tautz, D., and Pfeifle, C. (1989). A non-radioactive in situ hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene *hunchback*. Chromosoma *98*, 81-85.

Wang, C., Dickinson, L. K., and Lehmann, R. (1994). Genetics of *nanos* localization in *Drosophila*. Developmental Dynamics *199*, 103-115.

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

Wharton, R. P., and Struhl, G. (1991). RNA regulatory elements mediate control of Drosophila body pattern by the posterior morphogen *nanos*. Cell *67*, 955-967.

Figure 5.1. A typical polysome profile

A post-mitochdrial extract from wild-type embryos was prepared and polysomes were fractionated on a 20-45% sucrose gradient as described in Materials and Methods. Elution of the gradient is plotted with the Y-axis showing UV absorption at 254 nm. The double hash mark indicates a 5-fold change in scale necessary to detect the less abundant polysome fractions. The inferred content of portions of the gradient, as well as the composition of the pooled fractions, is indicated along the bottom of the figure. The top, lighter portion of the gradient is towards the left of the figure.

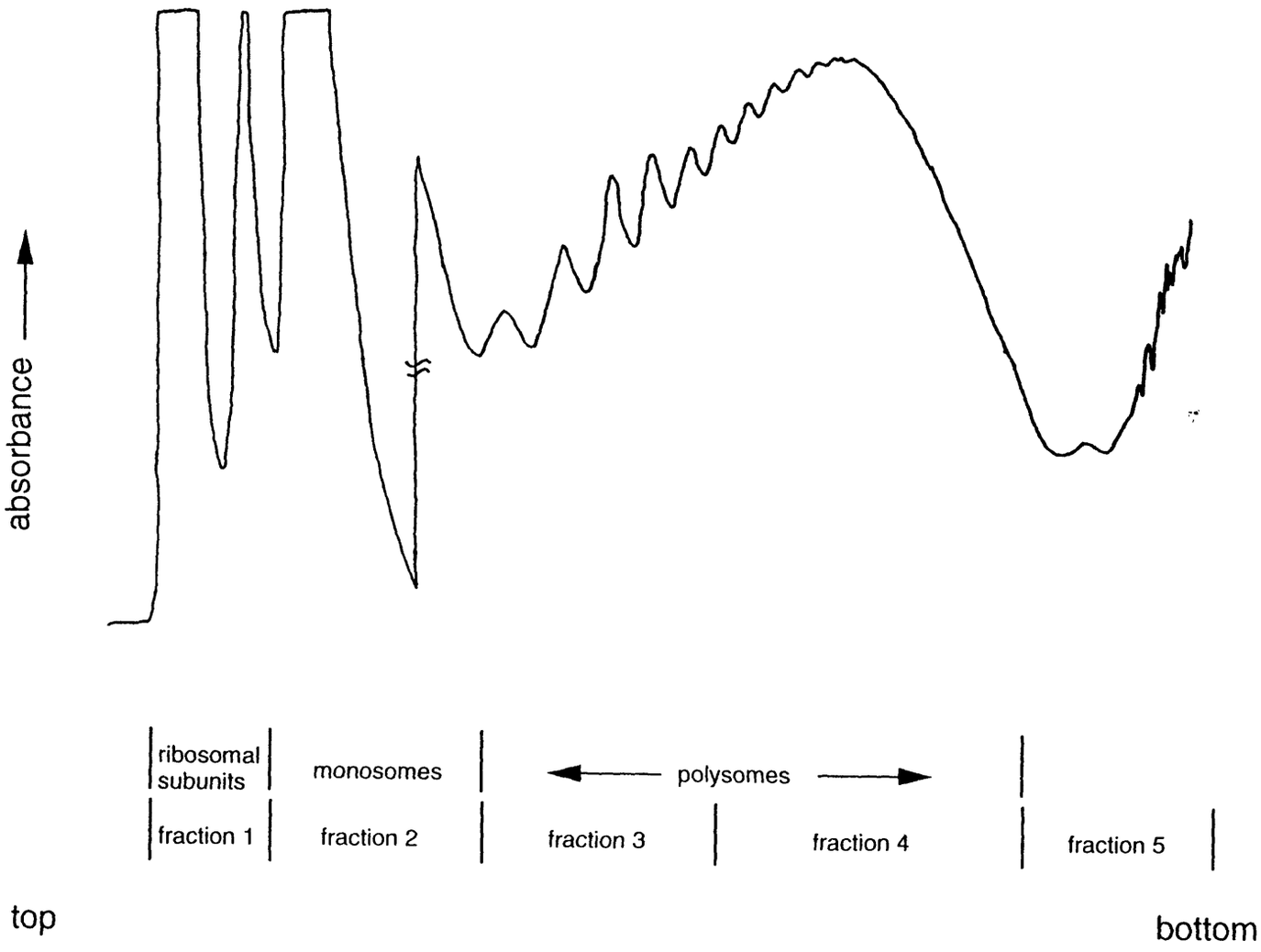
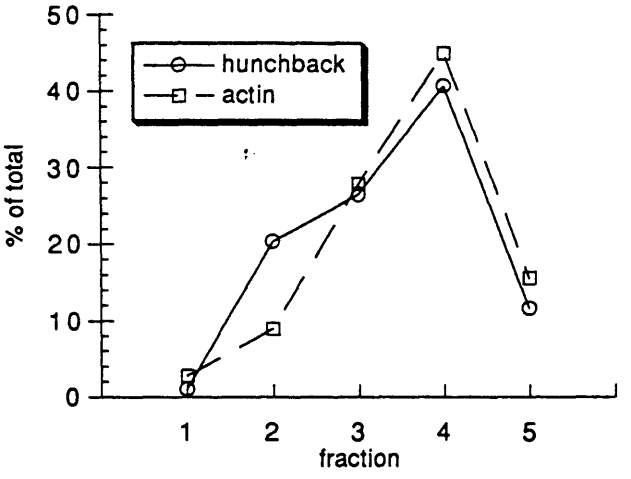

absorbance
ribosomal subunits
monosomes
polysomes
fraction 1
fraction 2
fraction 3
fraction 4
fraction 5
top
bottom

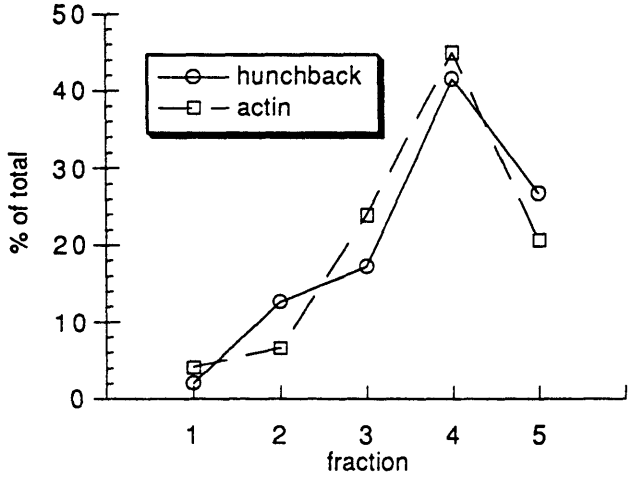
Figure 5.2. Nanos-dependent polysome distribution of maternal hunchback RNA

Polysome gradients were prepared from embryos derived from wild-type, *nos*^{BN}, and *nos-bicoid* mothers. Fractions were collected as indicated in the previous figure and RNA was prepared and analyzed as described in Materials and Methods. The blots were probed for maternal *hunchback* and actin transcripts, and the quantified hybridization signals are plotted as a percentage of the total signal summed across all the fractions. A.) Wild-type embryo extract. B.) Extract of embryos derived from mothers homozygous for the *nos*^{BN} allele. C.) Extract of embryos derived from mothers carrying the *nos-bcd* transgene.

wild-type



nos^{BN}



nos-bcd

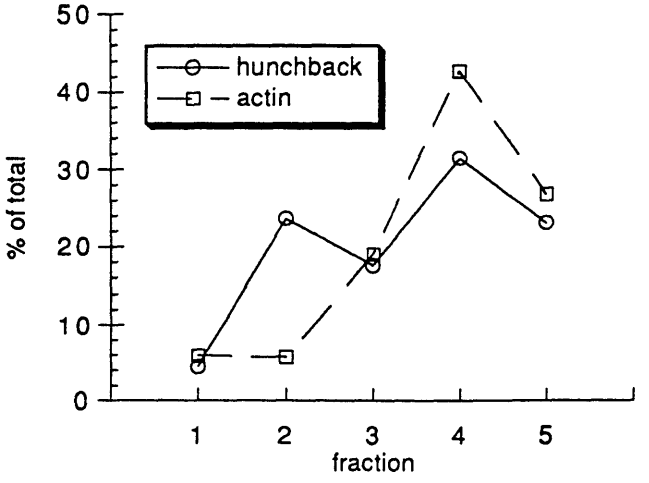


Figure 5.3. Nanos-dependent polysome distribution of bicoid RNA

The same RNA blots as in the previous figure were reprobed for the *bicoid* mRNA. A.) Wild-type embryo extract. B.) Extract of embryos derived from mothers homozygous for the nos^{BN} allele. C.) Extract of embryos derived from mothers carrying the *nos-bcd* transgene.

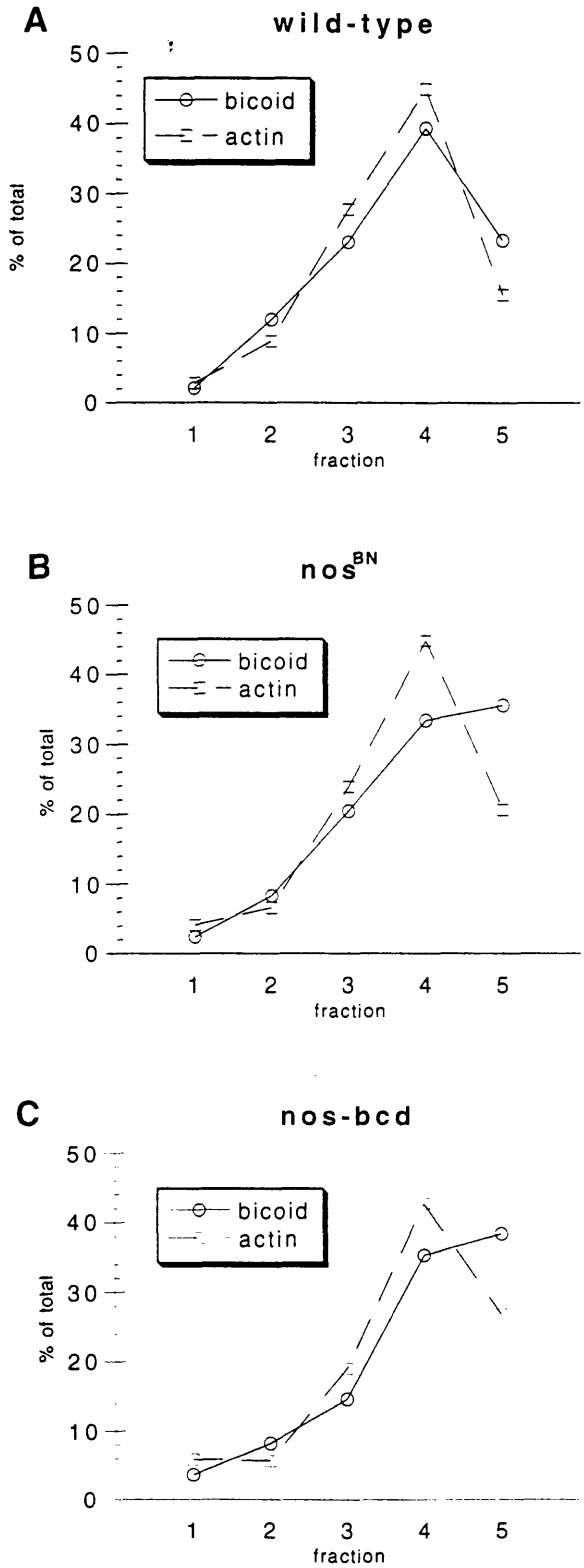
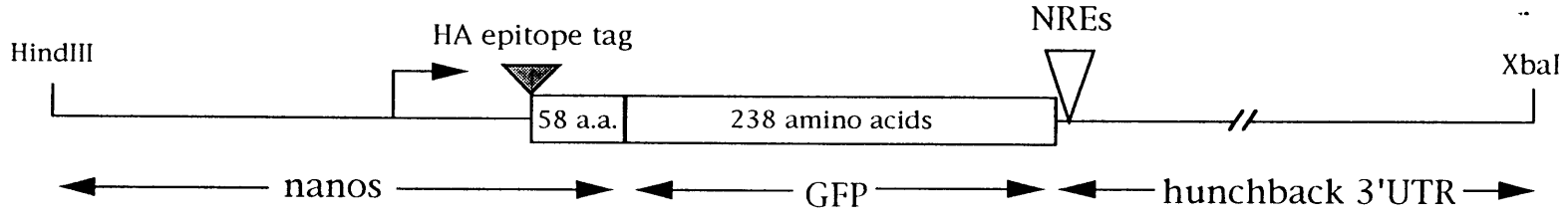

A
wild-type
bicoid
actin
% of total
fraction
B
nos^BN
bicoid
actin
% of total
fraction
C
nos-bcd
bicoid
actin
% of total
fraction

Figure 5.4. A transgenic GFP-containing reporter construct

A schematic diagram indicating the structure of the *nanos* promoter/green fluorescent protein/*hunchback* 3'UTR transgenic reporter construct. The restriction endonuclease sites bounding the construct are indicated. The *nanos* transcriptional start site is denoted by the arrow, and the positions of the hemagglutinin epitope tag and the NRE *nanos* regulatory element sequences are indicated with triangles. The boxes represent coding sequences, with the number of encoded amino acids indicated. The captions underneath indicate the extent of the denoted sequence regions. Drawing is made to scale.


HindIII
HA epitope tag
NREs
XbaI
58 a.a.
238 amino acids
nanos
GFP
hunchback 3'UTR

Chapter 6

Conclusions and future prospects

The studies described in this thesis have shown that *nanos*, a maternal-effect Drosophila gene required for abdomen specification, encodes an RNA which is localized to the posterior pole of the embryo. This localized source of RNA is translated to form a posterior-to-anterior protein gradient. *nanos* is one of a family of maternal-effect posterior group genes, all of which are required for abdomen formation. However, the majority of the posterior group genes act to specify abdomen solely through ensuring the proper localization and translation of *nanos*. Mutations in these genes disrupt localization of the *nanos* RNA, suggesting that they may encode components of the localization machinery. Indeed, the observation that oskar protein localization precedes *nanos* RNA localization in all situations thus far examined, including ectopic localization to the anterior, strongly suggests that oskar protein itself directs the localization of *nanos* RNA to the posterior pole (Ephrussi and Lehmann, 1992). The observation that nos protein translation varies with the dosage of *oskar* further suggests that oskar protein also acts to promote the translation of *nanos* (Ephrussi and Lehmann, 1992; Smith et al., 1992). In addition, unlocalized *nanos* RNA is not translated (Gavis and Lehmann, 1994). These results indicate that oskar protein nucleates the formation of a ribonucleoprotein complex including *nanos* RNA, factors necessary for its translation, and components of the poleplasm.

With the exception of *pumilio* and *nanos* itself, the numerous genes of the posterior group all constitute attractive potential components of the posterior RNA localization machinery. The additional phenotypes of several of these genes (*cappuccino, spire, valois, mago nashi, pipsqueak,* and *orb*) suggest a more general role in the localization process (Boswell et al., 1991; Christerson and McKearin, 1994; Lantz et al., 1994; Manseau and Schüpbach, 1989; Schüpbach and Wieschaus, 1986a; Siegel et al., 1993). However, a few genes, including *staufen, oskar, vasa,* and *tudor,* encode RNAs or proteins enriched or localized at the posterior pole, and thus may play a more specific role (Bardsley et al., 1993; Ephrussi et al., 1991; Hay et al., 1988; Kim-Ha et al., 1991; Lasko and Ashburner, 1990; St. Johnston et al., 1991). *Staufen* encodes a double-stranded RNA binding protein, and associates with injected RNAs in embryos, while *vasa* encodes an RNA helicase related to eIF-4A (Ferrandon et

al., 1994; Liang et al., 1994). However, as of yet, none of these factors has been shown to interact directly with any other localized component. This suggests that several factors in the localization complex remain to be identified.

An alternative approach to understanding *nanos* RNA localization is the dissection of the *nanos* 3'UTR to identify the sequence elements which specify localization. However, similar studies of *oskar* and *bicoid* localization signals have identified extensive redundant elements, and have not generally shed much light on the actual mechanism of RNA localization (Kim-Ha et al., 1993; Macdonald, 1990; Macdonald et al., 1993). Ultimately, the RNA localization apparatus will involve the cytoskeleton, which is presumably used as a framework for translocating and tethering RNAs. Indeed microtubules have been implicated in the localization of *bicoid* and *oskar* RNAs during oogenesis (Clark et al., 1994; Pokrywka and Stephenson, 1991). Finally, an exciting new area of study may be the translational control of *nanos* itself, and the link between RNA localization and translational activation. The specific cis-elements required for repression of unlocalized or activation of localized *nanos* RNA translation remain unidentified. As for trans-acting factors, proteins like the newly identified bruno (Kim-Ha et al., 1995) may mediate translational repression, while the RNA-binding proteins vasa and staufen may play a role in translational activation. Although activation of *nanos* translation at egg activation does not involve polyadenylation (Sallés et al., 1994), it remains to be seen whether adenylation state plays a role in the translational inactivation of unlocalized *nanos* RNA.

nanos specifies abdomen by acting in conjunction with *pumilio* to repress translation of the maternal *hunchback* RNA. *nanos*-dependent translational repression is mediated through a pair of bipartite sequence elements in the *hunchback* 3'UTR, the *nanos* response elements (NREs) (Wharton and Struhl, 1991). Recently it has been shown that pumilio protein, as well as a 55kD protein found in embryo extracts, bind directly to NRE sequences (Murata and Wharton, 1995). Since these factors bind even in the absence of *nanos*, it is possible that they act as targeting factors which promote a direct or indirect association of nanos protein with the *hunchback* mRNA. *nanos* then somehow acts to repress translation of this RNA. Clearly we need to understand more about the mechanism of *nanos* function.

One approach is detailed study of the nanos protein. A saturation mutagenesis of the nanos polypeptide is currently in progress. This screen utilizes a transgene which maternally expresses *nanos* in an unlocalized fashion in embryos, resulting in a dominant female sterile phenotype. Mutated females carrying this transgene will produce viable progeny only if the transgenic copy of nanos has lost its function (G. Arrizabalaga, personal communication). This screen therefore comprises a powerful saturation screen for additional *nanos* alleles. A precise definition of functional regions of the nanos polypeptide can only facilitate the search for a biochemical function for the protein.

Other approaches to understanding the mechanism of *nanos*-mediated translational repression include attempts to demonstrate the formation of specific ribonucleoprotein complexes containing nanos, pumilio, and/or 55kD proteins, and *hunchback* RNA. These studies can be carried out in wild-type embryo extracts, in extracts from embryos expressing transgenes designed to facilitate the detection of specific species, or in partially purified extracts. Also, polysome fractionation analysis as described in this thesis should offer not only an basic understanding of the mechanism by which *nanos* represses *hunchback* translation, but may also provide a tool for isolating translational regulatory complexes. There is no doubt that the study of *nanos*-mediated translational control would greatly benefit from the development of an in vitro system which recapitulates *nanos*-dependent repression of NRE-containing transcripts. Recently, sequence-specific translational regulation has been demonstrated for the 15-lipoxygenase (Lox) RNA (Ostareck-Lederer et al., 1994). Translation of Lox RNA in a reticulocyte lysate translation assay is repressed by the addition of a purified sequence-specific RNA binding protein. The availability of such an in vitro assay for the *nanos/hunchback* system should allow the detailed elucidation of the factors and mechanisms involved.

REFERENCES

Bardsley, A., Macdonald, K., and Boswell, R. E. (1993). Distribution of tudor protein in the Drosophila embryo suggests separation of function based on site of localization. Development *119*, 207-219.

Boswell, R. E., Prout, M. E., and Steichen, J. C. (1991). Mutations in a newly identified *Drosophila melanogaster* gene, *mago nashi*, disrupt germ cell formation of mirror-image symmetrical double abdomen embryos. Development *113*, 373-384.

Christerson, L. B., and McKearin, D. M. (1994). *orb* is required for anteroposterior and dorsoventral patterning during Drosophila oogenesis. Genes & Development *8*, 614-628.

Clark, I., Giniger, E., Ruohola-Baker, H., Jan, L. Y., and Jan, Y. N. (1994). Transient posterior localisation of a kinesin fusion protein reflects anteroposterior polarity of the Drosophila oocyte. Current Biology *4*, 289-300.

Ephrussi, A., Dickinson, L. K., and Lehmann, R. (1991). *oskar* organizes the germ plasm and directs localization of the posterior determinant *nanos*. Cell *66*, 37-50.

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

Ferrandon, D., Elphick, L., Nüsslein-Volhard, C., and St Johnston, D. (1994). Staufen protein associates with the 3'UTR of bicoid mRNA to form particles that move in a microtubule-dependent manner. Cell *79*, 1221-1232.

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

Hay, B., Jan, L. Y., and Jan, Y. N. (1988). A protein component of Drosophila polar granules is encoded by vasa and has extensive sequence similarity to ATP-dependent helicases. Cell *55*, 577-587.

Kim-Ha, J., Kerr, K., and Macdonald, P. M. (1995). Translational regulation of *oskar* mRNA by bruno, a newly-identified RNA binding protein, is essential. Cell *in press*.

Kim-Ha, J., Smith, J. L., and Macdonald, P. M. (1991). oskar mRNA is localized to the posterior pole of the Drosophila oocyte. Cell *66*, 23-35.

Kim-Ha, J., Webster, P. J., Smith, J. L., and Macdonald, P. M. (1993). Multiple RNA regulatory elements mediate distinct steps in localization of *oskar* mRNA. Development *119*, 169-178.

Lantz, V., Chang, J. S., Horabin, J. I., Bopp, D., and Schedl, P. (1994). The *Drosophila orb* RNA-binding protein is required for the formation of the egg chamber and establishment of polarity. Genes & Development *8*, 598-613.

Lasko, P. F., and Ashburner, M. (1990). Posterior localization of vasa protein correlates with, but is not sufficient for, pole cell development. Genes & Development *4*, 905-921.

Liang, L., Diehl-Jones, W., and Lasko, P. (1994). Localization of vasa protein to the *Drosophila* pole plasm is independent of its RNA-binding and helicase activities. Development *120*, 1201-1211.

Macdonald, P. M. (1990). *bicoid* mRNA localization signal: phylogenetic conservation of function and RNA secondary structure. Development *110*, 161-171.

Macdonald, P. M., Kerr, K., Smith, J. L., and Leask, A. (1993). RNA regulatory element BLE1 directs the early steps of *bicoid* mRNA localization. Development *118*, 1233-1243.

Manseau, L. J., and Schüpbach, T. (1989). cappuccino and spire: two unique maternal-effect loci required for both the anteroposterior and dorsoventral patterns of the Drosophila embryo. Genes & Development *3*, 1437-1452.

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

Ostareck-Lederer, A., Ostareck, D. H., Standart, N., and Thiele, B. J. (1994). Translation of 15-lipoxygenase mRNA is inhibited by a protein that binds to a repeated sequence in the 3' untranslated region. The EMBO Journal 1476-1481.

Pokrywka, N. J., and Stephenson, E. C. (1991). Microtubules mediate the localization of *bicoid* RNA during Drosophila oogenesis. Development *113*, 55-66.

Sallés, F. J., Lieberfarb, M. E., Wreden, C., Gergen, J. P., and Strickland, S. (1994). Coordinate initiation of *Drosophila* development by regulated polyadenylation of maternal messenger RNAs. Science *266*, 1996-1999.

Schüpbach, T., and Wieschaus, E. (1986a). Maternal-effect mutations altering the anterior-posterior pattern of the Drosophila embryo. Wilhelm Roux's Archives of Developmental Biology *195*, 302-317.

Siegel, V., Jongens, T. A., Jan, L. Y., and Jan, Y. N. (1993). *pipsqueak*, an early acting member of the posterior group of genes, affects *vasa* level and germ cell-somatic cell interaction in the developing egg chamber. Development *119*, 1187-1202.

Smith, J. L., Wilson, J. E., and Macdonald, P. M. (1992). Overexpression of *oskar* directs ectopic activation of *nanos* and presumptive pole cell formation in Drosophila embryos. Cell *70*, 849-859.

St. Johnston, D., Beuchle, D., and Nüsslein-Volhard, C. (1991). *staufen*, a gene required to localize maternal RNAs in the Drosophila egg. Cell *66*, 51-63.

Wharton, R. P., and Struhl, G. (1991). RNA regulatory elements mediate control of Drosophila body pattern by the posterior morphogen *nanos*. Cell *67*, 955-967.

Appendix A

The role of *pumilio* in posterior determination

AUTHOR'S NOTE

The work described in this appendix has been published as Barker, D.D., Wang, C., Moore, J., Dickinson, L.K., and Lehmann, R. (1992) "Pumilio is essential for function but not for distribution of the *Drosophila* abdominal determinant Nanos" Genes and Development **6**, 2312-2326.

INTRODUCTION

Genetic analysis has identified a set of maternal-effect genes required for *Drosophila* abdominal development known as the posterior group genes (reviewed in St. Johnston, 1993). Of these genes, most have a second, grandchildless phenotype. Only two posterior group genes (*nanos (nos)* and *pumilio (pum)*) are specific for abdomen development. Embryos derived from females mutant for either of these genes develop pole cells normally, but fail to form a segmented abdomen. Both *nos* and *pum* are required for the suppression of maternal *hunchback* (*hb*) RNA translation in the posterior half of the embryo (Barker et al., 1992; Macdonald, 1992). *nanos* encodes a localized determinant. *nos* RNA is localized to the posterior pole of early embryos, and this localized RNA is the source for a gradient of nanos protein which is highest at the posterior, and extends to at least 30% egg length (0% = posterior pole) (Barker et al., 1992; Smith et al., 1992; Wang and Lehmann, 1991). When *nos* RNA is mislocalized to the anterior of embryos, it directs the formation of a secondary abdomen in mirror image to the first (Gavis and Lehmann, 1992). This latter result demonstrates the importance of restricting *nos* expression to the posterior half of the embryo.

By contrast, *pum* RNA and protein are found ubiquitously throughout the early embryo, although some enrichment can be detected in the poleplasm (Barker et al., 1992; Macdonald, 1992). Other data corroborate this suggestion that *pum* is required for processes beyond its maternal role in abdomen formation. Flies mutant for some *pum* alleles (Lehmann and Nüsslein-Volhard, 1987a), as well as those which completely lack *pum* (Barker et al., 1992) show bristle defects as well as reduced viability and fertility. The polypeptide sequence of *pum* offers no obvious clues to its function, although an eight-repeat sequence motif found in sequences from a wide range of species has been noted (Barker et al., 1992) , D. Barker, personal communication). In order to clarify the role of *pum* in specifying abdomen, we tested whether *nos* can function in the complete absence of *pum*.

RESULTS/DISCUSSION

Earlier studies of *pumilio* had suggested that its function was not absolutely required for abdomen formation. Injection rescue experiments using wildtype posterior poleplasm (Lehmann and Nüsslein-Volhard, 1987a) or in vitro transcribed *nanos* RNA (Wang and Lehmann, 1991) showed that overexpression of *nos* can partially rescue abdominal segmentation in *pum* mutant embryos. However, these experiments were carried out using an ethylmethanesulfonate (EMS) -induced *pum* allele (pum^{680}) which is a probable hypomorph. Subsequently, two chromosomal rearrangements (In(3R)Msc ("Msc"), and T(3;1)FC8 ("FC8")) have been found to disrupt the *pumilio* transcribed region. Flies transheterozygous for these aberrations completely lack *pum* function, and accordingly are subviable and show strong scutellar bristle defects. Embryos derived from females transheterozygous for Msc/FC8 completely fail to develop abdomen. Injection of in vitro transcribed *nos* RNA has no effect on the abdominal phenotype of Msc/FC8 embryos (Table A.1). As a positive control, the same transcription reaction product was used to inject nos^{L7} embryos, which were fully rescued.

The ability of *nanos* RNA to rescue the abdominal phenotype of pum^{680} embryos indicates that overexpression of *nos* can bypass a partial loss of *pum* function. However, the Msc/FC8 result shows that *pum* is absolutely required for abdomen formation. Both *pumilio* and *nanos* are equally required for *hb* RNA repression and abdomen formation, despite the fact that in early embryos nanos protein is spatially restricted and pumilio protein is not. *pumilio* function is required for *nos*-specified abdomen formation even in embryos containing *nos* at the anterior (E. Gavis, personal communication). Several experiments have attempted to further distinguish the function of *pumilio* from that of *nanos* in abdomen formation. It had been previously suggested (Lehmann and Nüsslein-Volhard, 1987a) that *pum* is involved in the transport of nanos protein from the posterior pole to the prospective abdomen. However, the distribution of nanos protein is not affected by pumilio mutations (Barker et al., 1992; Smith et al., 1992). A search for *pum*-dependent post-translational modification of the nanos protein has also been negative (D. Barker, personal communication). Recently published data (Murata and Wharton, 1995) shows that pumilio protein from embryo

extracts, as well as pumilio protein translated in vitro, bind to the NRE sequences found in the 3'UTR of the *hb* RNA, as assayed by UV crosslinking and gel mobility shift. These results suggest that *pum* plays a rather direct role in the control of *hb* RNA translation by binding to its 3'-UTR. Presumably, this binding occurs throughout the embryo, and the additional presence of nanos protein causes or triggers an inhibition of *hb* RNA translation. Interestingly enough, neither nanos protein/*hunchback* RNA complexes nor nanos protein/pumilio protein complexes were detected (Murata and Wharton, 1995). The DNA sequence of the 13 existing EMS-induced *pum* alleles, coupled with functional assays of these mutant proteins, should provide significant insight into *pumilio* function.

MATERIALS AND METHODS

For the RNA injections, recipient embryos lacking *pumilio* were derived from mothers transheterozygous for the chromosomal rearrangements In(3R)Msc and T(3;1)FC8 (Barker et al., 1992). Control recipient embryos were derived from mothers homozygous for the nos^{L7} allele (Lehmann and Nüsslein-Volhard, 1991). RNA was transcribed and injected into embryos as previously described using the *nanos* cDNA plasmid pN5 (Wang and Lehmann, 1991).

REFERENCES

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

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

Lehmann, R., and Nüsslein-Volhard, C. (1987a). Involvement of the pumilio gene in the transport of an abdominal signal in the Drosophila embryo. Nature *329*, 167-170.

Lehmann, R., and Nüsslein-Volhard, C. (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.

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

Smith, J. L., Wilson, J. E., and Macdonald, P. M. (1992). Overexpression of *oskar* directs ectopic activation of *nanos* and presumptive pole cell formation in Drosophila embryos. Cell *70*, 849-859.

St. Johnston, D. (1993). Poleplasm and the posterior group genes. In The development of *Drosophila melanogaster*, M. Bate, and Martinez Arias, A., eds. (Cold Spring Harbor: Cold Spring Harbor Press).

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

Table A.1. RNA injection rescue of *pumilio* mutant embryos

			# of segments formed (% of total)		
recipient embryos	# injected	# developed	0-1	2-4	5-8
In(3R)MSC/ Tp(3;1)FC8	220	60	100	-	-
nos^{L7}	122	60	3	-	97

A plasmid containing a *nanos* cDNA was transcribed in vitro and the resulting RNA was injected into the prospective abdomen of embryos derived from females of the indicated genotype ("recipient embryos") Rescue is scored as the percentage of the total number of embryos scored which form a given number of abdominal segments.