

***nanos* is an evolutionarily conserved organizer of anterior-posterior polarity**

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SUMMARY

In *Drosophila melanogaster*, *nanos* functions as a localized determinant of posterior pattern. *Nanos* RNA is localized to the posterior pole of the maturing egg cell and encodes a protein that emanates from this localized source. *Nanos* acts as a translational repressor and thereby establishes a gradient of the morphogen Hunchback. Here we show that the mechanism by which *nanos* acts in *Drosophila* is a common developmental strategy in Dipteran insects. We used cytoplasmic transplantation assays to demonstrate that *nanos* activity is found in posterior poleplasm of five diverse Dipteran species. Genes homologous to *nanos* were identified from *Drosophila virilis*, the housefly *Musca domestica*, and the midge *Chironomus samoensis*. These

genes encode RNAs that are each localized, like *nanos*, to the embryonic posterior pole. Most importantly, we demonstrate that these homologues can functionally substitute for *nanos* in *D. melanogaster*. These results suggest that *nanos* acts in a similar pathway for axis determination in other insects. Comparison of the *Nanos* sequences reveals only 19% overall protein sequence similarity; high conservation of a novel zinc finger near the carboxy terminus of the protein defines a region critical for *nanos* gene function.

Key words: *nanos*, Diptera, anterior-posterior polarity, *Drosophila*, evolution

INTRODUCTION

As the mechanisms of early patterning events in *Drosophila* development become increasingly well understood, it is of great interest to determine their generality in other organisms. The extraordinary evolutionary conservation of the homeotic complex genes reveals that some features of the *Drosophila* body plan are widely shared with other segmented animals (Kenyon, 1994). However, the morphological diversity of different embryos suggests that the earliest events of axis formation and the establishment of a segmented body plan might occur by a variety of mechanisms (Gurdon, 1992).

In *Drosophila*, establishment of the embryonic longitudinal axis is under the control of two localized determinants, *nanos* (*nos*) and *bicoid* (*bcd*) (for review, see St. Johnston and Nüsslein-Volhard, 1992). *nos* and *bcd* RNAs are synthesized maternally and are localized during oogenesis to the posterior and anterior poles of the egg, respectively. Bcd protein translated at the anterior pole diffuses to form an anterior to posterior gradient, and Nos protein translated at the posterior pole forms a posterior to anterior gradient. Despite these similarities in the initial establishment of the Bcd and Nos protein gradients, the two proteins function by strikingly different mechanisms. While Bcd activates zygotic transcription of several genes, including *hunchback* (*hb*), in the anterior half of the embryo (Driever and Nüsslein-Volhard, 1988; Struhl et al., 1989), Nos represses translation of the uniformly distributed maternal *hb* RNA in the posterior, leading to an anterior to posterior Hb protein gradient complementary to that of Nos

(Tautz, 1988; Tautz and Pfeifle, 1989). Thus, using distinct mechanisms, both *nos* and *bcd* regulate *hb* and establish qualitatively similar gradients of Hb protein. Hb in turn plays a central role as a transcription factor in the subdivision of the embryo into defined body regions along the anterior-posterior axis (Hülskamp et al., 1990; Struhl et al., 1992), reviewed in (Hülskamp and Tautz, 1991).

Conservation of the *hb* DNA binding domain in insects, molluscs and annelids suggests that *hb* may have a widespread role in embryonic patterning (Sommer et al., 1992). In contrast, *bcd* activity, as tested by cytoplasmic transplantation, and *bcd* gene homologues have so far been identified only in species closely related to *D. melanogaster* within the Brachyceran suborder of the Diptera (Macdonald, 1990; Schröder and Sander, 1993; Sommer and Tautz, 1991). Since the Hb gradients resulting from the activities of *bcd* and *nos* are very similar, only one of these two means of establishing a gradient is, in principle, necessary. Transcriptional activation of anterior genes by *bcd* has been shown to require synergistic activation by *hb* (Simpson-Brose et al., 1994), leading to the suggestion that the role of *bcd* as a primary determinant of polarity evolved recently as *bcd* acquired some of the patterning functions performed by *nos* and maternal *hb* in other species. Experimental manipulations of eggs from different insect orders suggest a widely conserved role for localized posterior, but not anterior determinants in insects (Sander, 1976). Thus it is possible that the *nos-hb* regulatory interaction might be the fundamental mechanism for initiating the anterior-posterior axis in insects.

The mechanism by which *nos* regulates *hb* mRNA translation is not yet known. Sequences in the 3' untranslated region (3'UTR) of the *hb* RNA called nanos response elements (NREs) have been shown to mediate *nos*-dependent translational repression (Wharton and Struhl, 1991), but sequences in the Nos protein that are important for function have not yet been identified (Wang and Lehmann, 1991). To determine if *nos* is part of an evolutionarily conserved mechanism of axis determination, and to use phylogenetic sequence comparisons as a tool to identify important structural features of the Nos protein, we have investigated *nos* structure and function within the Dipteran insect order. Genes with sequence similarity to *nos* were isolated from *Drosophila virilis*, the housefly *Musca domestica* and the midge *Chironomus samoensis*, which are separated from *D. melanogaster* by approximately 60, 100 and 200 million years of evolution, respectively. Early embryonic development in these Dipteran species is morphologically very similar, and the expression patterns of the *nos* homologues are conserved. We find that the *nos* homologues retain function despite divergence of surprisingly large regions of the protein sequences. The carboxy-terminal region is highly conserved among the *nos* homologues and defines a novel zinc finger, critical for function. Our results show that *nos* is a conserved organizer of anterior-posterior patterning in the Diptera, and suggest it should perform a similar function in other insects as well.

MATERIALS AND METHODS

Cytoplasmic transplantation

Cytoplasmic transplantation was carried out as described by Lehmann and Nüsslein-Volhard (1991). *M. domestica* pupae were obtained from Carolina Biological Supply and raised according to the provided instructions. Eggs were collected on fresh chicken liver, and were used as donors as described for *Drosophila*. *C. samoensis* embryos were collected from cultures maintained by Dr. Klaus Kalthoff at the University of Texas at Austin. At about 1.5 hours of development, one of the four cleavage nuclei reaches the posterior pole to form the first pole bud, which divides to form two pole cells (Kuhn et al., 1987). *C. samoensis* donors were either at the two pole cell stage or younger. Because of differences in the sizes of donor embryos, the ratio of number of poleplasm donors to recipients was 1:1 for *Drosophila*, 1:3 for *Musca*, and 2.5:1 for *Chironomus* donors.

After injection, recipient embryos were allowed to develop for 2 days at 18°C. The resulting larvae were mounted in a 1:1 mixture of Hoyer's mountant and lactic acid (Wieschaus and Nüsslein-Volhard, 1986). Cuticles were scored for abdominal segmentation by counting ventral setal belts or dorsal hairs. Since rescue was frequently asymmetric either laterally or dorsoventrally, any part of a segment was counted as one segment.

Library screens

A *D. virilis* genomic library was obtained from John Tamkun and Mary Prout, and a *C. samoensis* genomic library from Klaus Kalthoff. A *M. domestica* genomic library was prepared using genomic DNA from adult flies (Carolina Biological Supply). The *D. virilis* library was screened using a 2.2 kb full length *nos* cDNA fragment, [³²P]dCTP labeled by random hexamer priming, in 30% formamide, 5×SSPE, 1% SDS, 1×Denhardt's, 0.1 mg/ml sonicated salmon sperm DNA, 10% dextran sulfate at 42°C. Washes were in 2×SSPE, 0.5% SDS at 42°C. Phages containing inserts with similarity to *nos* were not obtained by low stringency screening from *M. domestica* or *C. samoensis* libraries under the conditions described above, or by using

a probe corresponding to the conserved C-terminal region of *nos* in 25% formamide conditions.

Polymerase chain reaction cloning

To amplify *Md nos* and *Cs nos* sequences, the following PCR primers were used (written in 5' to 3' orientation with redundant nucleotides in parentheses; the corresponding Nos amino acid sequences are in brackets):

J2: TG(T,C)GTGTT(T,C)TG(T,C)GA(A,G)AA(T,C)AA [CVFCENN]
J5: GC(T,C)TT(T,A,G)ATGGC(A,G)TC(T,C)TCCAT [MEDAIKA].

PCR conditions were, 50 mM KCl, 10 mM Tris, pH 8.2, 2 mM MgCl₂, 0.2 mM each dNTP, 0.005 mM each primer, 500 ng genomic DNA, 5 units Taq polymerase (Perkin Elmer-Cetus), in a 50 µl volume with mineral oil overlay. Cycling was 94°C for 1 minute, 50°C for 1 minute, 72°C for 2 minutes, for 40 cycles. Amplified fragments were isolated from agarose gels using the Mermaid kit (Bio101), treated with T4 polymerase to blunt the ends, and cloned into *EcoRV*-cut pBluescript SK+ (Stratagene).

DNA sequencing

A 4442 nucleotide *XbaI* to *BamHI* fragment of *Dv nos*, a 4592 nucleotide *NsiI* to *Clal* fragment of *Md nos*, and a 4552 nucleotide *EcoRV* to *PstI* fragment of *Cs nos* (Fig. 4) were sequenced using standard methods. All three genomic sequences have been submitted to the EMBL and Genbank databases.

Whole-mount RNA and protein detection

Whole-mount in situ RNA hybridization with digoxigenin-labeled RNA probes (Tautz and Pfeifle, 1989) was performed as described by Gavis and Lehmann (1992). Embryos from all species were treated in the same way. RNA probes were transcribed from the following *nos* subclones (see Fig. 4): *Dm* 2.2 kb N5 cDNA (Wang and Lehmann, 1991); *Dv* 4.5 kb *XbaI-BamHI* fragment; *Md* 1.1 kb *EcoRI* fragment; and *Cs* 4.5 kb *EcoRV* to *PstI* fragment.

Antibody staining was carried out as described (Gavis and Lehmann, 1992) using Nos antiserum prepared against a peptide antigen corresponding to the C-terminal 13 amino acids of Nos (Wang et al., 1994).

cDNA cloning

Cs nos cDNAs were synthesized by reverse transcription (RT) of pole cell stage embryo total RNA, followed by PCR amplification (Kawasaki et al., 1988). The primers were designed to amplify sequences corresponding to the fourth amino acid through the stop codon of the *Cs* Nos open reading frame. *Md nos* cDNAs corresponding to *Md* Nos amino acids 16 to 399 were synthesized from female and 0- to 2-hour embryo total RNA. The cDNAs were completely sequenced by standard techniques.

To generate a *Cs nos* transcription template suitable for injection rescue experiments, the *Dm nos* cDNA clone N5 (Wang and Lehmann, 1991) was first modified as follows to create pNB40-N5:Nde tag RI. The sequence around the initiator methionine was altered to create an *NdeI* site at the ATG followed by the nine amino acid hemagglutinin epitope tag (Kolodziej and Young, 1991), incorporating the *Dm nos* codon bias. Following the tag, a single nucleotide change was introduced into the third *nos* codon, which does not change its coding potential, to create a unique *BspEI* restriction site. The new sequence reads, beginning at nucleotide 254 (Wang and Lehmann, 1991): TTTTCCAT ATG TAC CCC TAC GAT GTG CCC GAT TAC GCC TTC CGG AGC AAC. An *EcoRI* restriction site was introduced by PCR immediately following the *nos* stop codon (Gavis and Lehmann, 1992). A *BspEI* to *EcoRI* cassette encoding *Cs* Nos amino acids 4 through the stop codon was then introduced into the pNB40-N5:Nde tag RI vector backbone to create pNB40-CH. A version of this clone called pNB40-CH3 contains a 4 nucleotide frameshift deletion in the fifth amino acid of the *Cs nos* open reading

frame. This control RNA shows no *nos* rescuing activity when injected at a concentration of 3 mg/ml ($n=25$).

A *Md nos* cDNA template encoding *Md nos* amino acids 4 to the stop codon was engineered essentially as described above for *Cs nos*. *BspEI* and *EcoRI* restriction sites were introduced at the fourth codon and after the stop codon, respectively, by PCR from cloned genomic DNA. An internal fragment containing the introns was replaced by the corresponding fragment of the *Md nos* cDNA, and the *BspEI-EcoRI* cassette was cloned into the pNB40-N5:Nde tag RI vector. The RNAs transcribed from the *Md* and *Cs nos* templates share the same *Dm nos* 5' and 3'UTRs, and differ only in their protein coding sequences.

RNA injections

RNA was transcribed in vitro with SP6 polymerase from linearized templates, precipitated, resuspended in water and quantitated by UV absorption. Three-fold serial dilutions of RNAs were made in water and were injected as described (Wang and Lehmann, 1991). Injected embryos from mothers of the genotypes *nos^{L7}*, *nos^{BN}*, or *nos^{RC/nos^{BN}}* all gave equivalent results, and the results were pooled.

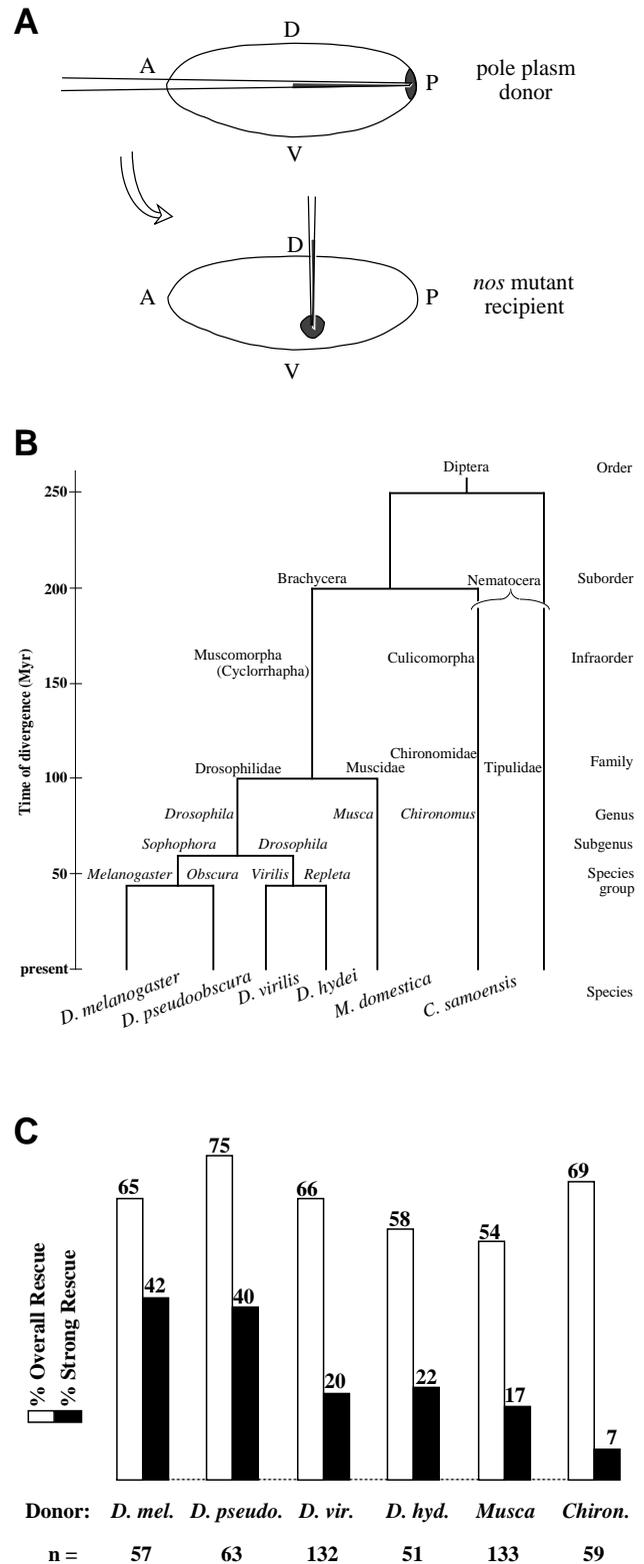
RESULTS

nos activity in other Dipteran species

To determine whether a *nos*-like activity is present at the posterior pole of other Dipteran insect species, posterior poleplasm from *Drosophila*, *Musca* or *Chironomus* donor embryos was transplanted into *D. melanogaster nos* mutant embryo hosts (Fig. 1). Embryos from *nos* mutant females develop with a normal head, thorax and telson (posterior terminal structures), but completely lack abdominal segmentation (Lehmann and Nüsslein-Volhard, 1991). Embryos injected with posterior poleplasm from each of the species assayed developed with partial or complete abdominal segmentation (Fig. 1C). Rescue frequencies were scored either as *overall*

Fig. 1. Cross-species cytoplasmic transplantation. (A) Cytoplasm was withdrawn from the posterior pole of various donor embryos and injected into the prospective abdominal region of early cleavage stage (pre-pole cell) *D. melanogaster* recipient embryos from *nos^{L7}* mutant females. A, anterior; P, posterior; D, dorsal; V, ventral. (B) A partial phylogenetic tree showing the relationships between the Dipteran species used in these experiments. Times of divergence (in millions of years; Myr) are approximate, and are based on the fossil record (Hennig, 1981; Kukalova-Peck, 1991; McAlpine, 1989) and on immunological relatedness of larval serum proteins (Beverley and Wilson, 1984). The bracket signifies that the suborder Nematocera is thought to be a paraphyletic classification; it groups together some families less closely related to each other than to some families in the suborder Brachycera. The crane flies (family Tipulidae) are thought to be the least derived family relative to the common ancestor of the Diptera (McAlpine, 1989). The species studied were the *Drosophilids* *D. pseudoobscura*, *D. hydei* and *D. virilis*, the housefly *Musca domestica*, and the midge *Chironomus samoensis*, which has been the subject of previous studies on anterior-posterior patterning (Elbetieha and Kalthoff, 1988; Yajima, 1964). (C) Results of cytoplasmic transplantations. 'n' is the number of injected embryos that developed a cuticle and could be scored for rescue. Numbers above the histogram bars are the percentage of scored embryos with 1-8 abdominal segments (overall rescue, open bar) or 5-8 abdominal segments (strong rescue, filled bar). Anterior pole cytoplasm or cortical cytoplasm at 50% egg length from *Musca* donors was also tested for *nos* rescuing activity. Neither anterior ($n=46$) nor lateral ($n=49$) cytoplasm could rescue the *nos* phenotype.

rescue, which includes embryos with any abdominal segmentation, or *strong* rescue, which includes only those embryos with 5 or more abdominal segments. The frequency of overall rescue is similar for each donor source, varying between 54% and 75%. The frequency of strong rescue, in contrast, decreases with increasing evolutionary distance of the donor, from 40%



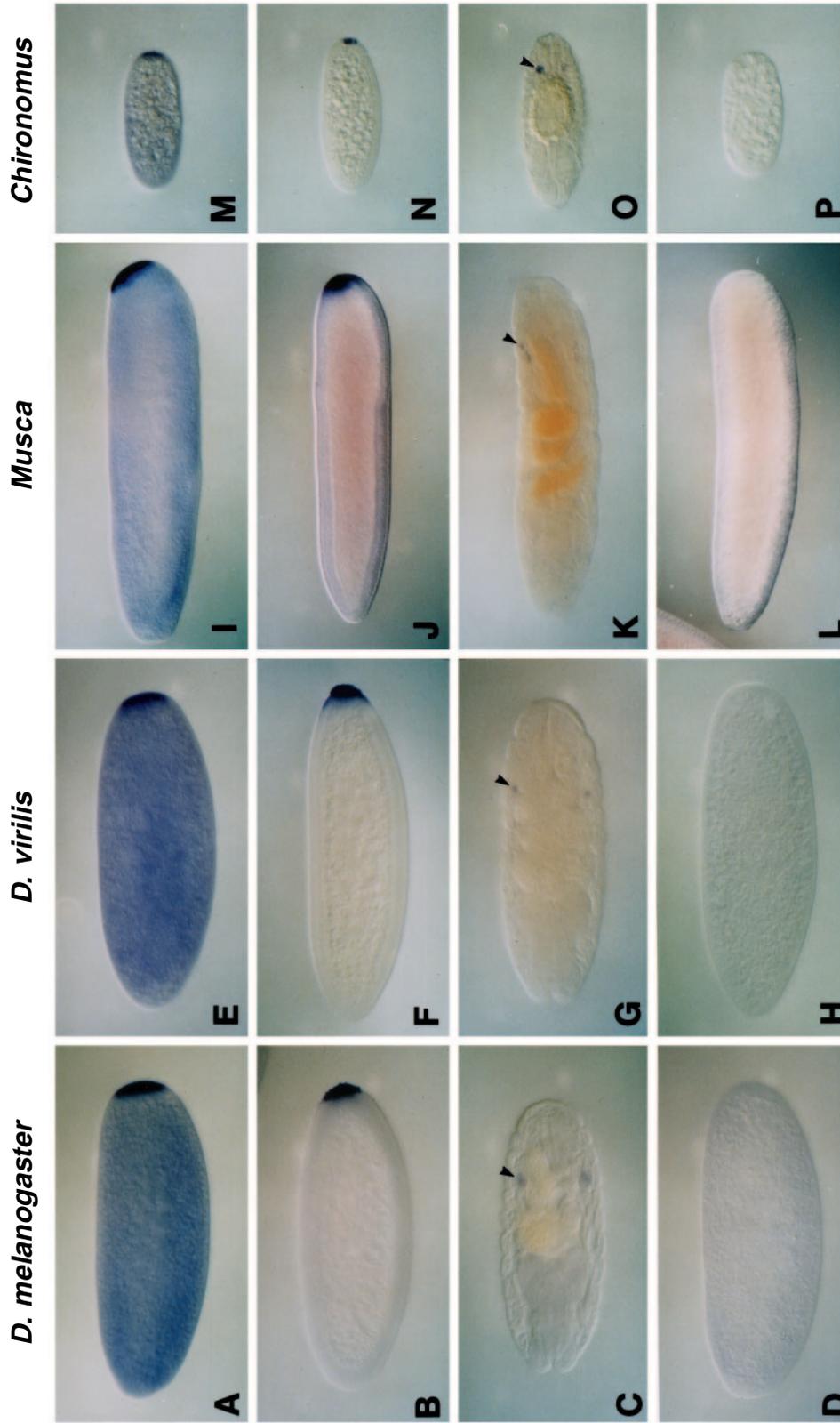


Fig. 2. Whole-mount in situ hybridization to *nos* RNAs in *D. melanogaster* (A-D), *D. virilis* (E-H), *M. domestica* (I-L), and *C. samoensis* (M-P), using species-specific *nos* probes. Top row (A, E, I, M), embryos in early cleavage stages. Specific posterior poleplasm staining is observed in each species. Not all of the *nos* RNA is localized, and a significant uniform stain is visible in early embryos (compare with D, H, L, P). Second row (B, F, J, N), embryos are at syncytial blastoderm stage. RNA staining is restricted to the pole cells. Third row (C, G, K), late-stage embryos after gut morphogenesis has taken place. Embryo shown in O is at a slightly earlier stage, before dorsal closure. Staining is observed in the germ cells within the gonads (arrowheads). Fourth row (D, H, L, P), early cleavage stage embryos hybridized with species-specific *nos* sense control probes. No staining is observed. All embryos are oriented anterior-left, dorsal-up, except C, G, K, O, which are dorsal view. *M. domestica* embryos are at 0.5 \times magnification relative to the others.

for *D. pseudoobscura* to 7% for *C. samoensis* (Fig. 1C). We conclude from these results that each of the Dipteran species tested contains a posterior activity that can functionally replace *nos*. To test whether this activity was *nos*, we used molecular techniques to isolate *nos* gene homologues.

Conservation of the *nos* expression pattern

A *D. melanogaster nos* cDNA was used as a probe on genomic libraries under low stringency hybridization conditions (Materials and Methods), and a single class of clones with sequence similarity to *nos* was isolated from a *D. virilis* library. Comparison of the predicted *D. virilis nos* (*Dv nos*) and *D. melanogaster nos* (*Dm nos*) protein coding sequences revealed relatively low overall similarity, with a region of high conservation near the carboxy (C) terminus (see below). The C-terminal sequences were used to design degenerate oligonucleotides that allowed the amplification of DNA fragments with sequence similarity to *nos* from *M. domestica* and *C. samoensis* genomic DNA by the polymerase chain reaction (PCR) (Materials and Methods). Subsequently, complete *nos* genes were obtained from *M. domestica* and *C. samoensis* genomic libraries by screening with the PCR fragment probes.

If genes with sequence similarity to *nos* function as posterior determinants, their expression patterns should be similar to that of *Dm nos*. The *nos* clones were used as probes on developmental RNA blots, and they detect single transcripts of 2.4, 2.5 and 1.8 kb in RNA prepared from *D. virilis*, *M. domestica*, and *C. samoensis*, respectively. As in *D. melanogaster* (Wang and Lehmann, 1991), the RNAs are strongly expressed in females and in early embryos (data not shown). To determine if these RNAs were posteriorly localized, the *nos* clones were used as probes for whole-mount in situ hybridization to embryos (Fig. 2). In early embryos of each species, *nos* RNA is highly concentrated at the posterior pole, while a low level of RNA is distributed throughout the embryos. Each *nos* RNA is taken up with the posterior poleplasm into the pole cells, the germ cell precursors. The RNAs in each species are detected continuously in the pole cells as these cells migrate through the embryo and are incorporated into the gonads. Expression is not detected in tissues other than the germ cells in any of the species. The similar temporal and spatial expression patterns of these candidate *nos* RNAs and *Dm nos* RNA suggests that we have isolated true *nos* homologues.

The shape of the Nanos protein gradient is critical for the determination of pattern within the embryo (Ephrussi and Lehmann, 1992; Gavis and Lehmann, 1992; Smith et al., 1992). In *D. melanogaster* embryos this distribution is in part achieved by translational regulation, such that only posteriorly localized *nos* RNA is translated while unlocalized *nos* RNA is not translated (Gavis and Lehmann, 1994). To determine the distribution of Nos protein in the other insect species, we stained *D. virilis* and *M. domestica* embryos using a polyclonal antiserum directed against a short C-terminal peptide of the *Dm* Nos protein (Wang et al., 1994). This peptide sequence is conserved in *Dv* Nos and *M. domestica* Nos (*Md* Nos), but not in *C. samoensis* Nos (*Cs* Nos) (see below). The antiserum detects a posterior to anterior gradient of Nos in *D. virilis* and *M. domestica* embryos comparable to the gradient of Nos observed in *D. melanogaster* (Fig. 3). The conserved RNA and, at least in two cases, protein expression patterns support the

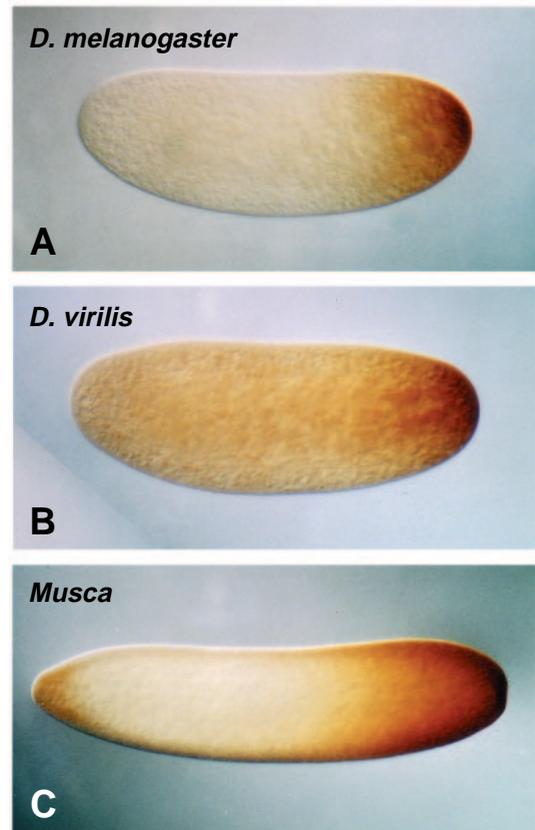


Fig. 3. Cross-species antibody detection of Nos protein.

(A) Distribution of Nos in *D. melanogaster*. An antigen with a similar posterior to anterior graded distribution is recognized by the anti-Nos antiserum in *D. virilis* (B) and *M. domestica* (C). *M. domestica* embryos frequently display a weak and variable anterior pole staining with the anti-Nos antiserum. Because no *nos* rescuing activity was detected in anterior *M. domestica* cytoplasm (Fig. 1), it is not clear if this anterior staining represents Nos protein or other cross-reacting material. Embryos are in nuclear cleavage cycles before pole cell formation, oriented anterior left, dorsal up. *M. domestica* is photographed at 0.5× magnification relative to the others.

conclusion that Nos functions as a localized determinant of posterior pattern in these species.

nos sequence comparisons suggest a novel Zn finger

To determine the extent of *nos* sequence similarity between the four species, the *nos* homologues were sequenced. Since all sequences required for proper expression and function of the *Dm nos* gene are contained in a 4.4 kb genomic fragment (Gavis and Lehmann, 1992), genomic DNA fragments of similar length were sequenced for *Dv*, *Md*, and *Cs nos* (Fig. 4A). The intron/exon structures of the *Md* and *Cs nos* transcripts were determined by cDNA sequencing (Materials and Methods). Overall sizes of the predicted Nos proteins are similar, but large regions have diverged extensively in sequence. Dot matrix comparisons between the predicted Nos protein sequences show that the region of greatest sequence similarity is near the C terminus of the protein (Fig. 4B). An alignment of the predicted Nos protein sequences is presented

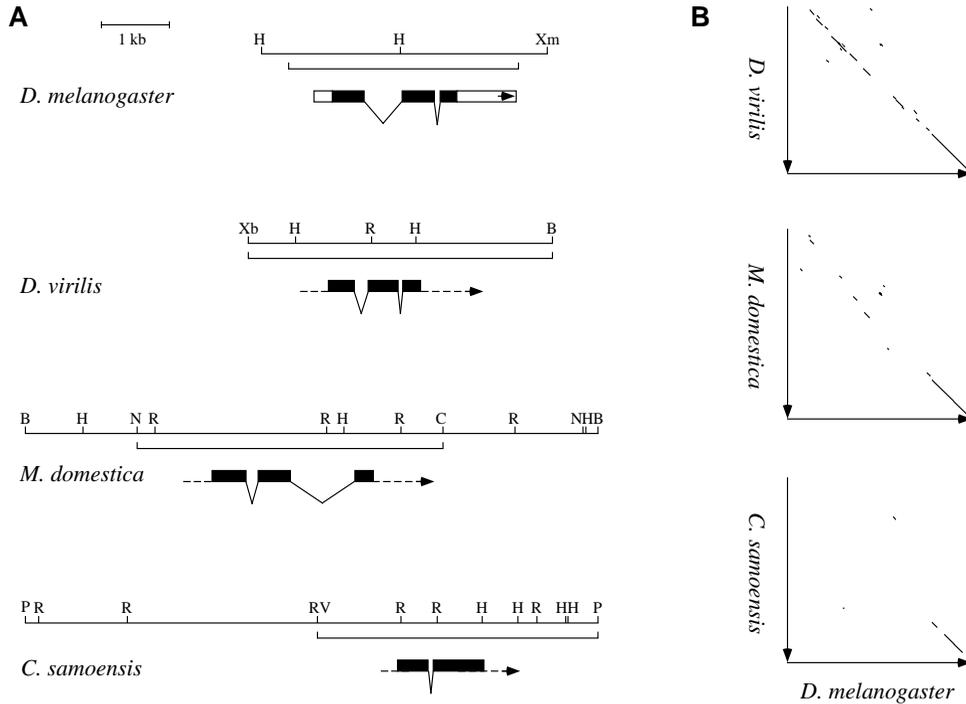


Fig. 4. (A) Genomic organization of the *nos* homologues. The upper line in each case is a partial genomic restriction map of the *nos* region (scale bar at upper left). Brackets below the maps indicate sequenced regions. Intron-exon structures of the genes are diagrammed below the genomic maps; filled boxes indicate coding sequences. The presence of two introns is conserved in each species except *C. samoensis*, which lacks the second intron. Open boxes in *D. melanogaster* indicate non-coding transcribed sequences. The approximate extents of the transcripts in the other species, based on transcript size, and assuming a similar gene structure to *D. melanogaster*, are indicated by dashed lines. Arrowheads denote the direction of transcription. The DNA fragments in these maps were used in P element constructs described in the text. Restriction sites: B, *Bam*HI; C, *Cla*I; H, *Hind*III; N, *Nsi*I; P, *Pst*I; R, *Eco*RI; RV, *Eco*RV; Xb, *Xba*I; Xm, *Xmn*I. (B) Dot matrix comparisons of predicted protein sequences of each of the *nos* homologues (Y axes), with *D. melanogaster* (X axes). A dot represents a match at 5/8 amino acid residues. Arrows on the axes indicate N- to C-terminal orientation of the protein sequences.

*Xmn*I. (B) Dot matrix comparisons of predicted protein sequences of each of the *nos* homologues (Y axes), with *D. melanogaster* (X axes). A dot represents a match at 5/8 amino acid residues. Arrows on the axes indicate N- to C-terminal orientation of the protein sequences.

in Fig. 5. Pairwise comparisons reveal a correlation between overall sequence divergence and time of evolutionary separation (Table 1, Fig 1B). Protein sequence similarity to *Dm* Nos ranges from 63% for *Dv* Nos to 30% for *Cs* Nos. Only 19% amino acid similarity, or 14% identity, is shared among all four Nos proteins.

Primary sequence alignment among all of the Nos homologues is restricted to two regions of the protein. The most extensive sequence conservation is in a 72 amino acid region near the C terminus (*Dm nos* residues 317 to 388). Pairwise protein sequence comparisons of this 72 amino acid region show that 72% to 97% of the residues are similar (Table 2). Among the completely conserved amino acids in this region are a series of cysteine and histidine residues (Fig. 5). They occur in the order CCHC, CCHC, and are likely to constitute a novel zinc-binding domain (see Discussion). Among sequences that have been reported in the databases, only the *Xenopus Xcat-2* gene has significant sequence similarity to *nos* (Mosquera et al., 1993). Similarity to Xcat-2 is found exclu-

sively in the C-terminal region of Nos, and the alignment is included in Fig. 5. Xcat-2 is 51% to 54% similar to each of the Nos homologues in the 72 amino acid region (Table 2), or 62% to 65% similar when only the first 60 amino acids of this region are compared. The precise spacing and conservation of Nos and Xcat-2 residues in this region, including the CCHC amino acids, is striking and argues that the region may form a discrete protein domain with a conserved function.

The second region of *nos* sequence similarity is a short stretch of 6 out of 11 amino acids (residues 170 to 181). In addition, a region rich in serine and threonine residues and a region rich in the basic amino acids asparagine and lysine are present in each species, and the location of these regions within Nos is conserved (Fig. 5).

Dv nos* is functional in *D. melanogaster

The significant divergence of the Nos protein sequence raises the question of whether the rescuing activity detected in the posterior poleplasm of each species is solely due to *nos*

Table 1. Nanos protein sequence comparisons

	<i>D. mel.</i>	<i>D. vir.</i>	<i>Musca</i>	<i>Chironomus</i>	
<i>D. mel.</i>	–	58	35	20	} Identity (%)
<i>D. vir.</i>	63	–	35	21	
<i>Musca</i>	44	42	–	20	
<i>Chironomus</i>	30	33	28	–	
	} Similarity (%)				

The larger of the two proteins in each pairwise comparison was used as the denominator in the calculations. Similarity is defined as identical plus conserved amino acids. Conserved residues are as in Fig. 5.

Table 2. Protein sequence comparisons among the Nanos C-terminal 72 amino acid regions

	<i>D. mel.</i>	<i>D. vir.</i>	<i>Musca</i>	<i>Chiron.</i>	<i>Xcat-2</i>	
<i>D. mel.</i>	–	92	76	61	40	} Identity (%)
<i>D. vir.</i>	97	–	81	65	42	
<i>Musca</i>	89	89	–	60	42	
<i>Chironomus</i>	75	78	72	–	42	
<i>Xcat-2</i>	51	54	51	53	–	
	} Similarity (%)					

The 72 amino acid sequences compared are indicated by brackets in Fig. 5.

but also for the presence of regulatory sequences required for correct transcription, RNA localization and translation of the *nos* genes in *D. melanogaster*.

RNA in situ analysis on embryos from females carrying the *Dv nos* transgene demonstrates that the *Dv nos* RNA is localized, like the endogenous *Dm nos* RNA, to the posterior pole (data not shown). Antibody staining shows that the transgenic *Dv Nos* protein is distributed in a posterior to anterior gradient comparable to that of the *Dm Nos* protein (data not shown). The quantities of the *Dv nos* RNA and protein, as judged by whole-mount staining intensity, appear equivalent to those of the endogenous *Dm nos*, and to the levels observed in *D. virilis*. Thus, all features of *nos* regulation including transcription during oogenesis, proper RNA localization at the posterior pole and restricted posterior translation are conserved between the *Drosophila* species. Indeed, small islands of sequence similarity are found between *Dv* and *Dm nos* both in the upstream region, which presumably contains transcriptional regulatory sequences, and in the 3' untranslated region (3'UTR) which has been shown to regulate posterior localization and translation of *Dm nos* RNA (data not shown; Gavis and Lehmann, 1992, 1994).

To determine whether the *Dv nos* transgene can functionally substitute for *Dm nos*, it was crossed into mutant *nos* females. *nos* is required for two processes, axis determination during embryogenesis, and production of egg chambers during oogenesis (Wang et al., 1994). The transgene complements both phenotypes, demonstrating that amino acid sequences required for both known functions of *nos* are conserved between the *Dv* and *Dm Nos* proteins (Tables 3, 4). However, the *Dv nos* transgene is less effective in complementing the *nos* abdominal phenotype than a *Dm nos* transgene (Table 4). Because *D. virilis* posterior poleplasm is likewise less effective than *D. melanogaster* posterior poleplasm in rescuing the abdominal phenotype (Fig. 1C), and because the transgenic RNA and protein distribution patterns appear equivalent to the endogenous *nos* patterns, the reduced activity of the *Dv nos* transgene is probably due to reduced *Dv Nos* protein activity in the *D. melanogaster* embryos rather than to a failure of the *Dv nos* transgene to be regulated properly.

Md* and *Cs nos* are functional in *D. melanogaster

P elements carrying the *Md* or *Cs nos* genes were unable to complement the *D. melanogaster nos* phenotype. In situ

hybridization detected only trace levels of *Md* or *Cs nos* RNAs in the transgenic embryos, and the RNAs are unlocalized (data not shown). These results suggest that *Md* and *Cs nos* regulatory elements have so diverged that they are unable to function in *D. melanogaster*. In fact, no DNA sequence conservation is found in the non-coding regions of these genes. An RNA injection assay was therefore used to test the function of the *Md* and *Cs nos* genes. This assay circumvents the requirement for correct regulation of *nos* transcription, localization or translation.

The protein coding sequences of a *Dm nos* cDNA were precisely replaced by the predicted *Md* or *Cs nos* coding sequences while maintaining the flanking *Dm nos* 5' and 3'UTR sequences (Materials and Methods). Like in vitro synthesized *Dm nos* RNA (Wang and Lehmann, 1991), in vitro synthesized *Md nos* RNA and *Cs nos* RNA are fully able to rescue the *nos* abdominal segmentation phenotype after injection into *nos* mutant embryos (Fig. 6). These experiments demonstrate that *nos* function is conserved between these species despite the fact that the *Md* and *Cs Nos* proteins share only 44% and 30% amino acid sequence similarity with *Dm Nos*. The results further suggest that *nos* RNA (and/or protein) is the active rescuing component in the cytoplasmic transplantation assay, and demonstrate that other species-specific factors are not required for the activity of these heterologous *Nos* proteins in *D. melanogaster*.

To compare the abilities of *Dm*, *Md* and *Cs nos* to rescue the *nos* mutant phenotype, the response to varying concentrations of injected RNAs was measured. For each RNA, an approximately ten-fold range was observed between the minimal concentration of RNA required for rescue and the concentration that achieved maximal rescue (Fig. 6). However, a 5-fold higher concentration of *Md nos* RNA, or a 7-fold higher concentration of *Cs nos* RNA than of *Dm nos* RNA is required to achieve 50% overall rescue (Fig. 6). *Md* and *Cs nos* RNAs

Table 4. Complementation of the *nos* abdominal segmentation phenotype by the *Dv nos* transgene

Transgene*	Hatch rate (%)†	% of embryos with given number of abdominal segments‡					
		8§	8¶	7	6	5	4
V2/+	88	2	29	25	25	15	4
V2/V2	99	35	41	11	11	2	0
M/+	98	100					

*V2 is the *Dv nos* transgene (see Table 3). The M transgene is a second chromosome P element insertion carrying the 4.4 kb *Dm nos* genomic DNA rescuing fragment with a hemagglutinin epitope tag inserted at the amino terminus of the *nos* coding sequences. Four independent transgenes gave the same results. (+) is a wild type or SM1 second chromosome lacking a transgene. The maternal genotype was *nos^{L7}* or *nos^{BN}* with the transgenes present in one or two copies as indicated. Control embryos from mutant *nos* females that lack the transgene fail to form any abdominal segments.

†Overnight egg collections were aged for 24 hours and eggs ($n \geq 240$) were scored for hatching. Larvae with as few as 4 abdominal segments are able to hatch. Hatch rates in the presence of single copies of three additional independent *Dv nos* P element inserts varied from 15% to 62%.

‡All hatched and unhatched larvae and embryos from 24 hour aged egg collections were mounted for cuticle preparations, and a random sampling of cuticles ($n \geq 52$) was scored for abdominal segmentation.

§Larvae with wild-type segmentation pattern.

¶Larvae had 8 segments, but at least one segment showed developmental defects.

Table 3. Complementation of the *nos* oogenesis phenotype by the *Dv nos* transgene

Maternal genotype		Eggs/day/female‡	No. of females tested
Transgene*	<i>nos</i> allele†		
V2/+	RC/Df	35±16	5
+/+	RC/+	26±5	7
+/+	RC/Df	0	15

*V2 is the strongest complementing *Dv nos* P element insertion, and maps to the second chromosome. (+) corresponds to an SM1 or wild type second chromosome lacking the transgene.

†*nos^{RC}* is a strong allele, Df is *DF(3R)DI^{x43}*, which is deleted for the *nos* locus, (+) corresponds to a TM3, *Sb Ser* third chromosome.

‡Females were placed in laying chambers 3 days after hatching and the number of eggs laid by each individual was counted for 10 days (average ± standard deviation).

are therefore 5- to 7-fold less active in the injection rescue assay, which is consistent with the weaker *nos* activity of transplanted *M. domestica* and *C. samoensis* poleplasm (Fig. 1C). Because the assay compared RNAs that differ only in their protein coding sequences, the difference in activities is likely to reflect a reduced ability of the *Cs* and *Md* Nos proteins to function in repressing translation of the *D. melanogaster hb* RNA.

DISCUSSION

nos homologues and establishment of the anterior-posterior axis

We have used cytoplasmic transplantation assays to demonstrate that *nos* activity is present in posterior egg cytoplasm of five Dipteran species, and we have isolated clones with sequence similarity to *nos* from three of the species, *D. virilis*, *M. domestica* and *C. samoensis*. These clones meet three criteria indicating that they are true *nos* homologues, or ortho-

logues: first, the expression patterns of the *nos* RNAs are indistinguishable from that of *Dm nos*. The RNAs are maternally expressed, localized to the posterior pole of early embryos and present exclusively in germ cells throughout embryogenesis. Second, the genes are similar in size, genomic organization and protein sequence. Using low stringency hybridization conditions for genomic DNA blots and library screens, as well as PCR with degenerate oligonucleotides, no other sequences with similarity to *nos* were detected in *D. melanogaster* or in the other three species. Thus, *nos* does not appear to be a member of a large gene family, and the isolated genes are the sequences most closely related to *nos* in their respective genomes. Finally, the isolated *nos* genes are able to substitute functionally for *Dm nos*. These results suggest that the *nos* homologues function like *Dm nos* as posterior determinants in their respective species and that a common *nos*-dependent mechanism is used to establish embryonic polarity in the Diptera.

Previous studies have identified *hb* homologues in *D. virilis* and *M. domestica*, and analysis of the expression patterns demonstrated that the maternal Hb protein in each species is expressed in an anterior to posterior concentration gradient from uniformly distributed maternal RNA (Lukowitz et al., 1994; Sommer and Tautz, 1991; Treier et al., 1989). This pattern is consistent with a role for *nos* in establishing the Hb maternal protein gradient. In addition, *bcd* gene homologues have been isolated from these species and exhibit similar RNA expression patterns to *Dm bcd* (Macdonald, 1990; Sommer and Tautz, 1991). Thus, the key components of anterior-posterior axis determination defined by genetic analysis in *Drosophila* seem to be conserved in the Brachyceran suborder of the Diptera. However, differences in the regulation of anterior and posterior determinants may exist in species of the Nematoceran suborder. In the Nematoceran *C. samoensis*, bicaudal embryos with two complete abdomens of opposite polarity can be obtained by UV irradiation of the anterior pole of the egg (Kalthoff, 1983). Anterior UV treatments of *D. melanogaster*, in contrast, do not result in bicaudal embryos, but rather in embryos lacking head and thorax that resemble *bcd* mutant embryos (Bownes and Sander, 1976). A model proposed by Kalthoff to explain the *C. samoensis* bicaudal embryos suggested that posterior determinants may be present throughout the embryo, while anterior determinants are enriched in the anterior (Kalthoff, 1983). On the basis of the posterior localization observed for *Cs nos* RNA, it appears that posterior determinant function is similar between this species and the other Dipterans. This implies that the difference in the developmental properties of *C. samoensis* and *D. melanogaster* embryos is not due to a difference in the distribution of the posterior activity. In *D. melanogaster*, bicaudal embryos result from the removal or inactivation of both the *bcd* and *hb* mRNAs at the anterior. This can occur by a variety of mechanisms, including genetic mutation of *bcd* and *hb* (Hülkamp et al., 1990), leakage of anterior cytoplasm from the embryo (Frohnhofer et al., 1986), and translational repression of *bcd* and *hb* by *nos*. The latter mechanism is possible because *nos* can translationally repress *bcd*, like *hb*, through NRE sequences in the *bcd* 3'UTR (Wharton and Struhl, 1991), and because *nos* can be ectopically expressed at the anterior by anterior localization of *nos* RNA or by unregulated translation of unlocalized *nos* RNA (Ephrussi et al., 1991; Gavis and

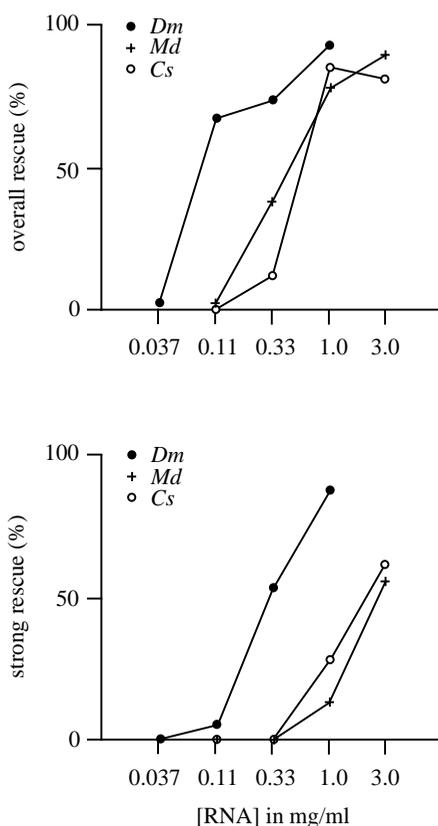


Fig. 6. Functional comparison of *Dm nos*, *Md nos* and *Cs nos* by RNA injection rescue. In vitro transcribed *Dm nos* RNA (●), *Md nos* RNA (+) or *Cs nos* RNA (○) were serially diluted and injected at the indicated concentrations. The data from the same set of experiments are graphed in two ways, showing overall rescue (% of embryos scored that have 1-8 abdominal segments) or strong rescue (% of embryos scored that have 5-8 abdominal segments). Data points represent pooled data from two to three experiments using independent preparations of RNA. Between 50 and 247 cuticles were scored for each data point.

Lehmann, 1992). Within the framework of the *D. melanogaster* model, the *C. samoensis* bicaudal phenotype could be interpreted as a result of a high UV sensitivity of anterior determinant RNAs, possibly *bcd* and *hb*, relative to *D. melanogaster*, or to high UV sensitivity of a repressor of *nos* mRNA translation in the anterior. Anterior determinant RNA could also be distributed in a more shallow gradient relative to *D. melanogaster*, which might explain why a *bcd*-like activity has not been detected in Nematoceran species.

Molecular studies have begun to explore the conservation of *Drosophila* patterning genes in non-Dipteran insects such as moths (Lepidoptera), beetles (Coleoptera) and locusts (Orthoptera), (for review see Nagy, 1994). These insects differ in the degree to which the body plan is determined at the time the germ band is first established. Dipterans are long-germ band insects; at the cellular blastoderm stage the germ band includes primordia for all of the body segments. In contrast, short-germ band insects generate some or all of their segments sequentially during a growth phase after cellularization. Fundamental differences must exist in the mechanisms by which a segment is formed within a field of syncytial blastoderm nuclei versus within the context of a growth zone of dividing cells. However, a common feature of insect development is that the initial determination of polarity occurs in the early syncytial egg cell. Thus a similar mechanism of early axis patterning could occur in embryos of different germ band types. A role for posteriorly localized factors in patterning the egg has been demonstrated in many diverse species (Sander, 1976). The finding that *nos* is localized and functions as a posterior determinant in both suborders of the Diptera suggests that it also does so in closely related insect orders. Whether posterior determinant activities detected in more distantly related short-germ insects correspond to a *nos*-like gene activity remains to be tested.

In addition to its role in axis determination, *nos* is required for oogenesis, and females lacking *nos* produce very few eggs (Wang et al., 1994), (Table 3). The roles of *nos* in oogenesis and in pattern formation are separable by mutation in *D. melanogaster*, since some *nos* alleles with strong abdominal phenotypes have no effect on oogenesis (Wang et al., 1994). In addition, *nos* does not act through *hb* during oogenesis and thus may act through a different RNA target at that time (Wang et al., 1994). While the relationship between the two functions of *nos* is not understood, we find that the *nos* homologue RNAs in each species are present in germ cells throughout embryogenesis. At least one homologue, *Dv nos*, can substitute for *Dm nos* during oogenesis, and the similar expression patterns of *Md* and *Cs nos* suggest that in these species *nos* is also essential for oogenesis. Although the two *nos* functions may be separable and one might prove to be more ancient in evolution, our data point to conservation of both functions in the Diptera.

Nos protein structure

A number of *D. melanogaster* gene homologues have been isolated from *D. virilis*. Protein sequence identity between cognate genes from these two species is typically ~80%, but can vary widely (for examples, see O'Neil and Belote, 1992, and references therein). The 58% overall *Dm-Dv* Nos identity is relatively low, indicating that much of the Nos protein sequence is not highly constrained. Further comparisons including the more divergent *Md* and *Cs nos* sequences shows

that only 19% of the Nos amino acids are identical or conserved across all four species. The ability of the heterologous Nos proteins to function in *D. melanogaster* indicates that the four conserved regions within the protein are likely to be functionally important. Two of these regions are similar only in amino acid content, while a short region of 11 amino acids, and a longer carboxy-terminal 72 amino acid region have substantial primary sequence identity.

The 72 amino acid C-terminal region of Nos is highly conserved, with 67% sequence similarity across all 4 species. The first 60 amino acids of this region are also conserved with the *Xenopus* gene *Xcat-2* (Mosquera et al., 1993). In each of these sequences eight cysteine and histidine residues are conserved with invariant spacing. If it is correct to group these residues into two sequential zinc coordinating sets, as suggested by Mosquera (1993), then the order of the residues, CCHC, makes the Nos sequences most similar to the retroviral nucleocapsid class of zinc finger proteins (Schwabe and Klug, 1994). The nucleocapsid proteins specifically bind and package single stranded genomic viral RNA (Dannull et al., 1994). The conservation of the Nos CCHC region, together with the role of Nos in regulating translation of *hb* RNA, invites the hypothesis that the Nos and *Xcat-2* C-terminal sequences define a new class of zinc-binding proteins with RNA-binding properties. Preliminary evidence that Nos C-terminal protein produced in bacteria contains divalent cations and binds RNA in vitro supports this proposal (D. C., A. Hannaford and R.L. unpublished data). The two Nos motifs, C-X₂-C-X₁₂-H-X₁₀-C and C-X₂-C-X₇-H-X₄-C, differ in spacing both from each other and from the invariant nucleocapsid motif C-X₂-C-X₄-H-X₄-C; thus a detailed analysis will be required to determine the structure of these novel protein elements.

The function of the *Xcat-2* gene is unknown, but *Xcat-2* RNA is localized to the vegetal pole of the developing *Xenopus* oocyte. This, together with its structural similarity to *nos*, suggests that *Xcat-2* may act as a region-specific translational regulator in *Xenopus*. When tested for function by RNA injection assay in *D. melanogaster*, neither the entire *Xcat-2* RNA, nor a hybrid *Dm nos* RNA in which the conserved 53 amino acids of the *Dm nos* zinc finger domain (amino acids 319 through 371) have been replaced by the corresponding *Xcat-2* residues, can rescue the *nos* phenotype (C. Wang, M. L. King, and R. L. unpublished data). Apparently the few additional amino acid differences introduced by the *Xcat-2* sequences, as compared to the tolerated differences present in the Dipteran sequences, renders the hybrid Nos protein unable to regulate *hb*. This experiment, as well as the finding that three *nos* point mutations map to the C-terminal region (D. C. and R. L., unpublished data), confirms that sequences in the C-terminal region are essential for *nos* function. The functional importance of the other regions conserved among the Dipteran Nos sequences is not yet known. One possible role for the basic asparagine/lysine rich region might be to facilitate or contribute to an RNA-binding function of the adjacent C-terminal region. Another possibility is that some of the conserved Nos sequences might engage in protein-protein interactions with *pumilio*, a gene required for *nos*-dependent *hb* repression (Barker et al., 1992), or with components of the translational machinery.

Translational control is an important mechanism of gene regulation in the egg cell, which must store many maternal

transcripts in an inactive form. *nos* is an example of a spatially restricted, specific translational regulator acting during early development to initiate embryonic patterning. Localized translational repression plays a similar role in the generation of asymmetry in the early nematode embryo (Evans et al., 1994), and may thus have a widespread role in early embryonic patterning. Future work will determine if a *nos*-like molecule is involved in this process in other species, and how *nos* exerts its biochemical effect.

We are grateful to Klaus Kalthoff for hosting D. C. in Austin and for making the work with *C. samoensis* embryos possible, and to members of the Kalthoff laboratory: Michael Rebagliati for assistance with the transplantation experiments, and Michele Dick and Myrtha Laurel for helping with embryo collections and for providing the staged embryos used for *C. samoensis* RNA preparations. We thank Mary Prout and John Tamkun for the *D. virilis* library, K. Kalthoff for the *C. samoensis* library, and A. Hannaford and K. Wills for technical assistance. We appreciate comments on this manuscript from Liz Gavis, Charlotte Wang, Phil Zamore, Jamie Williamson and Steve Burden. D. C. was supported by a postdoctoral fellowship from the American Cancer Society and by the HHMI. R. L. is an Associate Investigator of the HHMI.

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1910 D. Curtis, J. Apfeld and R. Lehmann

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(Accepted 7 February 1995)