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Nanos Is the Localized Posterior Determinant in Drosophila

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Summary

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 pole plasm. 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 that 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, 1989; Struhl et al., 1989).

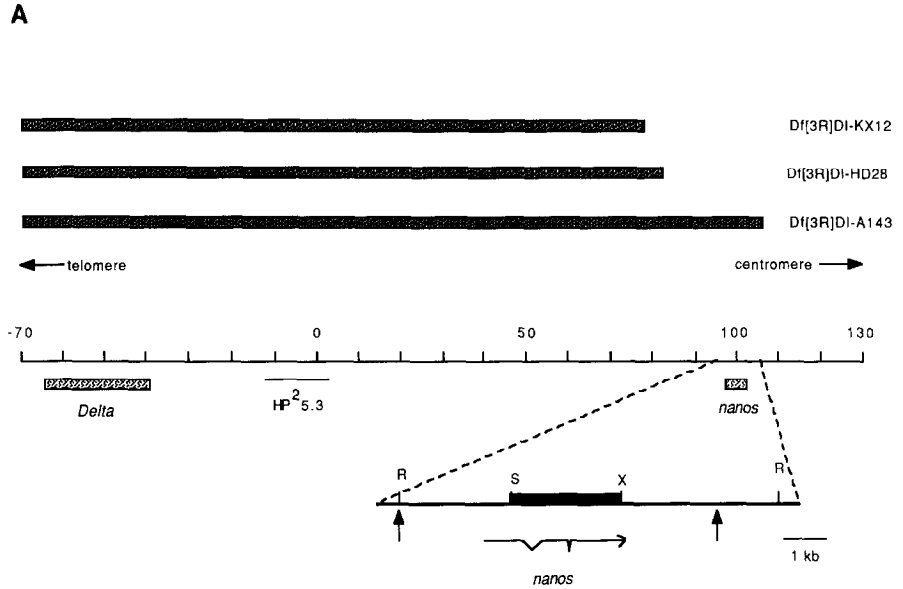
For the posterior system, at least nine genes (*nanos* [*nos*], *pumilio* [*pum*], *oskar* [*osk*], *vasa* [*vas*], *tudor* [*tud*], *staufen* [*stau*], *valois* [*vls*], *cappuccino* [*capu*], and *spire* [*spir*]) are required for abdomen formation (Boswell and Mahowald, 1985; Schüpbach and Wieschaus, 1986; Lehmann and Nüsslein-Volhard, 1986, 1987, 1991; Manseau and Schüpbach, 1989). 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, 1987, 1991). This result led to the hypothesis that the posterior pole plasm 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, 1987, 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 establishment of the posterior pattern predicted the existence of a maternally provided "determinant" localized to the posterior pole, the molecular nature of such a determinant remained unclear. In this article, we describe the isolation and molecular characterization of the *nos* gene, and we present evidence that *nos* RNA is functionally equivalent to the morphogenetic activity found in the posterior pole plasm and that nos protein acts at a distance to direct abdomen development.

Results

The *nos* Gene

nos maps cytologically to 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 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 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- to 2-hr-old embryos and which hybridizes weakly in 2- to 8-hr-old embryos. No transcript is detected in older embryos, larvae, or pupae (Figure 1B). The developmental profile of this transcript is consistent with the maternal mode of *nos* action and



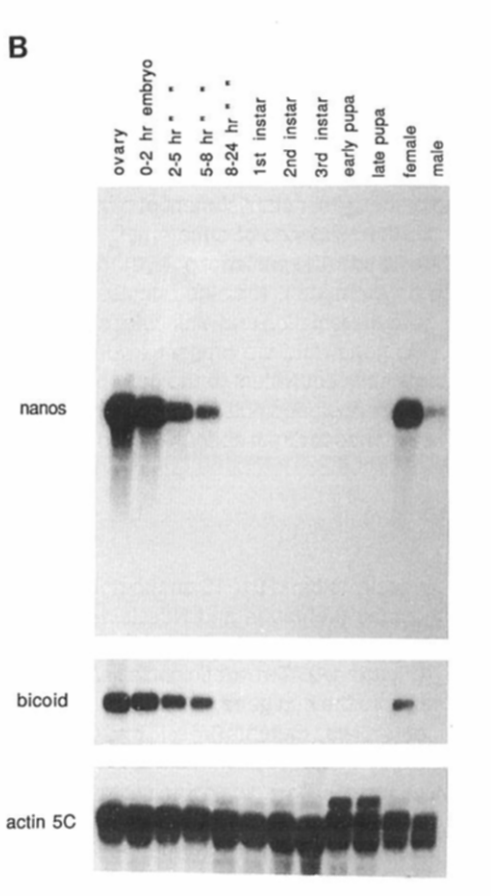


Figure 1. Molecular Cloning of *nos*

(A) Physical map of the *nos* region. Shaded bars at the top indicate DNA that is absent in the indicated deficiency chromosomes; the *Delta–nos* genomic region is represented by the central line; the location of the starting clone (HP²5.3) (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 kilobases 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 that 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). (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 that 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.

parallels that of another maternal-effect gene, *bcd* (Berleth et al., 1988; Figure 1B). Low levels of transcript are also detected in males. At present there is no genetic evidence of a role for *nos* in males. P element–mediated transformation of *nos* mutants with a 7.5 kb genomic DNA fragment (Figure 1A) that includes the 2.5 kb SalI–XhoI fragment rescues the abdominal phenotype of *nos* mutants (E. Gavis and R. L., unpublished data). 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, was sequenced (Figure 2). Comparison of cDNA and genomic sequences indicates that the *nos* primary transcript contains two 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 kd (Figure 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). No significant homology was found between nos and any protein listed in the latest available version of the PIR database (Altschul et al., 1990).

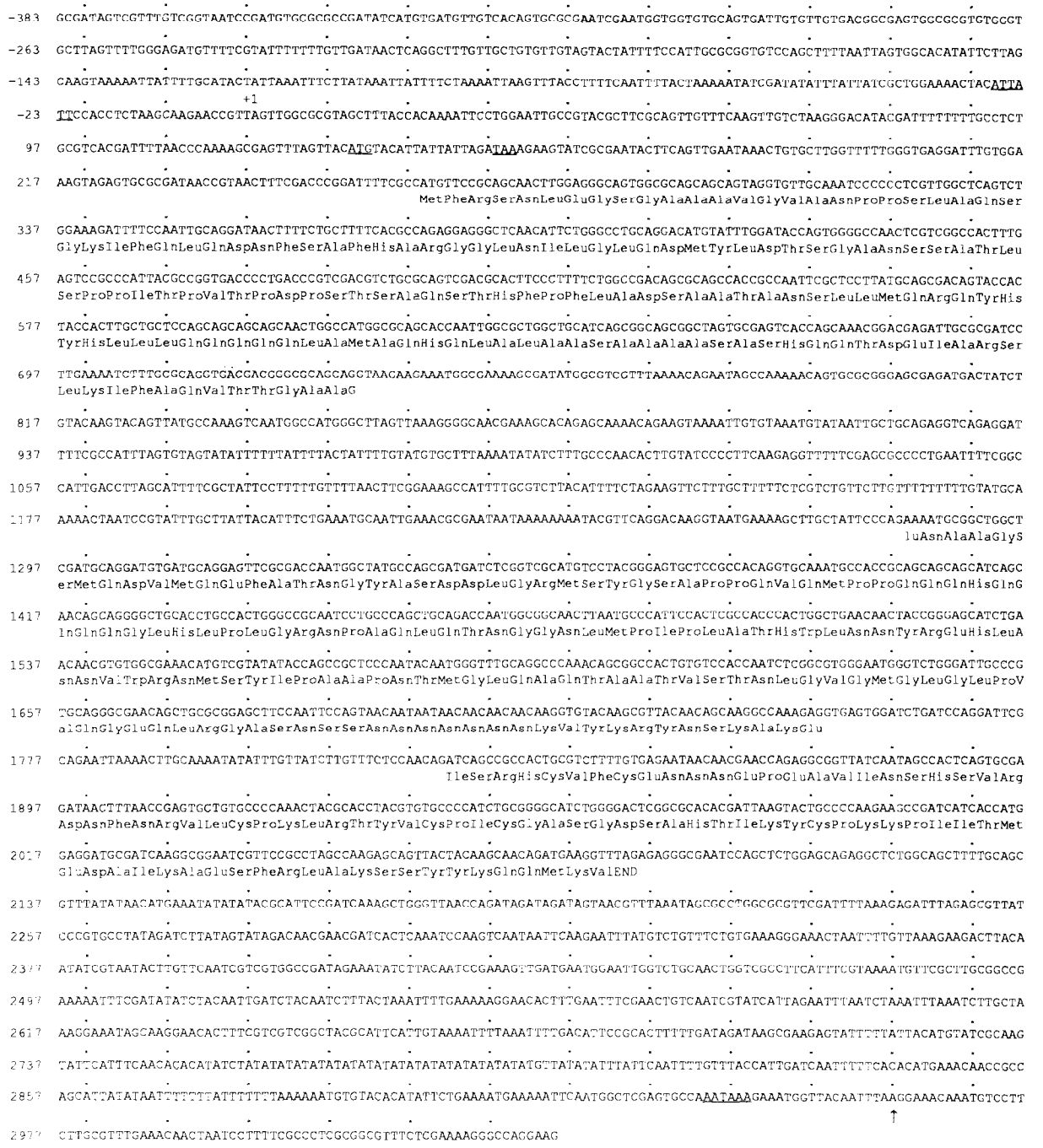


Figure 2. Sequence of the Predicted nos Polypeptide

This is a translation of the single large open reading frame encoded by the *nos* 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 two out of four 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 AATAAA polyadenylation signal is underlined, and the polyadenylation site is marked by an arrow.

Distribution of *nos* mRNA

A central prediction of the model for establishment of the 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 digoxigenin-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 3A). At the pole bud stage (stage 3), the transcript segregates into the nascent pole cells (Figure 3B). The transcript remains concentrated in the pole cells during blastoderm stages (stages 4 and 5; Figure 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 relative to earlier stages (stages 7 and 8, Figure 4D). While the transcript is still visible in the pole cells at the fully extended germband stage (stage 10, Figure 3E), localized staining is no longer detected once the pole cells have left the pocket formed by the posterior midgut invagination (stage 11, Figure 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 pole plasm and pole cells.

Synthetic *nos* RNA Is Biologically Active and Depletes Maternal *hb* 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 pole plasm, 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 4A). By contrast, sibling embryos injected with *nos* RNA show rescue of the abdominal segmentation defect (Figures 4B and 4C). Completely rescued embryos are indistinguishable from wild type, and they hatch and develop into normal, fertile adult flies.

The role of *nos* in abdominal segmentation is mediated by the maternally provided product of the *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* (*kni*) and *giant* (*gt*) (Hülskamp et al., 1990; Eldon and Pirrotta, 1991; Kraut and Levine, 1991). To see whether 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 4D and 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 1 hr and assayed for hb protein. These embryos were compared with embryos treated identically but injected with a *nos* RNA containing a frameshift mutation at amino acid 51 (Table 1). Uninjected or frameshift-injected (Figure 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 4E). In most of these embryos, hb protein can be seen at the anterior, farthest

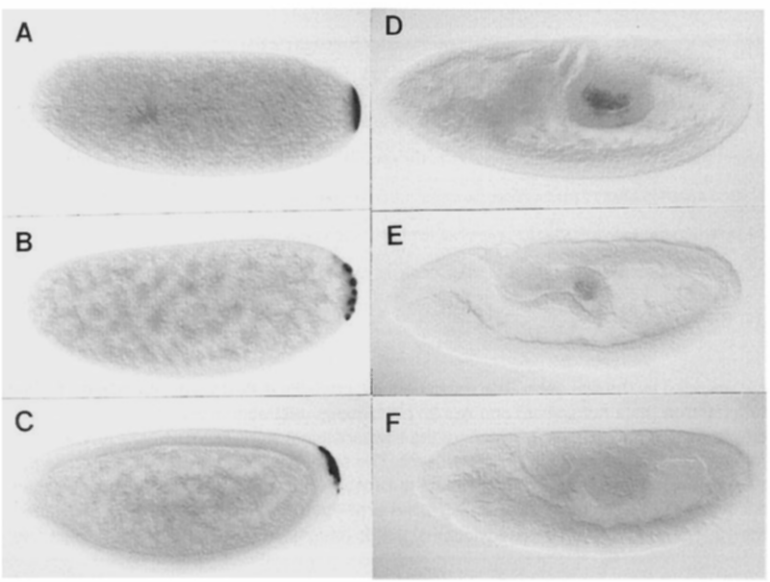


Figure 3. Whole-Mount In Situ Hybridization

The cDNA N5 (see Experimental Procedures) was used to prime synthesis of a uniformly labeled digoxigenin-UTP probe, which was hybridized to wild-type embryos.

(A) Early cleavage stage embryo (stage 2, ~0.5–1.3 hr). The probe recognizes a transcript that is localized to the posterior pole.

(B) Embryo at pole bud stage (stage 3, ~1.3–1.5 hr). The transcript is concentrated in the pole cells.

(C) Embryo at syncytial blastoderm stage (stage 4, ~1.5–2.5 hr). The transcript is restricted to the pole cells and lacking from the blastoderm, which will give rise to the somatic tissues.

(D) Embryo during early germband extension (stage 8, ~3.75–4.5 hr). Pole cells are carried inside the embryo and are clustered inside a pocket of cells that 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, ~4.5–5.15 hr). 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, ~5.15–6.25 hr). No specific staining for *nos* RNA can be detected. In all cases anterior is left, and dorsal is up. The final staining reaction was carried out longer in embryos shown in (D)–(F) than in those shown in (A)–(C). The developmental stages are as described in Campos-Ortega and Hartenstein (1985), and the duration of stages at room temperature (22°C) is according to Wieschaus and Nüsslein-Volhard (1986).

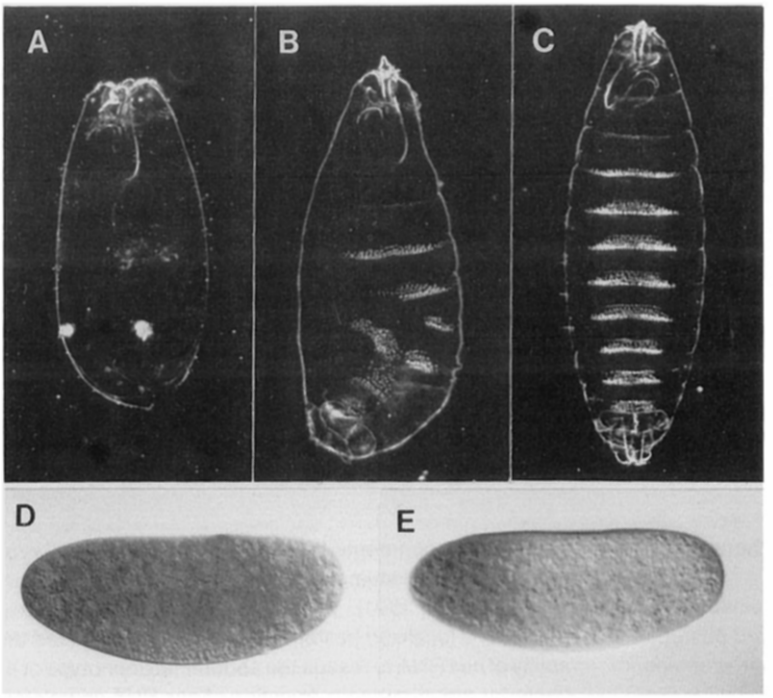


Figure 4. Rescue of the *nos* Mutant Phenotype by In Vitro Synthesized *nos* Transcript

(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 six 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) shows embryo injected with RNA synthesized from a template containing a frameshift mutation. (E) shows 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 shows 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.

from the site of injection (25%–50% egg length, where 0% is the 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 suggest that the phenotypic rescue of embryos by *nos* RNA injection is mediated by this interaction.

Table 1. Rescue of *nanos* Abdominal Phenotype by *nanos* Transcript

Donor Fraction	Embryos Scored (N)	Number of Abdominal Segments Formed (Percent Total) 0–1	2–5	6–8
Uninjected	29	100	–	–
No template	49	100	–	–
Frameshift template (1.7 μg/μl)[a]	79	96	4	–
pN5-RNA (0.03 μg/μl)	50	66	28	6
pN5-RNA (0.07 μg/μl)	45	15	49	36
pN5-RNA (0.13 μg/μl)	46	–	–	100

[a] Of the 79 cuticles scored, 3 developed more than one segment. Of these, 2 had two segments, and 1 cuticle had three 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 amino acid amino-terminal polypeptide.

nos 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 1). At the lowest concentration tested, most embryos show only partial rescue (Figure 4B). At the highest concentration tested, all embryos are completely rescued and form all eight abdominal segments (Figure 4C). RNA transcribed from a template containing a frameshift mutation fails to rescue the abdominal defect when assayed by injection (Table 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 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). Furthermore, optimal rescue is achieved if *nos* RNA is injected into the prospective abdominal region (25%–50% egg length) (Figure 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 pole plasm with respect to the spatial and temporal parameters of rescue (Lehmann and Nüsslein-Volhard, 1986, 1991).

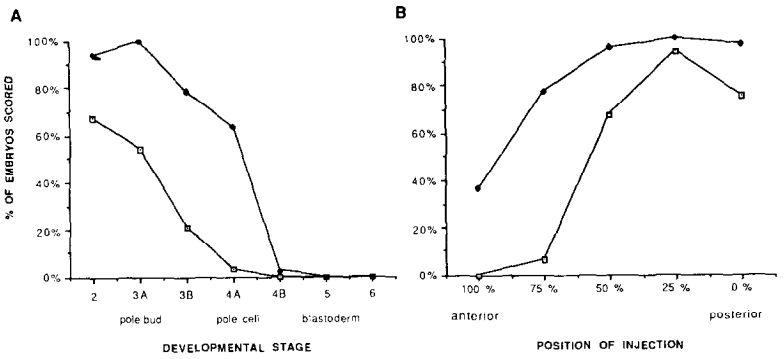


Figure 5. Developmental Stage and Position Dependence of Rescue

"Strong rescue" (open squares) indicates more than five abdominal segments, and "rescue" (solid squares) indicates two or more abdominal segments, as judged by the presence of abdominal ventral denticle belts. Fifteen to 80 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.

Synthetic *nos* 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 pole plasm, including the characteristic polar granules, and subsequently fail to form pole cells, the germline 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, 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). The germ cell defect, however, remains. These results indicate that *nos* acts as

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 (Percent 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	–	–
$pumilio^{660}$					
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	
$tudor^{WC8}/Df[2R]Pur^{P133a}$					
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.

[a] 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 $P \leq 0.004$.

a posterior determinant and suggest that these posterior group genes are dispensable for the translation and/or activity of nos protein. Since embryos from mothers mutant for these genes lack the specialized posterior pole plasm, we propose that localization of *nos* mRNA to the posterior pole depends on the presence of an intact pole plasm. The abdominal segmentation defect of these mutants may therefore be a secondary effect of failure to localize or stabilize pole plasm components, including the *nos* mRNA.

Embryos derived from *pum* mutant mothers have intact pole plasm and form germ cells. Cytoplasmic transplantation experiments have shown that the posterior pole plasm of *pum* embryos contains normal levels of rescuing activity for all posterior group mutants (Lehmann and Nüsslein-Volhard, 1987, 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), 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. Additional experiments are required to determine whether *pum* function involves intracellular transport, stabilization, abundance, or activity of nos protein.

Presence of *nos* at the Anterior Suppresses *bcd*

Previous studies have shown that transplantation of posterior pole plasm 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 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 6A and 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 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 that included reversal of abdominal segments. These results demonstrate that anteriorly intro-

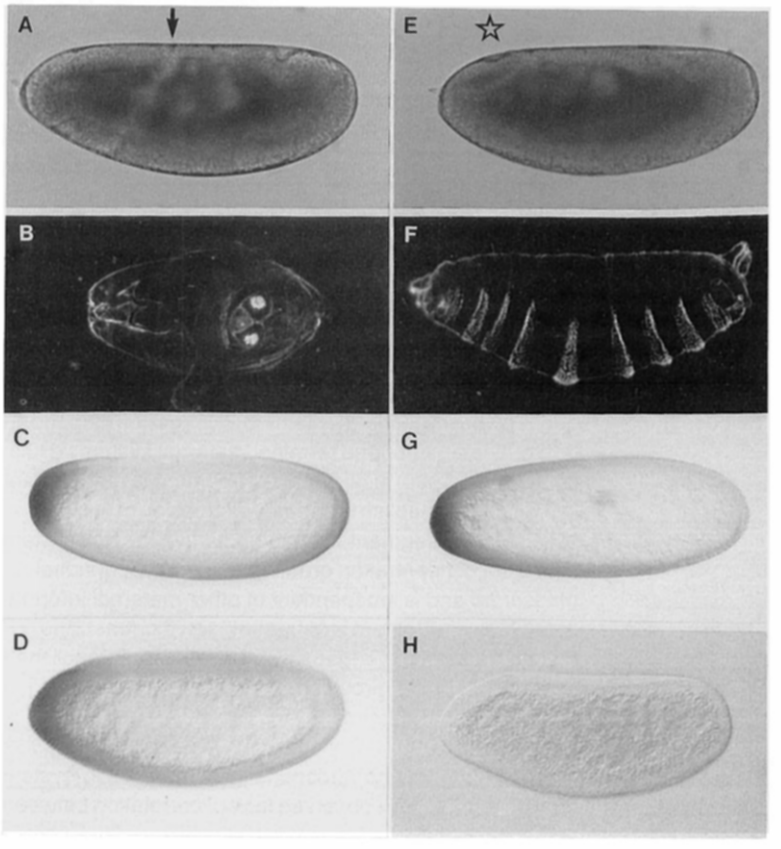


Figure 6. Ectopically Introduced *nos* Alters Embryonic Pattern

(A–D) Control embryos derived from nos^{L7} mutant females ([A] and [B], uninjected; [C] and [D], injected with in vitro synthesized *nos* RNA containing a frameshift mutation) show normal head development ([A] and [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] and [F]) and show normal levels and distribution of *bcd* RNA (G), while levels of bcd protein are reduced (H).

Top panel ([A] and [E]): living embryos during gastrulation (stage 7); the position of the cephalic furrow is indicated in (A) by an arrow. Star in (E) indicates the position of an ectopic posterior midgut invagination at the anterior. Second panel ([B] and [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] and [G]): syncytial blastoderm (stage 4) embryos stained for *bcd* RNA. Bottom panel ([D] and [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.

duced *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 *bcd* 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* and 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 frameshifted *nos* RNA (Figures 6D and 6H; Table 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 6C and 6G; Table 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 that are required for normal abdomen formation in Drosophila (Boswell and Mahowald, 1985; Schüpbach and Wieschaus, 1986; Lehmann and Nüsslein-Volhard, 1986, 1987, 1991; Manseau and Schüpbach, 1989). These studies revealed a factor concentrated in the pole plasm of wild-type embryos that rescues the abdominal defect of posterior group mutants (Lehmann and Nüsslein-Volhard, 1991). It was suggested that *nos* is involved in the synthesis of this factor (Lehmann and Nüsslein-Volhard, 1991). In this article, 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 to the anterior 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*), *kni*, and *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 *hb* gene products such that a concentration gradient of *hb* RNA and hb 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, 1990; Irish et al., 1989; Struhl, 1989).

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, "*nos*-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

Table 3. Effect of *nanos* on *bicoid*

Species	*nanos* Transcript Injected	N	Staining Pattern (Percent)			
			Wild type	Weak	None	Ectopic
bicoid RNA	Frameshift	30	47	30	10	13
	Wild type	29	31	38	27	34
bicoid Protein	Frameshift	49	61	25	14	
	Wild type	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.

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 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 6): first, *nos* suppresses *bcd* 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 6). In addition, in situ hybridization analysis of injected embryos shows that the stability and localization of *bcd* RNA is not significantly affected (Figure 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 the *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). Additional 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.

Experimental Procedures

Cloning of *nos*

The *nos* genomic region is defined by the chromosomal deficiencies *Df[3R]DI-HD28* and *Df[3R]DI-KX12*, which complement all *nos* mutants, and *Df[3R]DI-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]DI-A143* and *Df[3R]DI-KX12* interval contains, in addition to *nos*, three lethal complementation groups: *l(3)91Fb* (= *l(DIX43)c3*), *l(3)91Fe* (= *l(DIX43)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]DI-A143*, *Df[3R]DI-KX12*, and *Df[3R]DI-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]DI-A143* and *Df[3R]DI-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 cross-linked 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 *nos* probe. Blots were exposed to X-ray film with an intensifying screen for 48, 24, or 6 hr, for *nos*, *bcd*, or actin, respectively. Preparation of poly(A)$^+$ RNA as well as hybridization and washing of blots was 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 labeled with [α-^{32}P]dCTP by random primer oligolabeling and used to screen both lambda and plasmid cDNA libraries. Three independently isolated cDNA clones were subcloned into Bluescript vectors (Stratagene) and nested deletions were made using exonuclease III and mung bean nuclease according to manufacturers' protocols (Stratagene). These deletions were 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.

In Situ Hybridization

In situ hybridization was performed as described previously 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 β-globin 5′ untranslated leader sequence fused to the complete *nos* open reading frame (Brown and Kafatos, 1988). Methods for RNA synthesis were as described by Krieg and Melton (1984). The in vitro transcription products were ethanol precipitated and resuspended in diethyl pyrocarbonate–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 the 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 nucleotide 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, 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 diethyl pyrocarbonate–treated water. 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, 1987, 1991; Manseau and Schüpbach, 1989). In experiments in which rescued embryos were allowed to develop to 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; they were 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 hr 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 *bcd* cDNA probe for whole-mount in situ hybridization as described by Tautz and Pfeifle (1989).

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