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Extensive zygotic control of the anteroposterior axis in the wasp *Nasonia vitripennis*

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SUMMARY

Insect axis formation is best understood in *Drosophila melanogaster*, where rapid anteroposterior patterning of zygotic determinants is directed by maternal gene products. The earliest zygotic control is by gap genes, which determine regions of several contiguous segments and are largely conserved in insects. We have asked genetically whether early zygotic patterning genes control similar anteroposterior domains in the parasitoid wasp *Nasonia vitripennis* as in *Drosophila*. *Nasonia* is advantageous for identifying and studying recessive zygotic lethal mutations because unfertilized eggs develop as males while fertilized eggs develop as females. Here we describe recessive zygotic mutations identifying three *Nasonia* genes: *head only* mutant embryos have posterior defects, resembling loss of both maternal and zygotic *Drosophila caudal* function; *headless* mutant embryos have anterior and posterior gap defects, resembling loss of both maternal and zygotic *Drosophila hunchback* function; *squiggy* mutant embryos develop only four full trunk segments, a phenotype more severe than those caused by lack of *Drosophila* maternal or zygotic terminal gene functions. These results indicate greater dependence on the zygotic genome to control early patterning in *Nasonia* than in the fly.

Key words: *Nasonia*, *caudal*, *hunchback*, *engrailed*, *Ultrabithorax*, *abdominal-A*, *Drosophila*, Zygotic control

INTRODUCTION

The establishment of cell fates along the anteroposterior axis is well understood in *Drosophila melanogaster* (St. Johnston and Nüsslein-Volhard, 1992; Rivera-Pomar and Jäckle, 1996); yet the very rapid early embryogenesis of *Drosophila* is not shared with most other insects (Anderson, 1972). This makes insect embryonic patterning an attractive system for understanding the evolution of regulatory gene networks. Homologues of *Drosophila* embryonic patterning genes have been cloned and studied in other insects (Akam et al., 1994; Patel, 1994a; Tautz and Sommer, 1995; Brown and Denell, 1996; Rogers and Kaufman, 1997; Carroll, 1998). However, similarities in gene structure and expression do not guarantee the identity of gene functions. To address gene functions, we are studying anteroposterior patterning in the parasitoid wasp *Nasonia vitripennis*, an insect highly amenable to genetic analysis.

On first consideration, the Hymenopteran *Nasonia* and the Dipteran *Drosophila* appear very similar in their embryonic development, though the Hymenoptera diverged from the Diptera >200 million years ago. Embryos of both species produce larvae in about 1 day at 25°C (Bull, 1982; Campos Ortega and Hartenstein, 1985). In *Nasonia*, the fertilized egg gives rise to an embryo that undergoes syncytial and cellular blastoderm stages morphologically similar to those of *Drosophila*. Both *Nasonia* and *Drosophila* undergo the long germband mode of embryonic development. Despite these similarities, two observations suggest that the relative importance of maternal versus zygotic patterning functions may differ in the two insects. First, although postgastrulation events proceed with very similar timing, the time for early development differs substantially – at 25°C, the events preceding gastrulation take only about 3 hours in *Drosophila* but almost 10 hours in *Nasonia*. This difference in timing may allow for greater zygotic control of patterning in *Nasonia* than in *Drosophila*. Second, among the relatives of *Nasonia*, a polyembryonic mode of development has evolved in which a single fertilized egg gives rise to hundreds or thousands of progeny (Ivanova-Kasas, 1972). Polyembryonic development is likely to rely heavily on zygotic control of patterning. Polyembryony has arisen several times in the Hymenoptera, and the polyembryonic *Copidosoma floridanum* (Grbic et al., 1996) is in the same superfamily as *Nasonia*. These considerations pose the question – is early development substantially controlled by the zygotic genome in Hymenopterans?

We have addressed this question genetically, by isolating zygotic mutations that disrupt early anteroposterior patterning in *Nasonia*. The value of a combined genetic and molecular

approach to studying insect development has been established in the flour beetle *Tribolium castaneum* (Brown and Denell, 1996; Denell et al., 1996; Sulston and Anderson, 1996; Wolff et al., 1998). In *Tribolium*, patterning functions can be studied in a more primitive short germband embryo, using standard diploid genetics. In contrast, in *Nasonia*, unfertilized eggs develop as haploid males, so that embryonic lethal mutations covering the entire genome can be isolated as readily as mutations of X-chromosome genes in *Drosophila* (Whiting, 1967; Saul et al., 1967). For genetic analyses, *Nasonia* recessive lethal mutations can be carried in heterozygotes because fertilized eggs develop as diploid females.

Here we describe three mutations that we have isolated, defining three genes with roles in early *Nasonia* development. Each of these recessive zygotic mutations deletes pattern elements in several contiguous segments, as do gap gene mutations in *Drosophila*. However, each *Nasonia* gene affects a larger region of the embryo than does any zygotic gap gene function in *Drosophila*. In *Drosophila*, the most comparable phenotypes are produced either through the lack of maternal patterning functions or through the lack of both maternal and zygotic functions of key patterning genes. Thus, dependence on the zygotic genome to control early patterning is more extensive in the Hymenopteran *Nasonia* than in *Drosophila*.

MATERIALS AND METHODS

Nasonia genetics

The *head only (ho)*, *headless (hl)* and *squiggy (sq)* mutations were isolated in a screen for zygotic embryonic lethal pattern mutations that fail to hatch (Nüsslein-Volhard and Wieschaus, 1980). Wild-type males were mutagenized with 0.25% ethyl methanesulfonate (EMS) in 10% honey water for 3-12 hours and mated to females doubly or triply homozygous for genetic markers – *reddish-5 (rdh-5); scarlet-5219* (*st-5219)* or *purple*^{plum}*(pu*^{pm}*); rdh-5; st-5219.* Linkage relationships and genetic markers for *Nasonia (*previously known as *Mormoniella*) are described in Saul et al. (1967). *pu*^{pm} is an allele of *purple*. F_1 females were first set unmated – clutches with approximately 50% unhatched embryos (all males) were examined for cuticular phenotypes. Females bearing mutations of interest were mated to *rdh-5; st-5219* or *pu*^{pm}*; rdh-5; st-5219* males. >6800 genomes were screened as above. The mutations isolated were largely EMS-induced: 4.5% to 18% of F_1 females from EMS treatments carried new embryonic lethal mutations, compared to 0/168 for control females. Further details of this screen will be described elsewhere. The *squiggy* mutation was lost after the experiments described here were carried out.

To determine linkage relationships, F_2 females were sorted by marker genotype and assayed to determine which carried the lethal mutation of interest. If the lethal mutation did not show linkage to the original markers, lethal-bearing females were crossed to additional marker strains for two generations and evaluated similarly. Surviving males were also scored for consistent linkage data.

Complementation testing of lethal mutations is not straightforward in this haplo-diplo genetic system. The map positions of *ho*, *hl* and *sq* establish that these mutations identify three different genes. *ho* is <1 centimorgan from the adult morphological marker *mickey mouse*; *hl* is approximately 35 centimorgans from the nearest adult morphological marker *reverent; sq* is approximately 25 centimorgans from the eye-color marker *reddish-5*. Map positions for *ho* and *hl* were based on evaluation of >200 females and >200 males; the map position for *sq* is based on data from 20 females and 152 males. We also established that *hl* is not linked to *rdh-5* (based on 168 males), and that *sq* is not linked to *reverent* (based on >200 males). That *ho* and *mm* are not linked to *rdh-5* or to *reverent* have been confirmed through numerous mapping and double-mutant experiments (Saul et al., 1967; M. A. P., unpublished data). Map locations for *ho*, *hl* and *sq* were also reconfirmed each generation during stock maintenance.

Nasonia strains were maintained on pupae of *Sarcophaga bullata*; eggs were collected on pupae of *Sarcophaga* or of Calliphorid species. Mutant strains were maintained by selecting females phenotypically wild type for a linked marker gene, determining which females carried the embryonic lethal of interest by assaying male embryos, then crossing to males mutant for the linked marker. Some of our *headless* and *head only* lines eventually acquired consistently weakened phenotypes; except where noted, we describe lines with the original strong mutant phenotypes.

Analysis of *Nasonia* embryos

Except where noted otherwise, *Nasonia* embryos were raised at 28°C. For cuticle preparations, embryos were mounted in 90% lactic acid/10% ethanol, and cleared at 56°C. For observation of pole cells, living embryos were mounted in water on microscope slides with coverslips, without removal of the optically clear chorions. To fix, embryos (not dechorionated) were shaken in a 1:1 mixture of heptane: 4% formaldehyde in phosphate-buffered saline. Most of the heptane and formaldehyde were removed, leaving only the interface region. To devitellinize, embryos were shaken in −70°C 1:1 heptane:methanol, warmed rapidly under lukewarm tap water. The monoclonal antibody 4D9 (Patel et al., 1989) was used at 1:1 to detect ENGRAILED. The monoclonal antibody FP6.87 (Kelsh et al., 1994) was used at 1:7 to detect ULTRABITHORAX plus ABDOMINAL-A. Secondary antibodies were peroxidase-conjugated goat anti-mouse (Jackson ImmunoResearch), used at 1:250. Antibody incubations and nickle-enhanced diaminobenzidine (DAB) staining followed standard procedures (Patel, 1994b). To collect embryos for antibody staining, females heterozygous for the mutation of interest were identified by assaying phenotypes of unhatched progeny. When set unmated, females produce all male progeny. Genotypes used for antibody staining experiments were: (1) *ho, mm*$^{+}$*/ho*$^{+}$*, mm* and *st-318*$^{+}$*, ho / st-318, ho*$^{+}$, (2) *hl, rev*$^{+}$ */ hl*$^{+}$*, rev* and (3) *sq, rdh-5*$^{+}$ */ sq*$^{+}$*, rdh-5*. Control experiments indicated no embryonic abnormalities associated with the marker loci. For *ho* cold-sensitivity experiments, *st-318*$^{+}$*, ho / st-318, ho*$^{+}$ was used. Embryos were viewed on a Leica DMRB microscope using 20×/0.5 NA fluotar or 40×/0.7 NA fluotar objectives. Images were photographed using Kodak 160 ASA tungsten film or a DAGE-CCD camera with a DSP-2000 digital signal processor connected to a Mac Quadra 800 with a PDI Nubus frame grabber. Slides were scanned with a Nikon SuperCool Scan II. Adobe Photoshop was used for adjusting resolution and contrast of digitized images.

Analysis of *Drosophila* embryos

Drosophila stocks were maintained on instant *Drosophila* medium (Carolina Biological). Wild-type flies were Canton S. Fly embryos were raised at 25°C. Males from a balanced stock of the amorphic allele *hunchback*^{14F} (Lehmann and Nüsslein-Volhard, 1987) were crossed to Canton S females to generate *hb*/+ parents of homozygous *hunchback* mutant embryos. Embryos lacking *nanos* function were collected from *st nanos*^{53} *e / st nanos*^{18} *e* mothers. *nanos*^{18} is an amorphic allele and *nanos*^{53} is a strong allele (Lehmann and Nüsslein-Volhard, 1991; Gavis and Lehmann, 1992). In this experiment, cuticular mutant phenotypes indicated that abdominal development was only partially disrupted, and the embryo shown in Fig. 4 represents the most extreme 10% of the mutant phenotypes observed. Lack of maternal *caudal (cad)* function was assayed by generating germline chimeras with the yeast recombinase/dominant female sterile system, using the amorphic *cad*^{2} allele (Rivera-Pomar et al., 1995). *pr cad*^{2} *P* [*hs-neo; ry*$^{+}$*; FRT*]^{40A} / *CyO* females were crossed to *P* [*ry*$^{+}$*; hs-FLP*]^{12}; *P* [*w*$^{+}$*; Ovo*^{D1}]$^{2L\text{-}13X13}$ *P* [*hs-neo; ry*$^{+}$*; FRT*]^{40A} / *CyO* males, and larval

progeny were heat-shocked at 37°C. Cy^+ female progeny were crossed to cad^2 / CyO males; embryos were collected from this cross. Approximately half of the embryos had mild segmentation defects (as previously described for maternal loss of *cad* function) and about half had severe abdominal defects, as previously described for loss of both maternal and zygotic *cad* function (MacDonald and Struhl, 1986). The embryos with the most severe phenotypes were assumed to be those lacking both maternal and zygotic *cad* function; the embryo shown in Fig. 4 is an average representative of that class. *Drosophila* embryos were handled as described for *Nasonia*, except that fly chorions were removed with 50% bleach and embryos were devitellinized at room temperature.

RESULTS

Here we describe three recessive zygotic mutations that we have isolated in *Nasonia*. These define three genes affecting early anteroposterior patterning: *head only, headless* and *squiggy.* To examine the roles of these genes in segmentation, we have characterized their cuticular phenotypes as well as their effects on expression of the segment polarity gene *engrailed* and on trunk Hox genes. To interpret the relationship of the *Nasonia* genes to their *Drosophila* counterparts, we have also examined *Drosophila* mutant genotypes.

Wild-type *Nasonia*

In *Nasonia*, early development prior to gastrulation is morphologically very similar to that of *Drosophila* (Bull, 1982). As in *Drosophila*, the entire length of the *Nasonia* germband is formed from cells already present in the blastoderm at the initiation of gastrulation. Gastrulation initiates in *Nasonia* as the blastoderm pulls away ventrally from the vitelline membrane in the region of the anterior thorax and lateral folds appear in the gnathal region. As the germband extends, both head and tail extend around the dorsal side of the embryo, and the tail extends anteriorly on the dorsal side for only about 20% of the length of the embryo. Once the germband is extended, the gnathal lobes form, then segmental furrows appear gradually in an anteroposterior sequence. As segmentation proceeds, the stomadeum forms dorsally and then shifts gradually to an anterior position through stomadeal involution. Dorsal closure and germband retraction proceed concurrently.

Cuticular features of the first-instar larva are shown in Fig. 1A-C. The *Nasonia* larva, about 320 μm in length, has a distinct head and thirteen trunk segments (Fig. 1A; Azab et al., 1967; Bull, 1982). There is a denticle belt for each of the three thoracic and ten abdominal segments. Each denticle belt has finer denticles anteriorly and coarser denticles posteriorly. The denticle belt of the first thoracic segment does not extend

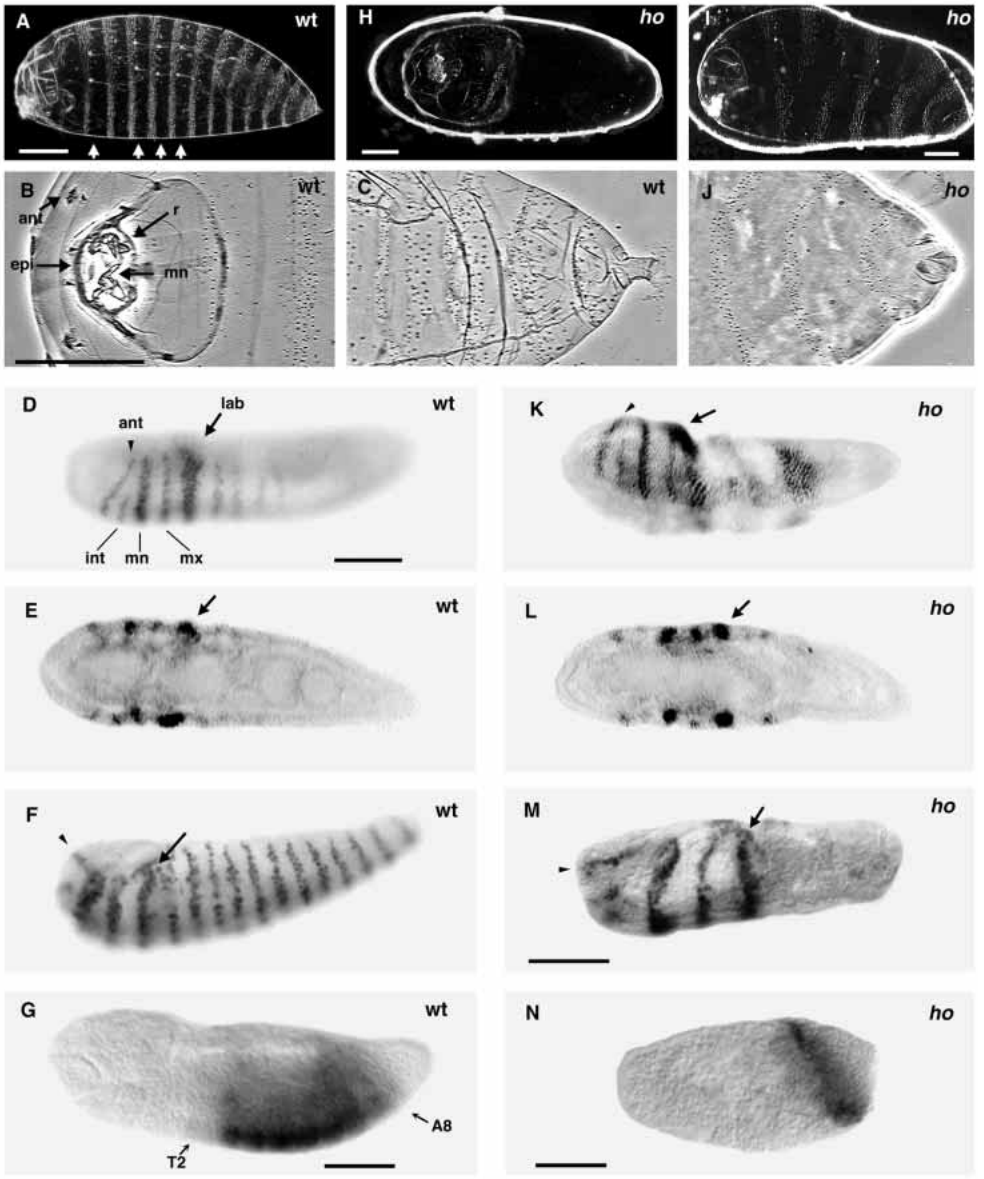

Fig. 1. Wild-type (wt) *Nasonia* and *head only* (*ho*) mutant phenotypes. Anterior, left. (A-G) Wild type. (A) First instar larval cuticle. Arrowheads: spiracle-bearing second thoracic and first three abdominal segments. (B) First instar ventral larval head, after Azab et al. (1967); mn, mandibles; r, chitinized rod; epi, epistoma; ant, antennal sensory papillae. (C) First-instar larval tail. (D,E) Initiation of EN expression; ant, antennal; int, intercalary; mn, mandibular; mx, maxillary; lab, labial. For all EN panels, arrowhead, antennal; arrow, labial. (F) Elaboration of EN expression, germband extending. (The tail of the embryo has become straightened during fixation.) (G) UBX-ABD-A in segmenting embryo. T2, second thoracic; A8, eighth abdominal. (H-N) *head only (ho)* mutant phenotypes. (H,I) Cuticular *ho* mutant phenotypes. (J) Tail of *ho* mutant embryo. (K,L) EN initiation in *ho* mutant embryos. (M) EN in *ho* mutant embryo with extending germband. (N) UBX-ABD-A in segmenting *ho* mutant embryo. Scale bars, 50 μm.

completely around the embryo. In the remaining trunk segments, there is little difference in the appearance of the dorsal aspect of the denticle belts from one trunk segment to the next, though the belts become narrower ventrally toward the posterior of the abdomen. The second thoracic segment and first three abdominal segments bear large spiracles laterally. Prominent features of the larval head include a dorsolateral pair of small truncate antennal papillae, and the anteroventral mouth parts, surrounded by a chitinized ring (Fig. 1B). The caudal region of the first instar larva is simple in structure, bearing a cuticular collar surrounding a tube-shaped anus that everts after hatching (Fig. 1C).

To follow expression of the segment polarity gene *engrailed (en)* in *Nasonia*, we used the monoclonal antibody 4D9 (Patel et al., 1989). In *Drosophila*, initiation of the EN pattern is controlled by pair-rule genes, which are controlled by gap genes and ultimately by maternal coordinate genes (Pankratz and Jäckle, 1993). The EN expression pattern is maintained by the segment polarity genes (Martinez Arias, 1993). In *Nasonia*, as gastrulation begins, the first EN stripes appear in the antennal, mandibular and labial segments, soon followed by stripes in the intercalary and maxillary segments (Fig. 1D,E). The antennal EN stripe slopes characteristically toward the posterior along the ventral-to-dorsal axis. After the head EN stripes are expressed strongly, the trunk EN stripes appear in succession from anterior to posterior. As the germband extends, there are five head stripes and twelve trunk stripes of EN expression (Fig. 1F). As the head extends dorsally, the head EN stripes develop characteristic morphologies seen in many insects (Flieg, 1990; Rogers and Kaufman, 1996), such as the formation of intercalary spots (not shown) and dorsal fusion of the maxillary and labial EN stripes.

To follow the expression of trunk Hox genes in *Nasonia*, we used the monoclonal antibody FP6.87 (Kelsh et al., 1994), which recognizes both ULTRABITHORAX (UBX) and ABDOMINAL-A (ABD-A). In *Drosophila*, the initiation of Hox gene patterning is controlled by early genes in the segmentation hierarchy, and maintenance of that pattern is controlled by homeotic gene cross-regulation and by the Polycomb-group genes (McGinnis and Krumlauf, 1992; Martinez Arias, 1993). Expression of UBX-ABD-A in *Nasonia* is similar to expression patterns in *Drosophila* and *Tribolium* (Kelsh et al., 1994; Castelli-Gair and Akam, 1995; Shippy et al., 1998). Weak expression extends from the posterior second through the third thoracic segments, and strong expression extends from the first through the seventh abdominal segments (Fig. 1G). The eighth abdominal segment stains more weakly than the anterior abdominal segments.

head only

In *head only* mutant embryos, head structures develop normally while the posterior is defective. Cuticular phenotypes of *head only* mutants typically range from embryos that have developed only a head and partial denticle belt, to those that have developed a limited number of abdominal segments (Fig. 1H,I) – in the latter, variable segmental fusions occur frequently in trunk segments. The *head only* mutant phenotype is sensitive to genetic background and is also cold-sensitive. Cold-sensitivity is most easily detected in lines with weaker mutant phenotypes. Table 1 compares *head only* embryos collected from the same mothers at 28°C and at 16°C, and shows that the frequency of hatching and the number of denticle belts are decreased at the lower temperature. Embryos with weak *head only* mutant phenotypes often hatch. These have missing abdominal segments and defective caudal structures (Fig. 1J).

Table 1. Cold-sensitivity of the *head only (ho)* mutant phenotype

	Hatching frequency	No. of denticle belts/*ho* embryo		
Temperature	Hatched *ho*/ all *ho* embryos	Range	Mean	No. of embryos scored
28°C	25/350	2-10	6.0	46
16°C	0/113	0-2	0.3	25

In *head only* mutant embryos, ENGRAILED (EN) initiates in anterior segments but fails to initiate normally in posterior segments (Fig. 1K, compare to 1D). EN expression is displaced posteriorly relative to the length of the embryo at the time of EN initiation (Fig. 1L, compare to Fig. 1E), indicating an alteration of the fate map. The variation in EN expression in *head only* mutant embryos parallels the variability of cuticular phenotypes. In extreme cases, EN stripes initiate in the head but not in the trunk. Fig. 1M shows an embryo undergoing germband extension – only the head EN stripes are present, though by this time trunk EN stripes should have appeared (as in Fig. 1F).

The trunk Hox genes UBX-ABD-A are expressed in a very narrow band in mutant *head only* embryos (Fig. 1N), with a strip of strong expression bordered anteriorly and posteriorly by weaker expression. The region of expression is very narrow from the time of UBX-ABD-A initiation (not shown). The size of the region posterior to the UBX-ABD-A domain appears to be similar in *head only* mutant embryos and in wild-type embryos (Fig. 1N,G).

Given the disruption of posterior development in *head only* mutant embryos, we asked whether pole cells are affected. Individual living embryos were followed from the time of pole cell formation until gastrulation, when mutant embryos can be distinguished from their phenotypically wild-type siblings. Pole cells in *head only* mutant embryos develop normally (not shown).

headless

headless mutant embryos have pattern deletions anteriorly and posteriorly. The *headless* cuticular mutant phenotype is shown in Fig. 2A-E. Fig. 2A,B shows ventral and dorsal views of the same embryo. *headless* mutant embryos have seven denticle belts surrounding the embryo. The spiracle pattern (Fig. 2B) identifies the widened anteriormost denticle belt on the ventral side (Fig. 2A) as that of the first abdominal segment. Dorsally, there are additional variably disorganized denticles anterior to the first abdominal denticle belt. Behind the seventh denticle belt are additional disorganized denticles. Based on the spacing and width of the segments that form approximately normally, we interpret the missing abdominal denticle belts as those of the posterior three abdominal segments eight through ten. In *headless* mutant embryos, the only consistent chitinized head structure is the anteriormost arch, the epistoma (Fig. 2C, compare to Fig. 1B), a labral derivative (Azab et al., 1967). The epistoma is often accompanied by a chitinized rod-like structure (Fig. 2D). The embryos also lack antennal sensory

papillae. The posterior ends of *headless* mutant embryos bear misshapen lobes of cuticle and defective analia (Fig. 2E, compare to Fig. 1C).

In *headless* mutant embryos, the anteriormost EN stripe initiates at a position corresponding to the gnathal region of a wild-type embryo (Fig. 2F). In older *headless* mutant embryos, there are seven EN stripes that wrap laterally and ventrally around the embryo (Fig. 2G). The anteriormost EN stripe is the widest, followed by six stripes and a posterior spot. The failure to initiate EN stripes in the anterior head, the trunk-like morphologies of the EN stripes, and the correspondence of the seven full EN stripes with the seven full abdominal denticle belts together indicate deletion of thoracic and gnathal segments, and of more anterior head segments including the antennal segment but not including labral derivatives. The posterior gap domain extends from the posterior seventh through the tenth and last abdominal segment. This leaves parasegments six through twelve.

In *headless* mutant embryos, the trunk Hox genes UBX-ABD-A are initiated (Fig. 2H) and maintained (Fig. 2I) in a domain that is expanded both anteriorly and posteriorly relative to that of wild-type embryos (Fig. 1G). Anteriorly, UBX/ABD-A expression extends through the region that would normally give rise to gnathal and more anterior head segments. Posteriorly, UBX-ABD-A extend almost to the posterior tip of the embryo. Pole cell formation is not affected in *headless* mutant embryos (not shown).

squiggy

In *squiggy* mutant embryos, both the anterior and posterior ends of the embryo fail to develop, leaving about four to nine trunk segments in the middle of the embryo (Fig. 3A,B). Fig. 3B shows an embryo with three adjacent spiracle-bearing segments, as in the first three abdominal segments of wild-type embryos. In this embryo, as in many, an additional ectopic spiracle has formed on what should be the fifth abdominal segment. This ectopic spiracle may represent a transformation to second thoracic segment identity, which would indicate mirror-image patterning of segment identity. In some embryos, the second thoracic segment also develops with its normal spiracle, forming a 1011101 mirror-image spiracle pattern on each side of the embryo in the second thoracic through fifth abdominal segments (not shown). However, the individual denticle belts of the developed segments do not have mirror-image polarity – rather, they have the normal anteroposterior sequence of fine to coarse denticles. In any given collection of *squiggy* mutant embryos, approximately 30-50% have phenotypes similar to those shown above, while mutant siblings have only fragments of poorly developed cuticle.

Despite the variability of *squiggy* cuticular mutant phenotypes, the pattern of EN expression in *squiggy* mutant embryos is quite consistent. EN fails to initiate normally in the head region and the first EN stripe appears in what would be the posterior gnathal region of a wild-type embryo (Fig. 3C). When EN expression is elaborated (Fig. 3D), every *squiggy* mutant embryo has four EN trunk stripes that are approximately normal in size and spacing. EN stripes posterior to these are closely spaced and only one cell in width, for up to four segments. EN patterning anterior to the four normal EN stripes is variable, and includes circular patterns of expression as in Fig. 3D, or solid EN spots (not shown).

UBX-ABD-A expression is also affected in *squiggy* mutant embryos (Fig. 3E). Whereas in wild-type embryos there is weak expression of UBX-ABD-A in the posterior thorax (Fig.

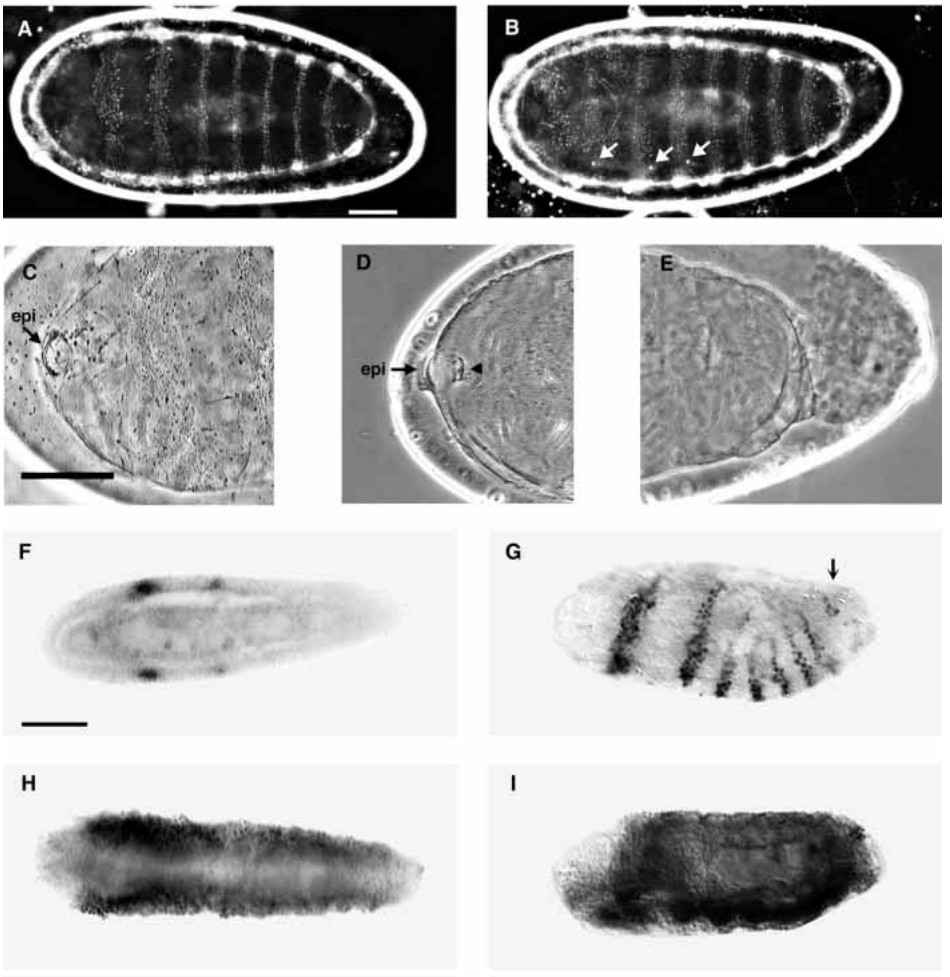

Fig. 2. *headless (hl)* mutant phenotypes. Anterior, left. (A,B) Ventral and dorsal views, respectively, of the same *hl* mutant embryo. Arrows, spiracles. (C,D) Heads of *headless* mutants epi, epistoma; arrowhead, cuticular rod. (E) Tail of *hl* mutant embryo. (F,G) EN at initiation (F) and in the extending germband (G), of *hl* mutant embryos. Arrow, posterior EN spot. (H,I) UBX-ABD-A in *hl* mutant embryos at initiation (H) and in segmented embryo (I). (H) At the extending germband stage, but has become straightened during fixation. Scale bars, 50 μm.

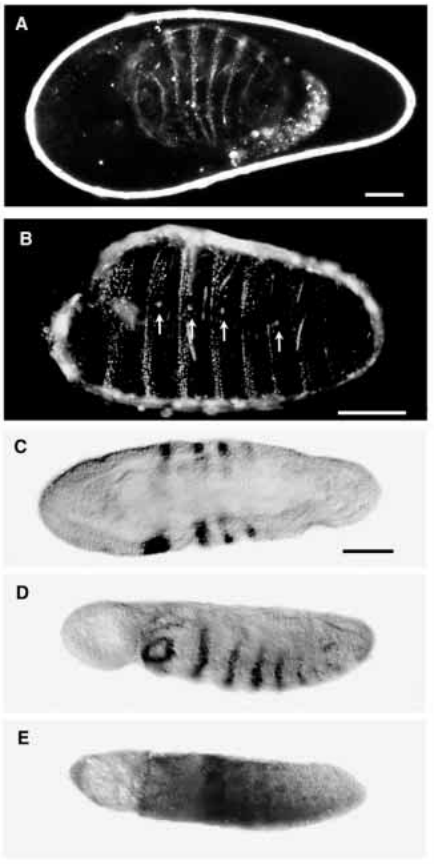

Fig. 3. *squiggy (sq)* mutant phenotypes. Anterior, left. (A,B) Cuticular *sq* mutant phenotypes. Arrows, spiracles. (C) EN initiation, (D) elaboration of EN expression and (E) UBX-ABD-A expression in *sq* mutant embryos Scale bars, 50 μm.

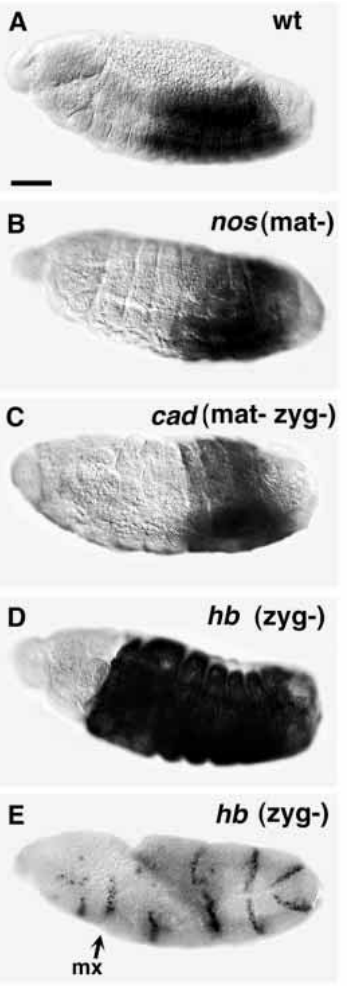

Fig. 4. *Drosophila* wild-type and mutant embryos. Anterior, left. (A-D) UBX-ABD-A expression. (A) Wild type. (B) Embryo lacking maternal *nanos* (*nos*) function. (C) Embryo lacking maternal and zygotic *caudal* (*cad*) function. (D) Embryo lacking zygotic *hunchback* (*hb*) function. (E) EN expression in embryo lacking zygotic *hb* function; mx, maxillary. Scale bar, 50 μm.

1G), *squiggy* mutant embryos have an expanded anterior region of weak UBX-ABD-A expression. Soon after UBX-ABD-A initiation, the region of strong UBX-ABD-A expression in *squiggy* mutant embryos is narrow, only one segment in width (Fig. 3E), with weaker expression more posteriorly. This weak posterior UBX-ABD-A expression may account for the mirror-image spiracle patterning described above. In older *squiggy* mutant embryos, the limit of UBX-ABD-A expression extends almost to the posterior tip of the embryo (not shown).

These results show that *squiggy* mutant embryos have a central domain that segments approximately normally. This domain is centered around the anterior abdomen and is about four segments in width, with variable development of up to four or five additional trunk segments. Regions anterior and posterior to the central trunk domain fail to segment and fail to differentiate position-specific cuticular structures. Pole cell formation is not affected in *squiggy* mutant embryos (not shown).

Comparison with *Drosophila* mutants

The *Nasonia* phenotypes described above suggest comparisons with the following *Drosophila* genes (see Discussion): (1) for *head only* – posterior group genes and *caudal*, and (2) for *headless* – *hunchback*. To evaluate these comparisons, we examined effects of the *Drosophila* genes on the molecular markers characterized in the *Nasonia* mutants. Fig. 4A-D shows UBX-ABD-A expression in wild-type and mutant *Drosophila* embryos at the end of germband retraction. UBX-ABD-A expression is similarly affected in younger *Drosophila* embryos, for all three mutant genotypes (not shown).

In *head only* mutant *Nasonia* embryos, the domain of UBX-ABD-A expression is narrowed, but not displaced posteriorly (Fig. 1N). In *Drosophila* embryos lacking maternal *nanos* (*nos*), the UBX-ABD-A domain is also narrowed and does not extend to the posterior tip of the embryo (compare the wild-type *Drosophila* embryo in Fig. 4A to the *nanos* mutant in Fig. 4B), consistent with previous results for *Ubx* (Irish et al., 1989). In *Drosophila* embryos lacking both maternal and zygotic *caudal (cad)*, the UBX-ABD-A domain is also narrowed and does not extend to the posterior tip of the embryo (Fig. 4C). The effect of *Nasonia head only* on UBX-ABD-A is therefore qualitatively consistent with both the *nanos* and *caudal* results shown here.

In *headless* mutant *Nasonia* embryos, UBX-ABD-A

expression is expanded (Fig. 2H,I). Fig. 4D shows the lesser degree to which the UBX-ABD-A expression domain is expanded anteriorly in *Drosophila* embryos lacking zygotic *hunchback (hb)* function. In *hunchback* mutant embryos, UBX-ABD-A expression is also expanded posteriorly, to a similar degree to that in *headless* mutant embryos. These results are consistent with those previously described for UBX (White and Lehmann, 1986).

Fig. 4E shows a *Drosophila* embryo lacking zygotic *hunchback* function at the extended germband stage, with seven trunk stripes of EN expression. EN is also expressed in the maxillary and more anterior head segments.

DISCUSSION

We have screened for mutations affecting cuticular patterning in *Nasonia*, and we have identified *head only*, *headless* and *squiggy*, three genes that control early anteroposterior patterning (Fig. 5A). For each of these *Nasonia* genes, a recessive zygotic mutation has much more extensive effects on embryonic patterning than does the loss of any zygotic gap gene function in *Drosophila*.

We have determined through linkage analysis that the three mutations described here identify three different genes (see Materials and Methods). All three mutations are recessive and are therefore likely to be loss-of-function alleles. Given a loss-of-function allele for a gene, the defective regions of mutant embryos identify regions in which the wild-type function of the gene is normally required. Recessive gain-of-function (neomorphic) mutations are not so straightforward in interpretation, but these are usually rare. Ethyl methanesulfonate (EMS) was used as the mutagen, therefore the mutations described here are probably point mutations.

head only

head only mutant embryos lack all segmentation posterior to the head, in the strongest manifestation of the phenotype, and have only a narrow domain of UBX-ABD-A expression. *head only* differs from *Drosophila* gap genes with respect to the extent of pattern deleted (Fig. 5A,B) and effects on UBX-ABD-A. In *Drosophila*, neither *Krüppel* nor *knirps* affects a domain as large as that of *head only* (Pankratz and Jäckle, 1993). Moreover, the wild-type functions of *Krüppel* and *knirps* are not required for the positive regulation of *Ubx* or *abd-A* in *Drosophila* (Ingham et al., 1986; Irish et al., 1989; Casares and Sánchez-Herrero, 1995).

Zygotic *head only* is more similar to *Drosophila* posterior-group genes than to gap genes, although the fly posterior-group genes function maternally. Specifically, the *nanos* and *pumilio* posterior group genes resemble *head only* in impairing abdominal development without abolishing pole cell formation (St. Johnston, 1993; Fig. 5C), and a lack of *nanos* function narrows the domain of *Ubx-abd-A* expression. However, since neither thorax nor telson are affected, the region impaired by *Drosophila* posterior-group genes is more limited than that affected by *head only*.

The phenotype of embryos lacking both maternal and zygotic *Drosophila caudal* corresponds most closely to that of *head only*. In *Drosophila*, *caudal* is expressed zygotically in a posterior domain, regulated by *hunchback*; in addition, maternal *caudal* mRNA is translationally repressed by maternal *bicoid* in the anterior, generating a gradient of *caudal* protein (Levine et al., 1985; MacDonald and Struhl, 1986; Mlodzik and Gehring, 1987; Schulz and Tautz, 1995; Dubnau and Struhl, 1996; Rivera-Pomar et al., 1996). Function of *caudal* is needed for activation of abdominal gap genes (Rivera-Pomar et al., 1995) and for control of hindgut development (Wu and Lengyel, 1998). The elimination of only maternal *caudal* function produces weak segmentation defects, and the elimination of only zygotic *caudal* produces variable defects of analia and other posterior structures. However, the elimination of both maternal and zygotic *caudal* produces larvae with extremely defective posterior development – those with the most extreme phenotype lack posterior thoracic and abdominal segments, and have defective analia (MacDonald

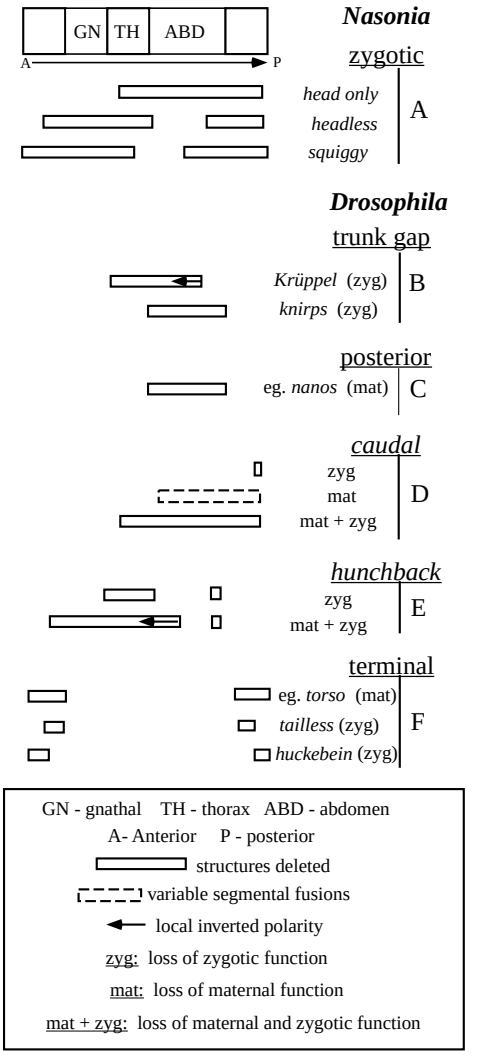

Fig. 5. Comparison of segments deleted in *Nasonia* and *Drosophila* mutants. (A) Phenotypes of the *Nasonia* mutants *head only*, *headless* and *squiggy*. (B) *Drosophila* abdominal gap gene phenotypes (Pankratz and Jäckle, 1993). (C) *Drosophila caudal* phenotypes (MacDonald and Struhl, 1986). (D) *Drosophila* posterior group gene phenotypes (St. Johnston, 1993). (E) *Drosophila hunchback* phenotypes (Lehmann and Nüsslein-Volhard, 1987). (F) *Drosophila* terminal gene phenotypes (Sprenger and Nüsslein-Volhard, 1993).

and Struhl, 1986; Fig. 5D). Lack of maternal and zygotic *Drosophila caudal* function narrows the UBX-ABD-A domain, as in *head only* mutant embryos.

Only *caudal* in *Drosophila* controls a region as large as that affected in *head only* mutant embryos. *caudal* is widely conserved in invertebrates and vertebrates (Murtha et al., 1991). The simplest interpretation of *head only* is that this may be a mutation in *Nasonia caudal*, which controls functions zygotically that are jointly controlled by both maternal and zygotic *caudal* in *Drosophila*.

headless

headless is similar to *Drosophila hunchback* in controlling the patterning of both anterior and posterior embryonic regions (Bender et al., 1987; Lehmann and Nüsslein-Volhard, 1987; Fig. 5A,E). *Drosophila hunchback* is expressed zygotically in both anterior and posterior embryonic domains; in addition, maternal *hunchback* mRNA is translationally repressed by *nanos* in the posterior, generating a maternal gradient of *hunchback* protein (Pankratz and Jäckle, 1993; St. Johnston, 1993). *hunchback* is evolutionarily conserved in insects (Sommer et al., 1992).

In both *headless* and *hunchback* mutants, posterior abdominal segments are deleted. In *Drosophila hunchback*, the posterior deletion spans from the posterior seventh through the eighth and last full abdominal segment. In *Nasonia headless*, the deletion spans from the posterior seventh through the tenth and last abdominal segment, and terminalia are also defective (Fig. 5A,E).

In both *headless* and *hunchback* mutants, the anterior gap domain includes the three thoracic segments, plus part of the head (Bender et al., 1987; Lehmann and Nüsslein-Volhard, 1987). In *Drosophila* embryos lacking zygotic *hunchback* function, the anterior pattern deletion extends only into the labial segment. In contrast, in *Nasonia headless* mutant embryos the deletion extends further anteriorly, through the gnathal and antennal segments, though the most anterior labral cuticular derivatives are present.

In *Nasonia headless* as in *Drosophila hunchback*, the trunk pattern elements remaining include the denticle belts of the first through seventh abdominal segments and the trunk EN stripes anterior to each of those denticle belts. For both *headless* and zygotic *hunchback*, the remaining trunk pattern spans parasegments six through twelve.

The effects of *headless* on UBX-ABD-A bear out the interpretation that *headless* is comparable to *Drosophila hunchback*, except that more anterior regions of the embryo are affected in *headless* than in fly embryos lacking zygotic *hunchback* function. In *headless* mutant embryos, UBX-ABD-A expression is expanded anteriorly through and beyond the region that would develop into the gnathal head segments in wild-type embryos. In fly embryos lacking zygotic *hunchback*, the UBX-ABD-A domain shows a more limited anterior expansion that extends only slightly into the gnathal region of the embryo (White and Lehmann, 1986). In both *headless* and *hunchback* mutant embryos, the UBX-ABD-A domain also expands posteriorly.

In *Drosophila*, *hunchback* is expressed both maternally and zygotically. Although the zygotic function alone is sufficient to direct normal embryonic development, both maternal and zygotic *hunchback* products are functional. When both maternal and zygotic *hunchback* functions are eliminated, the resulting mutant phenotype is stronger than the zygotic loss-of-function *hunchback* phenotype described above (Lehmann and Nüsslein-Volhard, 1987; Simpson-Brose et al., 1994). When both functions are removed, embryos lack all gnathal segments (Fig. 5E), and the anterior abdominal segments have reversed polarity. These embryos are similar to *headless* mutant embryos anteriorly, lacking all gnathal segments, although the abdominal reversed polarity phenotype does not resemble *headless*.

A novel class of *hunchback* alleles (class V alleles) was described by Lehmann and Nüsslein-Volhard (1987). These are recessive neomorphic (gain-of-function) alleles causing homeotic transformations of head or thoracic segments into abdominal segments, superimposed on weaker or stronger gap phenotypes. UBX expression is expanded further anteriorly in class V homozygous embryos than in embryos homozygous for a null *hunchback* allele (White and Lehmann, 1986). However, the homeotic phenotypes of class V fly *hunchback* alleles do not correspond to *Nasonia headless* phenotypes: the lack of EN expression in the head region of *headless* mutant embryos indicates that head segments are deleted rather than merely homeotically transformed.

The zygotic *headless* phenotype best resembles that of *Drosophila* embryos lacking both maternal and zygotic *hunchback*. The simplest interpretation is that *headless* may be a mutation in the *Nasonia hunchback* gene, which controls functions zygotically that are jointly controlled by both maternal and zygotic *hunchback* in *Drosophila*.

squiggy

squiggy mutant embryos have severe defects both anteriorly and posteriorly, leaving only four consistently developed trunk segments. This cuticular phenotype differs substantially from the phenotypes of maternal terminal group genes in *Drosophila* (Fig. 5A,F), such as *torso*, in which loss-of-function maternal-effect mutations delete pattern elements from both ends of the embryo (Sprenger and Nüsslein-Volhard, 1993). The terminal structures deleted in *torso* embryos are anterior to the gnathal segments and posterior to the seventh abdominal segment, and are thus limited compared to those of the zygotic *squiggy* mutant embryos. The terminal gap genes *tailless* and *huckebein* are zygotic targets of the *Drosophila* terminal pathway (Fig. 5B, Sprenger and Nüsslein-Volhard, 1993). These two genes control overlapping subsets of the limited maternal terminal gene domains described above, and therefore also affect regions much more limited than those affected by *squiggy*.

The extensive zygotic control of terminal development by *squiggy* appears to be a departure from *Drosophila* developmental mechanisms. The *Drosophila* maternal terminal gene patterning system is not known to be widely conserved, and the follicle cell types that express *torsolike* do not appear to be conserved even in the lower Diptera (Sander, 1996). Terminal patterning in insects may therefore be subject to considerable evolutionary flexibility.

Zygotic control of early patterning

head only, *headless* and *squiggy* share a common theme: the zygotic *Nasonia* phenotypes are more extreme than those of *Drosophila* gap genes and all three genes appear to control processes zygotically that are partially or fully subject to

maternal control in the fly. The *head only* and *headless* phenotypes are most like those of fly embryos lacking both the zygotic and the maternal gradient contributions of *caudal* and *hunchback*, respectively. We are currently testing linkage of *Nasonia caudal* and *hunchback* homologues to these *Nasonia* mutations and investigating *Nasonia caudal* and *hunchback* expression. In the beetle *Tribolium castaneum*, both *caudal* and *hunchback* have maternally loaded transcripts as well as zygotic expression (Wolff et al., 1995; Schulz et al., 1998). Since flies are more closely related to wasps than to beetles, the combined maternal and zygotic expression of *caudal* and *hunchback* appear to have preceded the divergence of the Diptera and the Hymenoptera.

The control of early development can evolve rapidly (Raff, 1996). For example, *bicoid* diverges rapidly within the Diptera (Schröder and Sander, 1993) and can carry out few of its transcriptional functions without its more conserved partner, *hunchback* (Simpson-Brose et al., 1994). Moreover, zygotic expression of *hunchback* in the anterior gap domain is positively regulated by *bicoid* in *Drosophila* but appears to be positively regulated by *caudal* in *Tribolium* (Wolff et al., 1998).

There is recent molecular evidence for zygotic expression in other insects of patterning functions supplied maternally in *Drosophila*. *ftz-f1*, needed maternally in *Drosophila* for pair-rule patterning, is expressed zygotically in a pair-rule pattern in *Tribolium* (S. Brown and R. Denell, personal communication).

Our results also indicate evolutionary flexibility of insect early development, contrasting the extensive zygotic control of *Nasonia* axial patterning with the combined maternal and zygotic control (or strictly maternal control) of comparable functions in *Drosophila*. Primarily zygotic control of Hymenopteran patterning would be favorable for the evolution of polyembryonic development. Further study of Hymenopteran embryogenesis should lead to greater understanding of flexibility in the evolution of early developmental processes.

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