A major role for zygotic *hunchback* in patterning the *Nasonia* embryo

Mary Anne Pultz[1,¶], Lori Westendorf[1,*], Samuel D. Gale[1,†], Kyle Hawkins[1], Jeremy Lynch[2], Jason N. Pitt[1,‡], Nick L. Reeves[1,§], Jennifer C. Y. Yao[1], Stephen Small[2], Claude Desplan[2] and David S. Leaf[1]

[1]Department of Biology, Western Washington University, Bellingham, WA 98225, USA
[2]Department of Biology, New York University, New York, NY 10003, USA
*Present address: Seattle Genetics, Bothell, WA 98021, USA
†Present address: Program in Neurobiology and Behavior, University of Washington, Seattle, WA 98105, USA
‡Present address: Program in Molecular and Cellular Biology, University of Washington, Seattle, WA 98105, USA
§Present address: Division of Biological Sciences, University of California – San Diego, San Diego, CA 92093, USA
¶Author for correspondence (e-mail: pultz@biol.wwu.edu)

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Summary

Developmental genetic analysis has shown that embryos of the parasitoid wasp *Nasonia vitripennis* depend more on zygotic gene products to direct axial patterning than do *Drosophila* embryos. In *Drosophila*, anterior axial patterning is largely established by *bicoid*, a rapidly evolving maternal-effect gene, working with *hunchback*, which is expressed both maternally and zygotically. Here, we focus on a comparative analysis of *Nasonia hunchback* function and expression. We find that a lesion in *Nasonia hunchback* is responsible for the severe zygotic *headless* mutant phenotype, in which most head structures and the thorax are deleted, as are the three most posterior abdominal segments. This defines a major role for zygotic *Nasonia hunchback* in anterior patterning, more extensive than the functions described for *hunchback* in *Drosophila* or *Tribolium*. Despite the major zygotic role of *Nasonia hunchback*, we find that it is strongly expressed maternally, as well as zygotically. *Nasonia* Hunchback embryonic expression appears to be generally conserved; however, the mRNA expression differs from that of *Drosophila hunchback* in the early blastoderm. We also find that the maternal *hunchback* message decays at an earlier developmental stage in *Nasonia* than in *Drosophila*, which could reduce the relative influence of maternal products in *Nasonia* embryos. Finally, we extend the comparisons of *Nasonia* and *Drosophila hunchback* mutant phenotypes, and propose that the more severe *Nasonia hunchback* mutant phenotype may be a consequence of differences in functionally overlapping regulatory circuitry.

Key words: *Nasonia*, Hunchback, Axis formation, Evolution of development, Hymenoptera, *Drosophila*

Introduction

The patterning of insect embryos is controlled by a spectrum of well-conserved to rapidly evolving genes. The molecular genetics of axis formation has been examined in a variety of insect embryos (Tautz and Sommer, 1995; Dearden and Akam, 1999; Lall and Patel, 2001; Lynch and Desplan, 2003b) and provides a framework for exploring the fundamental principles of regulatory gene evolution.

Anteroposterior axis formation is best understood in *Drosophila*, where early embryogenesis takes place extremely rapidly and depends heavily on maternal input (St. Johnston and Nüsslein-Volhard, 1992; Rivera-Pomar and Jäckle, 1996). The *bicoid* homeodomain morphogen is provided maternally as mRNA localized to the anterior of the oocyte (Berleth et al., 1988). Bicoid synergizes with Hunchback, a zinc-finger protein, in controlling anterior development (Simpson-Brose et al., 1994). *hunchback*, a gap gene, transcriptionally controls other gap genes, as well as pair-rule and homeotic genes (Pankratz and Jäckle, 1993; Simpson-Brose et al., 1994; Tautz and Sommer, 1995; Casares and Sánchez-Herrero, 1995; Fujioka et al., 1999; Shimell et al., 2000; Wu et al., 2001; Clyde et al., 2003). *hunchback*, in contrast to *bicoid*, is provided maternally as unlocalized mRNA, and is expressed zygotically under the control of *bicoid* and other transcriptional regulators (Bender et al., 1988; Schröder et al., 1988; Tautz, 1988; Margolis et al., 1995). Although not essential, maternal *hunchback* does control some head-determining functions in wild-type *Drosophila*, as embryos lacking all maternal and zygotic products have a larger anterior gap than those lacking only zygotic *hunchback* (Lehmann and Nüsslein-Volhard, 1987). Maternal *hunchback* must be translationally repressed by *nanos* for normal posterior development (Hülskamp et al., 1989; Irish et al., 1989; Struhl, 1989).

To better understand the evolution of anteroposterior patterning, we have taken advantage of the haplo-diploid genetic system of the wasp *Nasonia vitripennis* to screen for mutations affecting cuticular morphology. In haplo-diploids, fertilized eggs develop as diploid females while unfertilized eggs develop as haploid males, facilitating a screen of the genome for recessive zygotic mutations (Pultz and Leaf, 2003). We identified about one-fourth to one-third of the genes required to pattern the *Nasonia* embryo, including representatives of gap, pair-rule and Polycomb-group genes with varying degrees of functional

similarity to known *Drosophila* genes (Pultz et al., 2000). Three zygotic mutations caused extensive disruptions of early patterning, more severe than the defects caused by any known zygotic mutation in *Drosophila.* One of the *Nasonia* mutations, originally named *headless*, deletes all of the head except the most anterior labral segment, as well as thoracic and posterior abdominal segments (Fig. 1A) (Pultz et al., 1999). The *headless* mutant phenotype suggested a similarity to *Drosophila hunchback*. Zygotic loss of *hunchback* in *Drosophila* causes a gap deletion from the posterior labial segment – the posterior border of the head – through the thoracic segments, and also affects posterior abdominal segments (Fig. 1A) (Bender et al., 1987; Lehmann and Nüsslein-Volhard, 1987). We hypothesized that the *headless* mutant phenotype is caused by a mutation in *Nasonia hunchback*, and that zygotic *hunchback* plays a more extensive role in embryonic patterning in *Nasonia* than in *Drosophila* (Pultz et al., 1999).

Non-dipteran insects must initiate embryonic patterning using different methods from those of *Drosophila*. Although *bicoid* controls the development of head, thorax and anterior abdomen in *Drosophila*, the *bicoid* gene has apparently arisen only relatively recently, within the higher Diptera (Stauber et al., 1999; Brown et al., 2001; Stauber et al., 2002). *bicoid* encodes a homeodomain protein with a key lysine at position fifty (K50) of the homeodomain, and is hypothesized to have usurped functions originally controlled by *orthodenticle*, which also encodes a K50 homeodomain protein; in addition, *hunchback* is hypothesized to have played a more extensive role in patterning the anterior of more ancestral insects (Wimmer et al., 2000; Lynch and Desplan, 2003a). Parental RNA interference experiments in the beetle *Tribolium* have indicated that the *orthodenticle* gene plays a major role in patterning the anterior of this non-Dipteran insect; when both *Tribolium orthodenticle* and *Tribolium hunchback* (Wolff et al., 1995) are knocked down, very little remains of the segmental patterning in the *Tribolium* embryo (Schröder, 2003). The potential role of *hunchback* as an ancestral morphogen has also been tested by manipulating *hunchback* expression in *Drosophila*, where increased levels of *bicoid*-independent *hunchback* have been shown to be capable of patterning the abdomen and even the thorax in the absence of *bicoid* (Hülskamp et al., 1990; Struhl et al., 1992; Schulz and Tautz, 1994; Wimmer et al., 2000).

Studies of *hunchback* in representatives of more ancestral insects have provided an intriguing perspective on the evolution of this key regulatory gene. In the milkweed bug *Oncopeltus*, *hunchback* mRNA is expressed both maternally and zygotically, and embryos with knocked-down *hunchback* function exhibit transformations of gnathal and thoracic segments to an abdominal identity, as well as impaired germ-band development (Liu and Kaufman, 2004). By contrast, in the more ancestral grasshopper, *Schistocerca*, *hunchback* is provided maternally to the embryo as protein, rather than as mRNA, through release from the posteriorly located oocyte nucleus, suggesting that its function may be to distinguish embryonic from extra-embryonic cells in that short-germ embryo (Patel et al., 2001). A later graded expression of *Schistocerca* Hunchback is provided zygotically, consistent with a concentration-dependent role in axial patterning. These results indicate that the ancestral *hunchback* axis-determining function in insects is likely to be zygotic, and that the expression and function of maternal *hunchback* has significantly changed during insect evolution.

Here, we focus on the role of *hunchback* in the hymenopteran *Nasonia vitripennis*. *Nasonia* has long-germ embryos, with a syncytial mode of early development that is morphologically similar to *Drosophila* embryogenesis – although unlike *Drosophila*, *Nasonia* does not have the highly derived condition of extremely rapid early development. In fact, approximately three-fold more time is allocated to early development (prior to gastrulation) in *Nasonia* than in *Drosophila* (Fig. 1B) (Bull, 1982; Campos Ortega and Hartenstein, 1985) – allowing more time for the zygotic genome to control early development. The Hymenoptera have evolved diverse embryonic developmental strategies. These include embryos with derived holoblastic cleavage (Grbic and Strand, 1998), as well as several independently evolved cases of polyembryony, in which a single fertilized egg develops into hundreds or thousands of clonal progeny (Strand and Grbic, 1997; Grbic, 2000). Syncytical long-germ development is considered to be ancestral in the Hymenoptera (Strand and Grbic, 1997), so *Nasonia* can be considered to be a representative of the ancestral mode of development within this clade.

We show that the severe *Nasonia headless* zygotic-mutant phenotype is caused by a mutation in *Nasonia hunchback*, and we describe the expression of *Nasonia hunchback* mRNA and protein. We also compare molecular mutant phenotypes of *Nasonia* embryos lacking zygotic *hunchback* to those of *Drosophila* embryos lacking both maternal and zygotic *hunchback*. We propose that the divergent mutant phenotypes for the same gene in two different species may, in large part, be due to changes in the functionally overlapping genetic regulatory network.

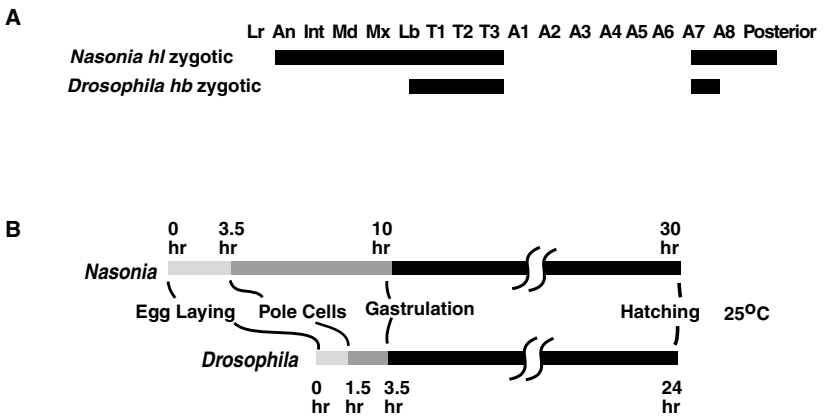

Fig. 1. Comparison of mutant phenotypes and embryonic timing. (A) Comparison of *Nasonia* zygotic *headless* (*hl*) and *Drosophila* zygotic *hunchback* (*hb*) mutant phenotypes. The black bars indicate regions with pattern deletions. (B) Comparative timing of embryogenesis in *Nasonia* and *Drosophila*. At 25°C, development from gastrulation to hatching is completed in about 20 hours in both insects, but approximately threefold more time is allocated to early development, prior to gastrulation, in *Nasonia*.

Materials and methods

Meiotic mapping of *Nasonia hunchback* to *Nasonia headless*

The highly conserved middle zinc-finger region of *Nasonia hunchback* was cloned from *Nasonia vitripennis* (*Nv*) and from *Nasonia giraulti* (*Ng*) using degenerate forward (5′-CGCGAATT-CAARCAYCAYCTNGARTAYCA-3′) and reverse primers (5′-AT-ATGCGACRTGRCARTAYTTNGTNGCRTA-3′) with the following PCR cycling conditions: 94°C for 30 seconds, 60°C for 60 seconds and 72°C for 120 seconds, for 32 cycles.

To identify a *Ng*-specific *hunchback* single nucleotide polymorphism, a *Ng*-specific forward primer (5′-CCATCTGCGC-AAGCA-3′), and a species non-specific reverse primer (5′-GC-AGTCGCAGCACCT-3′) were used to amplify a *Ng hunchback* fragment with the following PCR cycling conditions: 95°C for 30 seconds, 58°C for 60 seconds, 72°C for 60 seconds for 33 cycles.

To determine linkage, *Nv headless*-bearing females were crossed to *Ng* males cured of *Wolbachia* with antibiotics, kindly provided by Jack Werren (University of Rochester, NY, USA). The *Nv headless*/*Ng headless*$^{+}$ F1 hybrids were sorted from their *Nv headless*$^{+}$/*Ng headless*$^{+}$ control sisters by assaying their embryos. Experimental and control females were set unmated, then DNA was prepared from single surviving adult F2 males (Gloor et al., 1993). DNA that failed to amplify with the *Ng*-specific primer was shown to support amplification with species non-specific primers.

Analysis of genomic DNA from *headless* mutant embryos

The deletion in *Nasonia hunchback* was characterized by isolating DNA from 30-50 selected *headless* mutant embryos (Gloor et al., 1993). PCR amplification with primers at the 5′ and 3′ ends of the coding region generated a product approximately 1.5 kb shorter than the wild-type product, indicating a deletion. The mutant product was cloned and sequenced. The precise size of the deletion was 1497 bp, consistent with the PCR analysis. Identical sequences across the breakpoint were obtained from two independently amplified reactions.

Collection and fixation of *Nasonia* embryos and ovaries

When *Nasonia* embryos are collected from virgin females, all embryos are precisely staged – there are no older embryos from previously fertlized eggs as in *Drosophila*. Embryos after gastrulation were fixed as described in Pultz et al. (Pultz et al., 1999). Most of the blastoderm embryos were shaken in heptane for 2 minutes, then an equal volume of methanol was added and they were shaken for an additional 2-3 minutes at room temperature. Later, we found that sufficiently dry blastoderm embryos can also be fixed in 1:1 heptane: 4% formaldehyde in 1×PBS, improving morphology. Very early embryos (0-3 hours old) cannot be effectively devitellinated with methanol. These were fixed for 1 hour in heptane pre-saturated with 37% formaldehyde, then hand-peeled on double-stick tape in 1×PBS. Older hand-peeled embryos with a known expression pattern were included as a positive control. All embryos were males, collected from virgin mothers. To avoid cross reactivity of the anti-*Nasonia hunchback* antibody with endosymbiotic bacteria, we used wild-type *Nasonia vitripennis* cured of *Wolbachia* (a gift from Jack Werren), and we cured the *hunchback*^{hl} stock of *Wolbachia* by treating the mothers for two generations with rifampicin. Ovaries were dissected from mothers, fixed for 10 minutes in 8% formaldehyde, dehydrated and stored in methanol or ethanol until used for antibody staining or in situ hybridization, respectively.

In situ hybridization

Nasonia hunchback mRNA was visualized using an anti-sense RNA probe, as described previously (Jiang et al., 1991). The probe, about 1100 bp in length, extended from exon 2 through the central zinc-finger region (see Fig. 3). As a negative control, a probe was prepared from the opposite strand of the same fragment, and was applied to samples of all ages of embryos and tissues analyzed. No staining was observed with the negative controls.

Anti-*Nasonia* hunchback antibodies

A 125-amino-acid region beginning at amino acid 79 and terminating before the NF1 zinc finger was PCR-amplified using forward (5′-GTTGTTGAATTCGCTGGGATAAAATCGTA-3′) and reverse (5′-GTTGATAAGCTTGGGCAGCTCGAATCC-3′) primers, then cloned into the *Eco*R1 and *Hin*dIII sites of pGEX-KG, producing a GST-hunchback fusion protein. The fusion protein was isolated as described by Leaf and Blum (Leaf and Blum, 1998) and injected into rabbits for the production of polyclonal antiserum.

Antibody-staining experiments

The anti-*Nasonia* Hunchback antibodies were used at a dilution of 1:1000 to stain *Nasonia* embryos. All staining patterns observed in wild-type embryos – of cellular blastoderm age and older – were verified to be absent in *hunchback*^{hl} mutant embryos. The FP6.87 monoclonal antibody (Kelsh et al., 1994), which recognizes conserved epitopes on both Ultrabithorax (Ubx) and Abdominal-A (Abd-A) proteins was used at a dilution of 1:7 to stain *Nasonia* and *Drosophila* embryos. The anti-*Drosophila hunchback* guinea pig polyclonal antibody (Kosman et al., 1998) was used at a dilution of 1:400. All antibodies were visualized with horseradish peroxidase-labeled secondary antibodies and diaminobenzidine substrate, as described by Pultz et al. (Pultz et al., 1999).

Drosophila crosses

To analyze maternal Hunchback expression, embryos were collected from parents heterozygous for Df (3R*) p^{25}, which deletes the 5′ end of the *hunchback* transcription unit and does not produce *hunchback* mRNA (Bender et al., 1988). To analyze Ubx-Abd-A expression in embryos lacking both maternal and zygotic *hunchback*, we used a *hb*^{FB} FRT strain kindly provided by Ernst Wimmer (Georg-August-University Göttingen, Germany), collecting the embryos from FLP-bearing *hb*^{FB} FRT/ovo^{D} mothers that had been heat shocked as larvae to induce clones homozygous for the null *hunchback* mutation in their ovaries (Dang and Perrimon,1992). These females were crossed to *hb*^{14F}/TM3 males, such that approximately 50% of the offspring lacked both maternal and zygotic *hunchback*, whereas the other 50% lacked only maternal *hunchback*. Because maternal *hunchback* is not needed by *Drosophila* embryos in the presence of zygotic *hunchback*, half of the embryos showed a wild-type pattern of Hox gene expression. The other half, lacking both maternal and zygotic *hunchback*, were severely defective. The mutant phenotypes were confirmed using cuticle preparations.

Results

Linkage testing of *Nasonia hunchback?*

We began by cloning a small genomic fragment of the *Nasonia vitripennis* (*Nv*) *hunchback* gene containing the four middle zinc fingers, which are highly conserved in insects (Sommer et al., 1992, Patel et al., 2001). Next, to investigate whether the *Nv-hunchback* sequence maps to the *headless* (*hl*) mutation, we took advantage of the fact that *N. vitripennis* can be crossed to a sibling species, *N. giraulti* (*Ng*). Fertile F1 hybrids can be generated by removing *Wolbachia*, an endosymbiont responsible for cytoplasmic incompatibility (Breeuwer and Werren, 1990), which generates asynchronous cell cycles of the male and female pronuclei (Tram and Sullivan, 2002). The estimated divergence time of approximately 200,000 years (Campbell et al., 1993) between the sibling species enhances the likelihood that a single nucleotide polymorphism (SNP) for

mapping can be identified in a short and highly conserved sequence. After identifying such an SNP in *Nasonia hunchback*, we used an *Ng*-specific primer (Fig. 2A,B) to analyze the surviving adult male progeny from virgin *Nv hl/Ng hl*$^{+}$ experimental mothers and *Nv hl*$^{+}$*/Ng hl*$^{+}$ control mothers (Fig. 2C, Materials and methods). We found that 105/105 surviving sons of the experimental mothers were hemizygous for the *Ng-hunchback*$^{+}$ allele, as would be expected if the *headless* mutant phenotype were due to a lesion in *Nv hunchback*. (In the sons of the control mothers, *Nv* and *Ng* alleles segregated approximately equally: 9 *Ng* to 6 *Nv*.) These results led us to examine the DNA of *headless* mutant embryos for a lesion in the *Nasonia hunchback* gene.

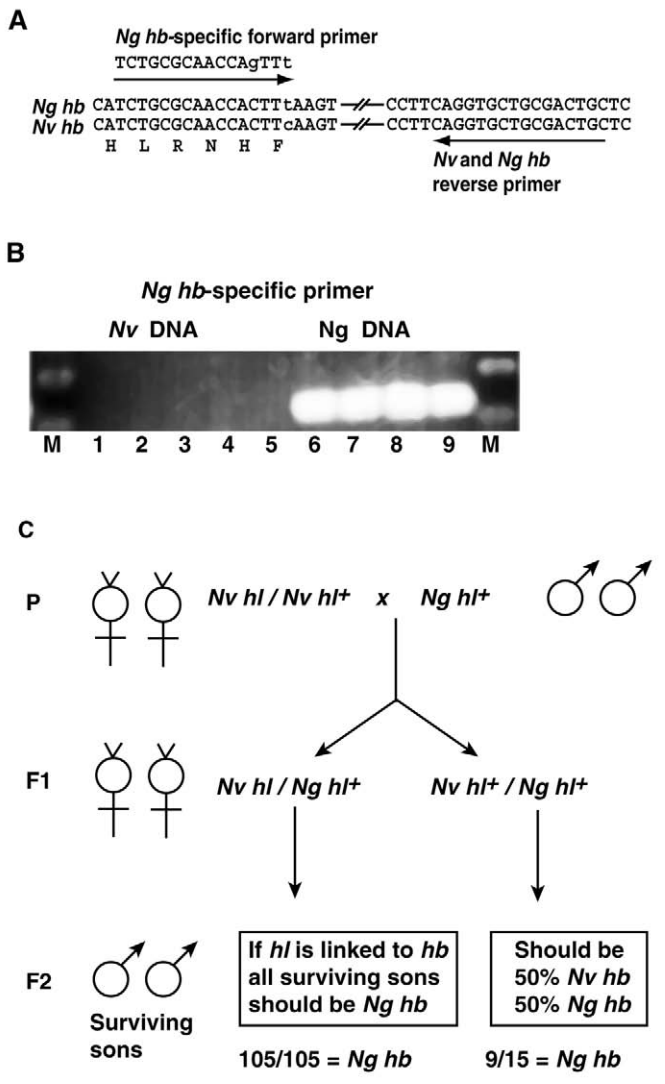

Fig. 2. Linkage analysis of the *headless* mutation. (A) Primers used for mapping *N. vitripennis* (*Nv*) and *N. giraulti* (*Ng*) *hunchback*. Lowercase letters on the Ng-specific primer indicate sites of mismatch: a T/C SNP at the 3′ end and a destabilizing mismatch four bases from the 3′ end. (B) PCR controls with *Nv* and *Ng* genomic DNA demonstrating the efficacy of the *Ng*-specific primer. (C) Strategy for inter-specific cross to test linkage of *Nv hunchback* to *headless*. If *hunchback* is linked to *headless* then surviving hemizygous sons of the experimental F1 mothers should all have *Ng hunchback*.

headless mutant embryos have a deletion in *Nasonia hunchback*

Nasonia Hunchback shares with other *hunchback* proteins a set of four-conserved zinc fingers in the middle of the protein and two zinc fingers at the C terminus (Fig. 3A). In addition, *Nasonia* Hunchback has an additional N-terminal zinc finger, not found in *Drosophila* or *Tribolium* Hunchback, which is similar to the Nf-1 zinc finger of *Schistocerca* and *Oncopeltus* Hunchback (D.S.L. and M.A.P., unpublished). As in *Drosophila* and *Tribolium*, *hunchback* appears to be transcribed in *Nasonia* from more than one promoter (Fig. 3A; D.S.L. and M.A.P., unpublished).

To identify the *headless* mutation, genomic DNA from *headless* mutant embryos was amplified and sequenced, revealing a deletion of 1497 bp after the first 40 amino acids of the predicted protein-coding sequence (assuming that translation starts in exon 2). As shown in Fig. 3B, this deletion disrupts the reading frame for the protein-coding sequence. This most likely defines a null allele for the *Nasonia hunchback* gene (see Discussion), consistent with our hypothesis that the very severe *headless* mutant phenotype is caused by a loss of zygotic *Nasonia hunchback*. Consequently, we re-designated *Nasonia headless* (*hl*) as *Nasonia hunchback*^{hl}.

Does *Nasonia hunchback* have candidate Nanos-response elements?

The translational regulation of *Drosophila hunchback* is mediated by the binding of Pumilio to Nanos Response Elements (NREs) within the 3′ untranslated region (UTR), and the subsequent recruitment of Nanos and Brain Tumor to form a quarternary complex (Murata and Wharton, 1995; Sonada and Wharton, 2001; Wang et al., 2002). Figure 3C shows candidate NREs from *Nasonia hunchback*, which are similar to the conserved Box A and Box B of the *Drosophila hunchback* NREs. The canonical *hunchback* NREs have a characteristic spacing of three to four bases between Box A and Box B. By contrast, the candidate NREs of *Nasonia hunchback* have 12-16 bases separating Box A and Box B, reminiscent of the structure of a candidate NRE found in the 3′ UTR of *Drosophila* cyclin B1 mRNA (Wang et al., 2002). In the germline, Pumilio and Nanos translationally repress *Drosophila* cyclin B1 expression (Nakahata et al., 2001). The presence of candidate NREs is of interest in light of the difference between the mRNA and protein expression of *Nasonia hunchback* described below.

Wild-type expression of *Nasonia hunchback*

To determine whether *Nasonia hunchback* is expressed maternally, and how embryonic expression compares with that of other insects, we examined *Nasonia hunchback* mRNA expression, and we raised an antibody against part of *Nasonia* Hunchback (HB-GST, Fig. 3). We found that *hunchback* mRNA is supplied to the embryo maternally, from high-level expression in the oocyte (Fig. 4), and as an mRNA that is dispersed throughout the egg (data not shown) and not yet translated, as no protein could be detected in 0-1 hour embryos (data not shown).

The timing of key morphological events during embryogenesis at 28°C is summarized in Fig. 5A. Just before pole cell formation, *Nasonia* Hunchback is expressed ubiquitously (data not shown). An anterior to posterior gradient

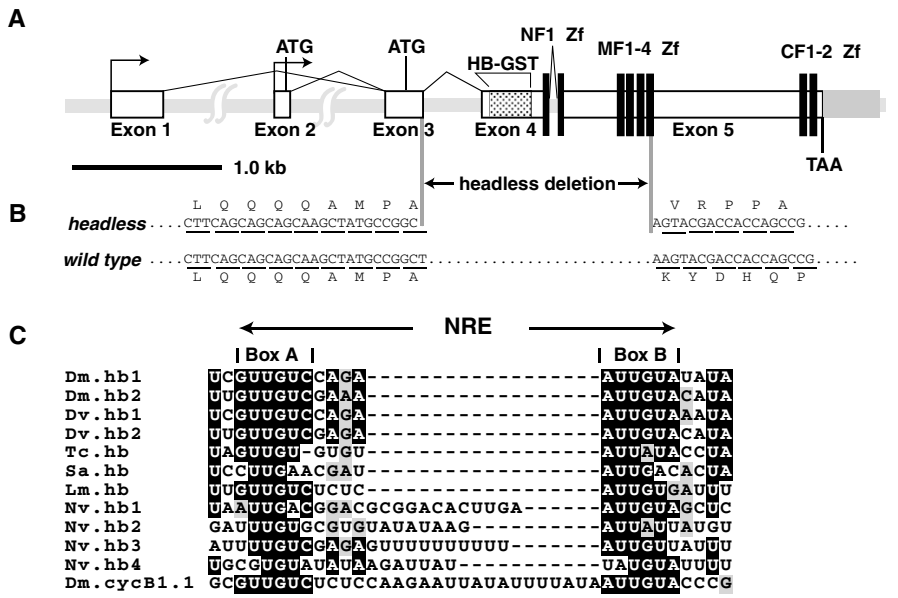

Fig. 3. *Nasonia hunchback* gene structure and the *headless* (*hb*^{hl}) deletion. (A) *Nasonia hunchback* gene structure [GenBank accession numbers: DQ116756 (cDNA), DQ116757 (cDNA), DQ116758 (genomic)]. Exons are boxed. Arrows indicate putative transcription start sites. ATG indicates putative initiating methionines. NF1 Zf, MF 1-4 Zf, and CF1-2 Zf refer to C2H2 zinc fingers, and are indicated as bars. The NF1 Zf is interrupted by an intron. The HB-GST region, against which the anti-*Nv*-Hunchback antibody was raised, is indicated as a stippled box in exon 4. TAA indicates the stop codon. The shaded box indicates a 3′ UTR. (B) A deletion in *Nasonia hunchback* in the DNA from *headless* (*hb*^{hl}) mutant embryos. The open reading frames from *headless* and wild-type genomic DNA show that the breakpoints of the 1.497 kb *hb*^{hl} deletion generate a frameshift mutation in *Nasonia hunchback* (GenBank accession number: DQ116759). The dotted lines on the wild-type *Nv hb* indicate contiguous sequence. (C) The alignment of candidate NREs from the 3′ UTR of *Nasonia hunchback* (Nvhb.1-4) with NREs of *D. melanogaster* (Dm), and with candidate NREs from *hunchback* genes of other insects: *D. virilis* (Dv), *Tribolium* (Tc), *Locusta* (Lm) and *Schistocerca* (Sa), as well as with Dm.cycB1.1.

of *Nasonia* Hunchback begins to form soon after the nuclei migrate to the surface and begin dividing at the surface of the embryo (Fig. 5C). The protein is localized to nuclei. During the next two hours (at 28°C), until the beginning of cellularization, the embryos continuously express an anterior domain of *Nasonia* Hunchback, with a sharpening border (Fig. 5E). However, throughout this period of graded *Nasonia* Hunchback expression, we did not observe a parallel gradient of *Nasonia hunchback* mRNA expression. Rather, the embryos express a low ubiquitous level of *hunchback* mRNA, superimposed with a small anterior and a larger posterior domain of expression during the cell cycles just after pole cell formation (Fig. 5B). This is followed by restriction of the posterior domain to the posterior, with incipient expression at the center of the embryo (Fig. 5D). The difference between the lack of *Nasonia* Hunchback at the posterior and the continuous presence of the mRNA at the posterior, throughout this two-hour period, indicates that *Nasonia hunchback* must be translationally controlled.

During the later stages of blastoderm development, the expression of *Nasonia* Hunchback appears to follow the expression of the mRNA, first expressed as anterior and posterior domains (Fig. 5F,G), then retracting from the anterior and anterodorsal region, and from the posterior. The anterior Hunchback domain diminishes in intensity in both the mRNA and protein expression prior to the onset of gastrulation, whereas the posterior stripe is still strongly expressed (Fig. 5H,I).

Just prior to gastrulation, a narrow stripe of *Nasonia hunchback* mRNA expression appears on the dorsal side of the embryo (Fig. 6A). Upon germ-band extension, this dorsal expression domain appears to be associated with serosa development (Fig. 6B-D). Although in many primitive insects the serosa develops from the anterior, in *Nasonia* the serosa, an extra-embryonic membrane, begins to develop in the dorsal region of the embryo, then expands anteriorly and ventrally to eventually envelop the entire embryo (Bull, 1982). Finally, *Nasonia* Hunchback is also expressed in a patterned subset of cells in the central nervous system, most strongly during the period of head involution (Fig. 6E,F).

We investigated whether *Nasonia hunchback* function is needed for serosa formation, by comparing living wild-type

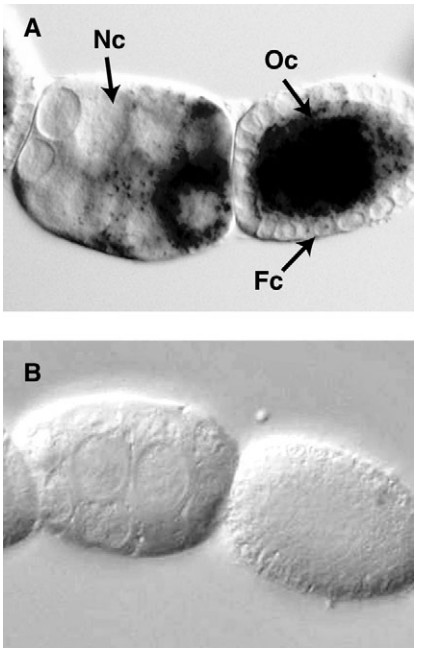

Fig. 4. Maternal expression of *Nasonia hunchback.* (A) *Nasonia hunchback* mRNA is loaded from the nurse cells (Nc) – of which there are 15, as in *Drosophila* – into the maturing oocyte (Oc). The non-staining cells surrounding the oocyte are the follicle cells (Fc). (B) Negative-control staining using a sense *Nasonia hunchback* probe.

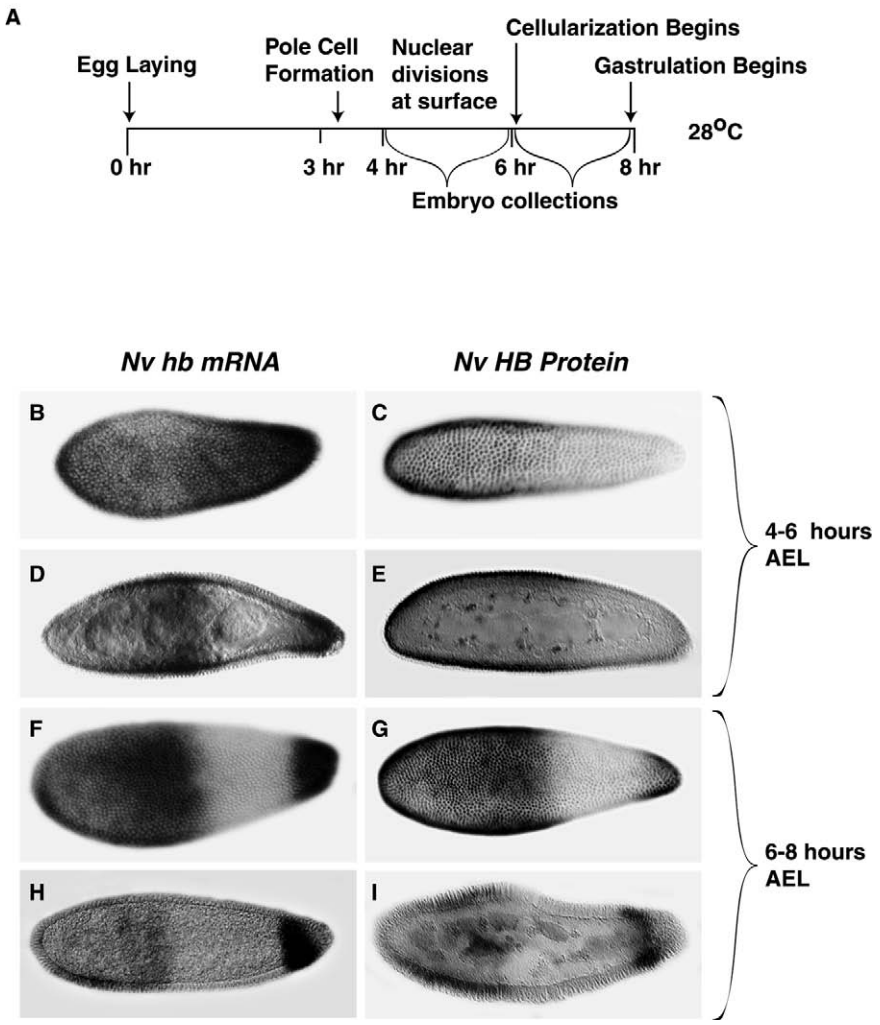

Fig. 5. *Nasonia hunchback* expression during blastoderm development. (A) Timeline of *Nasonia* embryogenesis at 28°C. The embryos in B and C are from the same two-hour egg collection as the embryos in D and E. (B) mRNA expression soon after the nuclei begin dividing at the surface of the embryo. (C) Anterior nuclear gradient of protein expression after the nuclei begin dividing at the surface of the embryo. (D) The next phase of mRNA expression after that shown in B, localized in a posterior and central domain. (E) Anterior Hunchback domain with sharper boundary several cell cycles later than is shown in C. (F,G) Subsequent mRNA and protein during early cellularization. (H,I) mRNA and protein expression shortly before gastrulation.

and *hunchback*^{hl} embryos (data not shown). We found that the serosa still forms apparently normally in the mutant embryos, indicating that although zygotic *Nasonia* Hunchback is expressed relatively early and strongly in this tissue, it is not necessary for its morphological determination.

Does maternal *Nasonia hunchback* contribute an anterior protein domain?

To investigate whether maternal *hunchback* mRNA contributes to the anterior expression of *Nasonia* Hunchback, we determined whether Hunchback expression could be detected in *hunchback*^{hl} mutant embryos, which lack zygotic Hunchback. Because the anti-*Nasonia* Hunchback antibody was generated against a region of the protein that was completely deleted in *hunchback*^{hl} mutant embryos (Fig. 3), any protein detected with this antibody in the mutant embryos must be maternally derived. Male embryos were collected from *hunchback*^{hl}/+ virgins, so one-half of the embryos should express no zygotic Hunchback. In a collection of such embryos, aged from about cycle 10 to cellularization (4-6 hours AEL at 28°C; Fig. 5A), all embryos (55/55) expressed Hunchback in an anterior domain (see Fig. 5C,E). The youngest embryos in the collection all appeared to have a similarly strong Hunchback expression, whereas three of the oldest embryos in this collection, approaching the beginning of cellularization, had barely detectable levels of the Hunchback gradient. A control for this experiment was a collection of older embryos from the same mothers, in which only half of the embryos expressed Hunchback, as expected during the purely zygotic phase of expression (see below). These results indicate that maternally derived *Nasonia hunchback* mRNA contributes to a gradient of Hunchback in *Nasonia* embryos prior to cellularization.

How late does maternal Hunchback persist?

Why does a lack of zygotic *hunchback* result in more severe consequences in *Nasonia* than in *Drosophila*, despite graded maternal Hunchback expression in both species? We hypothesized that because of the longer period of early development in *Nasonia* (Fig. 1) maternal Hunchback does not overlap temporally with zygotic Hunchback to the same extent that it does in *Drosophila*. To test this hypothesis, we examined Hunchback in *Nasonia hunchback*^{hl} mutant embryos, and compared them with *Drosophila* embryos lacking zygotic Hunchback, during the period when maternal Hunchback is decaying. Specifically, we examined whether residual maternal Hunchback is detected near the onset of cellularization, when both *Nasonia* and *Drosophila* embryos begin to express Hunchback zygotically in a posterior cap (in addition to the anterior domain).

In a tightly staged collection of male *Nasonia* embryos from *hunchback*^{hl}/+ virgins, we observed 34 embryos expressing Hunchback in the anterior and incipient posterior caps (Fig. 7A), while 31 sibling embryos had no detectable Hunchback expression (Fig. 7B). In a control experiment, all of 50 *Nasonia* wild-type embryos of a similar age clearly showed the zygotic Hunchback expression pattern. These results show that in *Nasonia* embryos, maternal Hunchback does not persist into the period of posterior cap expression, but our characterization of maternal Hunchback in earlier embryos (see the previous section above) indicates that it is weakly expressed just prior to that time.

To examine maternal Hunchback in *Drosophila*, we made

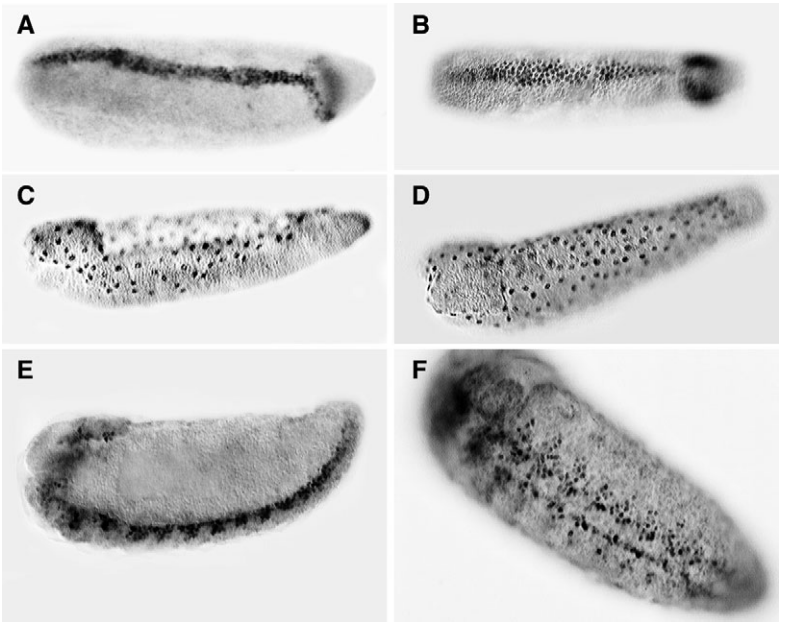

Fig. 6. *Nasonia* Hunchback in serosa and nervous system of wild-type embryos. (A) A dorsal stripe of mRNA expression initiates shortly before gastrulation. (B) Protein expression in the nuclei of the developing serosa, soon after germ-band extension. (C,D) Continued protein expression in the serosa as it begins to expand to envelop the entire embryo. (E) Protein expression in the nervous system, seen here during head involution. (F) Ventral view of embryo shown in E.

synchronous collections of embryos from *hunchback*$^{-}$/+ parents (see Materials and methods) and from wild-type parents, such that the youngest embryos were just beginning to express the zygotic posterior Hunchback cap (Fig. 7C). The progeny of the wild-type parents all expressed Hunchback in a strong anterior domain, as well as in the posterior cap. However, in 25% of the progeny of heterozygous parents (57/228), we found either weak staining only in anterior nuclei – presumably from residual maternal expression – or no detectable staining. Specifically, we observed 27 progeny of the heterozygous parents with only weak anterior staining (maternal expression only; Fig. 7D) and 30 with no staining. The 90 youngest siblings with strong anterior staining (mostly zygotic expression) showed incipient posterior-cap staining. (The remaining 81 siblings with zygotic expression were older, exhibiting either strong posterior cap staining or resolution of the posterior cap into a posterior stripe.) Because 27 is close to one-fourth of 117 (27 maternal plus 90 youngest zygotic), these results indicate that in *Drosophila*, maternal Hunchback perdures into the period of incipient zygotic posterior cap expression, during early cellularization. This is in contrast to *Nasonia* maternal Hunchback, which appears to decay before the equivalent stage of posterior cap expression during early cellularization. This timing difference may contribute to *Nasonia*'s stronger dependence on zygotic *hunchback*. However, the experiments described below indicate that maternal *hunchback* cannot fully account for the difference in functions covered by zygotic *hunchback* in *Nasonia* and *Drosophila*.

How does maternal and zygotic loss of *Drosophila hunchback* compare to *Nasonia hunchback*^{hl}?

To better understand the greater essential zygotic role of *hunchback* in *Nasonia* than in *Drosophila*, we examined the effects of *hunchback* mutant genotypes on Hox gene expression. In previous work (Pultz et al., 1999), we had compared *Nasonia hunchback*^{hl} to *Drosophila* embryos lacking zygotic *hunchback* for their effects on trunk Hox gene expression, *Ultrabithorax* (*Ubx*) and *abdominal-A* (*abd-A*), and demonstrated that the ectopic expression of Ubx-Abd-A extends more anteriorly in the *Nasonia headless* mutant embryos. Here, we extend this comparison to *Drosophila* embryos lacking both maternal and zygotic *hunchback*. For these analyses, as previously, we used the phylogenetically cross-reactive monoclonal antibody generated by Kelsh et al. (Kelsh et al., 1994), which recognizes epitopes on both Ubx and Abd-A. Expression of Ubx-Abd-A in a wild-type *Drosophila* embryo is shown in Fig. 8A; expression in a *Drosophila* embryo lacking zygotic *hunchback* is shown in Fig. 8B. As reported previously (White and Lehmann, 1986; Pultz et al., 1999), the trunk homeotic genes are derepressed both anteriorly and posteriorly when zygotic *Drosophila hunchback* function is eliminated. This derepression does not extend anteriorly into the maxillary segment. By contrast, Fig. 8C shows Ubx-Abd-A expression in *Drosophila* embryos lacking both maternal and zygotic *hunchback*, generated using germ line clones (Dang and Perrimon, 1992). In these embryos, no maxillary lobe develops, and the Hox

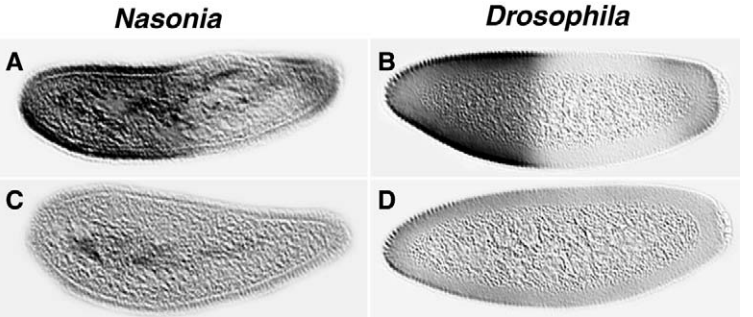

Fig. 7. How late is maternal Hunchback expressed in *Nasonia* and *Drosophila*? (A) Zygotic expression of *Nasonia* Hunchback during the onset of cellularization and the beginning of posterior cap expression. (B) Lack of residual maternal Hunchback expression in similarly aged *hunchback*^{hl} mutant embryo. The embryos in A and B were from a very tightly staged collection, and, therefore, are very similar in age (see Materials and methods). (C) Zygotic expression of *Drosophila* Hunchback, during the onset of cellularization and the beginning of posterior cap expression. (D) Residual maternal expression in nuclei at the surface of a *Drosophila* embryo lacking zygotic *hunchback*, very similar in age to the embryo in C. The A,B and C,D embryo pairs were photographed together in the same frames.

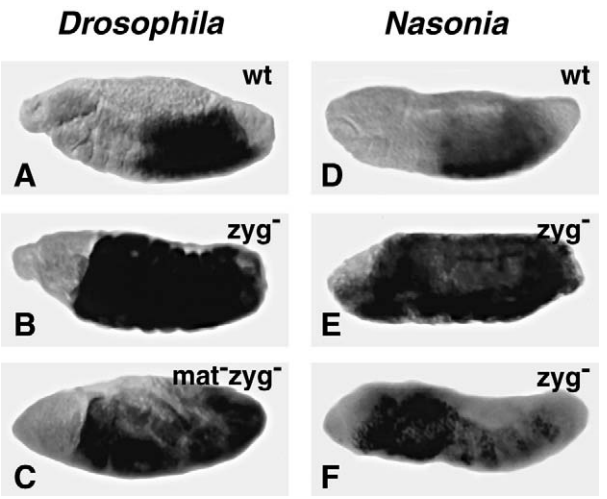

Fig. 8. Comparison of *hunchback* mutant phenotypes. All embryos are stained with the FP6.87 antibody (Kelsh et al., 1994), which recognizes both Ultrabithorax (Ubx) and Abdominal-A (Abd-A) proteins. The embryos in A,B,D and E are segmented. The embryos in C and F are younger, at the age of onset of Ubx-Abd-A expression. (A) Wild-type *Drosophila* embryo. (B) *Drosophila* embryo lacking zygotic *hunchback* function. (C) *Drosophila* embryo lacking both maternal and zygotic *hunchback* function. (D) Wild-type *Nasonia*. (E) *Nasonia hunchback*^{hl}, segmented embryo. (F) *Nasonia hunchback*^{hl} as Hox gene expression is initiating, to ensure that no head rearrangements have yet taken place.

gene expression extends slightly further anteriorly than with loss of only zygotic *hunchback* function, but much of the head is still clear of the trunk Hox gene expression. In wild-type *Nasonia* embryos, the trunk Hox gene expression (Fig. 8D) is very similar to that of wild-type *Drosophila* embryos. In *hunchback*^{hl} mutant embryos, as in *Drosophila* embryos lacking *hunchback* zygotic function, the Hox genes are derepressed anteriorly as well as posteriorly (Fig. 8E,F). However, the ectopic expression of trunk Hox genes in *Nasonia hunchback*^{hl} mutant embryos extends much further anteriorly than in *Drosophila* embryos lacking zygotic *hunchback*, and even appears to extend further anteriorly than in *Drosophila* embryos lacking both maternal and zygotic *hunchback*. This indicates that zygotic *hunchback* in *Nasonia* controls more functions than all *hunchback*, both maternal and zygotic, in *Drosophila.*

Discussion

hunchback^{hl} and maternal expression of *Nasonia hunchback*

We show here that the *Nasonia headless* mutant phenotype, which we described in Pultz et al. (Pultz et al., 1999), is caused by a 1.5 kb deletion in the *Nasonia hunchback* gene. This deletion begins after 40 amino acids of the predicted reading frame and introduces a frameshift mutation, disrupting the remaining reading frame such that the mutant protein lacks all zinc fingers (Fig. 3). Therefore, we have renamed *headless* (*hl*) as *hunchback*^{hl}. Hülskamp et al. (Hülskamp et al., 1994) describe several amorphic (functionally null) alleles of *Drosophila hunchback* including *hb*^{FB}, a 10 bp deletion that introduces a frameshift mutation after the first 150 amino acids, and *hb*^{14F}, which introduces a stop codon at amino acid 236. As in *Nasonia* Hunchback^{hl}, *Drosophila* Hunchback^{FB} and Hunchback^{14F} lack all zinc fingers. These comparisons indicate that *Nasonia hunchback*^{hl} can be considered to be a null allele.

The *hunchback*^{hl} mutant phenotype (hemizygous progeny of heterozygous mothers) reveals that zygotic *hunchback* is essential in *Nasonia* for development of almost the entire head, as well as the thorax and the posterior abdomen. In *Drosophila*, when only zygotic *hunchback* is removed (in homozygous progeny of heterozygous parents), the posterior labial segment and thorax – plus a small posterior abdominal region – are deleted, but underlying maternal *hunchback* still patterns part of the head. When both maternal and zygotic *hunchback* are removed, the anterior defects expand further into the head (Bender et al., 1987; Lehmann and Nüsslein-Volhard, 1987).

The above comparison of mutant phenotypes, the observation that more time is allocated to early development in *Nasonia*, and evidence from *Schistocerca* that the *hunchback* axial patterning function may originally have been zygotic (Patel et al., 2001), together suggested that *hunchback* might be expressed only zygotically in *Nasonia*. However, we have found *hunchback* mRNA in ovaries and in very early embryos, indicating that *hunchback* is transcribed maternally in *Nasonia*, as in *Drosophila* and *Tribolium* (Wolff et al., 1995), although it is not translated maternally as in *Schistocerca* (Patel et al., 2001). Moreover, by examining Hunchback expression in *hunchback*^{hl} mutant embryos, we found that maternal *hunchback* mRNA appears to be solely or primarily responsible for directing the synthesis of the early anterior Hunchback domain during the first cell cycles after pole cell formation in *Nasonia* embryos.

The finding that *Nasonia hunchback* is expressed maternally – even though zygotic *hunchback* controls more extensive patterning in *Nasonia* than in *Drosophila* – raises the question: what, if any, is the function of maternal *hunchback* in *Nasonia*? One possibility is that maternal *hunchback* is necessary as a positive regulator of zygotic *hunchback*; for example, in *Drosophila*, the parasegment 4 expression of *hunchback* is under positive regulation by *hunchback* gene products (Hülskamp et al., 1994; Margolis et al., 1995). If autoregulation were the sole role of maternal *hunchback*, then eliminating both maternal and zygotic *hunchback* would produce the same defects as eliminating only the zygotic gene products. Attempts to eliminate both maternal and zygotic *hunchback* function in *Nasonia* with parental RNA interference have only rarely yielded embryos with a phenotype as strong as that of *hunchback*^{hl} mutant embryos (J.L. and C.D., unpublished). This suggests that the maternal gene products may not control additional anteroposterior patterning functions.

Nasonia hunchback mRNA expression at the posterior

In contrast to the largely conserved expression of *Nasonia hunchback* protein (see below), the expression of *Nasonia hunchback* mRNA differs from that of *Drosophila*. In *Drosophila*, *hunchback* maternal mRNA at the posterior of the embryo degrades as the mRNA is being translationally controlled by Nanos, generating a gradient in both the mRNA and the protein expression (Bender et al., 1988; Schröder et al., 1988; Tautz, 1988). Similar posterior degradation of the

maternal mRNA has also been observed in the housefly *Musca domestica* (Sommer and Tautz, 1991). By contrast, throughout the two-hour period when *Nasonia* embryos are expressing an anterior Hunchback domain (from just after the nuclei arrive at the surface until the onset of cellularization), there is a substantial domain of *hunchback* mRNA at the posterior end of the embryo. The absence of Hunchback at the posterior of the embryos indicates that *Nasonia hunchback* is under translational control, presumably by Nanos. *Schistocerca hunchback* also appears to be translationally controlled at the posterior of the embryo (Lall et al., 2003). *Nasonia hunchback* does not have a canonical NRE such as is found in *Schistocerca*, *Locusta*, *Tribolium* and *Drosophila hunchback* mRNAs; however, *Nasonia hunchback* does have candidate NREs that are similar in structure to the *Drosophila melanogaster* cyclin B1 NRE, which is translationally regulated by Pumilio and Nanos in the germline (Nakahata et al., 2001).

Comparative timing and expression of *Nasonia* and *Drosophila* Hunchback

Because *Nasonia* and *Drosophila* differ in the extent of essential zygotic *hunchback* function and in the timeline for early development, we compared the overall timing, as well as pattern of Hunchback expression in the wild-type embryos. Our comparative observations of wild-type Hunchback expression in *Drosophila* (not shown, see Materials and methods) and *Nasonia* indicate that the dynamics of expression largely correlate with the same morphological markers during blastoderm development, rather than with absolute developmental time. In both *Nasonia* and *Drosophila*, just before pole cell formation, Hunchback is expressed ubiquitously, then this expression is replaced by an anteroposterior gradient with a progressively sharpening border as the cells begin dividing at the surface of the embryo. Very early in the process of cellularization, in both *Nasonia* and *Drosophila* embryos, a posterior cap of Hunchback begins to be expressed in addition to the anterior domain of expression. In both organisms, during the cellular blastoderm period, the posterior cap resolves into a posterior stripe and expression retracts from the anterior. However, the expression patterns of Hunchback in *Nasonia* and *Drosophila* are not entirely identical. The anterior domain of Hunchback persists into the early stages of germ band extension in *Drosophila* but not in *Nasonia*, and at gastrulation, a dorsal stripe is expressed in *Nasonia* that is not present in *Drosophila*.

Dorsal *Nasonia* Hunchback expression appears to be associated with development of the serosa. Expression in extraembryonic membranes is an aspect of Hunchback expression that has been described for other insects, including *Schistocerca* and *Drosophila* (Patel et al., 2001), as well as the mosquito *Anopheles gambiae* (Goltsev et al., 2004). We find that despite zygotic expression in the serosa, *Nasonia* zygotic *hunchback* function is not necessary for serosa formation. The dorsal expression domain suggests that zygotic *hunchback* might be positively regulated in *Nasonia* by the *zerknüllt* (*zen*) homeobox gene. In *Drosophila*, *zen* is dorsally expressed during blastoderm development and is required for development of the amnioserosa (Wakimoto et al., 1984; Doyle et al., 1986). *zen* is also expressed in the anterior and dorsal regions that give rise to extraembryonic membranes in other insects (Falciani et al., 1996; Stauber et al., 2002); in *Tribolium*, anterior expression of *zen1* has also been shown to specify serosa cell fates, differentiating them from those of the more posterior germ rudiment (van der Zee et al., 2005). *Nasonia* Hunchback is also strongly expressed in the nervous system, approximately during the period of head involution, in a pattern that appears to be similar to that observed in the nervous system of other insects (Woff et al., 1995; Rohr et al., 1999; Patel et al., 2001).

Because maternal *hunchback* is partially redundant with zygotic *hunchback* in *Drosophila* (Lehmann and Nüsslein-Volhard, 1987), we also examined the timing of the maternal component relative to the zygotic component in *Nasonia* and *Drosophila*. We found that maternal Hunchback expression in *Drosophila* appears to overlap with a slightly later zygotic phase of expression than in *Nasonia*. This timing difference may contribute to *Nasonia*'s greater reliance on zygotic *hunchback*. However, the strikingly different essential roles of *hunchback* in *Nasonia* and *Drosophila* call for further explanation.

Are phenotypic differences revealing changes in functionally overlapping gene functions?

We have considered several possible explanations to account for the observation that the zygotic *hunchback* loss-of-function phenotype is more severe in *Nasonia* than in *Drosophila*. As discussed above, we first hypothesized that *Nasonia* lacks maternally provided *hunchback* function, but this explanation was ruled out, as *Nasonia* does have strong maternal Hunchback expression. Second, we found that more limited perdurance of maternal Hunchback during the blastoderm stage of *Nasonia* may contribute to the differential function. Third, we consider here that *Nasonia* Hunchback might also regulate more downstream genes, either by DNA-binding or protein-protein interactions, than *Drosophila* Hunchback. In this regard, it is notable that *Nasonia* Hunchback has an N-terminal zinc finger (NF-1) that is lacking in *Drosophila*. However, the function of NF-1 is not understood, and N-terminal zinc fingers of Hunchback have been independently discarded in number of insect taxa including Hymenoptera (Apis) and Orthoptera (Cricket). Finally, *Nasonia* and *Drosophila* may differ in the degree to which other genes are redundant or synergistic with Hunchback function.

Our analysis of Hox gene expression in *Drosophila* embryos indicated that even when both maternal and zygotic *hunchback* products are removed, the defects are not as extensive as the zygotic defects of *Nasonia hunchback*^{hl}. Consistently, cuticular analyses of *Drosophila* embryos lacking both maternal and zygotic *hunchback* show that the deleted region extends forward only through the maxillary segment (E. Wimmer, personal communication); however, all gnathal plus at least two pregnathal segments are deleted in *Nasonia hunchback*^{hl} (Pultz et al., 1999). This raises the question of whether the absence of a *bicoid* gene in *Nasonia* could potentially be responsible for the extent of the defects observed with a loss of zygotic *Nasonia hunchback*.

When the dose of maternal *bicoid* was reduced by half in *Drosophila* embryos that also lacked all maternal and zygotic *hunchback* (E. Wimmer and C.D., unpublished), the array of head segments deleted (all except the labrum) was identical to the region deleted in *Nasonia hunchback*^{hl} mutant embryos.

Importantly, these 'headless' *Drosophila* mutant embryos can be rescued by a single zygotic *hunchback*$^{+}$ allele, indicating that although zygotic *Drosophila hunchback* is not usually needed to pattern multiple head segments, it is sufficient to do so (in the context of a remaining half dose of *bicoid* expression). In this comparison, *Drosophila hunchback* appears to be functionally similar to *Nasonia hunchback* in the range of segments that it can pattern, although this was not originally obvious from single-mutant analyses.

The roles of genes with overlapping functions, such as *orthodenticle* and *bicoid*, have changed during the course of evolution as *hunchback* has continued to control anterior development. Our finding that *hunchback* is responsible for controlling more of the anterior development in *Nasonia* than in *Drosophila* may indicate that the Hunchback protein has changed its interactions with downstream regulatory genes. Alternatively, the evolution of overlapping gene functions may be sufficient to account for the changing responsibilities of *hunchback* during the evolution of insect embryos.

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