Function of *bicoid* and *hunchback* homologs in the basal cyclorrhaphan fly *Megaselia* (Phoridae)

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The *Drosophila* gene *bicoid* functions at the beginning of a gene cascade that specifies anterior structures in the embryo. Its transcripts are localized at the anterior pole of the oocyte, giving rise to a Bicoid protein gradient, which regulates the spatially restricted expression of target genes along the anterior–posterior axis of the embryo in a concentration-dependent manner. The morphogen function of Bicoid requires the coactivity of the zinc finger transcription factor Hunchback, which is expressed in a Bicoid-dependent fashion in the anterior half of the embryo. Whereas *hunchback* is conserved throughout insects, *bicoid* homologs are known only from cyclorrhaphan flies. Thus far, identification of *hunchback* and *bicoid* homologs rests only on sequence comparison. In this study, we used double-stranded RNA interference (RNAi) to address the function of *bicoid* and *hunchback* homologs in embryos of the lower cyclorrhaphan fly *Megaselia abdita* (Phoridae). *Megaselia-hunchback* RNAi causes *hunchback*-like phenotypes as observed in *Drosophila*, but *Megaselia-bicoid* RNAi causes phenotypes different from corresponding RNAi experiments in *Drosophila* and *bicoid* mutant embryos. *Megaselia-bicoid* is required not only for the head and thorax but also for the development of four abdominal segments. This difference between *Megaselia* and *Drosophila* suggests that the range of functional *bicoid* activity has been reduced in higher flies.

Body axis formation in insects is best understood in *Drosophila*. Polarity along the anterior–posterior axis of the *Drosophila* embryo rests on maternally derived protein gradients, which emanate from prelocalized mRNAs in the pole regions of the egg (1). Anteriorly localized maternal *bicoid* mRNA encodes a homeodomain protein (Bicoid) that specifies anterior development in a concentration-dependent manner through spatially restricted activation of genes required for segmentation (2, 3). Loss of Bicoid activity results in embryos without a head and thorax and with variable deletions and fusions of segments in the anterior abdominal region. The head and thorax are replaced by duplicated posterior terminal structures of reversed polarity (4). The morphogen-like function of Bicoid requires cooperation with the zinc finger type transcription factor Hunchback in determining the anterior segment pattern; posteriorly, Bicoid complements the homeodomain transcription factor *caudal* in a partially redundant manner (5–8).

Because *hunchback* and *caudal* homologs of the red flour beetle *Tribolium castaneum* are regulated in a Bicoid-dependent manner in transgenic *Drosophila* embryos, it has been postulated that a *bicoid*-like gene is active in the *Tribolium* embryo (9). In the lower dipteran species *Smittia* (Chironomidae, Nematocera), irradiation experiments with UV light provide indirect evidence for an anteriorly localized morphogen (10, 11). However, a general role of Bicoid in anterior patterning of insect embryos contrasts with the fact that *bicoid* orthologs have been found only in cyclorrhaphan flies (12–14). Moreover, different developmental processes between cyclorrhaphan and noncyclorrhaphan Diptera suggest differences in anterior patterning among those species (15–17). Finally, genetic redundancy in patterning the *Drosophila* embryo led to the proposition that *hunchback* fulfills a *bicoid*-like function in lower insects (5, 18). This hypothesis implies that an ancestral *bicoid* gene performed fewer or different patterning functions than *bicoid* in *Drosophila*.

Recently, on the basis of sequence similarity, the most basal *bicoid* ortholog within the phylogenetic tree of the Diptera (19, 20) was identified in the lower cyclorrhaphan fly *Megaselia abdita* (Phoridae) (14). Sequence comparison suggests that *bicoid* emerged by duplication of the Hox class 3 gene *zerknüllt*, which specifies extraembryonic tissue in insects (14). If *bicoid* derives from *zerknüllt*, it must have undergone functional adaptations that might be apparent when comparing *bicoid* gene functions in *Drosophila* and *Megaselia* embryos.

Herein, we present a functional analysis of the *Megaselia-bicoid* gene (*Ma-bcd*) and of a newly identified *hunchback* homolog (*Ma-hb*) in *Megaselia* embryos. In the absence of genetic tools for *Megaselia*, we made use of double-stranded RNA (dsRNA)-mediated gene silencing (RNAi; refs. 21–25), which reduces transcripts in a sequence-specific manner (26–32). The results support the proposal that the range of *bicoid* function became gradually restricted to anterior body parts in the course of dipteran evolution.

Materials and Methods

Fly Culture and Injection of Embryos. The *M. abdita* Schmitz (Phoridae, Aschiza, Cyclorrhapha, Brachycera, Diptera) culture was provided by Klaus Sander (Albert-Ludwigs-Universität, Freiburg, Germany). Animals were kept on wet paper towels sprinkled with aquarium fish food "TetraRubin" (Tetra, Melle, Germany). For injection experiments, 30-min egg depositions at 21°C were used. Eggs were collected on ice, dechorionated in 50% (vol/vol) commercial bleach, attached to a coverslip with heptane glue, air dried on silica gel (Merck) for ≈6–7 min at 19°C and covered with 10S Voltalef oil (Atochem). *Drosophila* eggs were injected at ≈95% egg length (0% = posterior pole). *Megaselia* eggs were injected in either the anterior or the posterior pole at ≈95% or 5% egg length, because a distinction of the posterior from the anterior pole of *Megaselia* eggs was not possible under the dissecting microscope. A transjector (Eppendorf, no. 5246) and femtotips (Eppendorf) were used. The injection volume was below 100 pl as estimated from injections in oil.

Cloning of *Ma-hb*. A PCR clone of *Ma-hb* was obtained with the primer pair AARCACCAYYTNGARTAYCA (12) and TGRCARTAYTTNGTNGCRTA (N is A, C, G, or T; R is G or A; Y is C or T) on genomic *Megaselia* DNA. *Ma-hb* cDNA from adult *Megaselia* females was amplified by 3′ and 5′ rapid ampli-

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Abbreviations: RNAi, double-stranded RNA interference; dsRNA, double-stranded RNA.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AJ295635).

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fication of cDNA ends on a template prepared with the Marathon cDNA Amplification Kit (CLONTECH). The predicted ORF spans 1,863 bp; the 5′ untranslated region includes 236 bp; and the 3′ untranslated region includes 53 bp. The *Ma-hb* sequence is deposited in GenBank under accession no. AJ295635.

RNAi. *Ma-bcd*, *bicoid*, *Ma-hb*, and *hunchback* cDNA portions of the ORFs 1–1.5 kilobases in length were PCR amplified with the primer pairs TAATACGACTCACTATAGGGAGACCACTGTTTACGAGAAAATGGC/TAATACGACTCACTATAGGGAGACCACTCAATTGAAACAGTAGGC, TAATACGACTCACTATAGGGAGACCACTCCCATCCGCATCCG/TAATACGACTCACTATAGGGAGACCACGCCTCTCGTCCAGG, TAATACGACTCACTATAGGGAGACCACAATGCAGAATTGGGAATC/TAATACGACTCACTATAGGGAGACCACAATTCTCCTATGGGAAAC, and TAATACGACTCACTATAGGGAGACCACGATGCAGAACTGGGAGAC/TAATACGACTCACTATAGGGAGACCACCATACTTGCGCAGATGCA, respectively. The PCR primers contained a T7 promoter at their 5′ end, which was used for simultaneous sense and antisense RNA synthesis. RNA was phenol extracted, precipitated, and dissolved in water or injection buffer (100 nM $NaPO_4$, pH 7.2/5 mM KCl). All probes were checked on an agarose gel for the formation of dsRNA before injection. If necessary, probes were denatured and reannealed in injection buffer. To this end, the probes were submerged in a 250-ml beaker of water at 95°C for 1 min and allowed to cool to ambient temperature for several hours. For *Ma-hb*, two additional dsRNA probes covering 1 kilobase of the 5′ or the 3′ half of the cDNA were prepared from equimolar amounts of sense and antisense RNA strands generated with SP6 and T7 RNA polymerase, respectively. *Megaselia* dsRNA was injected at a concentration of 7–8 μM. *Drosophila* dsRNA was injected at concentrations of 7–9 μM (*hunchback*) and 1 or 3 μM (*bicoid*). *bicoid* dsRNA injections into *Drosophila* embryos at a concentration of 7 μM did not increase the phenotype but led to a high percentage of unspecific defects.

Immunocytochemistry. Hybridization to *Megaselia* embryos was done with DNA probes at 48°C as described for *Drosophila* (33) with the following modifications. To burst the vitelline layer, −80°C cold methanol was used in the devitellinization step, and the embryos were heated in methanol for 1 min to +56°C. The temperature shock was repeated two or three times during the following methanol washes. Engrailed and Even-skipped staining was done with the monoclonal antibodies 4D9 (34) and 2B8 (35), respectively. The developmental stage of early embryos was determined by nuclear Hoechst 33258 staining (1 μg/ml).

Results and Discussion

Distribution of *bicoid* and *hunchback* Transcripts in *Megaselia* and *Drosophila*. *Megaselia* and *Drosophila* eggs are the same size, and embryonic development of the two species is very similar as judged by both morphological criteria and molecular markers (14, 16, 36). In the *Drosophila* embryo, the maternal *bicoid* transcripts are localized in a narrow anterior cap, which disappears during the blastoderm stage (ref. 37; Fig. 1 *A* and *B*). In young *Megaselia* embryos containing four nuclei as visualized by Hoechst staining of DNA, *Ma-bcd* transcripts are also detected in a narrow cap (Fig. 1*C*). Transcripts in preblastodermal embryos, however, extend further posterior (Fig. 1*D*), suggesting that prelocalized transcripts have spread from the anterior pole. Thus, the patterns of localized *bicoid* transcripts in *Megaselia* and *Drosophila* are similar at the beginning of development but differ at subsequent stages when *bicoid* protein (Bicoid) is known to activate its zygotic target genes.

In *Drosophila*, Bicoid activates *hunchback* in the anterior half of the embryo, and the posterior boundary of this expression

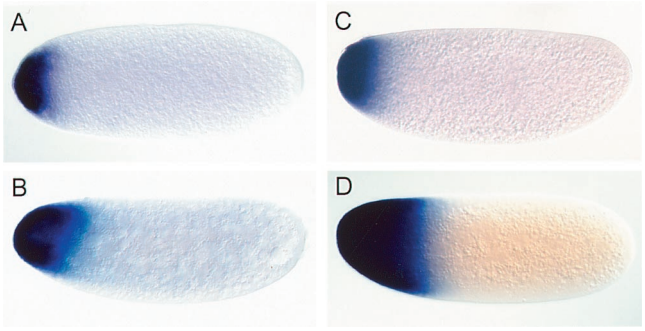

Fig. 1. Expression patterns of *bicoid* and *Ma-bcd* in early embryos. (*A* and *B*) Transcript distribution of *bicoid* in *Drosophila*. (*C* and *D*) Transcript distribution of *Ma-bcd* in *Megaselia*. Embryos are shown at the four-nuclei stage (*A* and *C*) and at preblastoderm stage (*B* and *D*).

domain is set by the concentration of Bicoid (2), serving as a measure for Bicoid's morphogenetic activity (38). In view of the different distribution of *bicoid* transcripts at the time when *hunchback* expression occurs, we asked whether this difference results in an altered expression pattern of the target gene *hunchback*. To address this question, we cloned the *Megaselia hunchback* gene and generated a cDNA by independent 3′ and 5′ rapid amplification approaches (see *Materials and Methods*). The rapid amplification of cDNA ends products were sequenced and compared with genomic DNA. The sequence alignment shown in Fig. 2 establishes the identity of the *hunchback* homolog, termed *Ma-hb* (Fig. 2).

Fig. 3*A* shows that *Ma-hb* transcripts accumulate in the nurse cells and the oocyte of ovarian follicles, indicating that they are maternally expressed. At the beginning of embryogenesis, the transcripts are distributed throughout the egg (Fig. 3*B*). Like in *Drosophila*, the maternal transcripts subsequently disappear from the posterior half of the embryo (Fig. 3*C*) and zygotic expression of *Ma-hb* occurs in the anterior half of the embryo, showing a sharp on/off boundary (Fig. 3*D*). Transcripts accumulate also in a posterior cap. At blastoderm, the transcripts disappear from the dorsoanterior position and the posterior pole, and expression resolves in three anterior stripes (Fig. 3 *E* and *F*). The only notable difference to the *hunchback* expression pattern in *Drosophila* is therefore the maintenance of transcripts in an anterior ventral position (Fig. 3*E*). After gastrulation, *Ma-hb* is expressed in the central nervous system as found with *Drosophila* (not shown). These observations indicate that, although *Ma-bcd* transcripts extend posteriorly, the pattern of zygotic *Ma-hb* expression is not correspondingly altered. Thus, the blueprint of the anterior portion of the *Megaselia* embryo, as visualized by *Ma-hb* expression, is not expanded posteriorly. This finding suggests that the putative gradient of Bicoid protein in *Megaselia* provides the same spatial information as in *Drosophila*.

RNAi Induced Phenocopies of *bicoid* and *hunchback* Mutations in *Drosophila*. In the absence of genetic tools that allow us to establish gene functions in *Megaselia*, we adopted the RNAi technique to assess the function of *Ma-bcd* and *Ma-hb*. Injection of dsRNA into insect embryos has already been demonstrated to cause phenocopies of zygotically expressed segmentation genes (39–41). However, genes that are expressed and required earlier than the genes of the zygotic segmentation cascade, such as maternally derived *bicoid* or maternally and zygotically expressed *hunchback*, have not yet been examined by this technique. To test the suitability of RNAi with respect to *bicoid* and *hunchback* function, we injected *bicoid* dsRNA into *Drosophila* embryos. These embryos developed as phenocopies corresponding to the phenotypic series of *bicoid* mutations (ref. 4; Table 1).

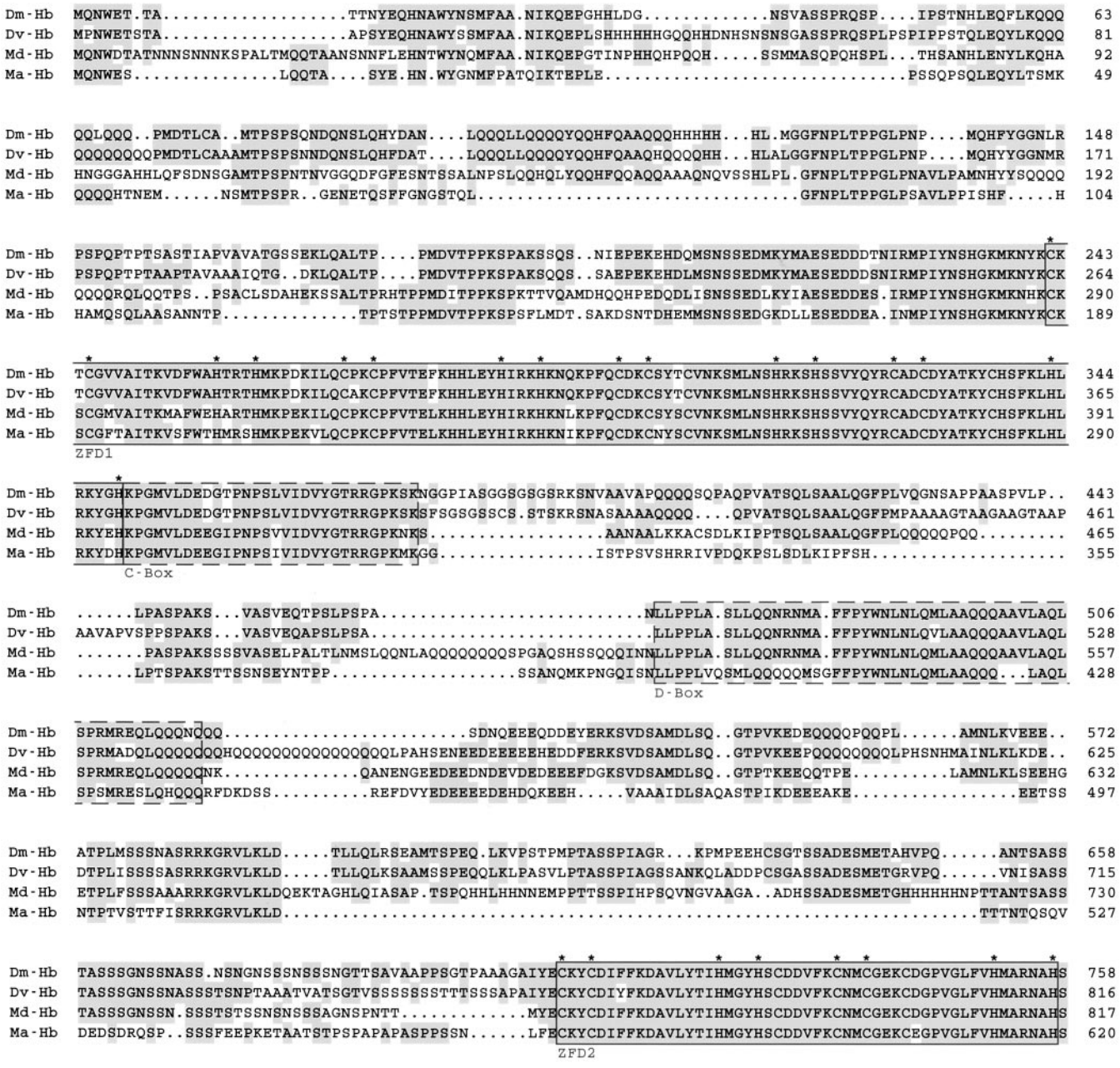

Fig. 2. Alignment of Hunchback sequences from *Drosophila melanogaster* (Dm-Hb; ref. 54), *Drosophila virilis* (Dv-Hb; ref. 55), *Musca domestica* (Md-Hb; ref. 56), and *M. abdita* (Ma-Hb). Identical amino acids are shaded in gray; dots denote gaps. Asterisks mark the cysteine and histidine residues that are crucial for the formation of the zinc fingers. The numbers to the right refer to the last amino acid in each row. The highest similarity is seen in the zinc finger domains ZFD1 and ZFD2 (boxed) and in the C and D boxes (dashed boxes; ref. 57).

Strong phenocopies lack head and thorax and exhibit a duplication of posterior structures with reversed polarity. The duplicated structures include the hindgut, spiracles, and abdominal segments 9 to 7. These embryos resemble the phenotype of a null allele of *bicoid* (Fig. 4 *B* and *C*). Because control-injected embryos did not develop as phenocopies (Table 1), we conclude that *bicoid* RNAi specifically interferes with maternally derived *bicoid* activity and causes specific and reliable phenocopies of the *bicoid* mutant phenotype.

Drosophila embryos lacking maternal *hunchback* activity develop normally, whereas embryos with only one maternal *hunchback* copy and lacking zygotic *hunchback* activity fail to develop the thorax, the posterior-most gnathal (labial) segment, and parts of abdominal segment 8, which is fused to abdominal segment 7. In addition, they have a defective central nervous system (refs. 42 and 43; unpublished observations). A weaker phenotype is observed in embryos lacking zygotic hunchback activity, if two maternal hunchback copies are provided. Such embryos lack thoracic segments 2 and 3 in addition to fused abdominal segments 7 and 8 (44). Embryos lacking both the maternal and the zygotic *hunchback* activities develop two to three segments of abdominal identity in reversed polarity, followed by four segments in normal orientation (43). *hunchback* RNAi resulted in the deletion of the thorax, variable deletions and fusions in abdominal segments 1 to 3 and 8, and defects in the gnathal segments and in the central nervous system including

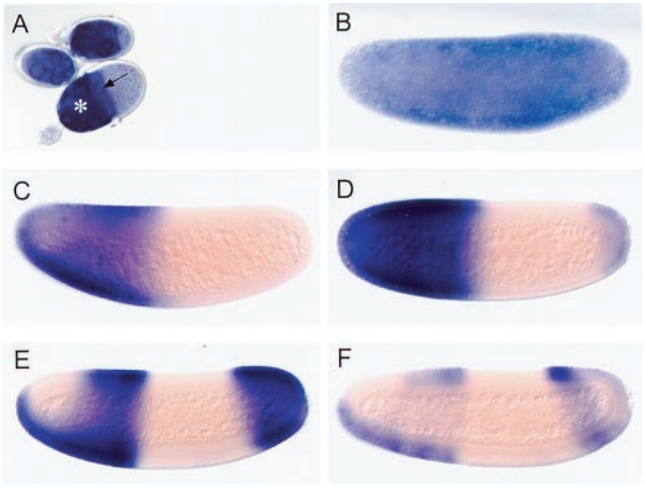

Fig. 3. Expression patterns of *Ma-hb* RNA in *Megaselia*. Transcript distribution in nurse cells (*A*; asterisk), the oocyte (*A*; arrow), and embryos at preblastoderm (*B*), progressively older syncytial blastoderm (*C–E*), and cellular blastoderm stage (*F*).

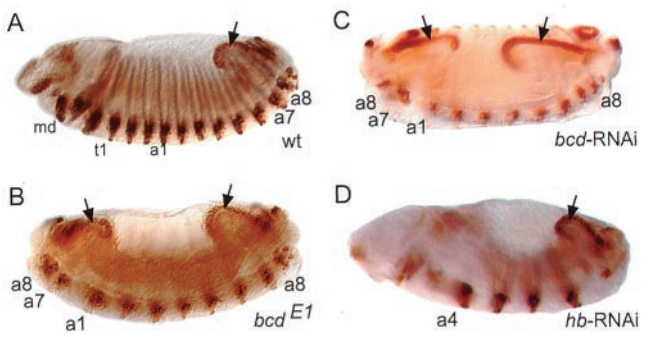

Fig. 4. RNAi in *Drosophila* embryos. Wild-type (wt; *A*), *bicoid*^{E1} mutant (*B*), *bicoid* RNAi (*C*), and *hunchback* RNAi (*D*) embryos were stained with Engrailed antibody to visualize segments and the hindgut (arrows). For description of phenotypes see text. md, t1, a1, a4, a7, and a8 designate mandibular, prothoracic, and various abdominal segments, respectively. Anterior is to the left; dorsal is up.

the brain (Table 1; Fig. 4*D*). Thus, the phenocopies resembled *hunchback* mutations, which in strength are intermediary between embryos lacking only zygotic *hunchback* activity and those that lack *hunchback* activity completely. Therefore, *hunchback* RNAi, in contrast to *bicoid* RNAi, does not cause phenocopies resembling the lack-of-function *hunchback* phenotype. This finding suggests that small amounts of *hunchback* mRNA escape RNAi-mediated degradation, providing sufficient *hunchback* activity to maintain the development of some anterior structures.

***Ma-hb* RNAi Causes *hunchback*-Like Phenotypes in *Megaselia*.** Based on the phenocopies produced by *bicoid* and *hunchback* RNAi in *Drosophila* embryos, we considered RNAi as a tool to address the function of the homologs in *Megaselia* embryos. *Ma-hb* RNAi resulted in the deletion of the thorax and variable defects in abdominal segments 1 to 3, and 8, the gnathal segments, and the central nervous system including the brain (Table 2; Fig. 5*E*). In addition, we found that the cephalopharyngeal head skeleton (which derives from several head segments; ref. 45) and spiracles (which derive from the eighth abdominal segment; ref. 46) were reduced (Fig. 5*F*). However, duplications of abdominal structures with reversed polarity, which are characteristic for *hunchback*-deficient *Drosophila* embryos, were not observed. To confirm the specificity of the RNAi-dependent *Ma-hb* phenotypes, we examined also phenotypes produced with dsRNA probes covering either the 3′ or the 5′ half of the cDNA, respectively (see *Materials and Methods*). The phenotypes obtained were not different from those described above. Thus, the *Ma-hb* phenotypes in *Megaselia* are similar to the *hunchback* phenocopies in *Drosophila*, which, however, corresponded only to a hypomorphic *hunchback* phenotype.

Table 1. Effect of *bicoid, hunchback,* and *Ma-bcd* RNAi and buffer injection in *Drosophila* embryos

	bicoid, 1 μM	*bicoid*, 3 μM	*hunchback*, 7–9 μM	*Ma-bcd*, 8 μM	Injection buffer
n	71	73	78	104	100
Percentage not developed	21	22	n.d.	8	7
Percentage mutant embryos	37	53	78	0	0

n, number of embryos injected; n.d., not determined. Percentage of mutant embryos was identified after Engrailed staining.

***Ma-bcd* RNAi Causes Symmetrical Double Abdomen in *Megaselia*.** We next asked whether *Ma-bcd* carries a *bicoid*-corresponding function in *Megaselia*. *Ma-bcd* RNAi caused a reduction of anterior segments including the cephalopharyngeal head skeleton (Table 2; Fig. 5). The strongest phenotypes lack the head, thorax, and three to four abdominal segments, which are replaced by a mirror image duplication of the remaining abdomen (Fig. 5 *D*, *H*, and *I*). Ectopic gastrulation movements at the anterior pole can be delayed with respect to gastrulation movements at the posterior pole, resulting in asymmetric germ band extension (Fig. 5*G*). The relative positions of the remaining abdominal segments at different developmental stages and the unambiguous identification of the reduced abdominal segment 9 suggest that abdominal segments 1, 2, and 3 and the dorsal part of abdominal segment 4 are missing. Dorsally, the symmetry plane lies most likely in abdominal segment 5, whereas in the nerve cord, it lies in the fourth abdominal neuromere (Fig. 5 *G–I*). Thus, such embryos resemble a *bicaudal* (47–49) rather than the *bicoid* phenotype. Injection of *bicoid* dsRNA into *Megaselia* embryos did not produce specific defects. In less than 1% of injected embryos presumably unspecific head defects but no duplications were observed (Table 2). These results indicate that *Ma-bcd* RNAi in *Megaselia* embryos causes the specific deletion of the anterior abdominal segments, which is not observed in the corresponding RNAi experiments with *Drosophila* or with *bicoid*- or *hunchback*-deficient *Drosophila* embryos (4). It is important to note, however, that in *Drosophila*, a symmetrical *bicaudal*-like phenotype had been observed when the combined activities of *bicoid* and *hunchback* are repressed in the anterior half of the embryos indicating synergistic effects of

Table 2. Effect of *Ma-hb, Ma-bcd, Ma-hb* + *Ma-bcd*, and *bicoid* RNAi in *Megaselia* embryos

	Ma-hb	*Ma-bcd*	*Ma-hb* + *Ma-bcd*	*bicoid*
n	602	322	108	377
Percentage hatched	47	53	n.d.	78
Percentage not hatched	53	47	n.d.	22
Percentage mutant cuticles	35	26	32	<1

n, number of embryos injected; n.d., not determined. Percentage not hatched includes mutant cuticles and cuticles without specific defects.

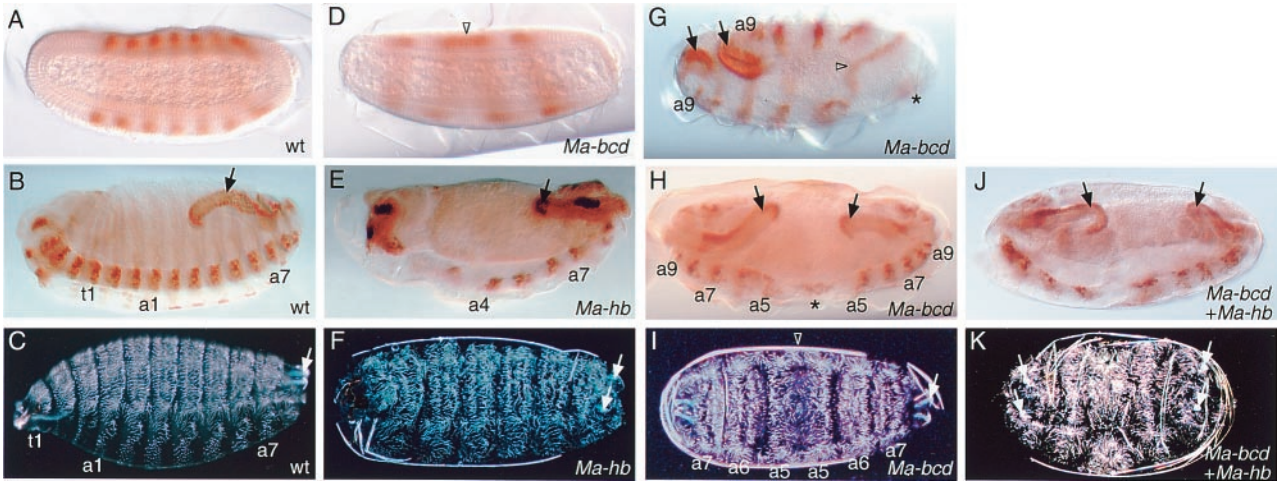

Fig. 5. RNAi in *Megaselia* embryos. Wild-type (wt; *A–C*), *Ma-bcd* RNAi (*D* and *G–I*), *Ma-hb* RNAi (*E* and *F*), and *Ma-bcd* + *Ma-hb* RNAi (*J* and *K*) embryos stained with Even-skipped antibody, which labels alternate segments (*A* and *D*), Engrailed antibody, which serves as a segmental marker (*B*, *E*, *G*, *H*, and *J*), and cuticle preparations (*C*, *F*, *I*, and *K*). Note dorsal fusion of abdominal segments in the symmetry plane (*D*, *G*, and *I*; triangle) and remnants of a putative a4 neuromere (*G* and *H*; asterisk). Hindgut (black arrows) and spiracular structures (white arrows) are duplicated in *Ma-bcd* RNAi embryos. For description of phenotypes, see text; for abbreviations, see legend to Fig. 4.

both genes (5). We therefore asked whether coinjection of *Ma-bcd* and *Ma-hb* dsRNAs into *Megaselia* embryos results in a more than additive extension of anterior deletions as compared with single dsRNA injections. We found that the phenotypes obtained after combined *Ma-bcd* and *Ma-hb* dsRNA injections were similar to the sum of effects observed in independent *Ma-bcd* dsRNA and *Ma-hb* dsRNA injections. In addition, the ventral nerve cord was more disorganized and interrupted between the symmetrical abdominal halves, and the gut was reduced in size (Fig. 5 *E*, *H*, and *J*). These results suggest only a weak synergistic effect of *Ma-bcd* and *Ma-hb* in the abdomen.

Concluding Remarks. Our findings provide evidence for conserved functions of *bicoid* and *hunchback* in *Megaselia* and *Drosophila* embryos. However, unlike *bicoid* mutant and RNAi-treated wild-type embryos of *Drosophila*, a deletion of three to four abdominal segments was observed after reducing *Ma-bcd* activity in *Megaselia* embryos. This result suggests that the activity range of *Ma-bcd* is extended consistent with the transcript distribution (Fig. 1). Possibly, *Ma-bcd* is repressing translation of a *Megaselia caudal* homolog more posteriorly than in the *Drosophila* embryo (50, 51) and/or *Ma-bcd* has a stronger influence on the activation of posterior gap genes such as *Krüppel* and *knirps* (7, 52). However, the additional requirement for *bicoid* might not be linked to increased *hunchback* function in the more primitive fly species, because the relevant expression of this gene is conserved (Fig. 3). It has been proposed that during insect evolution Bicoid gradually assumed morphogenetic functions that are carried out by Hunchback in insects more primitive than *Drosophila* (1, 5, 18, 53). With respect to Diptera, our results would rather argue in the opposite direction, because we have observed neither an increase of the functional range of *hunchback* activity in RNAi-treated *Megaselia* versus *Drosophila* embryos nor a corresponding reduction of *bicoid* activity in the lower fly species. Future studies directed at the identification, expression, and function of *bicoid* progenitors in noncyclorrhaphan Diptera will be required to test this hypothesis.

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