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SHORT COMMUNICATION

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Vasa expression and germ-cell specification in the spider mite *Tetranychus urticae*

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Abstract The specification of germ cells is an important process during the development of all animals. Expression of an evolutionarily conserved gene such as *vasa* can be used as a marker for germ cell fate. We have isolated a *vasa*-related gene from the two-spotted spider mite (*Tetranychus urticae*) and used it to examine the segregation of germ cells in this animal. In spider mites, *vasa* expression first appears in a group of cells that do not join the initial blastoderm surface. Instead, these cells remain in the interior of the blastoderm and then migrate to posterior regions of the embryo, where they form a cluster that appears in regions of the embryo consistent with the gonads. The expression pattern of this spider mite *vasa* homologue implies a novel process acts to specify germ cells in this species and that the specification of germ cells is an evolutionarily labile process.

Keywords *vasa* · Chelicerate · Evolution · Development

Introduction

The segregation of primordial germ cells (PGCs) is a process fundamental to the development of metazoans (Saffman and Lasko 1999). In many animals, the formation of PGCs appears to be regulated early in development by the presence of germ plasm, an electron-dense material that segregates into a specific cell lineage. In the fruit fly *Drosophila melanogaster*, the segregation of germ plasm, and the specification of PGCs, are intimately associated with the specification of posterior fate (Lasko and Ashburner 1990). However, this mode of specification is not conserved in metazoans (Saffman and Lasko 1999) and may not even be conserved in other insects (Chang et al. 2002).

DEAD box proteins are putative RNA unwinding proteins found to be present from viruses to mammals. Expression of *vasa*-like DEAD box proteins has been shown in PGCs of vertebrates, insects, *Caenorhabditis elegans*, a cnidarian, a planarian, an ascidian (reviewed in Mochizuki et al. 2001) and a chaetognath (Carre et al. 2002). As *vasa*-related genes appear to be expressed in the PGCs of most, if not all, metazoans (Mochizuki et al. 2001) they are good candidate germ cell markers.

The role of *vasa*-related genes in germ-line development is poorly understood. In *Drosophila*, *vasa* appears to be required for multiple processes during germ plasm assembly. It is required for the localisation of components of the germ plasm, the function of the Oskar protein, and localised translation of *nanos* RNA (reviewed in Mahowald 2001). In *C. elegans*, a family of *vasa*-related genes have been found, all of which appear to be involved in germ cell development (Gruidl et al. 1996). A Vasa-like protein has also been shown to be required for male germ cell development in the mouse (Tanaka et al. 2000).

We have made use of a *vasa*-like gene to examine the specification and movement of germ cells in the two-spotted spider mite (*Tetranychus urticae* Koch). Spider mites are chelicerates, an arthropod class including spiders, scorpions, mites and horseshoe crabs. The cloning and expression pattern of a *vasa*-related gene has not been previously reported for chelicerates. Segregation of germ cells in other chelicerates has been examined, but without markers for germ cell fate, these observations are based on morphological characteristics alone. Without markers, it is difficult to interpret when and where germ cells arise during development. In

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spiders, germ cells have only been identified at late stages of development (reviewed in Anderson 1973). In Opilones and scorpions, putative germ cells have been identified early in development (reviewed in Anderson 1973) but during early stages it is difficult to detect when and how they form using morphology alone.

The expression pattern we have observed using a spider mite *vasa*-related gene as a marker implies that germ cells are specified by a novel process in the spider mite. This process does not resemble germ cell segregation as described in insects such as *Bombyx mori* (Nakao 1999), *D. melanogaster* (Rongo et al. 1997) or *Schistocerca gregaria* (Chang et al. 2002).

Materials and methods

Spider mite culture

Two-spotted spider mites (*Tetranychus urticae* Koch) were cultured at 25°C on broad bean plants in a growth chamber with 40–60% humidity and a 16-h photophase.

Molecular cloning

Spider mite DNA was extracted using a Qiaquick kit (Qiagen). Degenerate PCR to amplify homologous *vasa* genes was performed using the methods of Chang et al. (2002). PCR products were cloned by ligation into pGEM-T Easy (Promega) following the manufacturer's instructions. Plasmid DNA was isolated with a Qiagen miniprep kit. Sequencing was performed using Big Dye chemistry (ABI) on a Perkin Elmer 377 DNA sequencer.

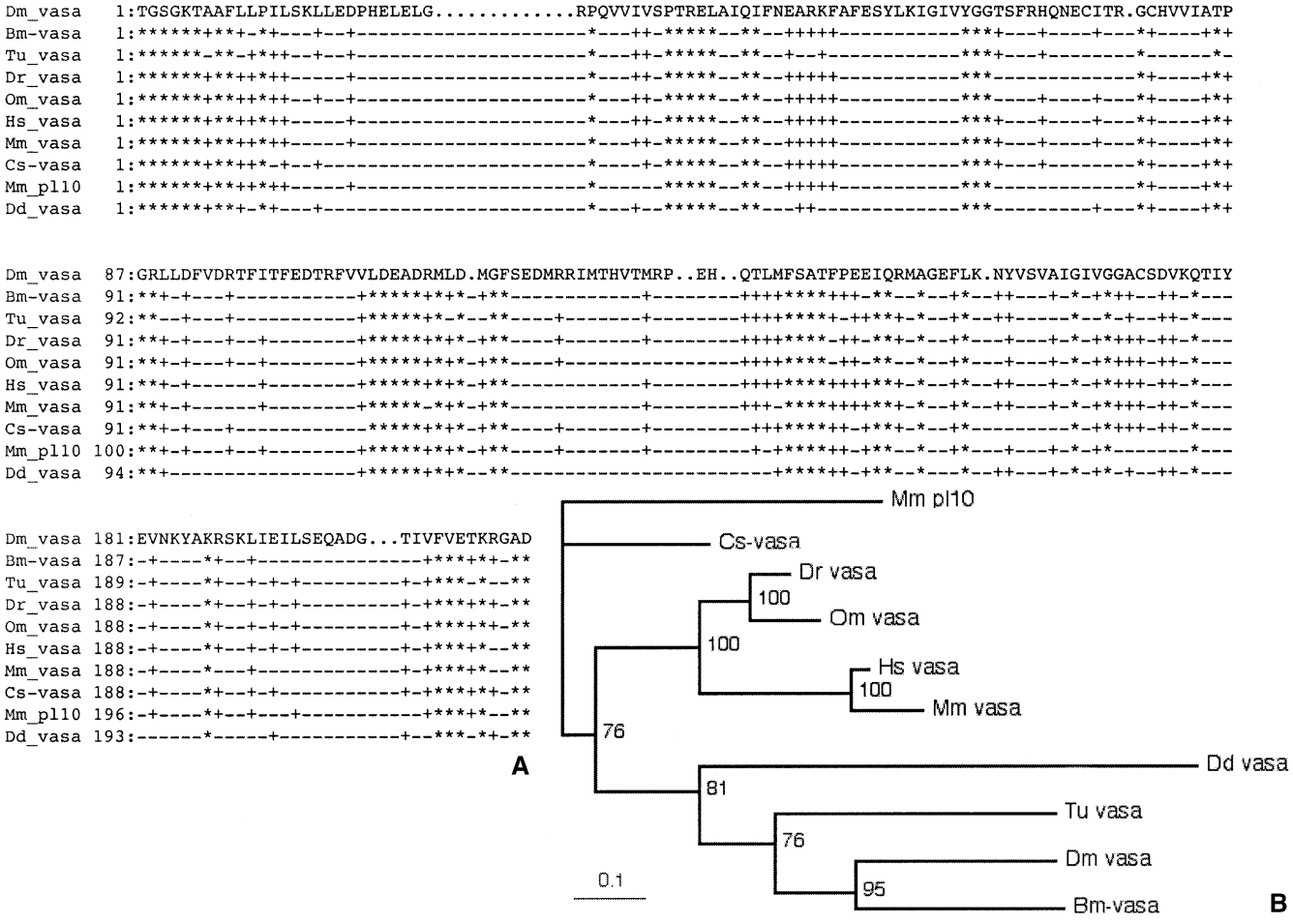

Fig. 1A, B Multiple alignment and phylogenetic analysis of Vasa-like sequences. **A** Multiple amino acid sequence alignment of the region cloned from *Tu-vasa* with other Vasa-like protein sequences. *Dm_vasa Drosophila melanogaster* vasa (accession number CAA31405), *Bm_vasa Bombyx mori* vasa (BAA19572), *Cs_vasa Ciona savigny* vasa (BAB12217), *Tu_vasa Tetranychus urticae* vasa, *Dr_vasa Danio rerio* vasa (NP_571132), *Om_vasa Oncorhynchus mykiss* vasa (BAA88059), *Mm_pl10* mouse PL10-related protein (P16381), *Hs_vasa* human vasa (AAF72705), *Mm_vasa* mouse vasa (BAA03584), *Dd_vasa Dugesia dorotocephala* PIVAS-1 *vasa*-related gene (BAB13313). *Asterisks* indicate all residues in the alignment are identical to the *Drosophila* sequence (*top*), *crosses* indicate residues identical to the *Drosophila* sequence, *dashes* indicate non-conserved residues. **B** Dendrogram of maximum likelihood analysis for the alignment shown in **A**. Tu_vasa clusters with other vasa-like proteins and not with the DEAD box helicase PL-10

Antibody staining and in situ hybridisation

Spider mite embryos were dechorionated with 50% bleach for 5 min, rinsed in tap water and fixed in PBS + 0.1% Tween + 4% formaldehyde for 15–30 min. Embryos were then washed in PTw (PBS + 0.1% Tween), and sonicated for 3 s in a sonic cleaning bath. Embryos were rinsed in PTw, re-fixed in PTw + 4% formaldehyde for a further 15 min and rinsed three times in PTw.

Antibody staining was performed using an antibody raised against *Drosophila* Distal-less (Dll) described in Panganiban et al. (1995). In situ hybridisation and antibody staining were performed using the methods of Dearden et al. (2002).

Staining with propidium iodide was performed after hybridisation. Embryos were treated with RNase (0.25 µg/ml for 1 h at 37°C), rinsed in PTw and then immersed in 0.25 µg/ml propidium iodide for 2 h. Embryos were then destained in three washes of PTw over 30 min and mounted in 70% glycerol. Confocal images were collected on a Zeiss LSM confocal microscope. Light images were collected using a Sony DXC-390P camera mounted on a Zeiss Axioplan II microscope and processed using Photoshop (Adobe).

Sequence analysis

Sequences were compared with the NCBI database using BLASTX searches (Altschul et al. 1990). Multiple alignments of strongly similar sequences were created using Clustal W (Thompson et al. 1994), and maximum likelihood analysis performed using TreePuzzle (Strimmer and von Haeseler 1996).

Results and discussion

Cloning and sequence analysis of spider mite *vasa* homologues

Degenerate PCR using primers directed towards *vasa*-like sequences was used to amplify fragments of *vasa*-like genes from two-spotted spider mite (*T. urticae*) genomic DNA. The product of the PCR was a single band of 763 base pairs that was ligated into pGEM-T Easy and transformed into *Escherichia coli*. Fifteen independent colonies from this transformation were randomly picked, and the plasmid DNA isolated and sequenced. All 15 clones contained an identical sequence with extensive similarity to *Drosophila vasa*. We designate the gene from which this sequence derives *Tu-vasa*. This sequence has been submitted to GenBank (accession number AY167036).

We prepared Clustal W multiple alignments using a highly conserved region of the DEAD box protein sequences from various metazoans, including a predicted translation of the amplified *Tu-vasa* fragment (Fig. 1). Maximum likelihood analysis of this alignment using TreePuzzle (Strimmer and von Haeseler 1996) resulted in 92.9% of quartets resolved. A dendrogram drawn from these results is shown in Fig. 1. The maximum likelihood analysis demonstrates that *Tu-vasa* clusters with other vasa homologues and not with a PL10-related DEAD box helicase. The clustering of *Tu-vasa* in our phylogenetic analysis and its expression pattern (see below) imply that we have cloned a homologue of *vasa* from spider mites.

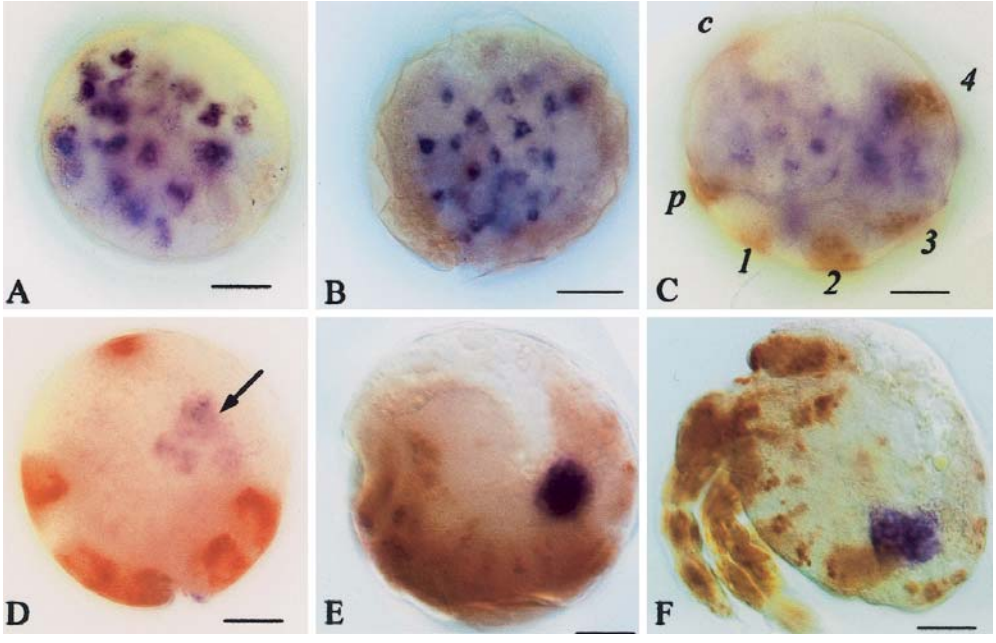

Fig. 2A–F Expression of *Tu-vasa* in whole-mount spider mite embryos. The embryos are hybridised for *Tu-vasa* RNA (*blue*) and stained for Distal-less protein expression (*brown*). *Scale bars* represent 50 µm. **A**, **B** Expression of *Tu-vasa* in two different blastoderm-stage embryos (17 h after egg laying – AEL). *Tu-vasa* RNA is present around nuclei that lie beneath the blastoderm surface. These cells appear to be randomly distributed and are not in a conserved pattern in different embryos. **C** Expression of *Tu-vasa* RNA in early morphogenising embryos (20–22 h AEL). Dll protein-expressing cells are present in the limb buds of the chelicerae (*c*), pedipalps (*p*) and the first four walking legs (*1–4*). *Tu-vasa*-expressing cells can be seen accumulating in posterior regions of the embryo, dorsal to the fourth walking leg domain of Dll expression. **D** Formation of a cluster of *Tu-vasa*-expressing cells (24 h AEL). *Tu-vasa*-expressing cells have formed a tight group, with a few stragglers, dorsal and posterior to the fourth walking leg limb bud. **E** Expression of *Tu-vasa* in a late nymph (34–36 h AEL). *Tu-vasa* is only expressed in a tight group of cells in the posterior of the germband, near the boundary between the prosoma and the opisthosoma. The group of cells lies dorsal to the nervous system (marked by expression of Dll in discrete groups of cells). **F** Expression of *Tu-vasa* in a hatched nymph (>39 h AEL). *Tu-vasa*-expressing cells remain in the posterior of the animal, dorsal to the nervous system. The placement of the cells in this nymph is close to the final position of the ovary in female adults

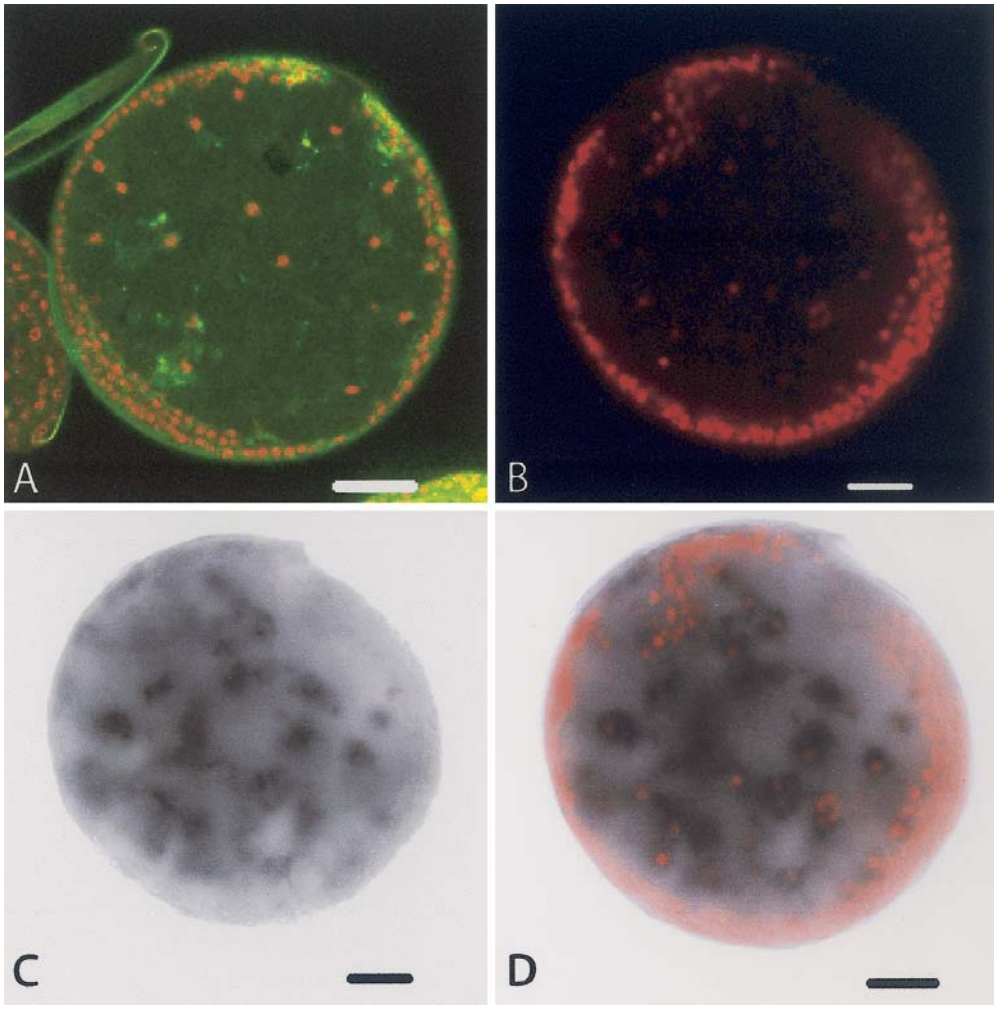

Fig. 3A–D Expression of *Tu-vasa* in cells internal to the blastoderm at early stages. *Scale bars* represent 50 μm. **A** Confocal section through a blastoderm stage embryo. Nuclei are stained *red* with propidium iodide, cortical actin is stained *green* with Oregon green phalloidin. A number of nuclei can be seen internal to the blastoderm surface. **B–D** Embryo hybridised for *Tu-vasa* RNA (*dark* in DIC images) and stained with propidium iodide to show nuclei. **B** Confocal section of a blastoderm stage embryo stained with propidium iodide (*red*). **C** DIC image of the same embryo hybridised for *Tu-vasa* RNA (*dark*). *Tu-vasa* RNA-positive cells can be seen in the centre of the egg, and not in the blastoderm. **D** Merged image of **B** and **C**. All nuclei stained with propidium iodide in the centre of the embryo are associated with a region of *Tu-vasa*-positive staining. As the nuclei are shown in confocal section, not all *Tu-vasa* RNA is associated with a visible nucleus

Expression of *Tu-vasa* during embryogenesis

To examine the expression pattern of the *Tu-vasa* gene we used transcripts of our PCR fragment labelled with DIG as probes for in situ hybridisation. To provide spatial information during the early stages of morphogenesis we co-stained our specimens with an anti-Dll antibody (Panganiban et al. 1995) that has been demonstrated to recognise limb bud tissue in early-stage spider mite embryos (Dearden et al. 2002). Limb buds form rapidly and simultaneously in the spider mite prosoma (excluding the chelicerae limb buds that form later) allowing anterior-posterior polarity to be determined from approximately 20 h after egg laying (AEL).

Two-spotted spider mite eggs are approximately 150 μm in diameter when laid. The eggs undergo nine cleavages, approximately one per hour, to form a blastoderm-stage embryo, with blastomeres around a yolky centre. The germband becomes visible by 20–23 h AEL, and hexapod nymphs hatch by 36 h (Dearden et al. 2002).

No maternal *Tu-vasa* RNA was detected in just laid eggs. *Vasa*-like genes are commonly, but not always (Chang et al. 2002), expressed maternally in insects. Expression of a maternal *vasa* homologue in the spider mite would likely be detectable using our *Tu-vasa* probe assuming a high level of similarity between possible multiple *vasa* homologues. Thus, we have no evidence for such a maternally expressed *vasa* in spider mites. Nevertheless, additional *vasa*-homologous genes may yet be present.

Tu-vasa RNA was first detected approximately 17 h AEL in blastoderm-stage embryos (Fig. 2). At this stage, *Tu-vasa* RNA is present in a population of cells that lie beneath the surface of the blastoderm (Fig. 2A). Cells expressing *Tu-vasa* appear to be distributed randomly, with no discernible anterior-posterior or dorso-ventral localisation. Cells in the outer blastoderm layer do not express *Tu-vasa*. To determine if all of the cells that do not join the blastoderm surface express *vasa* RNA, we examined spider mite embryos that were hybridised for *Tu-vasa* RNA and then stained with propidium iodine (to visualise the nuclei of the cells present). These embryos were examined under a confocal microscope to determine the co-localisation of *Tu-vasa* RNA and propidium iodide (Fig. 3). We examined over 100 embryos of the appropriate stage using this technique and never found a non-blastodermal, propidium iodide stained nucleus that was not associated with *Tu-vasa* RNA expression. Nor did we identify any cells included in the blastoderm layers that expressed *Tu-vasa*. This establishes a clear difference between non-blastodermal *Tu-vasa*-expressing cells, and blastodermal non *Tu-vasa* expressing cells (Fig. 3).

Tu-vasa-expressing cells migrate through the centre of the yolk when the germband first becomes visible. Co-staining for Dll protein demonstrates that these cells migrate toward posterior regions of the germband (Fig. 2C), and form a cluster of cells just posterior and dorsal to the fourth walking leg primordium (Fig. 2D). As the cluster of cells forms, *Tu-vasa* expression decreases but never fully disappears. Soon afterwards, *Tu-vasa* expression appears to increase, and the *Tu-vasa*-expressing cells form a tight cluster located dorsal to the nervous system (marked by Dll expression) and the fourth walking leg primordium (Fig. 2E). It is possible that the stronger phase of *Tu-vasa* RNA expression may be in a different group of cells from those that have migrated into the posterior. We believe this is unlikely given that *Tu-vasa* RNA never

completely disappears, nor do we see a large number of new cells beginning to express *Tu-vasa* RNA. *Tu-vasa* RNA is detectable in the posterior cluster of cells until after the nymphs have hatched (Fig. 2F). These cells are in the correct position to form gonads (Mothes-Wagner and Seitz 1984). The expression pattern of the *Tu-vasa* gene is thus consistent with it being localised in germ cells. We have been unable to determine the expression pattern of *Tu-vasa* in adult ovaries. The spider mite ovary is tiny and surrounded by a membrane that is impervious to RNA probes. We have been unable to remove this membrane thus far.

Primordial germ cell specification in the spider mite

Our results defining the early expression of *vasa* RNA in the spider mite imply that germ cell development occurs by a different process than that described for other arthropods (Rongo et al. 1997; Nakao 1999; Chang et al. 2002). The clear correlation of *vasa* expression with non-blastodermal cells implies that the PGCs may be specified by exclusion from the initial blastoderm of the embryo, a method of specification not described in other animals. Given that *vasa* expression begins at the blastoderm stage, it is possible that exclusion from the blastoderm cell layer serves to activate *vasa* expression and specify germ cell fate. In mites, lack of the kind of close contacts present between cells in the blastoderm could act as a trigger to PGC fate, thus specifying non-blastodermal cells as PGCs. It is also possible that germ cell specification occurs before the blastoderm stage but that *vasa* expression is activated later, or that another *vasa*-like molecule may define the germ-cell population earlier in development. We have not identified any cytological differences between the early blastomeres of the embryo before the formation of the blastoderm stage, but it is possible that earlier events specify PGC fate.

The initial distribution of *vasa*-positive cells in the mite embryo does not appear to be regulated by the polarity of the embryo. In *Drosophila*, the pathways that specify A/P polarity are associated with those of PGC specification. In *Schistocerca* it appears that D/V position may play a role in specifying PGC fate (Chang et al. 2002). In *Bombyx* the role of the axes in specification of PGCs, as determined by *vasa* expression, is unclear (Nakao 1999). The differences in PGC segregation between these four arthropod embryos indicates that a great deal of diversity exists in the developmental mechanisms determining germ cell fate. Thus the segregation of germ cells could serve as a specific target for the chemical control of pest arthropod species (such as the spider mite). Clearly, creating control methods that target non-conserved pathways or processes should reduce unwanted effects on non-target organisms. These data also imply that while *vasa* is an excellent marker for germ cell fate, the processes that activate this gene appear to be evolutionarily labile. Comparative studies of the regulatory sequences of *vasa*-homologous genes may enable a better understanding of the changes in this pathway during evolution and of the upstream activators of *vasa* expression. The small genome of the spider mite (Dearden et al. 2002) makes it an ideal candidate for the isolation of such promoter sequences.

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