Developmental expression of Apnanos during oogenesis and embryogenesis in the parthenogenetic pea aphid Acyrthosiphon pisum

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ABSTRACT Among genes that are preferentially expressed in germ cells, nanos and vasa are the two most conserved germline markers in animals. Both genes are usually expressed in germ cells in the adult gonads, and often also during embryogenesis. Both nanos-first or vasa-first expression patterns have been observed in embryos, implying that the molecular networks governing germline development vary among species. Previously we identified Apvasa, a vasa homologue expressed in germ cells throughout all developmental stages in the parthenogenetic and viviparous pea aphid Acyrthosiphon pisum. In asexual A. pisum, oogenesis is followed by embryogenesis, and both occur within the ovarioles. In order to understand the temporal and spatial distribution of nanos versus vasa during oogenesis and embryogenesis, we isolated a nanos homologue, Apnanos, and studied its expression. In adults, Apnanos is preferentially expressed in the ovaries. In early embryos, Apnanos transcripts are localized to the cytoplasm of cellularizing germ cells, and soon thereafter are restricted to the newly segregated germ cells in the posterior region of the cellularized blastoderm. These results strongly suggest that the Apnanos gene is a germline marker and is involved in germline specification in asexual A. pisum. However, during the middle stages of development, when germline migration occurs, Apnanos is not expressed in the migrating germ cells expressing Apvasa, suggesting that Apnanos is not directly associated with germline migration.

KEY WORDS: Acyrthosiphon pisum, germ cell, nanos, pea aphid

Introduction

The establishment of a germline lineage begins with the specification of germ cells and usually takes place during embryogenesis. Two distinctive strategies for specifying germ cells, namely “preformation” and “epigenesis”, have been described. In the “preformation” mode, germine determinants are synthesized during oogenesis and then they are asymmetrically localized to a subcellular cytoplasm (germ plasm) or a perinuclear region (nuage) within the oocyte (Eddy, 1975; Strome and Lehmann, 2007). In model organisms such as Caenorhabditis elegans (nematode), Drosophila melanogaster (fruit fly), Danio rerio (zebrafish), and Xenopus laevis (frog), embryonic cells inheriting maternal germ-line determinants from these structures become primordial germ cells. In the “epigenesis” mode, specification of germ cells does not depend on a preformed subcellular structure containing germine determinants. Instead, germ cells are derived from pluripotent progenitors responding to extraembryonic molecules secreted from adjacent somatic cells (Saffman and Lasko, 1999; Wylie, 1999; Extavour and Akam, 2003). For example, in the mouse Mus musculus proximal epiblast cells induced by signaling molecules such as Bone morphogenetic protein 4 (BMP4) and...
**A** Alignment of Apnanos and a nanos homologue from the cotton aphid *Aphis gossypii* (accession number: DR389642). DNA sequences of these two nanos homologues encode zinc-finger motifs conserved in Nanos-related proteins in metazoans. Nuclear acids identical in these two species are highlighted in black. Grey area indicates primer sequences adopted from the *Aphis nanos* sequences. Asterisks indicate different residues in the aligned region. Both of these two nanos fragments encode identical amino acid sequences. **B** Alignment of amino acid sequences in zinc-finger motifs of ApNanos protein and thirteen other Nanos homologues across invertebrates and vertebrates. Residues identical in all taxa are highlighted in black; those identical in at least 50% of taxa are highlighted in grey. Amino acid residues constituting the conserved double CCHC zinc-finger motifs are indicated with dots beneath sequences. Sequences are labelled with common names of species. Full names and GenBank accession numbers are as follows: pea aphid (*Acyrthosiphon pisum*, EU180023); grasshopper (*Schistocerca americana*, AAO38523) (Lalli et al., 2003); housefly (*Musca domestica*, AAA87461) (Curtis et al., 1995); honey bee (*Apis mellifera*, ABC41342) (Dearden, 2006); mosquito (*Aedes aegypti*, EAT35750) (Calvo et al., 2005); fruit fly (*Drosophila melanogaster*, AAA28715) (Wang and Lehmann, 1991); sea anemone (*Nematostella vectensis*, AAW29070) (Extauvour et al., 2005); jellyfish (*Podocoryne carnea*, AAU11513) (Torres et al., 2004); sea urchin (*Hemicentrotus pulcherrimus*, BA53723) (Fuji et al., 2006); planarian (*Schmidtea mediterranea*, ABO52809) (Wang et al., 2007); leech (*Helobdella robusta*, AAB63111) (Pilon and Weisblat, 1997); frog (*Xenopus laevis*, CAA51067) (Mosquera et al., 1993); zebrafish (*Danio rerio*, AAL15474) (Koprunner et al., 2001); mouse (*Mus musculus*, BAC82588) (Tsuda et al., 2003). **C** Detection of the expression of Apnanos mRNA in ovaries (O) and somatic (S) tissues with semiquantitative RT-PCR. A PCR fragment (arrowhead) corresponding to the expected size of the 167-bp Apnanos amplicon was identified in ovaries dissected from adult pea aphids. In remaining tissues, which were somatic, transcripts of mRNA were almost undetectable. Expression of Apatcin, an actin homologue in pea aphids, served as an internal control.

BMP8b from the extraembryonic ectoderm acquire germline competence at about embryonic day 6.25 (EE6.25) (Hayashi et al., 2007).

Although animals utilize different strategies to specify germ cells, they share some common factors to sustain germline survival and development. According to Extavour and Akam (2003), there are at least six gene homologues (*boule* *D.azl*, germ-cell-less, *nanos*, *pumilio*, *staufen*, *vasa*) required for germline development in *D. melanogaster*, a “preformation” organism, and in *M. musculus*, an “epigenesis” organism. Among these germ-line-related homologues, *nanos* and *vasa* are most conserved because they have been identified in germ cells of the five model organisms mentioned above and many non-model species across invertebrates and vertebrates (Extavour and Akam, 2003). Homologues of both *nanos* and *vasa* are specifically expressed in germ cells within the adult gonads in most animals, yet embryonic distribution of *nanos* and *vasa* varies among species. For example, mRNAs or proteins of *nanos* and *vasa* are components of maternal germ plasm in *C. elegans*, *D. melanogaster*, and *D. rerio* (Ikenishi, 1998; Subramaniam and Seydoux, 1999; Knaut et al., 2000; Koprunner et al., 2001), whereas in *X. laevis* it is *nanos* (*Xcat2*), rather than *vasa* (*XVLG1*), that is identified in the maternal germ plasm; *XVLG1* is first detectable in the germ cells of hatching tadpoles (Forristall et al., 1995; Ikenishi and Tanaka, 2000).

In *D. melanogaster*, posterior localization of *nanos* mRNA to the pole plasm (germ plasm) requires the RNA helicase Vasa, which promotes the translation of *nanos* mRNA (Gavis et al., 1996). The CCHC zinc finger motifs in *Drosophila* Nanos protein then guide abdomen formation and germline migration (Arrizabalaga and Lehmann, 1999). In other dipteran insects such as *D. virilis*, *Musca domestica* (housefly), and *Chironomus samoensis* (midge), homologues of *nanos* mRNA are also localized to the pole plasm, and they can functionally substitute for *nanos* in *D. melanogaster* (Curtis et al., 1995). This suggests that posterior localization and translation of *nanos* depends on Vasa and is conserved in dipters. In other non-dipteran insects such as the orthopteran *Schistocerca americana* (grasshopper) and the hymenopteran *Apis mellifera* (honeybee), *nanos* expressed in the posterior region of early embryos and *nanos* is regarded as...
a conserved posterior determinant in these insects. However, colocalization of nanos and vasa mRNA/protein occur neither to the posterior region of mature oocytes nor in newly-laid eggs in A. mellifera, S. americana, or the closely related S. gregaria, where a preformed germ plasm has not been identifiable (Chang et al., 2002; Lall et al., 2003; Dearden, 2006). In our previous studies we used cross-reacting antibodies against Nanos and Vasa to identify a preformed germ plasm in the posterior region of the syncytium in the parthenogenetic pea aphid Acyrthosiphon pisum. Additionally, we found that posterior localization of Nanos signals preceded that of Vasa signals during early embryogenesis in this species, which is different from the localization order of Nanos and Vasa in D. melanogaster (Chang et al., 2006). Taken together, this indicates that the hierarchical relationship between nanos and vasa in the molecular network governing germline development is not conserved, even within species relying on a preformed germ plasm to drive germ-cell formation. In effect, why animals need both nanos and vasa to sustain germline development and how they interact with other germline-specific components are still not clear.

We have cloned Apvasa, a Drosophila vasa homologue in parthenogenetic A. pisum, using it as a germline marker to monitor the migration of germ cells during embryogenesis. Apvasa is specifically expressed in germ cells throughout all developmental stages except for a short interval before the invasion of endosymbiotic bacteria (Chang et al., 2007). In order to understand how germline development was regulated by nanos and the regulatory relationship between nanos and vasa in A. pism, we cloned a pea-aphid nanos homologue and made riboprobes for in situ hybridization to detect its developmental distribution. In our previous immunostaining experiments the cross-reacting antibody against Drosophila Nanos only marked aphid germ cells until the blastoderm is formed, but after that germ cells become devoid of staining (Chang et al., 2006). We infer that either germ cells do not express Nanos or that the antibody cannot penetrate into the embryos after blastulation. We expect that under stringent in situ hybridization conditions the nanos riboprobes can have better penetration than that of Nanos antibody. In this report we analyze the developmental expression of nanos and discuss its potential roles in germline development.

Results

Isolation and characterization of a nanos homologue in parthenogenetic pea aphids

For animals lacking completely sequenced genomes the most common method used for isolating a nanos gene is via PCR cloning with degenerate primers designed from the conserved zinc-finger motifs. For example, Nvnos1 and Nvnos2, two nanos homologues in the sea anemone Nematostella vectensis, were cloned with this strategy (Extavour et al., 2005). In the pea aphid Acyrthosiphon pisum we attempted to amplify nanos-related genes using the same approach, but degenerate primers encoding amino acid sequences at both N and C termini of the zinc-finger motifs did not work for annealing temperatures of 40-60°C. As an alternative, we searched for nanos homologues in sequences from an Aphis gossypii/EST (expressed sequence tag) library created at USDA-ARS (United States Dept. Agriculture-Agricultural Research Service) and submitted to GenBank and from an A. pism EST database (http://urgi.versailles.inra.fr/). The A. pism EST database contained no nanos-like sequences, but we did identify a single nanos-like sequence in A. gossypii/that includes the same zinc-finger motifs used for the degenerate nanos primers. We used this sequence to design aphid-specific nanos primers that we successfully used to amplify and clone a 167 base-pair sequence from A. pism. This fragment differed from the A. gossypii/sequence at only three nucleotides (Fig. 1A), suggesting that DNA sequences encoding Nanos zinc-finger motifs in aphids are highly conserved. BLASTX searches clearly demonstrate that both Agnanos and Ananos are nanos homologues rather than genes encoding some other zinc-finger proteins. Additionally, comparison of the putative ApNanos with other Nanos proteins shows that ApNanos also contain amino acids constituting the highly conserved CCHC zinc-finger domain of Nanos (Fig. 1B). We then investigated gene expression using semi-quantitative RT (reverse transcription)—PCR in asexual adults of A. pism. This revealed that Ananos mRNA is preferentially expressed in ovarioles accommodating germ cells, while in somatic tissues it was expressed at a much lower level (Fig. 1C).

Expression of Ananos mRNA in germaria, oocytes and early embryos before gastrulation

We synthesized a DIG-labelled antisense Ananos riboprobe to detect the temporal and spatial distribution of Ananos mRNA in parthenogenetic A. pism during oogenesis and embryogenesis. Detection of Ananos expression was carried out with whole-mount in situ hybridization in this study. In the germarial lumen, a central space within the germarium, we identified an enrichment of Ananos mRNAs (Fig. 2A). Preferential expression of Ananos mRNA also took place in the trophic cord (Fig. 2B), a structure known to transport nutrients from the germarium to the developing oocytes (Blackman, 1987), suggesting that Ananos transcripts synthesized by the nurse cells (trophocytes) were transported to developing oocytes via this channel. Ananos transcripts aggregated to granular material in the cytoplasm of developing oocytes and in these oocytes they were also identified in the periphery of nuclei (stage 0-2; Fig. 2 A-D). A posterior accumulation of Ananos mRNA particularly appeared in the oocyte undergoing maturation division (stage 2; Fig. 2D). However, when the oocyte nucleus began to cleave synchronously, a cellular status categorized as the beginning of embryogenesis according to Miura et al. (2003), the posterior gathering of Ananos transcripts was not visible and the distribution patterns of Ananos mRNA became uniform (stage 3; Fig. 2E). A similar distribution pattern was identified in older embryos where the cleaved nuclei migrated toward the inner periphery of the syncytium (stage 4; Fig. 2F).

During formation of the blastoderm, expression of Ananos mRNA was down regulated in the anteriormost two thirds of the egg chamber, while in the posterior some Ananos transcripts aggregated to granules in the cytoplasm of the presumptive germ cells (stage 5; Fig. 2G). After blastoderm formation, expression of Ananos mRNA was restricted to the morphologically identifiable germ cells in the posterior region, whereas in other areas of the egg chamber Ananos mRNA was almost undetectable (early stage 6; Fig. 2H). Specific expression of Ananos mRNA continuously occurred within multiplying germ cells invaginated into the embryonic inner cavity (stage 6; Fig. 2I). Thereafter, expression
of *Apananos* mRNA in germ cells became weaker just before the incorporation of the maternal endosymbiotic bacteria (late stage 6; Fig. 2J). When bacteria had entered into the egg chamber, in the stage just before gastrulation, *Apananos* mRNA was undetectable in germ cells, bacteria, or other places in the embryo (stage 7; Fig. 2K).

**Identification of *Apananos* mRNA in migrating germ cells during gastrulation, katatrepsis and germ band retraction**

Germ cells start migrating out of the posterior egg chamber after gastrulation (stage 8). They stay at the dorsal region while the germ band is folding and elongating (stage 9-14); after that, when katatrepsis is initiated (stage 15), they start migrating from the dorsal to the anteriormost region of the egg chamber (Chang et al., 2007). During these developmental periods, we did not detect *Apananos* expression in migrating germ cells with the antisense *Apananos* riboprobe. The single *in situ* hybridization results were confirmed by the aid of double-labelled embryos with both antisense riboprobes of *Apananos* as well as *Apvasa*, a germline marker expressed throughout whole embryogenesis in asexual *A. pismum* (Chang et al., 2007). Figure 3A shows that co-localization of *Apananos* and *Apvasa in situ* signals occurs in the germarium and the stage 5 embryo; however, *Apvasa*, rather than *Apananos*, occurs in germ cells in the late blastula (late stage 6), the gastrulating embryo (stage 9) and the embryo undergoing germ band extension (stage 14). The absence of the preferential
expression of \textit{Apnanos} mRNA in germ cells continues in embryos undergoing kataterpesis. Figure 3B is an example showing that germ cells reaching the anterioriormost region of the egg chamber only express \textit{Apvasa}; co-localization of \textit{Apvasa} and \textit{Apnanos} mRNAs is not visible.

We again identified specific expression of \textit{Apnanos} mRNA in germ cells of stage-16 embryos, after kataterpesis was complete and the germ band was about to retract (Fig. 3 D,E). From this stage onward, germ cells expressed \textit{Apnanos} mRNA (Fig. 3 G,J). Compared with germ cells in the stage-15 embryo (Fig. 3B), germ cells in the stage-16 embryo were separated into subclusters due to the formation of germaria (Fig. 3F). As with the distribution pattern in germaria dissected from adult \textit{A. pisum} (Fig. 2 A-D), accumulation of \textit{Apnanos} transcripts occurred in the lumina of these newly formed germaria (Fig. 3F). While the germ band was retracting from dorsal anterior to dorsal posterior (stage 17; Fig. 3 G,H), and finally merging into the abdomen (stage 18; Fig. 3 J,K), germ cells expressing \textit{Apnanos} were located within the germaria and they migrated dorsally toward the midline of the abdominal cavity (Fig. 3 I,L).

**Expression of Apnanos mRNA in ovarioles dissected from mature embryos**

Parthenogenetic \textit{A. pisum} embryos are in fact pregnant before they are born. We also investigated the distribution of \textit{Apnanos} in developing ovarioles within mature embryos still in the mother. These mature embryos are already fully cuticularized so the \textit{Apnanos} riboprobe did not penetrate the embryonic cavity during our studies of earlier stage embryos in the mother. We therefore cut open the abdomen and exposed the ovarioles directly to the hybridization solution. These young ovarioles were not dissected out of the mature embryos until \textit{in situ} signals were developed to prevent them being washed off. We found that oocytes and embryos developed asynchronously between ovarioles and that embryos after stage 7 of development were not identifiable (Fig. 4). Distribution patterns of \textit{Apnanos} mRNA appeared similar to

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**Fig. 3. Whole-mount identification of Apnanos mRNA in embryos during mid and late embryogenesis.** Unless otherwise noted, anterior is to the left, dorsal is uppermost and all views are lateral. (A-C) Ovariole double probed with antisense \textit{Apvasa} and \textit{Apnanos} riboprobes. Color features of single and double in situ signals are indicated on the figure. (A) Colocalized signals of \textit{Apvasa} and \textit{Apnanos} appear in the germarium (G) and stage (St) 5 embryo. In embryos at St-6, 9 and 14 of development only \textit{Apvasa} transcripts are detected in germ cells (arrowhead). Expression of \textit{Apnanos} is not identifiable in embryos older than stage 5 of development in this preparation. (B) Stage 15, embryo undergoing kataterpesis. Arrow indicates the tip of the head migrating toward the anterior region of the egg chamber. Expression of \textit{Apvasa}, rather than \textit{Apnanos}, preferentially occurs in migrating germ cells (arrowhead) in the anterior region. (C) Nuclear staining of embryo in (B). (D-L) Embryos hybridized only with an antisense \textit{Apnanos} riboprobe. (D-F) Stage 16, embryo after kataterpesis. Head (Hd) flips to the anterior region of the egg chamber. (D) In situ signals of \textit{Apnanos} mRNA occur in germ cells (arrowhead) in the dorsolateral region. (E) Nuclear staining of (D). Posterior tip (arrow) of the abdomen (Ab) is close to the posteriormost part of the head (hollow arrow), indicating that germ band retraction has just initiated. Large cells of the serosal membrane are indicated with hollow arrows. (F) Magnification of the inset shown in (D). Arrowheads indicate the location of the presumptive germarial lumen, where the preferential expression of \textit{Apnanos} takes place. Dashed line marks the boundary of a germarium visible in the presented focal section. (G-I) Stage 17, embryo undergoing germ band retraction. (G) Germ cells (arrowhead) labelled with \textit{Apnanos} probes are migrating to the uppermost region of the embryo. (H) Nuclear staining of (G). Posterior tip (arrow) of the abdomen (Ab) is retracting to the posterior region of the embryo. (I) Magnification of the inset shown in (G). The expression pattern of \textit{Apnanos} mRNA is similar to that shown in (F). (J-L) Stage 18, germ band retraction completed. (J) Germ cells (arrowhead) labelled with \textit{Apnanos} probes are closer to the dorsal midline, but not visible from a lateral view. (K) Nuclear staining of (J). (L) Magnification of the inset shown in (J). Expression pattern of \textit{Apnanos} is similar to that described in (F) and (I). (M,N) Negative control (-Ctrl), embryos slightly older than stage 18, but not yet reaching the average size of St-19 embryos, were hybridized with a DIG-labelled \textit{Apnanos} sense riboprobe. In situ signals are not preferentially identified in germ cells (Gc; with visible germarium shape located dorsally) but background signals are detectable. In most embryos after germ band retraction, non-specific in situ signals can be identified in the terminal region of the rostrum (Rt; arrows in (G), (J), (M)) with either antisense or sense \textit{Apnanos} riboprobes. Other abbreviations: An, antenna; B, bacteria; E, eyes. T1-T3, the three thoracic segments. Scale bars: (F), (I), 10 µm; others, 50 µm.
those identified in ovarioles dissected from the adult (Fig. 2 A-I). For example, we identified an aggregation of Apnanos transcripts in the germarial lumina (Fig. 4 A-C), a uniform distribution in the syncytium (stage 4; Fig. 4C), and a down regulation of Apnanos expression in germ cells from stage 5 (Fig. 4A) to stage 6 of development (Fig. 4 B,C).

**Discussion**

We cloned Apnanos, a Drosophila nanos homologue in the parthenogenetic and viviparous pea aphid Acyrthosiphon pisum (Fig. 1 A,B). In adults, Apnanos mRNA (Apnanos) is preferentially expressed in the ovaries (Fig. 1C). In embryos, Apnanos transcripts are localized to the cytoplasm of cellularizing germ cells (stage 5; Fig. 2G), and thereafter Apnanos is restricted to the newly-segregated germ cells in the posterior region of the cellularized blastoderm (stage 6; Fig. 2H,I). These results strongly suggest that Apnanos is a germline marker and is involved in germ line specification in asexual A. pisum. However, from stage 7 to 15, the developmental period during which germline migration occurs (Chang et al., 2007), Apnanos is not identifiable in the migrating germ cells (Fig. 2K; Fig. 3A,B), suggesting that Apnanos is not directly associated with germline migration.

In our previous studies, we used a cross-reacting antibody against the Drosophila Nanos protein to identify presumptive germ plasm first localized to the posterior region of the oocyte undergoing maturation division (stage 2) (Hanyu-Nakamura et al., 2004; Chang et al., 2006). The Nanos signals remain localized in the posterior region during early embryogenesis and are finally incorporated into the morphologically identifiable germ cells at the posterior. This suggests that the specification of germ cells in asexual A. pisum depends on germ plasm expressing Nanos protein (Nanos) (Chang et al., 2006). Accumulation of Apnanos occurs in the posterior during stage 2 of development (Fig. 2D), which corresponds with the Nanos expression mentioned above. Nevertheless, unlike the distribution pattern of the Nanos protein, we did not detect the posterior localization of the Apnanos signal continuously in embryos from stage 3 to stage 4 of development, which is a period of nuclear division (Fig. 2E,F). Accordingly, we infer that the posterior localization of Nanos protein is via the translational control of Apnanos, or depends on a “posterior molecular anchor”, localized prior to Nanos, that can localize Nanos circulating to the posterior region. However, we cannot exclude the possibility that the Nanos signals detected by the cross-reacting antibody is not the protein transcribed from Apnanos. If this is the case, then there may be more than one nanos homologue in A. pisum.

In asexual A. pisum, migrating germ cells expressing Apvasa remain an integrated group from gastrulation to katatrepsis (Fig. 3 A,B), and they first appear as subclusters within germaria after the completion of katatrepsis (Fig. 3D) (Chang et al., 2007). Accordingly, this suggests that the coalescence between migrating germ cells and the somatic gonadal tissue takes place while germ cells are migrating from the anteriormost region of the egg chamber into the body cavity (Fig. 3 B,D), and that the formation of gonads, which will differentiate into the germarial primordia, is accomplished after the embryo flips (Fig. 3D) (Chang et al., 2007). Given that the re-expression of Apnanos occurs to germ cells within the newly formed germaria, we hypothesize that the synthesis of Apnanos mRNA may be induced by germarial somatic cells surrounding the germ cells. According to Blackman (1978) and Bünning (1985), undifferentiated germ cells within the germarium give rise to nurse cells and, under environmental conditions appropriate to asexual oogenesis, nurse cells at the posterior germarium further differentiate into oocytes. We thus surmise that Apnanos within the germ cells freshly incorporated into the germarium is involved in the production of oocytes (Fig. 3 D,G,J) and that Apnanos in the oocytes and embryos by stage 4 of development is associated with the maintenance of oogenesis and early embryogenesis (Fig. 2 C-F (ovarioles in adults); Fig. 4 A-C (ovarioles in embryos)). However, at present we do not have direct evidence via functional assay of Apnanos to support the above inference. In Drosophila melanogaster (fly) and Danio rerio (zebrafish), it has been demonstrated that nanos is required for production of oocytes (Forbes and Lehmann, 1998; Draper et al., 2007), suggesting that this feature may be conserved in A. pisum as well.

Besides the conserved character of being a germline marker, nanos genes in insects have been regarded as determinants that regulate posterior development (Curtis et al., 1995; Lall et al., 2003). Functional assays show that abdominal development is largely prevented in D. melanogaster mutants with nanos loss of function alleles (Gavis and Lehmann, 1992). In the grasshopper Schistocerca americana tools for genetic manipulation and knock-
down experiments have not yet been performed; nevertheless, nanos has been implicated as a posterior regulator by an empty patch of hunchback expression in the nanos-positive area (Lall et al., 2003). In addition, it has been proposed that nanos genes expressed in the posterior region of oocytes and early embryos in honeybees (Dearden, 2006), mosquitoes (Calvo et al., 2005), and wasps (Olesnicky and Desplan, 2007) are associated with posterior development. In asexual A. pismum, asymmetric localization of Apnanos mRNA occurs in the posterior region of the oocyte undergoing maturation division (stage 2; Fig. 2D), suggesting that Apnanos is also conserved in regulating posterior development. From stage 3 to stage 4 of development Apnanos is not localized to the posterior and there is no posterior to anterior gradient of Apnanos (Fig. 2 E,F). During the same developmental period localized Nanos signals are detectable in the posterior region (Chang et al., 2006), suggesting that it is the Nanos protein, rather than the nanos mRNA, that maintains the posterior development.

In D. melanogaster (Wang and Lehmann, 1991; Curtis et al., 1995), S. americana (Lall et al., 2003), Apis mellifera (Dearden, 2006), and A. pismum, where nanos expression has been studied throughout developmental stages, we find a common feature that nanos transcripts are again detectable within the germ cells that have just been incorporated into the presumptive gonads. However, the point at which nanos become undetectable varies in these four insect species. In S. americana and Ac. pismum, nanos becomes undetectable after germ cells are specified (Lall et al., 2003) (Fig. 2K; Fig. 3 A,B). In Ap. mellifera, this occurs after the formation of a posterior gradient of nanos during early embryogenesis, although whether germ cells are specified in early embryos is not clear (Dearden, 2006). In D. melanogaster, the breakdown of maternally-inherited nanos occurs in migrating germ cells in the midgut prior to germ band retraction (D. melanogaster developmental stage 10) (Wang and Lehmann, 1991; Curtis et al., 1995). Thus, of these four insects D. melanogaster is the only species in which nanos is detected in migrating germ cells (D. melanogaster developmental stage 6-10): pole cells lacking nanos activity fail to migrate to the gonads (Kobayashi et al., 1996). Whether nanos regulation of germline migration only takes place in Drosophila or is common to other insects requires further investigation. In our target insect, the asexual Ac. pismum, we propose experiments for knocking down Apnanos with tools such as RNA interference or antisense morpholino oligonucleotides in order to investigate how Apnanos affects germine specification and migration, and how Apvasa is expressed under the knock-down of Apnanos and vice versa.

Materials and Methods

Pea aphid culture

We reared parthenogenetic pea aphids, Acyrthosiphon pismum, on garden pea plants Pisum sativum at 15°C in a growth chamber with a long-day period (16 hours light/8 hours dark). Staging of aphid development follows the scheme established by Miura et al. (2003).

Cloning and reverse transcription (RT)-PCR of Apnanos

A partial nanos fragment of A. pismum was amplified from complementary DNA (cDNA) reverse transcribed from total RNA of A. pismum cDNA was synthesized using StrataScript reverse transcriptase (Stratagene), and RNA was purified with RNeasy Mini Kit (Qiagen). Experiments were carried out according to the manufacturer’s instructions. Primers were designed using the Aphis gossypii nanos sequence (Fig. 1) as follows: (1) forward: 5’-TGCGCAGTTCTGCAAGAAGCACC-3’ (CAFCKSN); (2) reverse: 5’-GGATTTTTTGGACAGACTAACG-3’ (RYCPK). Amplification parameters were: 95°C for 5 minutes, followed by 40 cycles at 94°C for 30 seconds, 50°C for 30 seconds, 70°C for 30 seconds and, finally, 70°C for 5 minutes. PCR products were cloned into the pGEM-T Easy Vector (Promega) for sequencing and in vitro transcription. Sequences were aligned using MacVector 8.0 (Accelrys). PCR cloning of an actin homologue in A. pismum (Apactin) was carried out under the same conditions as above, except that the annealing temperature was 54°C. Degenerate primers used for cloning Apactin were: (1) forward: 5’-GCACTCACAACACTGGGAYGAYATAGGA-3’ (FITNWDDME); (2) reverse: 5’-CTTCCGGATGTCCACGTCRACYTTCAT-3’ (MCVDDIRK).

GenBank accession number of Apactin: EU346758.

Semi-quantitative RT-PCR was performed using cDNA from ovaries and somatic tissues dissected from adult A. pismum. The concentration of cDNA was normalized to 250 ng for each PCR amplification. We tested various numbers of amplification cycles for Apnanos and found that: (1) After 40 cycles, PCR amplification was saturated—It was no longer possible to discriminate product intensity for the ovary or somatic groups in the agarose gel; (2) Under 22 cycles, PCR products became almost undetectable. The best detection of differential expression of Apnanos in both ovary and somatic tissues was obtained between 25 cycles of amplification. PCR conditions were the same as those used for cloning Apnanos except that the annealing temperature was 58°C. Primers used for semi-quantification of Apnanos mRNA were identical to those used in cloning Apnanos; primers used for semi-quantification of Apactin were: (1) forward: 5’-AATCCCTGTGACGAAAGC-3’ (LLETTA); (2) reverse: 5’-TTCGATGTTGACGGTACCC-3’ (GQVITIG).

Whole-mount in situ hybridization and microscopy

Aphid riboprobes for in situ hybridization were synthesized from linearized plasmids containing the 167 base pairs encoding the zinc-finger domain. For single in situ hybridization to detect the expression of Apnanos mRNA, Digoxigenin (DIG)-labelled UTP was incorporated into the probe with a DIG RNA Labeling Kit (SP6/T7) (Roche). Apvasa riboprobes for double in situ hybridization experiments were synthesized with Fluorescein Labeling Mix (Roche). Ovarioles were dissected from adult females or mature embryos in 1x phosphate buffered saline (PBS), then fixed in 3.8% formaldehyde in 1x PBS at 4°C overnight. Other steps for single in situ hybridization were based upon the protocol described in Chang et al. (2007). For double in situ we first developed Apnanos signals with NBT/BCIP (20 µl stock solution) in 1 ml 1x detection buffer containing 1 mM levamisole. Before applying antidigoxigenin antibody (Roche; Alkaline phosphatase (AP) conjugated), activity of the AP conjugated to the anti-DIG antibody was blocked with 0.1 M Glycine-HEPES (pH 2.2) in 0.1% Tween 20 for 10 minutes. Apvasa signals were developed with a Fast Red tablet (Roche). Ovarioles, whether dissected from adult females or mature embryos, were cleared in 70% glycerol in 1x PBS at room temperature for 6 hours or at 4°C overnight. Embryos older than stage 11 of development were mounted under a bridged coverslip. Samples were photographed with a Leica DMR connected to a Fuji FinePix S2 Pro digital camera. Nuclear staining was carried out with DAPI (2 ng/µl; Sigma) and pictures were taken with a Zeiss LSM510 META laser-scanning microscope.

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