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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, germline 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 germline 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 germline 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

Abbreviations used in this paper: Apnanos, Acyrthosiphon pisum nanos; Apvasa, Acyrthosiphon pisum vasa.

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BMP8b from the extraembryonic ectoderm acquire germline competence at about embryonic day 6.25 (E6.25) (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*/*Dazl*, *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 germline-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 dipterans. In other non-dipteran insects such as the orthopteran *Schistocerca americana* (grasshopper) and the hymenopteran *Apis mellifera* (honeybee), *nanos* is expressed in the posterior region of early embryos and *nanos* is regarded as

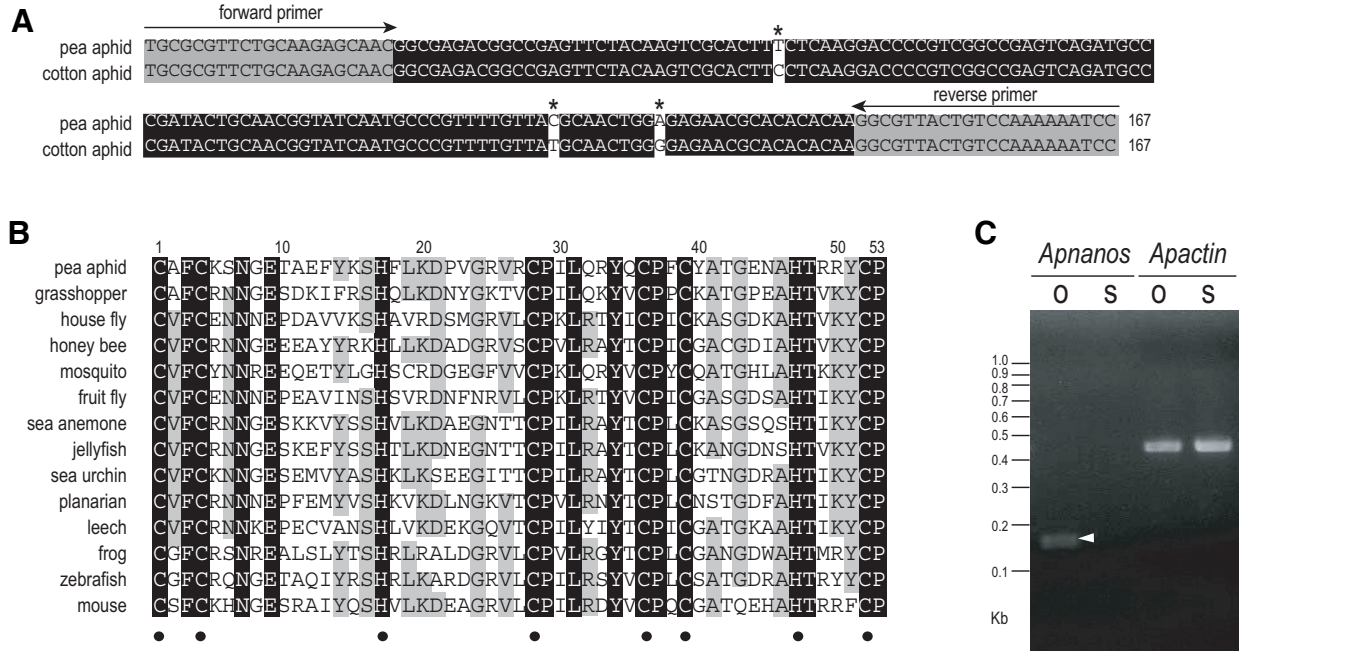

Fig. 1. Characterization of *Apnanos*. (A) *Alignment of* Apnanos *and a* nanos *homologue from the cotton aphid* Aphis gossypii *(accession number: DR389642). DNA sequences of these two* nanos *homologues encode zinc-finger motifs conserved in Nanos-related proteins in metazoans. Nucleic acids identical 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) (Lall* et al., *2003); house fly (*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) (Extavour* et al., *2005); jellyfish (*Podocoryne carnea, *AAU11513) (Torras* et al., *2004); sea urchin (*Hemicentrotus pulcherrimus, *BAE53723) (Fujii* 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) (Köprunner* et al., *2001); mouse (*Mus musculus, *BAC82558) (Tsuda* et al., *2003).* **(C)** *Detection of the expression of Apnanos mRNA in ovaries (O) and somatic (S) tissues with semi-quantitative 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 Apactin, an actin homologue in pea aphids, served as an internal control.*

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. pisum*, 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. pisum* EST database (http://urgi.versailles.inra.fr/). The *A. pisum* 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. pisum*. 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 *Apnanos* 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. pisum*. This revealed that *Apnanos* 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 Apnanos *mRNA in germaria, oocytes and early embryos before gastrulation*

We synthesized a DIG-labelled antisense *Apnanos* riboprobe to detect the temporal and spatial distribution of *Apnanos* mRNA in parthenogenetic *A. pisum* during oogenesis and embryogenesis. Detection of *Apnanos* 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 *Apnanos* transcripts (Fig. 2A). Preferential expression of *Apnanos* 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 *Apnanos* transcripts synthesized by the nurse cells (trophocytes) were transported to developing oocytes via this channel. *Apnanos* 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 *Apnanos* 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 *Apnanos* transcripts was not visible and the distribution patterns of *Apnanos* 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 *Apnanos* mRNA was down regulated in the anteriormost two thirds of the egg chamber, while in the posterior some *Apnanos* transcripts aggregated to granules in the cytoplasm of the presumptive germ cells (stage 5; Fig. 2G). After blastoderm formation, expression of *Apnanos* mRNA was restricted to the morphologically identifiable germ cells in the posterior region, whereas in other areas of the egg chamber *Apnanos* mRNA was almost undetectable (early stage 6; Fig. 2H). Specific expression of *Apnanos* mRNA continuously occurred within multiplying germ cells invaginated into the embryonic inner cavity (stage 6; Fig. 2I). Thereafter, expression

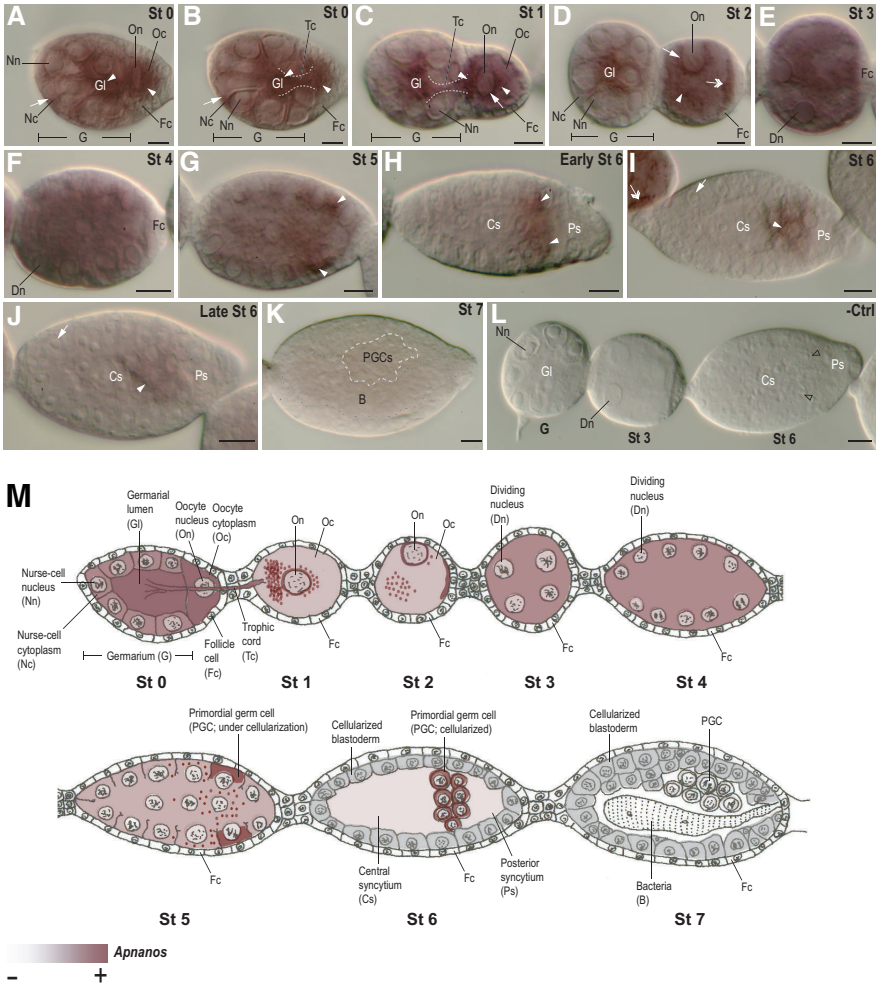

Fig. 2. Temporal and spatial distribution of *Apnanos* mRNA in germaria, developing oocytes and embryos by stage 7 of development. *Samples were whole mount hybridized with a DIG-labelled* Apnanos *antisense riboprobe. Anterior regions of germaria, oocytes and egg chambers (accommodating embryos) are to the left and posterior regions are to the right.* **(A,B)** *Germaria and developing oocytes (stage (St) 0).* **(A)** Apnanos *transcripts accumulate in the germarial lumen (Gl; arrowhead). Area of the germarium (G) is as indicated.* Apnanos *transcripts occur in the cytoplasm of nurse cells (Nc; arrow), and more weakly within the nuclei of the nurse cells (Nn). In oocytes not fully segregated from the germarium,* Apnanos *transcripts occur in the oocyte cytoplasm (Oc; arrowhead), and in lesser quantities in the oocyte nucleus (On). Follicle cells (Fc) are devoid of staining.* **(B)** *Another focal plane of (A), showing* Apnanos *expression in the trophic cord (Tc; highlighted with a dashed line).* **(C)** *Stage (St) 1, segregated oocyte. Preferential expression of* Apnanos *occurs in granules mostly in the anterior (arrowhead), as well as some that are posteriorly associated with the oocyte nucleus (On; arrowhead). In the peripheral region of the nucleus* Apnanos *transcripts also accumulate (arrow), and weak expression occurs in the oocyte cytoplasm (Oc). Follicle cells (Fc) have almost undetectable signals. The trophic cord (Tc; highlighted with a dashed line) is not clear in this focal plane.* **(D)** *Stage (St) 2, the oocyte nucleus (On) undergoing maturation division moves anteriorly.* Apnanos *transcripts occur primarily in the cytoplasmic granules (arrowhead), in the periphery (arrow) of the oocyte nucleus (On), and in the posterior region (double arrowhead).* **(E,F)** *Stages (St) 3 and 4, embryos undergoing nuclear division. In the stage 4 embryo, dividing nuclei (Dn) migrate to the inner periphery of the embryo.* Apnanos *transcripts are uniformly distributed in the cytoplasm.* **(G)** *Stage (St) 5, cellularization and blastoderm formation. Localization of Apnanos transcripts occurs in the cytoplasmic granules at the posterior (arrowhead), where formation of the primordial germ cells (PGCs) occurs.* In situ *signals are weaker from posterior to anterior.* **(H-J)** *Stage (St) 6, morphogenesis of germ cells.* **(H)** *An early St-6 embryo shortly after cellularization of blastoderm. Apnanos transcripts specifically occur in the cytoplasm (arrowhead) of primordial germ cells invaginating into the center of the embryo. In* **(I)**, Apnanos *mRNA is expressed in primordial germ cells (arrowhead), whereas in* **(J)** *expression of* Apnanos *is weaker. The embryo in (J) has more nuclei in the blastoderm (arrow) than (I), and is therefore slightly older. Double arrowhead in (I) indicates posterior localization of Apnanos in a stage-2 embryo (upper left), similar to that in (D). The anterior region of this embryo is not shown in (I).* **(K)** *Stage (St) 7, invasion of the maternal endosymbiotic bacteria (B; outlined with black dashed line). Expression of* Apnanos *is almost unidentifiable in primordial germ cells (PGCs, outlined with white dashed line) above the bacteria.* **(L)** *Negative control (-Ctrl), ovariole containing germarium (G), stage (St)-3 and 6 embryos were hybridized with a DIG-labelled* Apnanos *sense riboprobe. Only weak background signals are detected.* **(M)** *A schematic illustration of the expression of* Apnanos *shown in (A)-(K). Cellularized blastoderm in embryos at stages 6 and 7 of development is marked with grey color. Cs, central syncytium; Ps, posterior syncytium. Scale bars, 10 μm.*

of *Apnanos* 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, *Apnanos* mRNA was unidentifiable in germ cells, bacteria, or other places in the embryo (stage 7; Fig. 2K).

Identification of Apnanos *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 *Apnanos* expression in migrating germ cells with the antisense *Apnanos* riboprobe. The single *in situ* hybridization results were confirmed by the aid of double-labelled embryos with both antisense riboprobes of *Apnanos* as well as *Apvasa*, a germline marker expressed throughout whole embryogenesis in asexual *A. pisum* (Chang *et al.*, 2007). Figure 3A shows that co-localization of *Apnanos* and *Apvasa in situ* signals occurs in the germarium and the stage 5 embryo; however, *Apvasa*, rather than *Apnanos,* 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 *Apnanos* mRNA in germ cells continues in embryos undergoing katatrepsis. Figure 3B is an example showing that germ cells reaching the anteriormost region of the egg chamber only express *Apvasa*: co-localization of *Apvasa* and *Apnanos* mRNAs is not visible.

We again identified specific expression of *Apnanos* mRNA in germ cells of stage-16 embryos, after katatrepsis was complete and the germ band was about to retract (Fig. 3 D,E). From this stage onward, germ cells expressed *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 *A. pisum* (Fig. 2 A-D), accumulation of *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 *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 *A. pisum* embryos are in fact pregnant before they are born. We also investigated the distribution of *Apnanos* in developing ovarioles within mature embryos still in the mother. These mature embryos are already fully cuticularized so the *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 *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 *Apnanos* mRNA appeared similar to

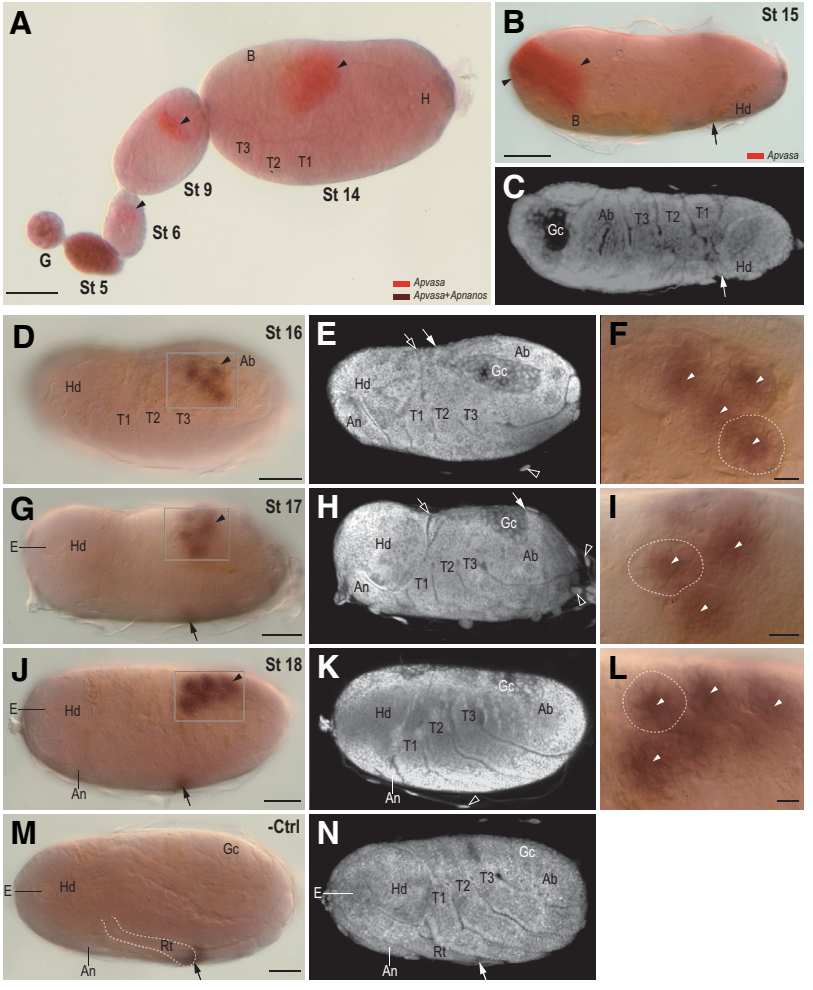

Fig. 3. Whole-mount identification of *Apnanos* mRNA in embryos during mid and late embryogenesis. *Unless otherwise noted, anterior is to the left, dorsal is uppermost and all views are lateral.* **(A-C)** *Ovariole double probed with antisense Apvasa and Apnanos riboprobes. Color features of single and double in situ signals are indicated on the figure.* **(A)** *Colocalized signals of* Apvasa *and* Apnanos *appear in the germarium (G) and stage (St) 5 embryo. In embryos at St-6, 9 and 14 of development only* Apvasa *transcripts are detected in germ cells (arrowhead). Expression of* Apnanos *is not identifiable in embryos older than stage 5 of development in this preparation.* **(B)** *Stage 15, embryos undergoing katatrepsis. Arrow indicates the tip of the head migrating toward the anterior region of the egg chamber. Expression of* Apvasa, *rather than* 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* Apnanos *riboprobe.* **(D-F)** *Stage 16, embryo after katatrepsis. Head (Hd) flips to the anterior region of the egg chamber.* **(D)** *In situ signals of* 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* 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* 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* Apnanos *mRNA is similar to that shown in (F).* **(J-L)** *Stage 18, germ band retraction completed.* **(J)** *Germ cells (arrowhead) labelled with* 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* 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* 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* Apnanos *riboprobes. Other abbreviations: An, antenna; B, bacteria; E, eyes. T1-T3, the three thoracic segments. Scale bars: (F,I, L), 10 μm; others, 50 μm.*

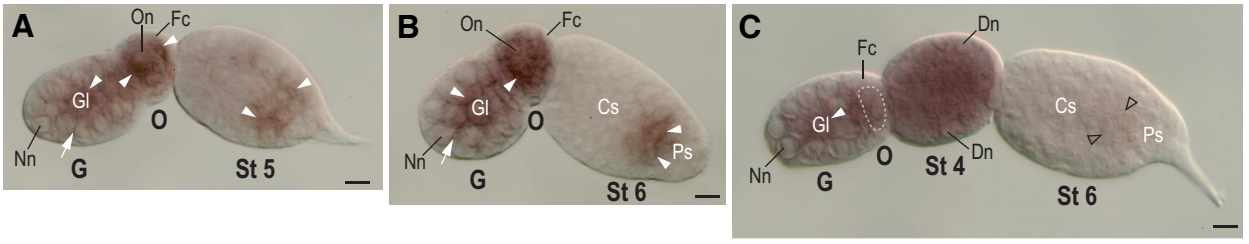

Fig. 4. Expression of *Apnanos* mRNA in ovarioles of mature embryos. *Ovarioles were dissected from fully cuticularized embryos at stage 20 of development, a stage just before larviposition. These ovarian tubules contained germaria, developing oocytes and embryos up to stage 6 of development. Anterior is to the left but note that the above structures in an ovariole cannot be aligned horizontally and straight in all preparations. For the exact orientation of each respective structure please refer to Fig. 2. All abbreviations and labels are also identical to those in Fig. 2. In all panels, distribution of* Apnanos *mRNA in the germaria (G) and developing oocytes (O) is similar to Fig. 2 A-C.* **(A,B)** *Expression of* Apnanos *occurs in the cytoplasm of germ cells (arrowhead) in stage (St)-5 and St-6 embryos respectively.* **(C)** *In an older St-6 embryo, expression of* Apnanos *is almost undetectable in germ cells (hollow arrowhead). The oocyte is not fully segregated from the germarium, and is not clear in this focal plane: its boundary is highlighted with a dashed line. Distribution of* Apnanos *in St-4 embryo is similar to that shown in Fig. 2F. Scale bars, 10 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. 2 H,I). These results strongly suggest that *Apnanos* is a germline marker and is involved in germline 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. 3 A,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. 2 E,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 Büning (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 developed; 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. pisum*, 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. pisum*, 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. pisum*, *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. pisum*, we propose experiments for knocking down *Apnanos* with tools such as RNA interference or antisense morpholino oligonucleotides in order to investigate how *Apnanos* affects germline 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 pisum,* 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. pisum* was amplified from complementary DNA (cDNA) reverse transcribed from total RNA of *A. pisum*. 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'- TGCGCGTTCTGCAAGAGCAAC-3' (CAFCKSN); (2) reverse: 5'- GGATTTTTTGGACAGTAACGCC-3' (RYCPKN). 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. pisum* (*Apactin*) was carried out under the same conditions as above, except that the annealing temperature was 45°C. Degenerate primers used for cloning *Apactin* were: (1) forward: 5'-GCATCATCACCAACTGGGAYGAYATGGA-3' (FITNWDDME); (2) reverse: 5'-CTTCCGGATGTCCACGTCRCAYTTCAT-3' (MKCDVDIRK). GenBank accession number of *Apactin*: EU346758.

Semi-quantitative RT-PCR was performed using cDNA from ovaries and somatic tissues dissected from adult *A. pisum*. 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 on 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 from 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'-AATCCTGTTGACCGAAGCCC-3' (ILLTEA); (2) reverse: 5'-TTCCGATGGTGATGACCTG TCC-3' (GQVITIG).

Whole-mount* in situ *hybridization and microscopy

Apnanos 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 apteriform adults 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*s we first developed *Apnanos* signals with NBT/BCIP (20 μl stock solution (Roche) in 1 ml 1x detection buffer containing 1 mM levamisole). Before applying anti-Fluorescein antibody (Roche; Alkaline phosphatase (AP) conjugated), activity of the AP conjugated to the anti-DIG antibody was blocked with 0.1 M Glycine-HCl (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 adults 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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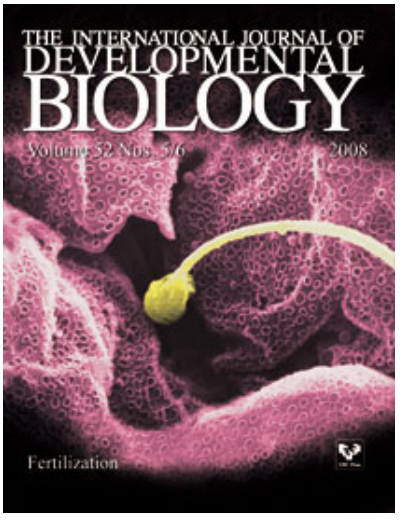

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