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Formation of a large Vasa-positive germ granule and its inheritance by germ cells in the enigmatic Chaetognaths

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SUMMARY

Chaetognaths (arrow worms) are abundant hermaphrodite marine organisms whose phylogenetic position amongst protostomes and deuterostomes is still debated. Ancient histological observations dating from a century ago described the presence in eggs of a large granule, presumed to be a germ plasm, and its probable inheritance in four primary germ cells (PGCs). Using videomicroscopy, electron microscopy and immunocytochemistry (labelling with anti-Vasa antibodies) we have followed the cycle of aggregation and dispersion of germ plasm and nuage material in eggs, embryos, PGCs and oocytes in several species of benthic (*Spadella*) and planctonic (*Sagitta*) chaetognaths. In these animals, germ cells and gametes can be observed in vivo throughout the 1-2 month life cycle.

After describing internal fertilization in live animals we show that the single large (15 μm diameter) germ granule forms by a spiralling aggregation movement of small germ islands situated in the vegetal cortex at the time of first mitosis. We also demonstrate that the granule forms autonomously in unfertilized activated eggs or fertilized egg fragments. Once formed, the germ granule first associates with the cleavage furrow and is segregated into one of the first two blastomeres. The germ granule is then translocated from the cortex to the mitotic spindle during 3rd cleavage and remains in the single most-vegetal blastomere until the 32-cell stage. At the 64-cell stage the germ granule is partitioned as nuage material into two founder PGCs and further partitioned into four PGCs situated at the tip of the archenteron during gastrulation. These four PGCs migrate without dividing to reach the transverse septum, then proliferate and differentiate into oocytes and spermatocytes of two ovaries and two testes. We noted that germ plasm and nuage material were associated with mitochondria, the nucleus, the spindle and the centrosome during some stages of development and differentiation of the germ line. Finally, we demonstrate that a Vasa-like protein is present in the germ granule, in PGCs and in the electron-dense material associated with the germinal vesicle of oocytes. These features stress the conservation of cellular and molecular mechanisms involved in germ cell determination.

Movies available on-line

Key words: Germ plasm, Germ cell, Vasa, Fertilization, Chaetognath, Arrow worm

INTRODUCTION

Chaetognaths, commonly called arrow worms, are among the most abundant marine planctonic organisms (Feigenbaum and Maris, 1984). About 200 species compose this phylum whose position amongst protostomes and deuterostomes is often debated (Telford, 1993; Telford and Holland, 1993; Telford and Holland, 1997; Ghirardelli, 1995; Nielsen, 2001). The most recent molecular analysis even proposes that the phylum chaetognath is situated between protostomes and deuterostomes (Giribet et al., 2000).

Chaetognaths are direct developers that become reproductive adults in about 1-2 months (Reeve, 1970; Reeve and Walter, 1972; Reeve and Cosper, 1974). This cycle can be achieved in the laboratory using benthic species (*Spadella*) (Goto and Yoshida, 1997). Gametogenesis is easily observed in these very transparent organisms where the two male and the two female gonads can occupy more than half of the body volume (Fig. 1A). For this reason chaetognaths were examined by biologists interested in the germ line more that a century ago. Butschli (Butschli, 1873) and then Hertwig (Hertwig, 1880) identified the four primary germ cells (PGCs) in juveniles. It was then reported that these PGCs derived from an early blastomere that contained a unique ‘cytoplasmic body’, detectable just before first cleavage (Elpatievsky, 1909). That this ‘cytoplasmic body’ or ‘germ granule’ may represent a germ cell determinant was emphasised by Wilson (Wilson, 1925) and more recently by Ghirardelli (Ghirardelli, 1968). It is important to note that these ancient observations based on histological sections and staining as well as a crude ablation experiments, never really proved that the germ granule in chaetognaths represents the equivalent of a germ plasm. Germ plasms comprise electron-dense cytoplasmic domains, particles or granules, observed in the vegetal or posterior pole

of oocytes, eggs or early embryos. Germ plasms have been shown to contain determinants for the germ line in *Drosophila* (polar granules), *Caenorhabditis* (P granules) and *Xenopus* (dense body or germinal granules) (Ikenishi, 1998; Wylie, 1999; Saffman and Lasko, 1999; Matova and Cooley, 2001). Germ plasms are also present in many other species of protostomes and deuterostomes (Beams and Kessel, 1974; Eddy, 1975). The germ plasm is inherited by the PGCs. PGCs are characterized by the presence of electron-dense material (called nuage) often associated with the nucleus and mitochondria. At present the only prevalent markers of germ plasm and nuage are proteins and mRNAs of the RNA helicase Vasa family. Vasa has now been identified as a component of germ plasm in *Drosophila*, *Xenopus*, *Caenorhabditis*, chicken, mouse, human, rat, zebrafish, ascidians and even planarians (Ikenishi, 1998; Wylie, 1999; Castrillon et al., 2000; Braat et al., 2000).

We noted that, in contrast with what was believed previously, the germ granule of chaetognaths can be easily observed in living eggs and early embryos, particularly in the very transparent *Sagitta* species. This prompted us to investigate fertilization and the formation, segregation and dispersal of the germ granule in eggs, embryos and germ cells of several species of *Sagitta* (plantonic species) and *Spadella* (benthic species). We also addressed whether a Vasa-like protein is present in the germ granule and in germ cells throughout the reproductive cycle of chaetognaths.

MATERIALS AND METHODS

Collecting chaetognaths

The planctonic species *Sagitta bipunctata*, *Sagitta setosa* and *Sagitta inflata* were most abundant in fall and winter in the bay of Villefranche sur Mer. They could be collected using a 100 μm mesh plankton net. Mature specimens were selected from plancton tows and used immediately. The benthic species *Spadella cephaloptera* was collected all year round by using a net that grazed over fields of *Posidonia*. *Spadella* is a sturdy benthic species that can be found in abundance from March through June and raised in small dishes using *Artemia* as food (Goto and Yoshida, 1997).

Imaging

Imaging in DIC optics was performed on a Zeiss axiophot microscope equipped with time-lapse video recording (Lhesa Newicon camera, Universal Imaging Image One acquisition software and Panasonic OMDR disc recorder).

Electron microscopy

Eggs, embryos and juveniles were fixed with glutaraldehyde and osmium as described (Eisenman and Alfert, 1982), embedded in Spurr resin, sectioned and post-stained with uranyle acetate and lead citrate. Sections were observed with a Hitachi electron microscope. Photos were digitized and colorized using Adobe Photoshop and Illustrator software programs.

Immunocytochemistry

Two anti-Vasa antibodies were used for immunoblotting and immunolocalisation: (1) Vasa monoclonal anti-mouse antibody (2L13) generated against *Xenopus* Vasa-like protein (XVLG1), kindly provided by Dr Komiya (Komiya et al., 1994), and (2) polyclonal anti-rabbit antibody generated against *Drosophila* Vasa protein, kindly provided by Drs Jan and Ackerman (Hay et al., 1988). We used dilutions of 1:5000 and 1:200, respectively. For immunoblotting, chaetognath eggs, embryos and juveniles were dissolved in SDS-containing sample buffer. The hybridized antibodies were revealed after the immunoperoxidase reaction using either a Vectastain peroxidase ElitePK-620 universal ABC kit (Vector) or the ECL procedure. For immunolocalizations, chaetognath eggs, embryos, juveniles or adults were fixed in a mixture of glutaraldehyde (0.1%) and formaldehyde (4%) in EPPS buffer (pH 8.0) in sea water, washed in buffer and stored in 80% ethanol at –20°C. Before performing immunolocalization with the Vectastain kit, eggs and embryos were bisected and adults and juveniles opened to allow penetration of the reagents.

RESULTS

Internal fertilization in *Spadella* and *Sagitta*

Chaetognaths are hermaphrodites that reproduce by copulation and exchange of sperm packets (Ghirardelli, 1968; Reeve and Walter, 1972). After migrating along tracks on the animal body, sperm are stored in seminal receptacles (colored in blue in Fig. 1) situated close to the transversal septum until mature oocytes (colored in pink in Fig. 1) are produced (Fig. 1A).

We could for the first time observe fertilization *in vivo* in the particularly transparent *Sagitta* species. Mature oocytes, about 300 μm in diameter, have a micropyle-like structure and a 'fertilization apparatus' consisting of two imbricated cells, called fertilization cells AFC1 and AFC2, according to Shinn (Shinn, 1997) and Goto (Goto, 1999). The AFC1/AFC2 cell complex connects the vegetal region of the oocyte to the ovarian wall and to the sperm duct (the complex of AFC1 and AFC2 cells is colored in yellow in Fig. 1).

At the end of oogenesis, the germinal vesicle migrates to the animal pole situated opposite the fertilization apparatus complex in the vegetal region (Fig. 1B). The GV breaks down and the first polar body is emitted (Fig. 1D). Immediately after the completion of meiosis I, bundles of spermatozoids diverging from the sperm duct cavity penetrate through the wall of the sperm duct and reach the fertilization apparatus complex (Fig. 1C,E,F). Sperm migrate towards the vesicle-filled distal end of the fertilization cell AFC2 (Fig. 1F). A single sperm then penetrates into the microtubule-filled central region of the AFC2 cell, which is coiled within the AFC1 cell (Fig. 1E). The microtubule bundles observed in the AFC2 cell examined by electron microscopy (Fig. 1G) cannot be detected after fertilization and are apparently replaced by large vacuoles (Fig. 1H). The sperm head then penetrates in the indentation of the proximal end of the AFC2 cell where the nucleus is located (Fig. 1E). As the 300 μm-long sperm penetrates the egg, the AFC2 cell resorbs at the level of the micropyle (Fig. 1I,J).

In planctonic *Sagitta* species, fertilized eggs detach from the ovarian wall, migrate in the oviduct and are laid as a clutch 10-15 minutes after internal fertilization while meiosis II is being completed. In contrast in the benthic species of *Spadella cephaloptera* fertilized eggs arrested in meiotic metaphase II can be stored in the ovary for several hours before being laid. In all species completion of meiosis II immediately follows egg laying.

Formation of a Vasa-positive germ granule during the first mitotic cell cycle

The formation of a large germ granule can be easily observed

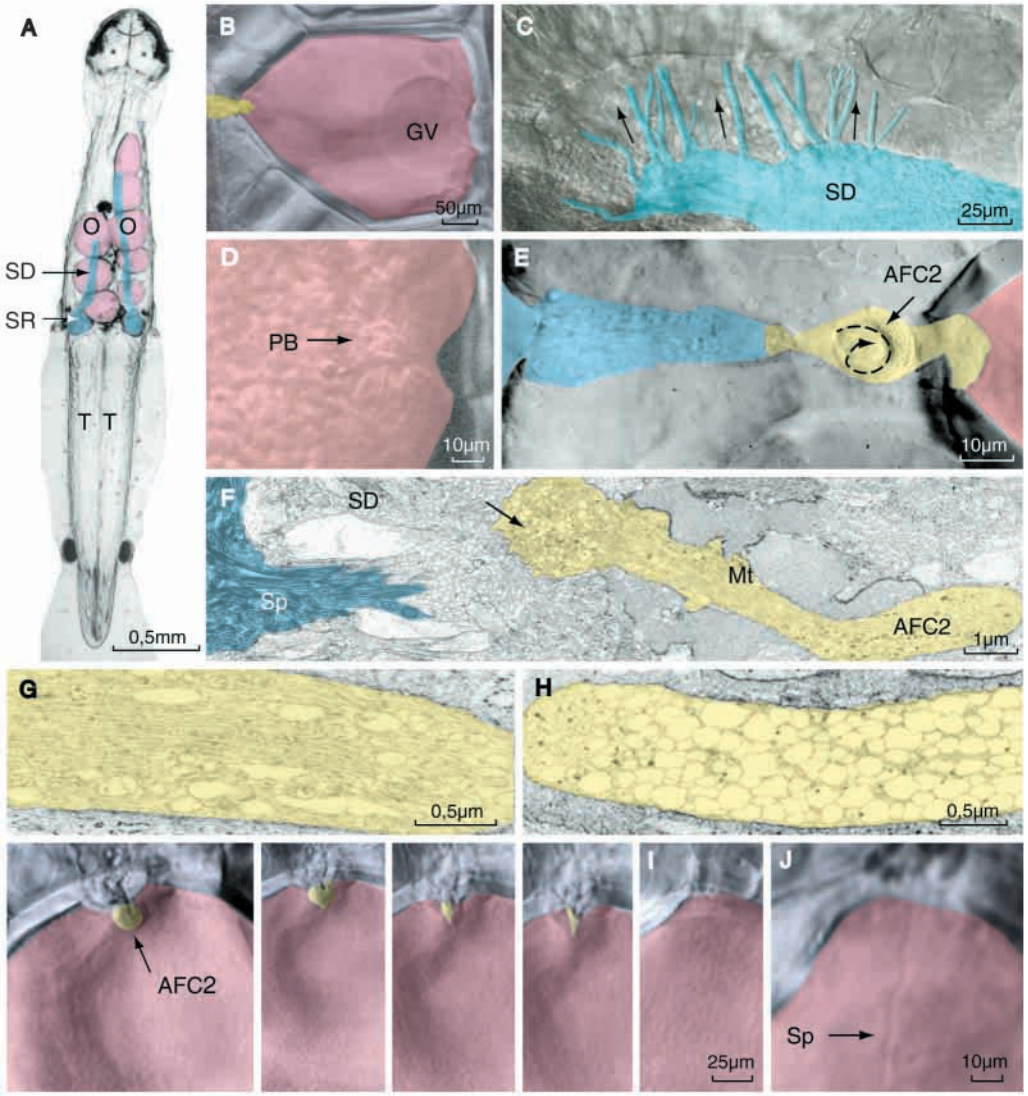

Fig. 1. Fertilization in chaetognaths. (A) *Spadella cephaloptera*: adult specimen with the two testes (T) and the two ovaries (O) in the posterior and anterior part of the body cavity, respectively. The two rows of maturing oocytes are colored pink and the two seminal receptacles (SR) and sperm ducts (SD) are colored blue. (B) *Sagitta inflata*: oocyte (pink) just before fertilization. The germinal vesicle (GV) has migrated to the animal pole opposite the fertilization apparatus (yellow). (C) *Sagitta inflata*: a portion of the sperm duct (SD) filled with sperm (blue). Bundles of sperm (arrows) traverse the sperm duct wall in the direction of oocytes. (D) *Sagitta inflata*: first polar body (PB) emission just before fertilization. (E) *Sagitta inflata*: a bundle of sperm (blue) reaches the fertilization apparatus. The AFC2 cell is colored yellow. The nucleated portion of AFC2 cell contacts the oocyte (pink) while the rest of the cell spirals (arrow) and contacts the sperm bundle (blue). (F) *Sagitta setosa*: bundles of sperm (Sp, blue) traverse the sperm duct (SD) wall in the direction of the microtubule (Mt)-filled cellular extension of the AFC2 cell rich in vesicles (arrow). (G,H) *Sagitta setosa*: close-up view of the cellular extension of the AFC2 cell showing the microtubule bundles before fertilization (G) and the abundance of vesicles just after fertilization (H). (I) *Sagitta bipunctata*: sequence of sperm penetration in the region of the AFC2 cell (only the nuclear portion is colored yellow). The AFC2 cell resorbs. (J) *Sagitta bipunctata*: same as I but at a higher magnification. The entering sperm (Sp) can be observed.

in living fertilized eggs at the time that male and female pronuclei meet in the center of the egg (Fig. 2A). The germ granule appears close (2-4 µm) to the vegetal surface of the egg, the region where internal fertilization has taken place (see preceding paragraph and Fig. 1I). The germ granule acquires clear boundaries at the time of the first mitotic metaphase (Fig. 2A). The germ granule measures about 15 µm in diameter (Fig. 2F), and is segregated into one of the two first blastomeres by the first cleavage furrow which bisects the egg equally along the animal-vegetal axis (Fig. 2A). In time-lapse observations the granule visibly forms in about 10 minutes by aggregation of small cortical particles localized in the vegetal cortex (Fig. 2B). The progressive recruitment of small cortical particles is achieved through a spiralling movement as if spooling cotton candy (Fig. 2B, see Movies).The speed of movement of the particles feeding into the granule is of the order of 15-25 µm/minute. The small cortical particles can be detected in the egg cortex after fertilization as electron-dense particles 0.3-0.5 µm in diameter (Fig. 2C,D). The bulk of the germ granule is clearly made of an accumulation of the electron-dense material making up the cortical particles. The germ granule also includes entrapped endoplasmic reticulum cisternae and mitochondria (Fig. 2E).

We wondered whether the germ granule of chaetognaths like germ plasms in insects, nematodes, amphibians, fishes and many other organisms contained a Vasa-like protein. Immunodetection by western blots using two different anti-Vasa antibodies yielded one major band (approx. 70 kDa apparent molecular mass), corresponding to the lower band of the doublet characteristic of the pattern observed in *Drosophila* (Fig. 2I). Immunolocalisation revealed that the Vasa-like protein was concentrated in the large germ granule (Fig. 2G,H).

The germ granule forms autonomously

We wished to know whether formation of the germ granule was dependant on specific events taking place before, during or after fertilization (such as maturation, fertilization, pronuclear

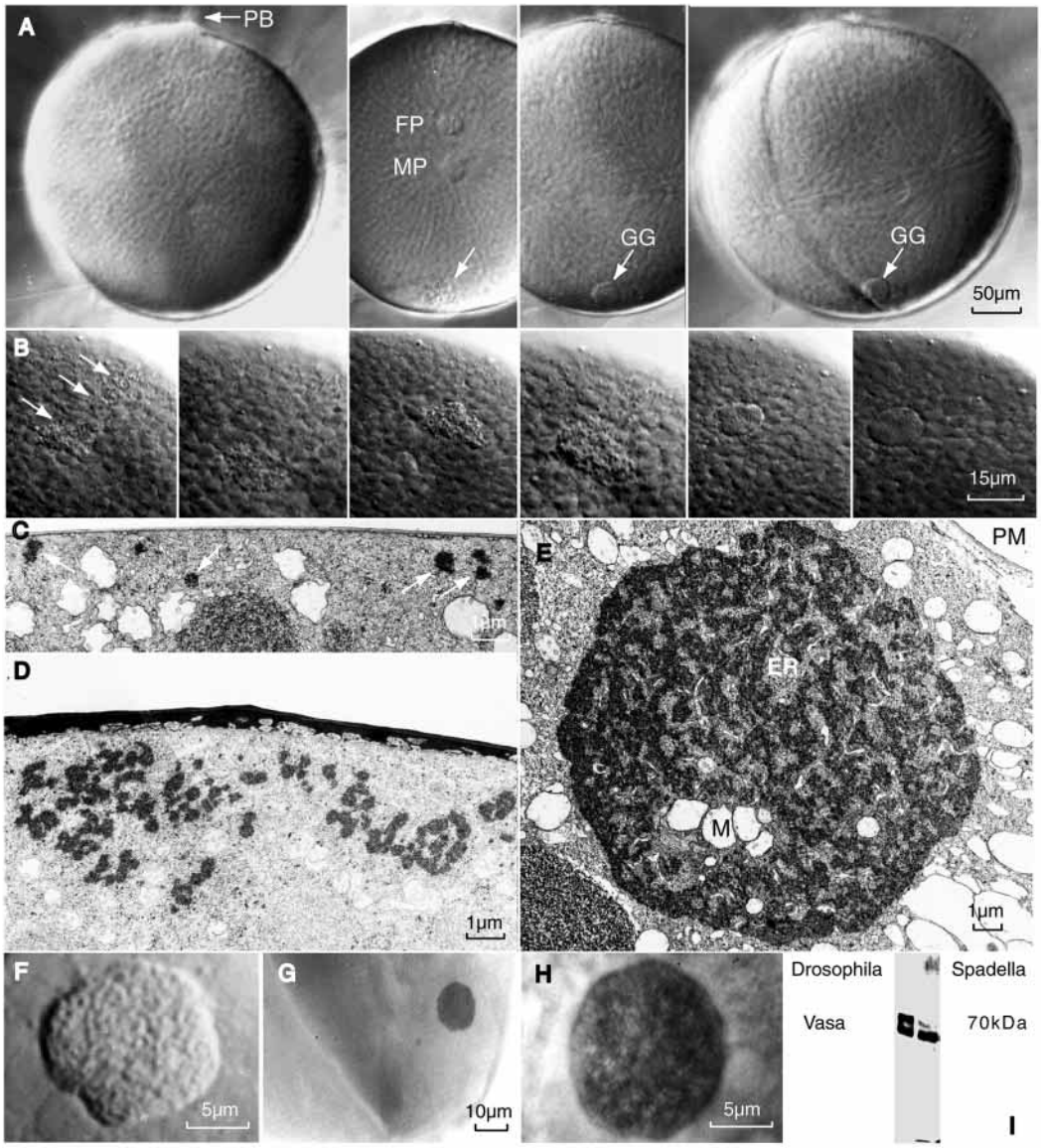

Fig. 2. Assembly and composition of the germ granule (see sequences on our web site at: http://www.obs-vlfr.fr/biomarcell.html, then click on Chaetognaths). (A) *Sagitta setosa*: During the migration of the male pronucleus (MP) and female pronucleus (FP) 25 minutes after fertilization aggregation of cortical particles (arrow) begins in the vegetal cortex and leads to the formation of the germ granule (GG). During cleavage the germ granule (GG) is segregated into one of the two first blastomeres. PB, polar body. (B) *Sagitta bipunctata*: sequence of images, spaced about 2 minutes apart, showing the aggregation of the small cortical particles (arrows) into a single germ granule. (C,D) *Sagitta bipunctata*: vegetal cortex just after egg laying (C) and during pronuclear migration (D). Electron-dense particles are first dispersed along the vegetal cortex (arrows in C) and gather together (D). (E) *Sagitta bipunctata*: thin section of the germ granule situated near the vegetal cortex at the 2-cell stage (PM, plasma membrane). The germ granule is a compact and well-delineated aggregate of electron-dense material derived from the cortical particles and contains endoplasmic reticulum (ER) and mitochondria (M). (F) *Sagitta setosa*: germ granule *in vivo* seen with DIC optics: a granular substructure can be observed. (G,H) *Spadella cephaloptera*: immunolocalization (peroxidase stained) of Vasa protein in the germ granule at the 2-cell stage. The substructure can be seen in H (*Drosophila* anti-Vasa, rabbit polyclonal antibody). (I) *Spadella cephaloptera*: immunoblot of *Drosophila* embryos (left) and *Spadella* embryos (right) showing a cross-reaction with the *Drosophila* anti-Vasa antibody. A major band of approx. 70 kDa can be seen in *Drosophila* and in *Spadella* samples.

migration, mitosis). To test whether fertilization played a role, we obtained unfertilized mature oocytes by dissecting the ovaries of *Sagitta bipunctata*. In a large proportion of these unfertilized eggs the meiotic cell cycle proceeds until two polar bodies are emitted. In these eggs, the female pronucleus generated at the animal pole migrates to the egg center, enlarges, breaks down and undergoes successive cycles of chromosome replication without cleavage, indicating that the egg activates without sperm. In this case, a large germ granule still forms in the vegetal hemisphere at the time that the envelope of the female pronucleus breaks down. This corresponds to the time the germ granule normally forms in the fertilized egg (data not shown).

To test whether the presence of male, female or zygote nuclei and centrosomes played a role in germ granule formation, we bisected fertilized eggs just after egg laying before the extracellular chorion hardened (Fig. 3). The germ granule always formed in the vegetal cortex of the bisected fragments, irrespective of the presence or absence of male pronucleus and centrosome, female pronucleus, or both (Fig. 3A,C,D).The size of the germ granule which formed was related to the size of the vegetal cortical region generated during the bisection (see Fig. 3A,B).

These experiments show that the formation of the germ granule is most likely linked to meitoic and mitotic cell cycle factors and is independent of factors brought by sperm (nucleus, basal body, activation factors). Furthermore, the spiralling movements that aggregate small particles to form the germ granule do not depend on the formation of a bipolar mitotic apparatus.

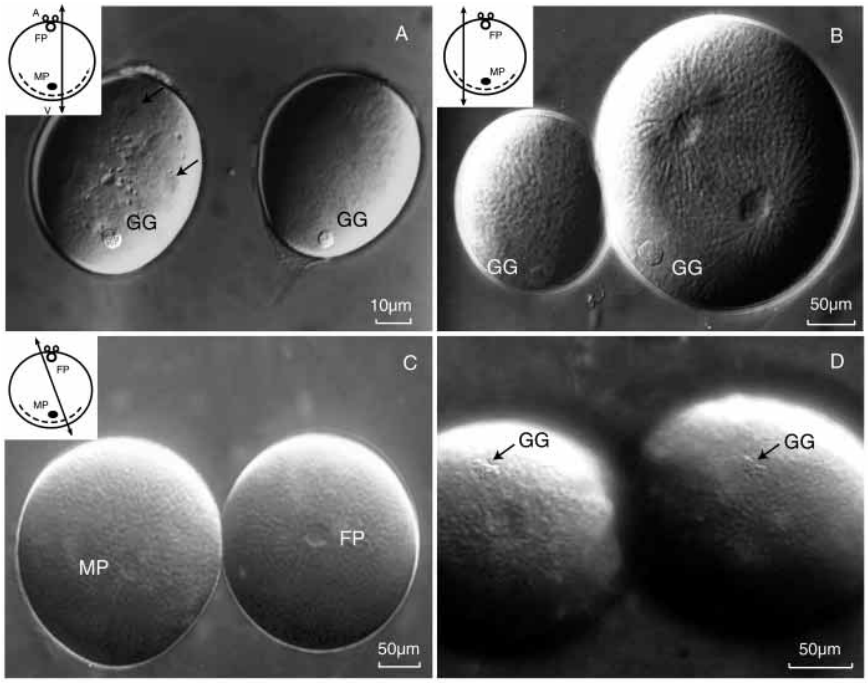

Fig. 3. Formation of germ granule in fertilized bisected egg fragments of *Sagitta bipunctata*. (A) Equal section along the animal-vegetal (AV) axis: the fragment containing the two pronuclei (left) has segmented into four closely apposed cells whose boundaries are not seen in the plane of focus shown (two of the four nuclei are indicated by arrows). The germ granule (GG) has formed in the vegetal cortex of both nucleated (left) and enucleated (right) fragments. (B) Unequal section along the AV axis: germ granules (GG) have sizes proportional to the size of vegetal hemisphere fragments (see inset). (C,D) Same two fragments in two different planes of focus after performing a slightly oblique section with respect to the AV axis (see inset). In C it can be seen that each fragment contains a pronucleus (MP, FP). In the tangential focal plane shown in D, two germ granules (GG) are seen forming in the cortex.

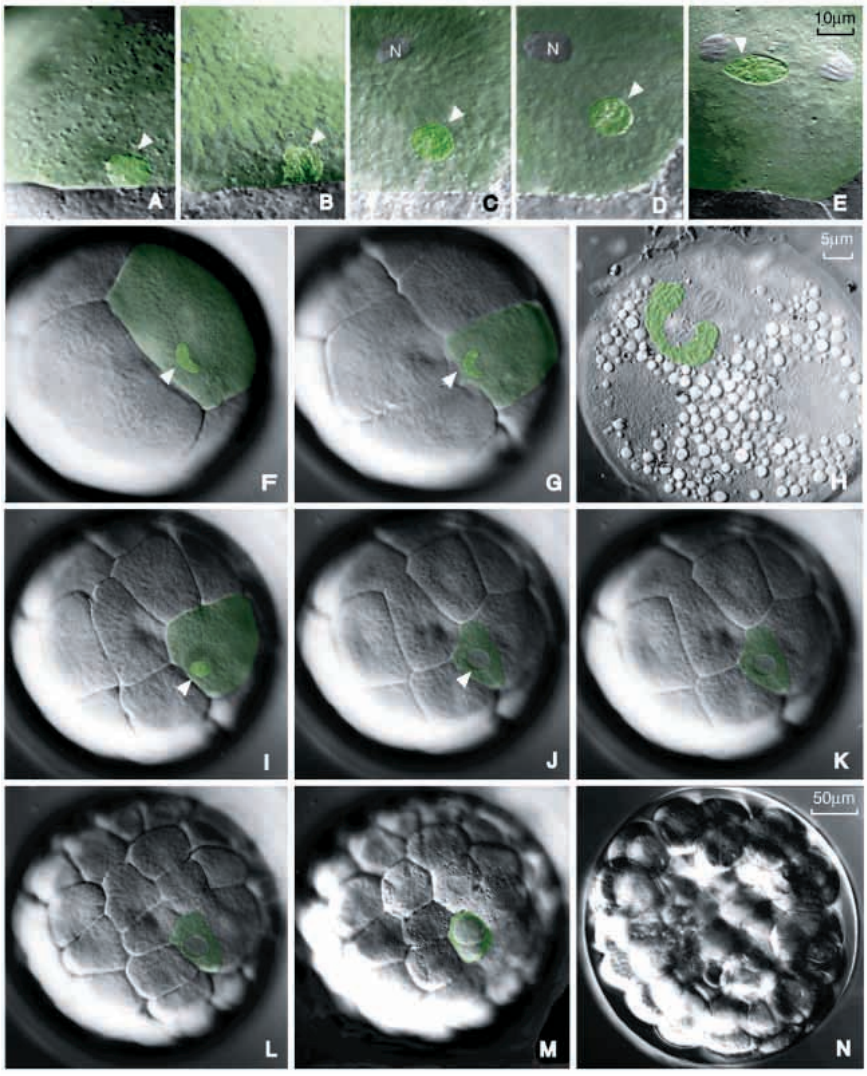

Fig. 4. Relocalisation of the germ granule during segmentation in *Sagitta inflata*. The germ line blastomere and the germ granule are coloured in two shades of green. (A,B) The germ granule (arrowhead) is cortical and juxtaposed to the cleavage plane during stages 2 (A) and 4 (B). (C-E) The sequence C to D shows the migration of the germ granule (arrowhead) towards the nucleus (N) during stage 4-8. During telophase in E, the germ granule is seen stretching towards one of the spindle poles. It is inherited by one of the two blastomeres at stage 8. (F) Stage 8. As in E, the germ granule (arrowhead) is lying on the side of the spindle and is closer to one of the spindle poles (compare to E). (G,H) Stage 16. The germ granule (arrowhead) forms a cap around one spindle pole (G; live observation in DIC) (H; thick section tangential to vegetal pole). (I) Stage-31 cells. All blastomeres have divided except for the larger cell containing the germ granule (light green). (J) Stage-32 cells. Unequal cleavage segregates the germ granule (arrowhead) into the smallest blastomere constituting the founder PGC. (K) Stage-32 cells. The germ granule cannot be seen in vivo but the founder PGC is identifiable because it remains in interphase with a large visible nucleus. (L) Stage-63 cells. All blastomeres divide except for the founder PGC. (M) Stage 64 cells. The founder PCG has divided into two cells identifiable by the continuous presence of an interphase nucleus. (N) Blastula stage.

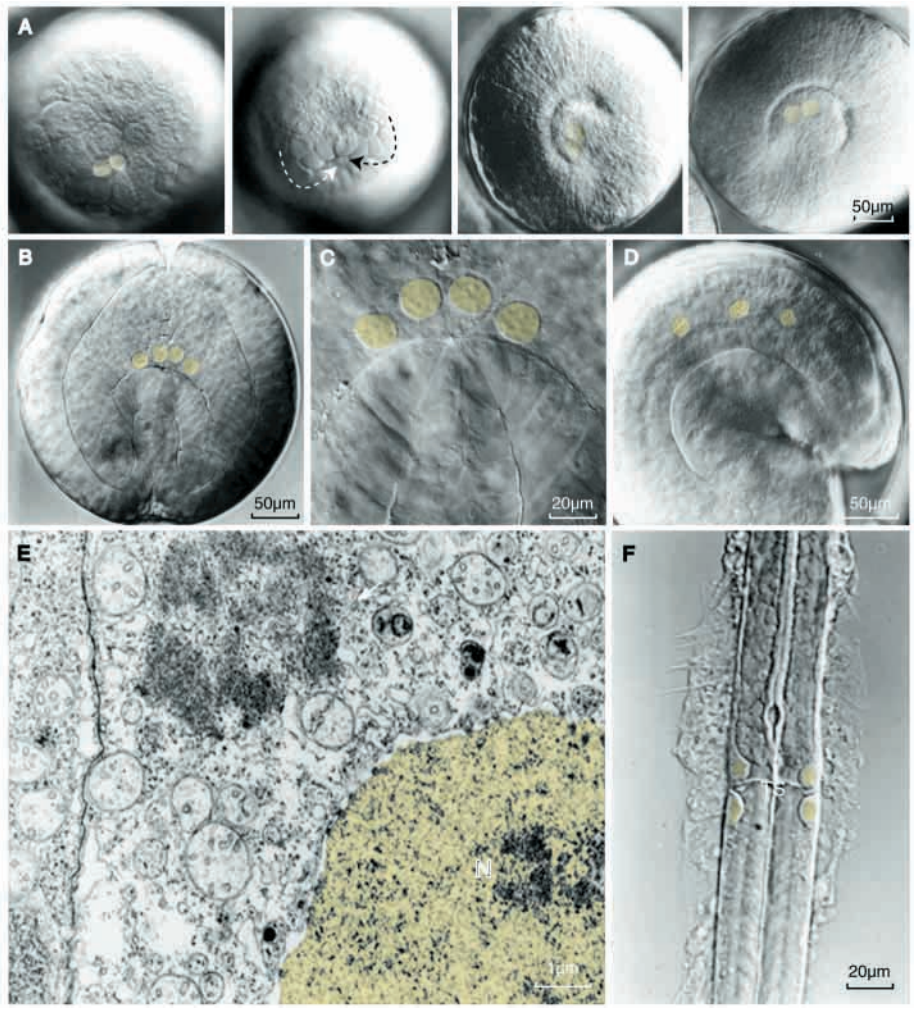

Fig. 5. PGCs during embryogenesis. (A) *Spadella cephaloptera*: sequence of four images showing the beginning of gastrulation. The first two PGCs are identified by the continuous presence of large interphase nuclei (colored ochre). During gastrulation these cells are the first to invaginate with the archenteron (arrows in second image) and to position themselves at the tip of the archenteron (last image of the sequence). (B,C) *Sagitta inflata*: late gastrula. The two first PGCs have divided once. They are identified by their well-delineated interphase nuclei. (D) *Sagitta bipunctata*: as the trunk of the embryo elongates, PGCs migrate (three PGCs are visible due to their large interphase nuclei). (E) *Sagitta bipunctata*: thin section through one of the PGC at gastrula stage (see sequence A). Many electron-dense islands (arrow) are dispersed in the cytoplasm. N, nucleus of the PGC. (F) *Sagitta bipunctata*: median portion of a juvenile showing the four PGCs positioned in pairs above and below the transversal septum (TS).

Germ granule inheritance during segmentation

Once the germ granule (colored in green in Fig. 4) is formed at the end of the first mitotic cycle, it remains as a single identifiable body in a single vegetal blastomere until stage 32 (approx. 2 hours after fertilization). The first cleavage furrow occurs tangentially to the cortically located germ granule. Cleavage segregates the single germ granule into one of the two first blastomeres (Fig. 2A, Fig. 4A). The germ granule remains in a cortical location during stages 2-4 (Fig. 4B). During stages 4-8 the granule moves away from the cortex towards the centrally located nucleus in one of the first four blastomeres (Fig. 4C,D). During the third and fourth cleavages the germ granule is stretched and pulled towards one of the mitotic poles (Fig. 4E,F). During stages 16-32 the germ granule material displays a characteristic cup-like shape surrounding one of the mitotic poles in the most vegetal blastomere (Fig. 4G,H). Starting at stage 16, cells undergo mitosis as a wave starting from the animal pole and progressing along the animal vegetal axis. As a consequence, the vegetal blastomere containing the germ granule is the last to divide (see sequence 4G-M). In addition, the blastomere containing the germ granule is the smallest as it results from asymmetric cleavage during stage 16-32 (Fig. 4J).

The mitotic wave which travels from the animal to the vegetal pole is again observed during stages 32-64 about 2 hours after fertilization (Fig. 4K-M). The blastomere containing the germ granule divides last into two cells of equal size (Fig. 4M). Although the germ granule cannot be observed *in vivo* at this stage we presume that it is partitioned equally between the two small vegetal blastomeres. These blastomeres do not divide between the blastula stage (Fig. 4N) and the end of gastrulation. They constitute the two founder PGCs of chaetognaths.

PGCs from gastrulation to hatching

Chaetognath embryos composed of about 300 cells start gastrulation by invagination of the cellular layer at the vegetal pole about 3 hours after fertilization (Fig. 5A). The two small PGCs present in the gastrula remain rounded at the periphery of the embryo and are easily recognized by the constant presence of their large interphasic nuclei. These two cells are first to invaginate during gastrulation. The primary invagination process takes 15 minutes and more complex cellular reorganizations lead to a characteristic late gastrula stage (Fig. 5B) such that after 12 hours, four cells with large nuclei are clearly identified at the tip of the archenteron in a position corresponding to that of the two original PGCs (Fig. 5A-C). Electron microscopy sections indicate that these four cells descend from the two first PGCs since they have inherited pieces of electron-dense material (nuage), which resemble the material constituting the bulk of the germ granule (Fig. 5E). During later embryogenesis and the extension of the trunk, the four PGCs migrate along the elongating body of the juvenile (Fig. 5D). About 48 hours after fertilization the juvenile hatches. A day later, four PGCs can be clearly identified. Two are positioned on one side and two on the other side of the transverse septum (Fig. 5F, Fig. 6A). The transverse septum partitions the anterior coelum which will harbour the two ovaries and the

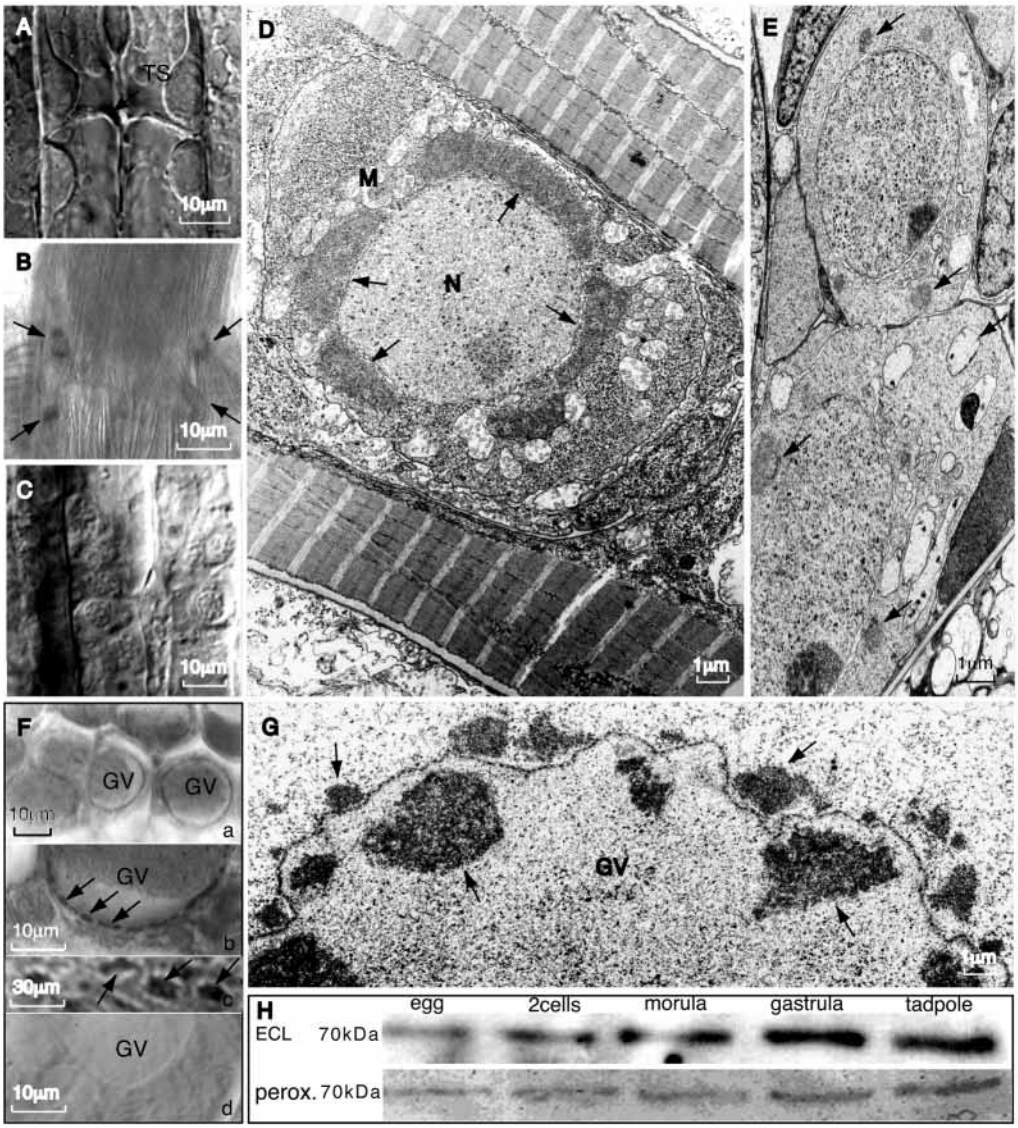

Fig. 6. Gametogenesis. (A) *Sagitta bipunctata*: two PGCs above the transversal septum (TS) will give the female germ cells of the two ovaries; the two PGCs below will yield the male germ cells of the two testes (see also Fig. 5F). (B) *Sagitta bipunctata*: the four PGCs (arrows) are labelled with *Drosophila* anti-Vasa antibody. (C) *Sagitta bipunctata*: the four PGCs have proliferated (4 days after fertilization). (D) *Sagitta bipunctata*: thin section of one of the four first PGCs. Characteristic electron-dense material (arrows) is sandwiched between the nucleus (N) and a ring of mitochondria (M). (E) *Spadella cephaloptera*: thin section of proliferating PGCs containing dispersed electron-dense material (arrows). (F) *Spadella cephaloptera*: *Drosophila* anti-Vasa antibody localization in oocytes (peroxidase detection). (a,b) The GVs of oocytes are surrounded by patches of Vasa-positive material. Three of these patches are indicated by arrows in b. (c) A higher magnification view of the Vasa-positive patches (arrows) at the level of the GV surface (tangential view). (d) Control; view of a GV stage oocyte exposed to HRP-labelled secondary antibody only. (G) *Sagitta inflata*: thin section of a portion of an oocyte GV showing the electron-dense material on both side of the nuclear pore-rich GV membrane (arrows). (H) *Spadella cephaloptera*: Immunoblots with *Drosophila* anti-Vasa polyclonal antibody using three eggs, three embryos or three juveniles, representing similar amounts of protein in each lane. An increasing amount of Vasa-like protein is visualized using two different detection methods (ECL/Vectastain peroxidase kit).

posterior coelum where the two testicles will develop (Fig. 1A).

Proliferation of primordial germ cells and gametogenesis

The two primordial male germ cells (posterior) and two primordial female germ cells (anterior) present on either side of the transverse septum of the juvenile are morphologically indistinguishable (Fig. 6A). The four cells display a ring of Vasa-positive labelling around their nuclei, which corresponds to the ring of electron-dense material (nuage) observed in electron micrographs (Fig. 6B,D). At that stage, the electron-dense perinuclear material in all four germ cells is itself surrounded by a ring of mitochondria. The four PGCs then proliferate into cells that remain clumped together (Fig. 6C). These cells contain many electron-dense granules about 1-2 µm in diameter (Fig. 6E). During oogenesis the anterior coelomic cavities fill up with oocytes of various sizes, which all display patches of Vasa-positive material around the germinal vesicles (Fig. 6Fa-c). No such Vasa-positive staining is seen around the GVs of control oocytes (Fig. 6Fd). The location of this Vasa-positive material corresponds well to the electron-dense material positioned on either side of the nuclear pores, as seen in thin sections (Fig. 6G). We deposited identical numbers of solubilized eggs, embryos and juveniles on SDS-polyacrylamide gels to examine the total content of Vasa-like protein during chaetognath development after electrophoresis. The total amount of protein deposited was constant, as judged from Coomassie Blue and Ponceau S staining patterns (data not shown). Immunoblots of these gels using two different detection methods (ECL and peroxidase) show that the Vasa-like protein (70 kDa; see Fig. 2I) increases in amount from the time of fertilization and segmentation through gametogenesis (Fig. 6H).

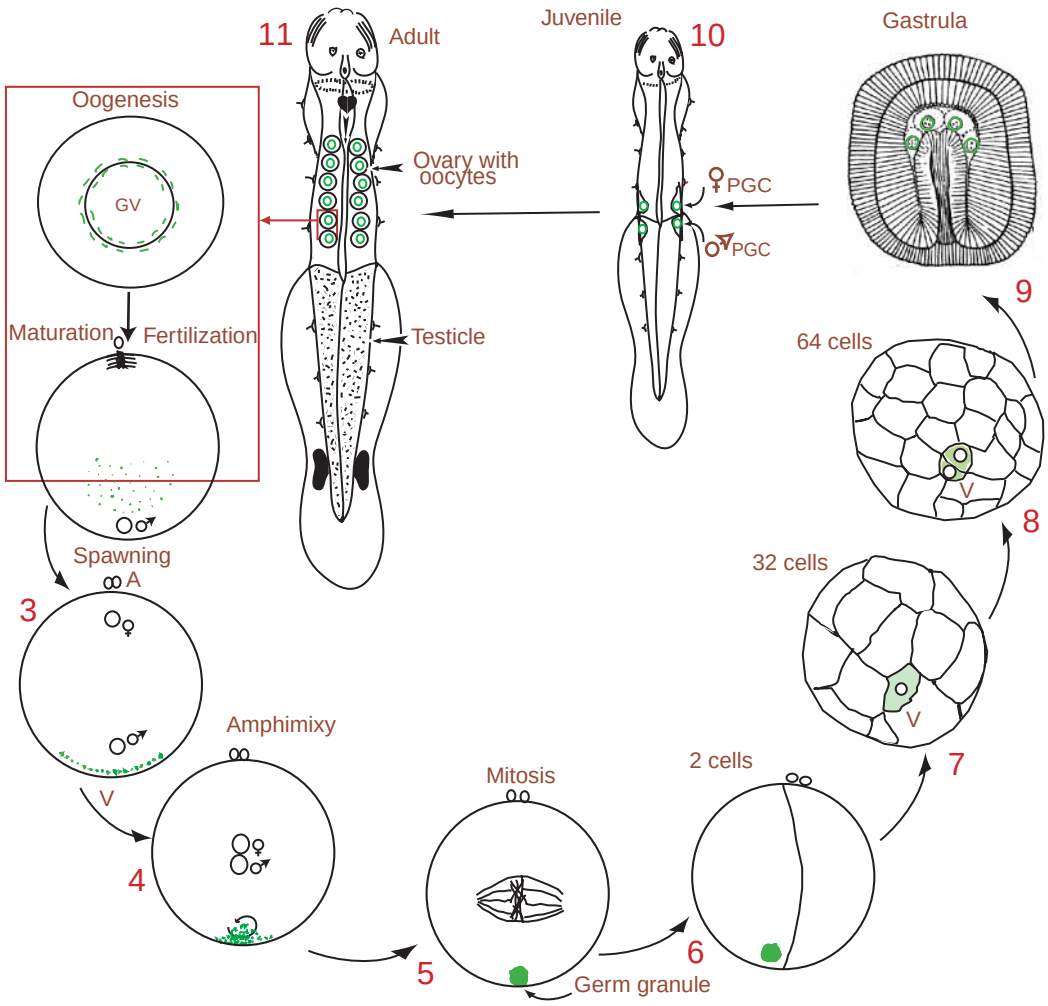

Fig. 7. Germ plasm and germ cells during the life cycle of chaetognaths. During oogenesis (1), germ plasm/nuage material (in green) is within and around the germinal vesicle (GV). During maturation and internal fertilization at the vegetal pole (2), germ plasm presumably fragments into minute granules. After spawning (3), many small granules line the vegetal cortex (V) and then aggregate during amphimixy (4). At mitosis (5), small germ granules aggregate into a single large granule. This large granule is segregated into one of the first two blastomeres and continues to be inherited by only one vegetal blastomere until the 32-cell stage (7). The germ granule then fragments and is distributed into two blastomeres at the 64-cell stage (8). The germ plasm is then found in the four presumptive PGCs at the tip of the archenteron in the gastrula (9). The four PGCs become the male (posterior) and female (anterior) germ cells in the juvenile (10), which give rise to the spermatocytes and the oocytes in the adult.

DISCUSSION

Our study confirms and extends ancient observations that chaetognaths have remarkable specializations with regard to germ plasm and the germ line (Butschli, 1873; Hertwig, 1880; Elpatievsky, 1909; Wilson, 1925; Ghirardelli, 1968). Most noticeably, a single large germ granule forms in the egg after fertilization and this single granule is inherited by a single blastomere until stage 32 (Fig. 7). We show that the origin, inheritance and descendance of this single germ granule can be traced through most stages of embryonic development, differentiation and oogenesis (Fig. 7). In addition, the two PGCs at the origin of sperm and the two PGCs that give rise to the oocytes can be traced during migration and proliferation in live embryos and juveniles.

Common themes in germ plasms and germ cells

Common themes concerning the presence, appearance and behavior of germ plasms, nuage and germ cells are found in over 80 species spanning eight phyla, reviewed elsewhere (Beams and Kessel, 1974; Eddy, 1975; Ikenishi, 1998; Wylie, 1999; Saffman and Lasko, 1999; Matova and Cooley, 2001). Commonalties emerge from observations made principally on one insect (*Drosophila*), one nematode (*Caenorhabditis*) and three vertebrates (*Xenopus, Zebrafish* and mouse) (Mahowald et al., 1979; Heasman et al., 1984; Hay et al., 1988; Wilsch-Brauninger et al., 1997; Pitt et al., 2000; Braat et al., 2000; Knaut et al., 2000; Schisa et al., 2001).

These general features are:

(1) Germ plasms are composed of ribonucleoprotein complexes forming electron-dense particles that undergo characteristic condensation, decondensation cycles generating granules or islands of various shapes and sizes. They constitute cytoplasmic domains related to similar domains called nuages in germ cells. Germ plasm and nuage ribonucleoprotein complexes contain at least one conserved RNA binding protein and/or its mRNA, Vasa.

(2) Germ plasms and nuages are often associated with mitochondria, ER and/or nuclear pores. Aggregation of germ plasms into larger or smaller domains and their associations with organelles vary with the state of development and differentiation of the germ line.

(3) When germ plasms are detected in oocytes they are located near or around the large germinal vesicle and near the posterior or vegetal pole region in the maturing oocyte and egg.

(4) Germ plasms are inherited by a few germ cell precursor blastomeres that are transcriptionally inactive. These cells become PGCs, which undergo migrations and divisions reaching gonadal tissues of various origins to form the gonad.

Although they have been separated from the above-

mentioned species for hundreds of millions of years chaetognaths fit many of these themes (1-4 outlined above).

(1) Chaetognath germ plasm contains a Vasa-like protein and RNAs

The germ plasm and nuage material found in chaetognath eggs, embryos and PGCs contain a Vasa-like protein, as do those of many other species. The protein is present during many (and probably all) stages of oogenesis, embryogenesis and differentiation of the germ line (Fig. 7), and we can guess from immunocytochemical observations that Vasa protein is synthesized in greater amounts during the phase of oocyte proliferation and oogenesis. Histological staining before and after RNAse digestion had previously suggested that the large germ granule of the chaetognath *Spadella* was RNA-rich (Ghirardelli, 1968). It will be interesting to examine the RNA content of the germ granule and of its precursors and descendants by more quantitative techniques, since it has been shown recently in *Caenorhabditis* that presence of RNAs is a highly dynamic property of germ plasms and nuage (Schisa et al., 2001).

(2, 3) Chaetognaths germ plasm undergoes transformations and associates with organelles

As in *Drosophila* and several nematodes and amphibians, germ plasm in chaetognaths undergoes clear cycles of aggregation and association with mitochondria, ER and nuclear pores (Mahowald et al., 1979; Goldstein et al., 1998; Savage and Danilchik, 1993; Houston and King, 2000; Pitt et al., 2000). During chaetognath oogenesis large quantities of germ particles associate with both sides of the nuclear membrane of the germinal vesicle, which is itself characterized by a high density of nuclear pores. Such an association with nuclear pores has been described in several species and recently analysed in detail in *Caenorhabditis elegans*, where it persists during early embryogenesis (Eddy, 1975; Pitt et al., 2000).

As the chaetognath oocyte matures, its germinal vesicle breaks down and the perinuclear aggregates of germ plasm disperse into small particles. Although we could not observe the dispersal directly, we presume that the small particles move vegetally during oocyte maturation to line up against the vegetal cortex. At that stage the small germ granules do not seem to have preferential associations with mitochondria or ER. In chaetognaths, as in nematodes, egg laying immediately follows internal fertilization. During the centering of male and female pronuclei, the small granules of germ plasm that line the vegetal cortex undergo one of the most spectacular reorganizations yet described. They aggregate into one large granule by a spiralling movement of the cytoplasm in the vegetal region of the egg. Some ER and mitochondria are present in the granule. They may become trapped during the spiralling movement and/or may participate in it. The spiralling movement takes place at a particular time in the cell cycle (the onset of mitosis) and may be driven by a sort of cortical/cytoplasmic flow. Such flows have been described in *Caenorhabditis elegans* (fountain flow) as male and female pronuclei migrate to the egg center. These coordinated cortical flows (toward the anterior) and cytoplasmic flows (toward the posterior) are thought to carry P granules to the future posterior pole of the embryo (O'Connel et al., 2000).

The process of germ granule aggregation in chaetognaths can be clearly dissociated from fertilization as it occurs spontaneously in unfertilized matured eggs. The formation of a single germ granule coincides with the centering and enlargement of the single female pronucleus, which depends on the completion of the meiotic cell cycle, which apparently activates the egg. We were able to create two granules instead of one in situations (fragments generated by bisections) where parts of the cytoplasm lacked nuclei and their attached centrosome. This argues against a role for astral microtubular structures during the vegetal localisation of germ plasm particles and their subsequent aggregation into a single granule in the center of the vegetal cortex. Aggregation of the germ particles (or islands) lying against the vegetal cortex have also been described in *Xenopus* eggs (Savage and Danilchick, 1993). They take place just before cleavage and apparently depend on MPF-driven surface contraction waves that start at the animal pole and propagate vegetally (Perez-Mongiovi et al., 1998; Perez-Mongiovi et al., 2000).

(4) The germ granule is inherited by vegetal blastomere precursors of PGCs that stop dividing

The single germ granule of chaetognaths is pushed into one of the two blastomeres by the cleavage furrow (Fig. 7). This unequal distribution of germ plasm into one of the first two blastomeres resembles that seen in nematodes or molluscans, although in these organisms it occurs by unequal and orientated cleavages.

The large vegetal germ granule translocates from the cortex towards the spindle of the precursor blastomere at the 4-8 cell stage and moves to one pole of the spindle during the 3rd and 4th cleavages. At 5th cleavage the germ granule caps one spindle pole and, after an unequal cleavage (stage 32-64), it is inherited by the smaller blastomere. We presume that the granule then fragments, to be localized in two and then four blastomeres with large nuclei found at the tip of the archenteron during gastrulation. The nuage material shows no particular association with ER, mitochondria or the nucleus at that stage.

Four migrating PGCs are at the origin of the two male and the two female gonads

The four PGCs in chaetognaths migrate without dividing along the trunk of the larvae. We have not yet examined these migrations but it will be interesting to see in which order the four PGCs reach their destination, since two PGCs will give rise to the two male gonads and the other two PGCs to the two female gonads. Once the four founder PGCs are positioned on either side of the transverse septum, the nuage material is detected as a ring sandwiched between the nucleus and a layer of peripheral mitochondria. At this stage there are no detectable ultrastructural differences between the two anterior PGCs, which will proliferate and differentiate into oocytes in the two ovaries, and the two posterior PGCs, which give rise to the spermatocytes in the two testes. At present we do not know if these precursor germ cells are predestined to give male and female gametes or if the nature of the surrounding tissues in the transversal septum region dictates their differentiation (Wylie, 1999). Ablations or laser inactivations of one out of two or two out of four germ cells should provide an answer to this question. These four PGCs in the transverse septum region

rapidly undergo 3-4 rounds of division, and it appears that the perinuclear nuage material is fragmented and distributed among the dozen PGCs thus produced. Oogenesis and spermatogenesis resumes within a month or two once the juvenile has grown to reproductive size. As in many species, electron-dense material characteristic of germ plasm is associated with the nuclear pores of the large oocyte nucleus (Beams and Kessel, 1974; Pitt et al., 2000).

The presence of a Vasa-like protein in the germ plasm and nuage in the germ cells of the enigmatic phylum of chaetognaths, which at present is thought to be situated between protostomes and deuterostomes, argues strongly that the existence and formation of germ plasms and germ cells rely on conserved cellular and molecular mechanisms.

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