Zebrafish vasa RNA but Not Its Protein Is a Component of the Germ Plasm and Segregates Asymmetrically before Germline Specification

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Abstract. Work in different organisms revealed that the vasa gene product is essential for germline specification. Here, we describe the asymmetric segregation of zebrafish vasa RNA, which distinguishes germ cell precursors from somatic cells in cleavage stage embryos. At the late blastula (sphere) stage, vasa mRNA segregation changes from asymmetric to symmetric, a process that precedes primordial germ cell proliferation and perinuclear localization of Vasa protein. Analysis of hybrid fish between Danio rerio and Danio rerigradei demonstrates that zygotic vasa transcription is initiated shortly after the loss of unequal vasa mRNA segregation. Blocking DNA replication indicates that the change in vasa RNA segregation is dependent on a maternal program. A symmetric segregation is impaired in embryos mutant for the maternal effect gene nebel. Furthermore, ultrastructural analysis of vasa RNA particles reveals that vasa RNA, but not Vasa protein, localizes to a subcellular structure that resembles nuage, a germ plasm organelle. The structure is initially associated with the actin cortex, and subsequent aggregation is inhibited by actin depolymerization. Later, the structure is found in close proximity of microtubules. We previously showed that its translocation to the distal furrows is microtubule dependent. We propose that vasa RNA but not Vasa protein is a component of the zebrafish germ plasm. Triggered by maternal signals, the pattern of germ plasm segregation changes, which results in the expression of primordial germ cell–specific genes such as vasa and, consequently, in germline fate commitment.

Key words: primordial germ cells • Danio rerio • nebel • asymmetric segregation • RNA localization

Introduction

A symmetric cell division contributes to the generation of cell diversity in many organisms (for reviews see Horvitz and Herskowitz, 1992; Rhyu and Knoblich, 1995; Knoblich, 1997; Jan and Jan, 1998, 1999). One way to achieve asymmetric cell division is to unequally partition intrinsic cell fate determinants from the mother cell to only one of the two daughter cells. Such a mechanism is used in mating type switching in yeast (Bobola et al., 1996; Sil and Herskowitz, 1996; Loom and Herskowitz, 1996; Long et al., 1997; Takizawa et al., 1997), in Drosophila and vertebrates during neurogenesis (Rhyu et al., 1994; Hirata et al., 1995; Knoblich et al., 1995; Spana and Doe, 1995; Spana et al., 1995; Shen et al., 1998; Schober et al., 1999; Wodarz et al., 1999), and in germline specification in Caenorhabditis, Drosophila, and X. enopus (Nieuwkoop and Sutasurya, 1979; Rongo et al., 1997; Seydoux and Strome, 1999).

In Caenorhabditis and Drosophila, germline determinants are asymmetrically localized as components of a special type of cytoplasm, the germ plasm (for reviews see Rongo et al., 1997; Seydoux and Strome, 1999). This cytoplasm consists of electron-dense material in association with fibrils, mitochondria, and often the nuclear envelope. Because of its cloudy appearance in ultrastructural studies, it has been termed nuage (Andre and Rouiller, 1957). In Drosophila, nuage is first detected in nurse cells, and it is thought to represent the precursor of polar granules (Mahowald, 1968, 1971). During oogenesis, the polar granules are recruited to the posterior of the oocyte and form the germ plasm (called pole plasm). Upon pole cell formation, the granules fragment and associate with the outer nuclear envelope as nuage and remain associated with the germ cells throughout the life cycle of the Drosophila. Similarly, germ-line cells in Caenorhabditis, X. enopus, and zebrafish also are characterized by nuage-like structures that segregate
to the primordial germ cells (PGCs) and, again, remain associated with the germline throughout development (Eddy, 1975; Selman et al., 1993; Braat et al., 1999; Seydoux and Strome, 1999).

Transplantation experiments in Drosophila and X. laevis together with genetic studies in Drosophila and Caenorhabditis demonstrate that germ plasm is required for germ cell induction (Illmensee and Mahowald, 1974; Okada et al., 1974; Nieuwkoop and Sutatsuya, 1979; Ehrussi et al., 1991; Ehrussi and Lehmann, 1992; Seydoux and Strome, 1999) and, in the case of Drosophila and Caenorhabditis, leads to transcriptionally silenced nuclei (Zalokar, 1976; Seydoux and Dunn, 1997; A saoka et al., 1998; V an Doren et al., 1998). Yet, the time point at which germline specification occurs is different among these species. In Drosophila and Caenorhabditis, the germline is separated from the somatic tissue immediately at the onset of embryogenesis, whereas, in X. enopus, restriction to the germline occurs shortly before gastrulation (for review see Wylie, 1999). In fish, the specification of germ cells is poorly understood. N. uage-like structures are detected during oogenesis and late embryogenesis, and the origin of germ cells has been traced to different germ layers in different fish (Hamaguchi, 1982; Wallace and Selman, 1990; Gevers et al., 1992; Selman et al., 1993; Braat et al., 1999). Therefore, it is difficult to assign a specific time point and event to germ cell specification in fish.

The recent identification of the zebrafish vasa orthologue indicates that germ cell precursors are separated early from somatic cells, and they can be traced throughout embryogenesis (Olsen et al., 1997; Yoon et al., 1997; Weidinger et al., 1999; Braat et al., 1999). The vasa gene was initially identified in Drosophila by genetic screens for maternal-effect mutations that affect anterior–posterior polarity. Embryos from mutant mothers lack localized polar granules and do not form pole cells (Schüpbach and Wieschaus, 1986; Lehmann and Nüsslein-Volhard, 1991). The vasa gene encodes an RNA helicase of the DEAD box family (Hay et al., 1988b; Lasko and Ashburner, 1988) that is expressed specifically in the germline throughout the life cycle (Lasko and Ashburner, 1990). In oocytes, vasa RNA is uniformly distributed in the cytoplasm, whereas the protein associates with polar granules and is asymmetrically segregated to the posterior pole (Hay et al., 1988a). A fter pole cell formation, vasa protein is exclusively found in the germline. The expression of the two vasa homologues in Caenorhabditis (ghl-1 and gh-2) is similar. The Vasa proteins are associated with the P granules, which are asymmetrically segregated to the germline precursors and restricted to the germline throughout the life cycle (Gruidl et al., 1996). Vertebrate vasa homologues have been identified and shown to be expressed in the germline at later stages of embryogenesis (Fujiwara et al., 1994; Komiya et al., 1994; Komiya and Tanigawa, 1995). In zebrafish, vasa RNA is found throughout oogenesis and embryogenesis (Olsen et al., 1997; Yoon et al., 1997; Braat et al., 1999). Strikingly, the vasa transcript is localized to the distal parts of the first cleavage furrows as granules and eventually ingresses into four cells of the blastula. The number of vasa RNA-positive cells remains constant until the sphere stage (cell cycle 13; all staging is according to Kimmel et al., 1995), where vasa RNA-positive cells increase in number and migration to the gonads begins. Recent studies have demonstrated that these cells are presumably the zebrafish PGCs because of their migration pattern, final location and morphology (Olsen et al., 1997; Yoon et al., 1997; Braat et al., 1999; Weidinger et al., 1999).

Initial insights into the mechanism of early zebrafish vasa RNA localization came from microtubule inhibition studies. Embryos treated with the microtubule depolymerizing agent nocodazole fail to translocate vasa RNA to the distal cleavage planes, a phenotype that is also observed in the maternal-effect mutation nebel (Pelegri et al., 1999). Embryos from homozygous nebel mutant mothers (referred to as nebel embryos) have reduced furrow-associated microtubule and cellularization defects (Pelegri et al., 1999). However, the nature of these localized vasa RNA structures, their mode of assembly, and restriction to a limited number of cells and the time point of germ cell specification have not yet been resolved.

In this paper, we show that vasa RNA is a component of a germ plasmlike structure. We also show that vasa RNA segregates asymmetrically to the future founder population of PGCs. The asymmetric segregation is impaired in embryos mutant for the maternal-effect gene nebel. We find that a change in vasa RNA localization at the sphere stage (cell cycle 13) precedes PGC proliferation, Vasa protein localization, and zygotic vasa transcription. This transition is independent of zygotic or nuclear signals, suggesting that maternal signals induce a change in germ plasm segregation, which results in germ plasm activation and consequently in germline specification.

Materials and Methods

cDNA Cloning

Poly(A)+ RNA was enriched from the 4-cell stage D. rerio and D. feugeri embryos using TriStar Reagent (A new aged Gentechology Systeme) and oligotex columns (Qiagen) according to the manufacturer's instructions. First strand cDNA synthesis was performed using Superscript II (Gibco-BRL Life Sciences) with the primer 5'-TCAAGTACAGCTGGAGCTGGCCTCACACCT-3' and 5'-GGCCCTAGATCCGCCTGAGGACCGCTGGCC-3'

Antibody Stainings

Poly(A)+ RNA was enriched from the 4-cell stage D. rerio and D. feugerei embryos using TriStar Reagent (A new aged Gentechology System) and oligotex columns (Qiagen) according to the manufacturer's instructions. First strand cDNA synthesis was performed using Super

Materials and Methods

Whole-mount In Situ Hybridization and Antibody Stainings

For Vasa protein stainings, embryos were fixed in Dent's fixative (80% methanol and 20% DMSO) at -20°C overnight. A fter dehydration and dechorionation, embryos were blocked in PBS with 0.2% Triton X-100 and 1% BSA (PB/1Ts) for 1 h at room temperature, incubated with the pri

1Abbreviations used in this paper: dpf, days postfertilization; FMA, furrow microtubule array; hpf, hours postfertilization; MBT, midblastula transition; PGC, primordial germ cell; RACE, rapid amplification of cDNA ends; RNA II, RNA polymerase II; UTR, untranslated region.
was purified on nickel agarose columns (Qiagen). The purified protein was overexpressed in Escherichia coli. The fusion protein was cloned into pRSETA (Invitrogen Corp.) to generate a His-tagged fusion protein. The antibody generation was performed using a stereomicroscope or a fluorescence microscope. For colocalization of vasa RNA and Vasa protein, in situ hybridization was performed as described in Ober and Schulte-Merker (1999). For double staining of Vasa protein and the nuclear membrane, embryos were treated as above but anti-lamin B antibody was added to the primary antibody incubation step (Pugh et al., 1997; Dagh et al., 1998) and detected with goat anti-mouse IgG Cy3 coupled to (Dianova). For immunodetection of Vasa protein in stained embryos was performed as described above. For double and triple detection of vasa RNA and β-catenin, RNA polymerase II, nuclear pore complexes and/or γ-tubulin, the embryos were stained for vasa RNA and proteins as described above using the mouse mAb bs anti-β-catenin, anti-γ-tubulin (Sigma-Aldrich), mAb 414 (Hiss Diagnostics), and H5/H714 (Hiss Diagnostics) at a dilution of 1:10,000. Colocalization of α-tubulin and vasa RNA was done as described in Pelegri et al. (1999) with the modification that 0.0025% glutaraldehyde and 5 mM magnesium sulfate were used in the fixation step. The dimeric cyanine nucleic acid stain 3 (TO-TO-3) and propidium iodide (Molecular Probes) were used for nuclear staining. The signal was detected using DAB (Boehringer Mannheim). For structural electron microscopy, an electron-dense substrate (see Materials and Methods) was used. Alkaline phosphatase detection was done as described in Ober and Schulte-Merker (1999). Hybrid embryos were collected for cDNA preparation and fixed for protein and RNA stains as described above. A control, D. rerio and D. feegradei embryos were collected in the same manner. Genomic DNA from both fish species was prepared as described in Yeon et al. (1997). Specific primers for vasa cDNA from both species were designed such that genomic contamination in the cDNA preparation was detected by the presence of an intron. For D. feegradei, the primer pair 5'-CTGCGCTCCATAGAAGA-3' and 5'-CATCACTGATGATCTCC ACCC-3' and, for D. rerio, the primer pair 5'-TTCTCAGCTGCACTCAC-3' and 5'-GGGAGATGTTTTGATGTT-3' were used. Detection of vasa transcripts was performed using a Taq polymerase (A mersham-Pharmacia; 94°C for 30 s, 60°C for 30 s, 73°C for 1 min and 35 amplification cycles).

**Electron Microscopy**

For vasa RNA and protein detection on electron microscopy sections, embryos were stained as described above with the modification that the proteinase K step was omitted in the case of in situ hybridization. The signal was detected using DAB or TBS (Boehringer Mannheim). For structural electron microscopy sections, embryos were fixed in 1% glutaraldehyde and 4% paraformaldehyde in PBS and freeze-substituted in Epon 812 (Rotth). All sections were contrasted using uranylacetate and osmium tetroxide and inspected using a Philips TEM 10 electron microscope.

**Drug Treatment of Embryos**

Latrunculin B (BioMol Research Laboratories) was prepared as a stock solution of 3 mg/ml in DMSO and used at a concentration of 30 μg/ml diluted in distilled water. Single cell embryos were coinjected with 50 nl of the indicated concentration and phenol red as a marker. A phidcolin (Sigma-Aldrich) was dissolved in 50% DMSO at a concentration of 2.5 mg/ml. For injections, the aphidcolin stock was diluted 1:10 in distilled water and 50 nl was injected in single cell embryos. A cinomycin D (Sigma-Aldrich) was dissolved at a concentration of 5 mg/ml in water and 50 nl of a 1:1,000 dilution in distilled water was injected in single cell embryos. A control, embryos were injected with the same amount of the solvent. Embryos were stained for vasa RNA by in situ hybridization as described above.

**Fish Maintenance and Mutant Strain**

Zebrafish were maintained as previously described (Mullins et al., 1994; Haffter et al., 1996). For all experiments, the Tubingen and TL strain were used. neb mutants were kept in the TL background. No difference between the Tubingen and TL strain was observed, although a difference in primordial germ cell number was reported for the AB strain (Weidinger et al., 1999).

**Determination of Germ Plasm–containing Cells**

Germ plasm–containing cells were determined by vasa RNA and/or protein expression using a stereomicroscope or a fluorescence microscope. Since the number of germ plasm–containing cells varied, embryos were taken from the same clutch during a particular time course analysis.

**Generation of D. rerio–D. feegradei Hybrid Fish and Detection of Zygotic Vasa Transcription**

To generate hybrid fish, D. rerio eggs were in vitro fertilized with D. feegradei sperm, which was isolated from the testes as described in Pelegri and Schulte-Merker (1999). Hybrid embryos were collected for cDNA preparation and fixed for protein and RNA stains as described above. A control, D. rerio and D. feegradei embryos were collected in the same manner. Genomic DNA from both fish species was prepared as described in Yeon et al. (1997). Specific primers for vasa cDNA's from both species were designed such that genomic contamination in the cDNA preparation was detected by the presence of an intron. For D. feegradei, the primer pair 5'-CTGCGCTCCATAGAAGA-3' and 5'-CATCACTGATGATCTCCACACC-3' and, for D. rerio, the primer pair 5'-TTCTCAGCTGCACTCAC-3' and 5'-GGGAGATGTTTTGATGTT-3' were used. Detection of vasa transcripts was performed using Taq polymerase (A mersham-Pharmacia; 94°C for 30 s, 60°C for 30 s, 73°C for 1 min and 35 amplification cycles).

**Results**

**vasa RNA Is a Component of Zebrafish Germ Plasm**

To understand the structural nature of localized vasa RNA aggregates in zebrafish embryogenesis, we performed transmission electron microscopy on four-cell embryos that are labeled for vasa RNA using in situ hybridization and an electron-dense substrate (see Materials and Methods). Utrathin sections of these embryos show that the transcript is restricted to a distinct, electron-dense structure, which is in close proximity to the indented furrow (Fig. 1, a and b). The densely labeled structure consists of porous elements embedded in an electron-dense matrix and resembles nuage-like structures found associated with germ cells and their precursors in many animals (Eddy, 1975). To investigate whether vasa RNA remains associated with these nuage-like structures in PG C S, we analyzed sections of embryos at later stages. We found vasa RNA to be associated with the same nuage-like structures in the 1,000-cell stage embryos (Fig. 1, c and d). In the 2.5-d postfertilization (dpf) embryos, we failed to detect vasa RNA in Vasa protein–positive cells (data not shown), presumably because of the too low vasa RNA transcript levels as judged by Northern analysis (Yoon et al., 1997, and our own observations). Based on the presence of the vasa RNA, which is embedded in electron-dense material that resembles nuage, we conclude that this structure is the zebrafish germ plasm.

**Germ Plasm First Associates with the Actin Cortex, and then with the Microtubule Network**

Because of the harsh nature of the in situ technique, few cytoplasmic structures are conserved around the nuage-like structures (Fig. 1, a-d). Therefore, we decided to section...
structurally preserved embryos to analyze germ plasm in a more natural context. Before aggregation of germ plasm at the distal ends of the first cleavage furrow, we always find nuage-like structures evenly distributed in small particles of 1 μm in diameter adjacent to or in the proximity of the actin cortex (Fig. 2 a). After aggregation in two- and four-cell embryos, the nuage-like particles have increased in size and form rodlike structures underneath the incomplete furrow. These aggregated particles are no longer seen in proximity to the actin cortex, but rather are seen in close association with microtubule and mitochondria (Fig. 2, b and c). Some small particles are still detected along the actin cortex, though fewer in number.

Since germ plasm particles are found close to the actin cortex in single cell embryos, we asked whether actin plays a role in initial germ plasm aggregation. To test this hypothesis, we treated single cell embryos with latrunculin B, an actin inhibitor (Spector et al., 1983). The treated embryos failed to aggregate germ plasm particles. Instead, we see germ plasm around the edges of the cortex (data not shown) and no accumulation at the cleavage planes. This observation is consistent with the idea that the initial aggregation of the germ plasm is actin dependent. However, we cannot exclude a secondary effect of actin inhibition on germ plasm aggregation since this agent also inhibits the formation of the cellular furrow (Rappaport, 1996).

**Vasa Protein Distribution during Oogenesis and Embryogenesis**

The observation that zebrafish vasa RNA is localized to the germ plasm raised the question whether Vasa protein is also a germ plasm component. To test this possibility, we raised rabbit anti-Vasa antibodies to characterize the subcellular distribution of Vasa protein during oogenesis and embryogenesis. The antiserum detects a band of 80 kD on
a Western blot, which is consistent with the calculated molecular mass of 77 kD (see Fig. 4 e). The preimmune rabbit serum does not cross-react with fish proteins of similar size. Anti-Vasa antibody–depleted serum fails to detect both the 80-kD Western band and primordial germ cells in whole-mount antibody staining (data not shown). We conclude that the anti-Vasa antibody is specific for Vasa protein.

In whole-mount double stainings for vasa RNA and the protein of stage I oocytes, vasa RNA is diffuse and cytoplasmic, whereas the protein is localized in patches around the germinal vesicle (Fig. 3, a and b). In stage II oocytes, vasa RNA is transiently localized to the cytosolic part of cup-shaped granules, presumably cortical granules, and then accumulates around the animal cortex of the maturing oocyte (Fig. 3 c). Vasa protein remains associated with the germinal vesicle until germinal vesicle breakdown between stage III and IV (Fig. 3, d and e). During late oogenesis, we detect uniform Vasa protein presumably because of its cytoplasmic localization. We cannot detect localized Vasa protein by whole-mount stainings before the late sphere (cell cycle 13) stage, although developmental Western analyses suggest that the protein is present at equivalent levels throughout embryogenesis (Fig. 4 e). In stainings at these early stages, Vasa protein shows a diffuse cytoplasmic distribution, suggesting that the maternal Vasa protein is neither localized nor confined to a subpopulation of cells (data not shown). Beginning at the sphere (cell cycle 12–13) stage, Vasa protein–positive cells display the same migration pattern as shown with in situ stainings against vasa RNA, suggesting colocalization of Vasa protein and RNA to the same cell population. Vasa protein is detected as a faint perinuclear staining in four clusters of one to four cells each in late sphere stage embryos (cell cycle 13; Fig. 4 a). Beginning at the dome stage (cell cycle 13–14), Vasa protein–positive cells can be followed with the nuclear dye propidium iodide (red) that does not stain oocyte nuclei. Stainings were analyzed by confocal microscopy and superimposed with DIC images. In stage I oocytes, vasa RNA is cytoplasmic (a), whereas Vasa protein is localized around the germinal vesicle (b). Later in stage II oocytes, the RNA localizes to cortical cuplike structures while the Vasa protein remains associated with the germinal vesicle (c). In stage III oocytes, vasa RNA enriches at the animal pole cortex (d). After germinal vesicle breakdown between stages III and IV, Vasa protein localization is lost while the RNA remains cortical (e). In 30 hpf embryos, vasa RNA is found in patches within the cytosol again and the protein resumes its perinuclear location (f).
throughout embryogenesis, as they migrate to form two clusters lateral to the paraxial mesoderm extending roughly from somites 2–4 at the 6 somite stage (Fig. 4b). At 24 hpf, the Vasa protein–positive cells are located in two clusters at the anterior part of the yolk extension, from where they extend posteriorly in two bilateral rows of cells dorsolateral to the forming gut (Fig. 4, c and d).

To address the structural nature of the perinuclear localization of Vasa protein and its possible colocalization with vasa RNA, we analyzed the Vasa protein distribution on its own and together with vasa RNA in 30 hpf embryos. Vasa protein is localized strictly perinuclear to granules. These granules do not colocalize with the nuclear pore as has been reported for Caenorhabditis (Pitt et al., 2000), and are distinct from vasa RNA–containing structures (Fig. 4, f–h). vasa RNA is partially perinuclear, partially cytosolic, and does not colocalize with Vasa protein (Fig. 3 f), a pattern reminiscent of what is seen at early oogenesis. From these observations, we draw two conclusions. First, we infer that Vasa protein is not a component of the zebrafish germ plasm. Second, we believe that while vasa RNA is subcellularly localized to the distal cleavage fur-
rows during early cell divisions (Olsen et al., 1997; Yoon et
al., 1997; and our own observations), its protein remains in
all cells. The accumulation of Vasa protein in PGCs at the
late sphere stage (cell cycle 13) may, therefore, occur by
de novo synthesis. Alternatively, perinuclear Vasa protein
accumulation also could reflect active recruitment of ma-
ternal protein.

Zygotic vasa Transcription Starts at Late Sphere Stage
(Cell Cycle 13)

To resolve the question of the initial origin of perinuclear-
localized Vasa protein at the late sphere stage (cell cycle
13), we analyzed the onset of zygotic transcription of vasa
RNA. To this end, we devised a strategy that allowed us to
monitor zygotic vasa RNA in the background of maternal
vasa RNA. First, we cloned the vasa homologue of D. feegradei,
a fish closely related to D. rerio (data not shown; accession number A F251800). Based on polymorphism in the 3′ UTR of these two species, we designed primers that are specific for either vasa transcript (data not shown and Fig. 5 b). Then, we created hybrid embryos from D. rerio females and D. feegradei males by in vitro fertilization (Fig. 5 a) and used reverse transcriptase-PCR to follow the ini-
tiation of paternal (i.e., zygotic) vasa transcription during
development. The localization pattern of vasa RNA and early embryogenesis of D. feegradei and the D. rerio–D.
feegradei hybrids resembles D. rerio (data not shown), and, therefore, it is likely that both species utilize similar
mechanisms for this process. Our reverse transcriptase-
PCR approach demonstrates that whereas we are able to
amplify maternal vasa RNA throughout early embryogen-
esis, paternal (i.e., zygotic) vasa RNA is first detectable
at the late sphere stage (cell cycle 13; Fig. 5 b). Therefore, zygotic vasa expression is initiated shortly after the begin-
ing of general zygotic transcription. The activation of the
vasa promoter at the late sphere stage (cell cycle 13) sug-
gests that PGCs adopt their fate at the onset of their pro-
iferation and migration period.

To analyze the relationship between the onset of zygotic
vasa transcription and localized Vasa protein, we deter-
mined the Vasa protein distribution in embryos at the
early sphere stage (cell cycle 12–13), which do not yet ex-
press zygotic vasa (early sphere stage, cell cycle 12–13).
Only a few embryos within such a clutch show Vasa pro-
tein accumulation in the four to five presumptive PGCs. In

contrast, all embryos at the dome stage (cell cycle 13–14),
which have begun transcribing vasa RNA, show Vasa pro-
tein accumulation in these four to five cells (data not
shown). These observations suggest that translation of ma-
ternal RNA provides a minor contribution to the localized
vasa protein, and that further Vasa protein accumulation
relies on translation of zygotically expressed vasa RNA. In
support of this observation, we also find weak Vasa pro-
tein accumulation in four to five cells in the late sphere
stage (cell cycle 13) embryos lacking genomic DNA and, hence, zygotic vasa contribution (see below).

Germ Plasm Does Not Transcriptionally Silence the Nucleus

Although the activation of the zygotic genome at mid-
blastula transition (MBT) occurs at 3.0 hpf (Kane and
Kimmel, 1993), our experiments indicate that zygotic vasa
transcription occurs approximately 1 h later, at ~4.0 hpf
(Fig. 5 b). This prompted us to ask whether presumptive
germ cell nuclei are, in general, transcriptionally quiescent
until the sphere stage (cell cycle 13). Transcriptional quies-
cence has been reported for germ cell nuclei of Drosophila
and Caenorhabditis (Zalokar, 1976; Seydoux and Dunn,
1997; A saoka et al., 1998; Van Doren et al., 1998). To ad-
dress this question, we used the H5 mAb. This antibody
specifically recognizes a phosphoepitope on the COOH-
terminal domain of the RNA polymerase II (RNA Pol II).
This epitope has been reported to reflect elongating and,
thus, transcriptionally active RNA Pol II, and is a marker for
actively transcribing nuclei (Warren et al., 1992; Bregman
et al., 1995; K im et al., 1997; Seydoux and Dunn, 1997; Pat-
turaj et al., 1998). No RNA Pol II signal is detected with
the H 5 antibody in the nuclei of 128-cell stage embryos
(Fig. 6 a), although we see an RNA Pol II signal with the H 4
mAb that recognizes a phosphoepitope that does not cor-
relate with transcriptional activity in extracts of pre- and
post-MBT embryos (Fig. 6 c). Shortly before MBT, at the
transition from the 256- to 512-cell stage, we see distinct
subnuclear staining of cells containing and lacking germ
plasm with the H 5 antibody that persists at least until late
epiboly (Fig. 6 b and data not shown). Since we can de-
tect no difference between cells with and without germ
plasm in H 5 RNA Pol II stainings, we suggest that transcrip-
tion is initiated at MBT in all nuclei irrespective of germ
plasm.
Germ Plasm Is Initially Asymmetrically, and then Symmetrically Segregated into Daughter Cells

We were interested in the reasons why the number of presumptive PGCs remains constant during initial cleavages while somatic cells increase dramatically in number. There are two possibilities to account for the lack in primordial germ cell proliferation until the sphere stage (cell cycle 13): either germ plasm–containing cells do not divide during early embryogenesis, as has been shown for Drosophila, or germ plasm is segregated asymmetrically to one daughter cell during cell division, as has been reported for Caenorhabditis and Xenopus (Whittington and Dixon, 1975; Seydoux and Strome, 1999) and suggested in zebrafish (Yoon et al., 1997; Braat et al., 1999). To address this question, we followed germ plasm–positive cells during early embryogenesis. In the 512-cell stage embryos, dividing cells segregate the germ plasm with one of the two daughter nuclei, whereas in sphere stage (cell cycle 13) embryos, germ plasm is inherited by both daughter cells (Fig. 7, d and e). In 1,000-cell stage embryos, we still see germ plasm as a tight structure restricted to one part of the cell (Fig. 7 a). Three-dimensional analysis shows that the germ plasm is punctate and tightly localized between the nucleus and the vegetal-most membrane, often forming a cuplike structure (data not shown). Beginning at the sphere stage (cell cycle 12–13) the germ plasm disintegrates and seems to spread into the cytoplasm (Fig. 7 b). Shortly before gastrulation (30% epiboly), the germ plasm is fully disintegrated and fills the cytoplasm evenly in little patches (Fig. 7 c).

Germ plasm is localized to one side of the division plane during early cell divisions, as seen in centriole and microtubule double stainings for cell polarity (Fig. 7 f and data not shown). Most frequently, we see the axis of cellular division to the animal-vegetal axis with the germ plasm segregated to the vegetal spindle pole. Therefore, the observed low number of vasa-positive cells in early embryogenesis is due to asymmetric localization of germ plasm to one daughter cell, which is a process that is discontinued at the beginning of the sphere stage (cell cycle 12–13) when germ plasm fills the cytoplasm. In addition, changes in the germ plasm segregation pattern are observed before the beginning of Vasa protein localization and zygotic vasa transcription, suggesting that this change is independent of Vasa protein function.

Germ Plasm Localization and Segregation Is Impaired in Maternal Nebel Mutants

Since initial vasa RNA segregation along the first two cellular cleavages is impaired in nebel mutant embryos, we were interested whether the nebel mutation affects germ plasm integrity and possibly also asymmetric germ plasm segregation (Pelegri et al., 1999). Sectioning nebel mutant embryos, we find that vasa RNA is still localized to nuage-like structures in the 8-cell stage embryos, although the germ plasm is seen in ectopic locations close to the center of the embryos instead of its wild-type location at the distal part of the cleavage furrows (Fig. 8, a and b). Therefore, the nebel mutation does not affect germ plasm integrity, but affects the movement of vasa RNA and other germ plasm components along the forming furrow.

To investigate possible defects in unequal germ plasm segregation in nebel embryos, we followed vasa RNA as a marker of germ plasm in nebel embryos from the 256- to the 1,000-cell stage. We find that, in some dividing cells, the asymmetric segregation of the germ plasm is defective, so that it is distributed prematurely to both daughter cells (Fig. 8 d). This results in additional germ plasm–containing...
cells in close proximity to their daughter cells, although they contain smaller amounts of germ plasm (Fig. 8c). Assessing the number of vasa RNA–positive cells at the 1,000-cell stage in nebel and wild-type embryos shows that, in nebel embryos, on average, seven cells carry germ plasm, compared with five in wild-type (Table I). Since the nebel phenotype is variable, we compared the frequency of embryos with additional germ plasm–containing cells. We find that the distribution is broader in nebel than in wild-type embryos, and a significant proportion of nebel embryos carry two to three times as many cells with germ plasm as wild-type embryos (Fig. 8, e and f). Therefore, we conclude that the nebel mutation affects germ plasm migration both early to the distal part of furrows and later during asymmetric segregation. This suggests a common mechanism for both processes.

The Change from Asymmetric to Symmetric Germ Plasm Segregation Is Independent of Zygotic Signals and the Nucleus

We investigated whether the signals triggering the end of asymmetric vasa RNA segregation at the late blastula stage are dependent on nuclear signals or whether they are dependent on the cytoplasm or the germ plasm itself. To address this question, we analyzed vasa RNA segregation in embryos where cell division occurs but which lack any detectable nuclei. In aphidicolin-treated embryos that lack any detectable nuclei, vasa RNA is localized in 1,000-cell stage embryos (g), starts to disperse into and eventually evenly fills the cytoplasm at early to late sphere stage (h and i).

Table I. Number of Presumptive PGCs during Zebrafish Embryogenesis

| Stage               | 1 cell | 128 cell | 1,000 cell | Sphere | Dome | 50 percent epiboly | 1 day |
|---------------------|--------|----------|------------|--------|------|-------------------|-------|
| Total cell number   | 1      | 128      | 1,000      | 8,000  | 12,000 | 24,000            | ?     |
| Average number of PGCs in wild-type* | 1.0 ± 0.7 | 5.0 ± 0.9 | 6.1 ± 1.3 | 10.3 ± 2.6 | 15.1 ± 4.0 | 29.9 ± 4.1 | 31.1 ± 9.4 |
| (n = 32)            | (n = 31) | (n = 24) | (n = 21)   | (n = 30) | (n = 27) | (n = 30)         |       |
| Average number of PGCs in nebel mutants*† | nd | 7.0 ± 1.8 | nd | nd | nd | 31.1 ± 9.4 |       |
| (n = 36)            | (n = 31) | (n = 24) | (n = 21)   | (n = 30) | (n = 27) | (n = 33)         |       |

*Sample size (n) and SD are indicated.
†nd, nondetermined.
colin to inhibit DNA replication and, as a consequence, nuclear division (Nagano et al., 1981; Raff and Glover, 1989). Treated embryos developed until the sphere to dome stage (cell cycle 12–14), and cell division was normal as judged by centriole stainings, although few to no nuclei were detectable (data not shown). Germ plasm segregation was also unaffected in treated embryos. In the 512-cell stage embryos, germ plasm tightly localized to one part of the cell and only four to five aggregates were observed (Fig. 7 g). This localization was lost during early to the late sphere stage (cell cycle 12–13; Fig. 7, h and i). At this stage, a greater number of vasa RNA–positive cells were observed, which is consistent with the idea that the asymmetric segregation program has been completed. Similarly, we did not see any effect on germ plasm segregation in embryos treated with the transcription inhibitor actinomycin D (data not shown). These experiments show that the signal(s) that trigger the switch from asymmetric to symmetric germ plasm segregation depend neither on the nucleocytoplasmic ratio nor on the activation of zygotic gene expression.

Discussion

The Nature of the Zebrafish Germ Plasm

In this paper, we describe a nuage-like structure that contains vasa transcripts (Fig. 1). The structure consists of po-
As has been observed by numerous workers in different species, germ plasm movement to distal cleavage planes in both bryos. Our previously reported studies attribute the loss of plasm translocation also is impaired in...of germ plasm to move distally suggestive of a role for this cytoskeletal network in vasa aggregation of cortical germ plasm particles (data not shown). A possible involvement of the actin network in...microtubule (Fig. 2). Disruption of the actin mesh by exposure to latrunculin B before furrow formation inhibits aggregation of cortical germ plasm particles (data not shown). A possible involvement of the actin network in these processes cannot be directly tested since actin inhibitors affect proper furrow formation. However, the colocalization of germ plasm aggregates with the actin network is suggestive of a role for this cytoskeletal network in vasa RNA recruitment at the furrow. In addition, depolymerization of the microtubule by nocodazole after furrow initiation results in a failure of germ plasm to move distally along the forming furrow (Pelegri et al., 1999). Germ plasm translocation also is impaired in neb mutant embryos. Our previously reported studies attribute the loss of germ plasm movement to distal cleavage planes in both the nebel- and nocodazole-treated embryos to a reduction of the furrow microtubule array (FMA; Pelegri et al., 1999). These observations suggest that the initial assembly of germ plasm particles into larger aggregates at the forming furrow is actin-mediated, and that the subsequent peripheral movement of germ plasm along the cleavage plane is FMA-dependent. The importance of the cytoskeleton for germ plasm assembly also has been shown in Drosophila and Xenopus. In Drosophila, the recruitment of the posterior pole plasm components is microtubule- and actin-dependent (Erdelyi et al., 1995; Pokrywka and Stephenson, 1995; Lantz et al., 1999). Flies fed on a diet supplemented with the microtubule polymerization inhibitor colchicine produce oocytes that fail to localize oskar RNA to the posterior pole. In wild-type flies, oskar RNA induces the assembly of germ plasm at the posterior pole, causing development of the abdomen and germline (Ephrussi et al., 1991; Ephrussi and Lehmann, 1992). oskar RNA localization to the posterior pole is also strongly reduced in tropomyosin II mutant oocytes, indicating a dependence of oskar RNA localization on the actin network (Erdelyi et al., 1995). In Xenopus, germ plasm assembly is microtubule-dependent (Ressom and Dixon, 1988). Injection of different microtubule inhibitors into activated eggs consistently abolished germ plasm aggregation, whereas actin depolymerization showed no effect on germ plasm assembly. The role of microtubules in X enopus germ plasm aggregation is substantiated by the requirement of a microtubule-dependent kinase-like protein, X klp1 (Robb et al., 1996). Although germ plasm assembly is cytoskeleton-dependent in Drosophila, Xenopus and zebrafish, there are different temporal requirements for actin and microtubule. In Drosophila, pole plasm components require an intact actin and microtubule network simultaneously, whereas, in Xenopus, the actin network is dispensable for germ plasm aggregation. In zebrafish, the initial germ plasm aggregation may be actin-dependent, whereas the subsequent segregation requires a functional FMA.

**Transport of Zebrafish Germ Plasm**

In the 1-cell stage embryos, we see numerous small germ plasm particles in close proximity to the cortical actin mesh, sometimes also in direct physical contact. After the first and second cleavage, the germ plasm aggregates and moves to a position underneath the distal part of the cleavage furrows, where it is found in close proximity with the microtubule (Fig. 2). Disruption of the actin mesh by exposure to latrunculin B before furrow formation inhibits aggregation of cortical germ plasm particles (data not shown). A possible involvement of the actin network in these processes cannot be directly tested since actin inhibitors affect proper furrow formation. However, the colocalization of germ plasm aggregates with the actin network is suggestive of a role for this cytoskeletal network in vasa RNA recruitment at the furrow. In addition, depolymerization of the microtubule by nocodazole after furrow initiation results in a failure of germ plasm to move distally along the forming furrow (Pelegri et al., 1999). Germ plasm translocation also is impaired in neb mutant embryos. Our previously reported studies attribute the loss of germ plasm movement to distal cleavage planes in both the nebel- and nocodazole-treated embryos to a reduction of the furrow microtubule array (FMA; Pelegri et al., 1999). These observations suggest that the initial assembly of germ plasm particles into larger aggregates at the forming furrow is actin-mediated, and that the subsequent peripheral movement of germ plasm along the cleavage plane is FMA-dependent. The importance of the cytoskeleton for germ plasm assembly also has been shown in Drosophila and Xenopus. In Drosophila, the recruitment of the posterior pole plasm components is microtubule- and actin-dependent (Erdelyi et al., 1995; Pokrywka and Stephenson, 1995; Lantz et al., 1999). Flies fed on a diet supplemented with the microtubule polymerization inhibitor colchicine produce oocytes that fail to localize oskar RNA to the posterior pole. In wild-type flies, oskar RNA induces the assembly of germ plasm at the posterior pole, causing development of the abdomen and germline (Ephrussi et al., 1991; Ephrussi and Lehmann, 1992). oskar RNA localization to the posterior pole is also strongly reduced in tropomyosin II mutant oocytes, indicating a dependence of oskar RNA localization on the actin network (Erdelyi et al., 1995). In Xenopus, germ plasm assembly is microtubule-dependent (Ressom and Dixon, 1988). Injection of different microtubule inhibitors into activated eggs consistently abolished germ plasm aggregation, whereas actin depolymerization showed no effect on germ plasm assembly. The role of microtubules in X enopus germ plasm aggregation is substantiated by the requirement of a microtubule-dependent kinase-like protein, X klp1 (Robb et al., 1996). Although germ plasm assembly is cytoskeleton-dependent in Drosophila, Xenopus and zebrafish, there are different temporal requirements for actin and microtubule. In Drosophila, pole plasm components require an intact actin and microtubule network simultaneously, whereas, in X enopus, the actin network is dispensable for germ plasm aggregation. In zebrafish, the initial germ plasm aggregation may be actin-dependent, whereas the subsequent segregation requires a functional FMA.

**A Change in Structure and Segregation of Germ Plasm Induces Germ Cell Specification**

During zebrafish embryogenesis, cell number and diversity increase as cells divide and adopt different identities. One way to determine diverse cell fate is to partition determinants unequally between daughter cells. Such a mechanism is employed in zebrafish germline specification. During the first cell divisions, vasa RNA-containing structure is the zebrafish germ plasm. Similar observations have been reported in Caenorhabditis, Drosophila, and Xenopus, where nuage-like structures are associated with the germline throughout the life cycle of these animals (M. ahowald, 1962, 1968, 1971; Whitington and Dixon, 1975; Wolf et al., 1983). However, in Drosophila and Caenorhabditis, the Vasa protein and its orthologues but not their corresponding RNAs are part of the polar granules/P granules (Hay et al., 1988a,b; Lascko and Ashburner, 1990; Gruidl et al., 1996). This is in contrast to zebrafish, where we find vasa RNA but not Vasa protein to be a component of the germ plasm (Fig. 3f).

In Drosophila, Vasa protein localization to the germ plasm is dependent on localized oskar RNA, the key component in germ plasm assembly and abdominal patterning (Ephrussi et al., 1991; Ephrussi and Lehmann, 1992). It is tempting to speculate that localization of vasa RNA to germ plasm may be a vertebrate variant to restrict Vasa protein expression via the localization of Vasa-like gene 1 RNA has not been reported, it cannot be concluded whether the restriction of Vasa protein expression via the localization of vasa RNA is a commonly used mechanism.
that affects the FMA and FMA-based translocation of germ plasm in 4-cell stage embryos (Pelegri et al., 1999). However, it is also possible that this phenotype is a secondary consequence of other nebel-associated phenotypes, such as the insertion of newly added membrane (Pelegri et al., 1999). Further work will be necessary to clarify this issue.

During the transition from unequal to equal cell division in presumptive PGCs, germ plasm fills the cytoplasm and changes from a tight to a more diffuse structure. The initial signals that trigger this change are of maternal and non-nuclear origin since germ plasm structure and segregation are unaffected in embryos where DNA replication is inhibited and cell division progresses normally until early dome stages (cell cycle 13–14; Fig. 7, g–i). Furthermore, these signals need to be linked tightly to the number of cell divisions to induce relocalization of germ plasm exactly at the late sphere (cell cycle 13) stage. In Xenopus, the ratio of chromatin to cytoplasm has been implicated in a similar process, which is the timing of MBT (Newport and Kirschner, 1982). The chromatin/cytoplasm ratio cannot account for the timing of germ plasm relocalization in zebrafish since the chromatin level does not change in replication-inhibited embryos during development. Rather, we believe that the timing owes to an unknown counting mechanism that may be related to the germ plasm itself or other cellular processes, such as the division of the centrioles, whose division and colocalization with the germ plasm is unaffected in cells lacking nuclear DNA.

Vasa protein begins to localize around the nuclei of presumptive PGCs at about the same stage as when vasa RNA asymmetric segregation is lost. From this stage on, vasa RNA and protein are now inherited by both daughter cells. A consequence, the number of PGCs starts to increase and we find that zygotic vasa transcription is initiated (Table I, Figs. 4 and 5). It is tempting to speculate that the beginning of zygotic vasa expression indicates a fate decision in cells that contain germ plasm to adopt a germ cell fate. This idea is supported by the fact that fate decision in cells that contain germ plasm to adopt a presumptive germ cell fate. In the absence of additional germ plasm, are required to specify germ cell fate. In the absence of additional germ cell markers, it is impossible to further substantiate the suggestion that the beginning of zygotic vasa transcription is indicative of germ cell fate specification. A through germ plasm repression early zygotic expression in PGCs of Drosophila and Caenorhabditis is essential for germ cell formation and in mice for male germ cell proliferation (Hay et al., 1988b; Lasko and Ashburner, 1992). However, the inheritance of vasa RNA alone does not suffice to induce zygotic vasa transcription since injection of vasa mRNA into 1-cell stage embryos did not yield an increase in PGCs, the vasa RNA was rapidly degraded (Weidinger et al., 1999). Therefore, it is likely that additional factors, possibly residing within the germ plasm, are required to specify germ cell fate. In the absence of additional germ cell markers, it is impossible to further substantiate the suggestion that the beginning of zygotic vasa transcription is indicative of germ cell fate specification. A through germ plasm repression early zygotic expression in PGCs of Drosophila and Caenorhabditis (Zalokar, 1976; Seydoux and Dunn, 1997; Asooka et al., 1998; van Doren et al., 1998), the delayed onset of zygotic vasa transcription is not a consequence of transcriptional repression of germ cell nuclei. We find that transcription initiated at the MBT in all nuclei irrespective of germ plasm. In Xenopus, similar changes in germ plasm localization shortly before gastrulation have been suggested to reflect germ plasm activation (Whittington and Dixon, 1975). If relocalization of germ plasm in zebrafish is linked to its activation, one possible consequence of this process may be the translation of stored maternal transcripts such as vasa RNA. Indeed, we see weak vasa protein signals in aphidicolin-treated embryos at the sphere stage (cell cycle 13; data not shown). This indicates that maternal vasa RNA is translated in the absence of zygotic transcription. Since Vasa protein is homologous to RNA helicases and is implicated in initiating translation of maternal transcripts in Drosophila (Styhler et al., 1998; Tomancak et al., 1998), it is tempting to speculate that newly translated Vasa protein facilitates translation of other germ cell–associated transcripts, including vasa RNA itself. Because the appearance of the Vasa protein also coincides with the activation of zygotic vasa transcription, it is also possible that protein products that are dependent on Vasa protein for their translation result in the expression of germ-line-specific genes and germ line fate commitment. However, in the absence of vasa mutations in zebrafish, we cannot rule out the possibility that Vasa protein does not play a role in germ cell specification, but rather is required during later stages of germ cell development. Evidence for germ cell inducing capacity of germ plasm comes from transplantation experiments in amphibians, where it has been shown that the amount of germ plasm determines the number of PGCs. When germ plasm is successively removed, the number of PGCs decreases accordingly. Total removal of germ plasm leads to sterility (for review see Nieuwkoop and Sutasurya, 1979). In nebel embryos, the germ plasm is often equally instead of unequally segregated to daughter cells (Fig. 8d). This results in more cells that carry smaller amounts of germ plasm at the 1,000-cell stage.

In summary, our observations suggest the following steps for germ cell determination in zebrafish. Unequal germ plasm partitioning separates four cells from the embryo that have the potential to form the germline. These presumptive PGCs turn into true PGCs (Whittington and Dixon, 1975) at the sphere stage (cell cycle 13) when they start expressing vasa RNA, a germ-line-specific marker. This presumptive fate commitment is preceded by the translational process of the germ plasm from its cortical to a more diffuse cytoplasmic location. The change in germ plasm localization is induced by a maternal program that is independent of the nucleocytoplasmic ratio and zygotic transcription. This maternal program results in Vasa protein localization around the nucleus and initiation of zygotic vasa transcription. Vasa protein accumulation may originate from maternal vasa RNA but is primarily dependent on zygotically derived transcripts. These events result in the expression of germ-line-specific markers and equal cell division that give rise to ~30 PGCs, the founder population of the germline. Upon migration to the gonads, the PGCs resume proliferation and spermatogenesis or oogenesis will begin (Yoon et al., 1997). Further analysis of maternal–effect mutants defective in vasa RNA segregation together with zygotic screens for germ line defects will shed more light on germ line formation in zebrafish.

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