Development 124, 3157-3166 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
DEV3705



# Zebrafish *vasa* homologue RNA is localized to the cleavage planes of 2- and 4-cell-stage embryos and is expressed in the primordial germ cells

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## SUMMARY

**Identification and manipulation of the germ line are important to the study of model organisms. Although zebrafish has recently emerged as a model for vertebrate development, the primordial germ cells (PGCs) in this organism have not been previously described. To identify a molecular marker for the zebrafish PGCs, we cloned the zebrafish homologue of the *Drosophila vasa* gene, which, in the fly, encodes a germ-cell-specific protein. Northern blotting revealed that zebrafish *vasa* homologue (*vas*) transcript is present in embryos just after fertilization, and hence it is probably maternally supplied. Using whole-mount in situ hybridization, we investigated the expression pattern of *vas* RNA in zebrafish embryos from the 1-cell stage to 10 days of development. Here we present evidence that *vas* RNA is a germ-cell-specific marker, allowing a description of the zebrafish PGCs for the first time. Furthermore, *vas* transcript was detected in a novel pattern, localized to the cleavage planes in 2- and 4-cell-stage embryos. During subsequent cleavages, the RNA is segregated as subcellular clumps to a small number of cells that may be the future germ cells. These results suggest new ways in which one might develop techniques for the genetic manipulation of zebrafish. Furthermore, they provide the basis for further studies on this novel RNA localization pattern and on germ-line development in general.**

Key words: zebrafish, *vasa* homologue, germ line, primordial germ cell, localized RNA, cleavage

## INTRODUCTION

Germ cells are a highly differentiated cell type whose unique role is to transmit genetic information between generations. For any model organism, identifying the germ cells, determining when and where they arise during embryonic development, and ultimately understanding the genetic basis for their determination are important questions. In recent years, much has been learned about the origin of germ cells in *C. elegans*, *Drosophila*, frog and mouse embryos (reviewed by Wei and Mahowald, 1994; Nieuwkoop and Sutasurya, 1979). In worms, flies and frogs, specialized cytoplasm containing specific RNAs and proteins, and known as germ plasm, or pole plasm (in *Drosophila*), is asymmetrically localized within the egg (reviewed by Eddy, 1975). During cleavage of the zygote, this specialized cytoplasm becomes segregated to cells that will become the germ cells (pole cells). In *Drosophila*, cytoplasmic transplantation has shown that pole plasm is sufficient to direct the development of ectopic pole cells at the site of injection (Illmensee and Mahowald, 1974, 1976; Okada et al., 1974). The application of genetics in *Drosophila* has permitted the identification of a number of genes essential for the determination of the germ line, including some of the genes whose RNA or protein products are localized to pole plasm in the egg and early embryo (reviewed by Williamson and Lehmann, 1996). Despite extensive progress, much remains to be learned about how germ-cell fate is determined, how germ cells migrate to the gonad and how these cells retain totipotency.

Besides intrinsic biological interest, studies of germ cells can have practical implications. In mice, it is possible to culture primordial germ cells (PGCs) (Matsui et al., 1992; Resnick et al., 1992) and return them to the embryo where they can participate in normal development, including contributing to the future germ line (Labosky et al., 1994; Stewart et al., 1994). Thus, identification of germ cells can open the way to genetic manipulation of the germ line, including, for example, homologous recombination and insertional mutagenesis.

The zebrafish is a model organism of great importance for the study of early vertebrate development, but little is known about the origin of the zebrafish germ line (Walker and Streisinger, 1983; Lin et al., 1992). In other teleosts, analyses of early germ-line development have relied almost exclusively on morphological identification of the PGCs (Wolf, 1931; Dildine, 1936; Johnston, 1951; van den Hurk and Slof, 1981; Hamaguchi, 1982; Lebrun et al., 1982; Brusle, 1983; Parmentier and Timmermans, 1985; Timmermans, 1989). In medaka (Hamaguchi, 1982) and rosy barb (Timmermans, 1989), for example, PGCs can first be identified by light microscopy at the 10- to 12-somite stages. Clearly, in order to better understand early germ-line development in teleosts, it would be helpful to identify genes expressed specifically in PGCs.

In *Drosophila*, the *vasa* gene was initially identified in

genetic screens for maternal-effect mutants that altered embryonic anterior-posterior polarity (Schupbach and Wieschaus, 1986). Females homozygous for a mutation in the *vasa* gene give rise to progeny that are sterile because they lack pole cells, the future germ cells. The *vasa* gene encodes an RNA helicase of the DEAD-box family of proteins that is specifically expressed in the germ-cell lineage (Hay et al., 1988a,b; Lasko and Ashburner, 1988). Transcripts of *vasa* are maternally supplied and are distributed throughout the egg cytoplasm; however, vasa protein is expressed only at the posterior end of the fly embryo in pole cells and zygotic transcription of the gene is limited to pole cells. In vertebrates, a *vasa*-like homologue has been cloned in frogs (Komiya et al., 1994), mice (Fujiwara et al., 1994) and rats (Komiya and Tanigawa, 1995), and has been shown to be expressed specifically in the germ line of older animals in these organisms, although its localization in early embryos has not been reported.

To identify a molecular marker for the germ-cell lineage in zebrafish, we cloned a *vasa*-like gene, which we designate zebrafish *vasa* homologue (*vas*), and used northern blotting and whole-mount in situ hybridization to analyze its RNA expression pattern in embryos and larvae. We find that, as in *Drosophila*, transcripts of *vas* are supplied maternally in the egg. In striking contrast to *Drosophila*, however, RNA in situ hybridization reveals that *vas* transcripts are precisely localized within the zebrafish embryo as early as the 2-cell stage. Specifically, we detected the RNA along the cleavage planes at the 2- and 4-cell stages. Examination of *vas* RNA in whole-mount in situ hybridization at many closely spaced time points suggests that during subsequent cleavages, this localized maternal RNA first condenses into subcellular clumps and then is segregated to a small number of cells, usually exactly four. Later during the somite stages, the number and position of the *vas*-expressing cells suggests that they are the zebrafish primordial germ cells. Although it remains to be proven, the data are strongly suggestive that the cells that initially inherit the maternally expressed *vas* RNA are the future zebrafish germ cells. We have found a novel mechanism of maternal RNA localization, on cleavage planes, that potentially serves to mark the future germ line from an extremely early stage of development.

## MATERIALS AND METHODS

### cDNA cloning

PCR was performed with the degenerate primers MACAQT (5′-ATGGCNTG(T/C)GCNCA(A/G)ACNG-3′) and MLDMGF (5′-(A/G)AANCCCAT(A/G)TCNAGCAT-3′ and 5′-(A/G)AANCCCAT(A/G)TC(T/C)AACAT-3′), Taq Polymerase (Boehringer Mannheim), and 1 μg of template prepared from zebrafish ovary (gift of M. Allende), using 35 amplification cycles (90˚C for 30 seconds, 55˚C for 1 minute, 72˚C for 1 minute). The adult zebrafish cDNA library was a gift of Robert Riggleman and Kathryn Helde. RT-PCR and 5′ RACE were performed using SuperScript II (GICBO-BRL Life Sciences). AP primer (GICBO-BRL Life Sciences) and the primers 5′-GGACGTGAGTGGCAGCAATC-3′ and 5′-GATAGCGCACTTTACTCAGG-3′ were used for RT-PCR and the primers, 5′-CCTGAACGAATCACCAGTCA-3′ and 5′-CCAGTCATTTTCCATGAGCTACC-3′, were used for 5′-RACE. RNA from adult fish was extracted by grinding frozen fish and using Tri Reagent (Molecular Research Center, Inc.) according to the manufacturer's instructions. Sequence alignment was accomplished by the Lasergene software (DNAStar, Inc.) and modified manually.

### Northern blot analysis

Northern blot analysis was performed under high-stringency conditions as previously described (Gaiano et al., 1996), using 15 μg total RNA per lane. The 0.83 kb probe was isolated from the *vas* cDNA by digestion with *Bgl*II and *Hin*dIII (corresponding to nucleotides 1728-2562 of *vas* cDNA, accession number AB005147).

### Whole-mount in situ hybridization

Embryos were maintained at 28.5˚C and staged according to hours and days postfertilization and morphological criteria (Kimmel et al., 1995). Following fixation in 4% paraformaldehyde-PBS, chorions were removed from the embryos by hand using forceps. In situ hybridization was performed essentially according to Allende et al. (1996), with the following modifications. After in vitro transcription using a 1.2 kb fragment from the 3′-end of *vas* cDNA (corresponding to nucleotides 1728 to 2865 of *vas* cDNA, accession number AB005147) or a 0.4 kb fragment (corresponding to nucleotides 1022 to 1405 of *vas* cDNA) as a template, the RNA probe was purified using NucTrap Push Columns (Stratagene), and then precipitated with ammonium acetate and ethanol. Proteinase K treatment was performed for 5 minutes at 10 μg/ml for 10- to 20-somite-stage embryos, 10 minutes at 10 μg/ml for 24 hour embryos and 30 minutes at 25 μg/ml for 3, 4 and 10 day larvae. Embryos younger than 10-somite stage were not treated with proteinase K. For double in situ hybridizations, both RNA probes were labeled with UTP-11 digoxigenin, and hybridization and detection reactions were carried out simultaneously. Embryos were cleared in glycerol, mounted under a bridged coverslip and photographed with a Nikon Microphot SA microscope.

For histological analysis, in situ hybridized embryos were stained for at least 36 hours, then processed for plastic sectioning as described (Allende et al., 1996). Sections were photographed with a Zeiss Axiophot microscope.

## RESULTS

### Isolation of zebrafish *vasa* homologue cDNA

To isolate a zebrafish homologue of the *Drosophila vasa* gene (Hay et al., 1988b; Lasko and Ashburner, 1988), we designed degenerate PCR primers to amplify the ATP-binding sequence conserved in DEAD-box family genes (Fig. 1). Similar degenerate primers to this region have been used to clone the *vasa* homologues of other vertebrates, *RVLG* (rat; Komiya and Tanigawa, 1995), *XVLG-1* (frog; Komiya et al., 1994) and *Mvh* (mouse; Fujiwara et al., 1994). DNA bands of approximately 400 bp were amplified by PCR using zebrafish ovary cDNA as a template. This DNA was cloned and analyzed by DNA sequencing. Among seven different clones, three were homologous to RNA helicases. Two of these were similar to *p68*, another RNA helicase with a DEAD-box (Ford et al., 1988), while the third was more similar to the vertebrate *vasa* genes. Southern hybridization using the latter DNA showed that it was a single copy in the zebrafish (data not shown). This 400 bp *vasa*-like DNA was therefore subsequently used to screen a zebrafish adult cDNA library.

Three clones were isolated by the cDNA library screening and the longest one was sequenced. The overall structure of this clone was highly homologous to the *vasa* genes of other animals. We found, however, that there was a frameshift mutation in the coding region and the clone lacked a complete 5′ end. 5′ RACE and RT-PCR using total RNA prepared from an adult female fish were performed and a longer cDNA sequence of the zebrafish *vasa* homologue was obtained. The single base pair deletion causing the frameshift mutation was

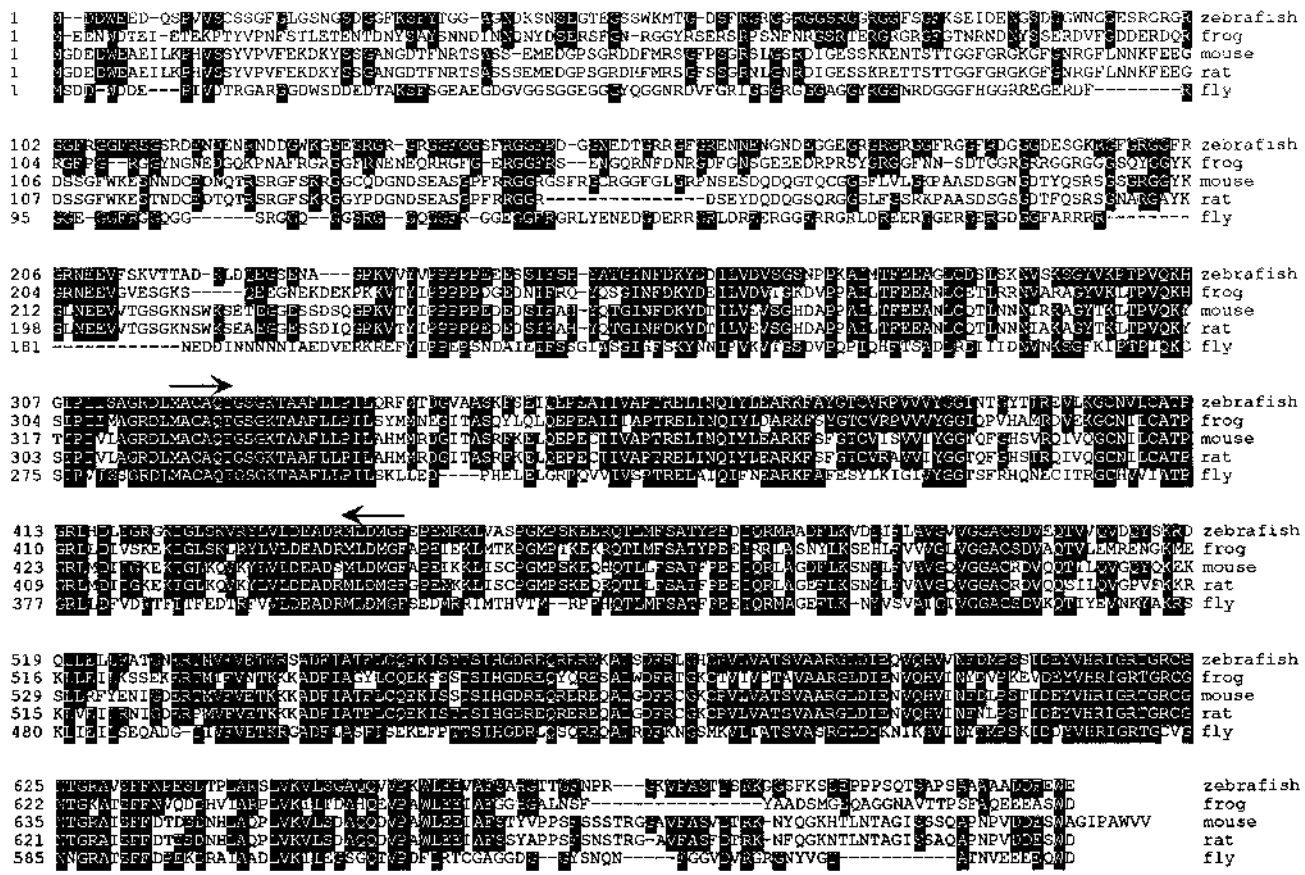


**Fig. 1.** Alignment of the zebrafish, frog, mouse, rat and fly vasa proteins. The predicted zebrafish (this work), *Xenopus* (Komiya et al., 1994), mouse (Fujiwara et al., 1994), rat (Komiya and Tanigawa, 1995) and *Drosophila* (Hay et al., 1988; Lasko and Ashburner, 1988) vasa protein sequences are aligned. The amino acids that are identical to the zebrafish sequence are highlighted. Arrows have been placed above the amino acids on which the degenerate PCR primers are based. **GenBank accession number:** The accession number for the sequence reported in this paper is AB005147.

present only in the cDNA library. The 2865 bp cDNA sequence assembled from the sequences of the cDNA, 5′ RACE and RT-PCR products encoded an open reading frame for a protein of 716 amino acids. Although we could not find a stop codon prior to the putative initiation codon, we think this 716 amino acid protein is likely to be a product of this gene because (1) the sequence around the putative initiation ATG codon completely matched the consensus sequence for eukaryotic translation initiation (Kozak, 1984) and (2) the length of the cDNA was consistent with that of the transcript detected by northern hybridization (see below).

The alignment of the zebrafish protein to the vasa proteins of other animals is shown in Fig. 1. The predicted amino acid sequence is 52.8%, 52.8%, 49.3% and 40.9% identical to the mouse, rat, *Xenopus* and *Drosophila* vasa proteins, respectively. The zebrafish protein also contains the eight regions that are conserved among DEAD protein family members (Linder et al., 1989; Fujiwara et al., 1994). While we can not exclude the possibility that, in zebrafish, there may be a closer homologue to the *Drosophila vasa* gene, the high degree of conservation in the overall structure of the protein and conserved helicase-domain between the zebrafish gene and the *vasa* family genes leads us to conclude that the gene that we cloned is likely to be a zebrafish homologue of the *vasa* gene. Thus, the gene represented here is designated zebrafish *vasa* homologue (*vas*).

## Northern blot analysis indicates that zebrafish *vasa* homologue is maternally supplied

Northern blot analysis was performed using a probe derived from the *vas* cDNA (see Materials and Methods). During embryonic development, the *vas* gene is expressed as a transcript of approximately 3.0 kb (Fig. 2). *vas* RNA is present in embryos just after fertilization (0 hour), indicating that the message is maternally provided. RNA continues to be detected at the beginning of gastrulation (6 hours) but, by 24 hours and up to 4 days, it was undetectable by northern analysis. As shown by in situ hybridization (see below), however, this is probably because the amount of *vas* transcript becomes too low relative to total RNA to be detected.

## Whole-mount in situ hybridization to detect the localization of zebrafish *vasa* homologue RNA

To determine the localization of *vas* RNA, whole-mount in situ hybridization was performed using digoxigenin-labeled RNA probes corresponding to regions of the *vas* cDNA (see Materials and Methods). The maternal *vas* message, detected by northern blot hybridization, was not detected in freshly fertilized eggs or in 1-cell-stage embryos by whole-mount in situ hybridization (data not shown). This is most likely due to a limit in the sensitivity of the whole-mount in situ hybridization protocol. *vas* transcript was detected from the 2-cell stage, however.

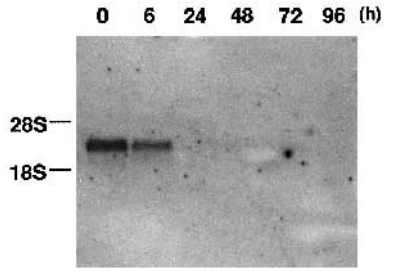


**Fig. 2.** Northern blot analysis shows that *vas* is maternally supplied. Total RNA from embryos at various developmental stages was blotted and probed with a *vas* cDNA fragment. Hours after fertilization are shown. Equivalent amounts of total RNA were loaded as judged by the amounts of 28S and 18S rRNA visible (not shown).

The zebrafish zygote undergoes a series of rapid, synchronous meroblastic cleavages so that the dividing embryo is situated on top of the non-cleaving yolk cell. As shown in Fig. 3A,B (see also Fig. 6 for schematic summary), *vas* transcript is first detected by in situ hybridization at the 2-cell stage (45 minutes postfertilization at 28.5˚C). Strikingly, the transcript is seen along the cleavage plane. It is not localized along the entire length of the plane, but in short stripes of expression, generally closer to the yolk than the center of the embryo. By the 4-cell stage (1 hour), expression along the first cleavage plane has become stronger and expression is detected in addition along the second cleavage plane, which is perpendicular to the first (Fig. 3C). Again, the expression does not extend along the entire length of the plane.

At the next two cleavages, *vas* expression along the first and second cleavage planes persists and remains strong. The expression that began as four lines along the two cleavage planes at the 4-cell stage starts to condense into clumps as early as the 8-cell stage (Fig. 3D) and, by the 32-cell stage (1.5 hours), *vas* expression is detected in four cells (data not shown). As can be seen in a section of an in situ hybridized 32-cell-stage embryo (Fig. 5A), the RNA appears as a single clump in the cytoplasm, considerably smaller in diameter than the cell in which it is contained. Weaker expression is often seen along other cleavage planes at the 8- and 16-cell stages or in other cells around the margin of 32- and 64-cell-stage embryos. This expression does not become as strong as the first four regions of expression and was not detected at later stages.

*vas* RNA remains localized to exactly four cells through the 1000-cell stage (3 hours) (Fig. 3E). By the dome stage (4.5 hours), however, when there are approximately 4000 cells in the embryo, the RNA is detected in four to twelve cells per embryo and is no longer subcellularly localized, but appears to fill the cytoplasm (Figs 3F, 4A,B). The midblastula transition, when zygotic transcription begins, occurs at approximately 3 hours postfertilization in zebrafish (Kane and Kimmel, 1993). *vas* expression detected at dome stage in multiple cells is probably due to new transcription from the embryonic genome and to division of the cells that first contained subcellularly localized *vas* RNA.

At the shield stage (6 hours), there are about 16-25 *vas*-expressing cells per embryo (Fig. 4C,D). They are usually in four separate groups, spaced around the embryo, and generally near the margin. It appears that the four cells that inherited maternal *vas* RNA have undergone at most three divisions to generate the numbers of positive cells that we detect. Double in situ hybridization of shield-stage embryos with a *goosecoid* probe, serving to mark the position of the shield (Statchel et al., 1993), indicates that the position of the *vas*-expressing cells is different relative to the shield in different embryos.

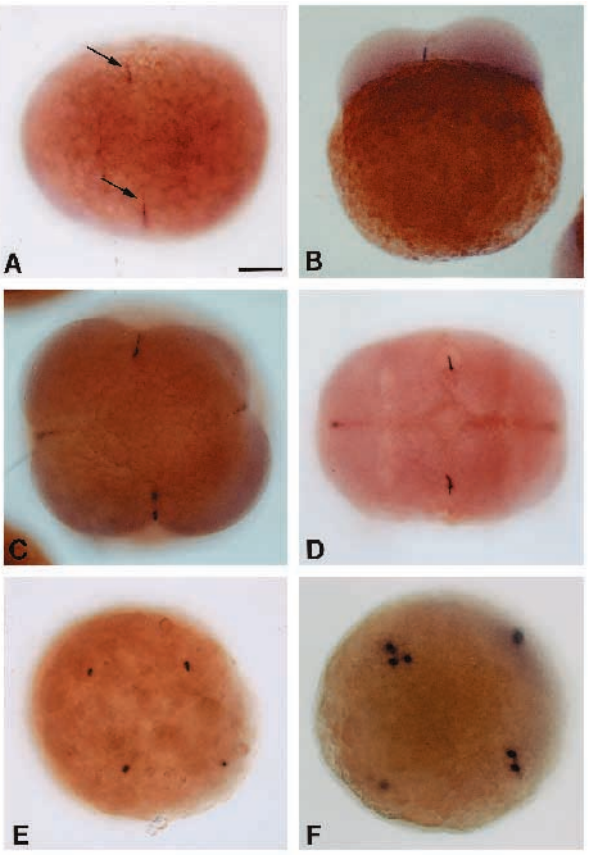


**Fig. 3.** Whole-mount in situ hybridization on early cleavage to dome-stage embryos. Embryos were hybridized with a *vas* cDNA fragment riboprobe. (See also Fig. 6 for schematic summary.) All panels show top views of embryos, except in B (side view). *vas* transcript is detected along the cleavage planes (arrows) of embryos at the 2-cell stage (A,B, same embryo) and 4-cell stage (C). These lines of expression persist through the 8-cell stage (D). *vas* RNA expression condenses into 4 subcellular clumps by the 32-cell stage (not shown), and remains in this configuration through the 1000-cell stage (E). By the 4000-cell stage (dome stage), *vas* RNA is no longer subcellularly localized and appears to fill the cytoplasm (F). There are 4-12 *vas*-expressing cells per embryo at the dome stage. Scale bar, 100 μm.

During the remainder of epiboly, when cells move to completely enclose the yolk, *vas* expression is detected in approximately 30 cells per embryo (Fig. 4E-H). Using zebrafish *Brachyury* as a marker for the notochord and the germ ring (Schulte-Merker et al., 1992), *vas*-expressing cells are seen to lie in positions that vary from embryo to embryo. In general, however, *vas* was found in cells that were approximately half way between the leading edge of the blastoderm and the anterior of the embryo. As epiboly progresses, the four groups of *vas*-expressing cells appear to move towards the dorsal side of the embryo and cluster into two groups on either side of the midline, the same distance to the right and left of the notochord. Although the clustering of the cells into two groups is generally completed by the beginning of somitogenesis (10

hours), variability is often seen within a clutch of embryos. For example, the *vas*-expressing cells are occasionally seen to trail down one side of the embryo, or extend along the length of the body.

During somitogenesis, the *vas*-expressing cells remain clustered at the level of the third to fifth somite, as determined by double in situ hybridization with *MyoD*, which is expressed in the somites (Weinberg et al., 1996) (Fig. 4I,J). Transverse sections of in situ hybridized embryos at this stage show that the *vas*-expressing cells are located in a peripheral region (Fig. 5B). They remain in this position throughout somitogenesis (Fig. 4K,L). By 24 hours, the cells are located where the yolk ball

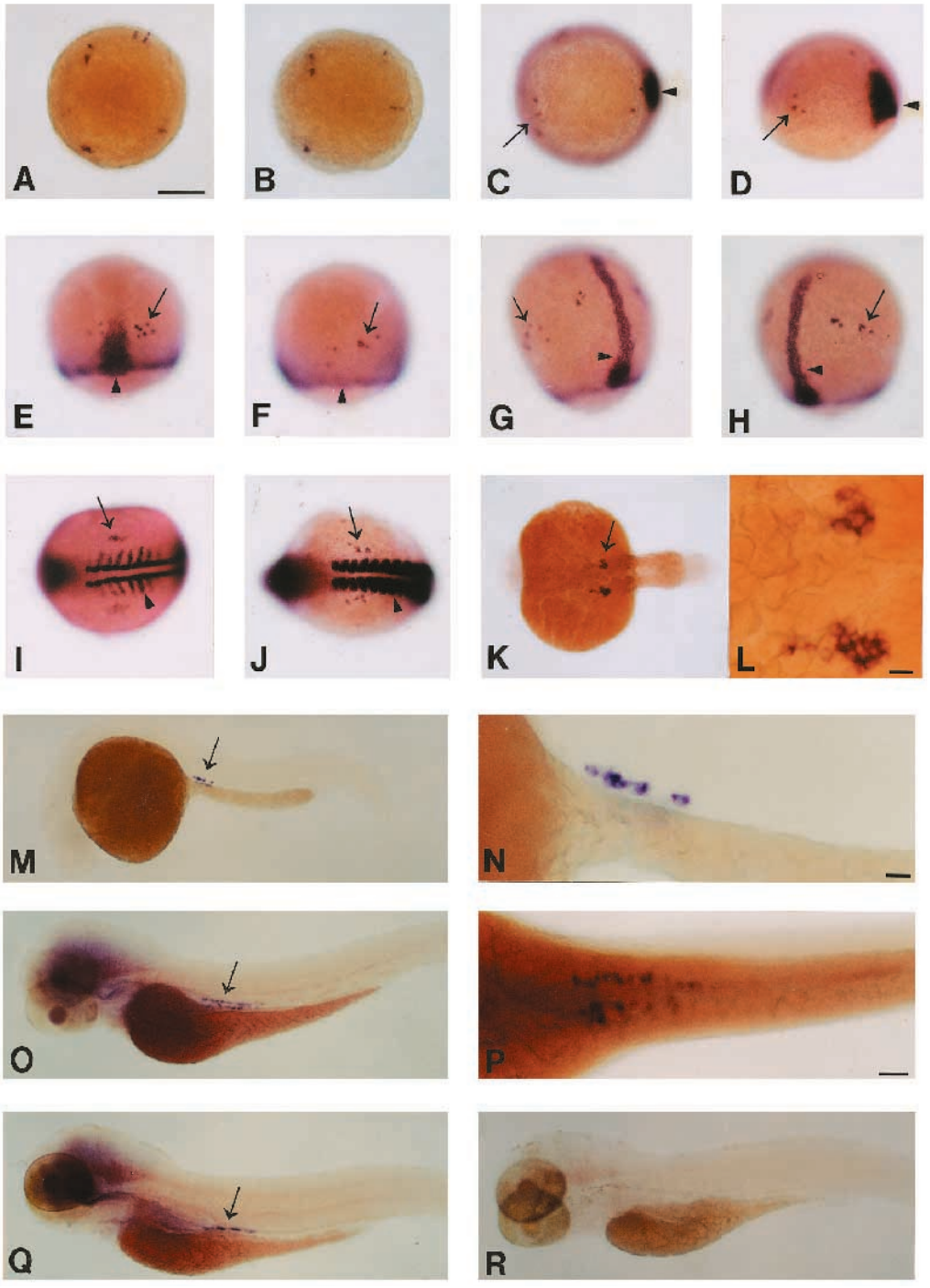


**Fig. 4.** Whole-mount in situ hybridization on older embryos and larvae. Embryos were hybridized with a *vas* riboprobe. The cells expressing *vas* are indicated with an arrow. In embryos that were simultaneously hybridized with *vas* and a second probe to mark the location of particular structures, the cells expressing the second gene are indicated with an arrowhead. In I-R, embryos and larvae are oriented such that anterior is to the left. (See also Fig. 6 for schematic summary.). (A,B) Top views of 2 different 4000-cell-stage (dome) embryos show that the location and numbers of *vas*-expressing cells are similar but not identical in different embryos. (C,D) Top view (C) and side view (D) of a single embryo double labeled with *vas* (arrow) and *goosecoid* (arrowhead). *Goosecoid* is expressed in the dorsal shield (Statchel et al., 1993). The position of the *vas*-expressing cells relative to the shield varies. (E-H) Embryos undergoing epiboly double labeled with *vas* (arrow) and *Brachyury* (arrowhead). *Brachyury* is expressed in the developing notochord and the germ ring of the embryo (Schulte-Merker et al., 1992). The dorsal view (E) and ventral view (F) of the same embryo at 70% epiboly show the *vas*-expressing cells are located around the margin of the embryo. These cells migrate towards the dorsal side of the embryo as seen in the left side view (G) and right side view (H) of one embryo at 90% epiboly. (I-J) Dorsal views of embryos at the 6-somite stage (I) and 10-somite stage (J) are double labeled with *vas* (arrow) and *MyoD* (arrowhead). *MyoD* is expressed in the somites (Weinberg et al., 1996). As seen in I, the *vas*-expressing cells have clustered on either side of the midline at the level of the third to fifth somite. These cells remain in the same relative position during somitogenesis (J). (K-L) Dorsal view of 20-somite embryo shows that *vas*-expressing cells remain in 2 clusters (K). Higher magnification view (L) reveals that *vas* RNA is present in the cytoplasm. (M-Q) *vas*-expressing cells (arrow) extend posteriorly in 2 bilateral rows during late embryonic and early larval development. (M,N) Side view of 24 hour embryo at low (M) and high (N) magnification. (O,P) Low magnification side view (O) and higher magnification dorsal view (P) of 3 day larva. (Q) Side view of 4 day larva. (R) Side view of a representative sense control (4 days). Scale bars, 200 μm except 20 μm in L, N and 50 μm in P.

meets the yolk tube, and relative to the somites, their position is unchanged (Fig. 4M,N). The *vas* transcript still appears to fill the cytoplasm (Figs 4L, 5C). As development proceeds, the *vas*-expressing cells extend posteriorly for a variable distance to form two bilateral rows of cells dorsolateral to the gut (Figs 4O,P,Q, 5D). Generally, the number of cells expressing *vas* transcript is approximately the same on the left and right sides of the animal.

The latest stage at which we performed whole-mount in situ hybridization is 10 days (data not shown). At this stage in development, the larvae are well developed and are swimming and feeding vigorously. The number of *vas*-expressing cells is seen to have increased greatly, while remaining in two bilateral rows of cells that appear to have coalesced into a gonad. The two rows of cells are located lateral to the caudal portion of the swim bladder, dorsolateral to the gut and ventral to the pronephric tubules (Fig. 5E).

## DISCUSSION

We identified a gene, zebrafish *vasa* homologue (*vas*), whose transcript serves as a marker for the zebrafish germ line. The position and number of *vas*-expressing cells during somitogenesis and onwards suggest that they are the zebrafish PGCs. Most strikingly, by the 4-cell stage, maternally supplied *vas* RNA is localized into four stripes located along the first two cleavage planes (Fig. 3A-D). These stripes condense into four subcellular clumps that persist during early cleavage stages (Fig. 3E). We believe that, soon after the midblastula transition, the four cells that inherit these clumps begin to express *vas* RNA zygotically and undergo several cell divisions. We recognize that further experiments will be necessary to confirm that the *vas*-expressing cells in early embryos are the founding population of germ cells. However, our in situ hybridization experiments were performed on many closely spaced time points and suggest that we are observing the origin, replication and migration of a single cell population, the zebrafish PGCs.

### *vas* is the homologue of the *Drosophila vasa* gene

Previously, putative homologues of *Drosophila vasa* (Hay et al., 1988b; Lasko and Ashburner, 1988) had been cloned in *Xenopus* (Komiya et al., 1994), mouse (Fujiwara et al., 1994) and rat (Komiya and Tanigawa, 1995). The predicted amino acid sequence of *vas* is highly similar to that of these vertebrate *vasa* genes (Fig. 1). Closer examination of the predicted amino acid sequences of these genes reveals the presence of signature amino acids, suggesting that these genes are true *vasa* homologues. These amino acids include tryptophan (W) residues near the start and stop codons, and a glycine (G)-rich region in the $NH_2$-terminal portion of *vas*, containing multiple arginine-glycine-glycine (RGG) repeats. Furthermore, *vas* encodes a protein that contains the eight conserved regions found in DEAD-box protein family members (Linder et al., 1989; Fujiwara et al., 1994), including *Drosophila* vasa and its vertebrate homologues. Therefore, we conclude that *vas* is probably the zebrafish *vasa* homologue.

### *vas* RNA is expressed in the zebrafish primordial germ cells

In other fish species, primordial germ cells have been identified by morphology (Wolf, 1931; Dildine, 1936; Johnston, 1951; van den Hurk and Slof, 1981; Hamaguchi, 1982; Lebrun et al., 1982; Brusle, 1983; Timmermans and Taverne, 1989).

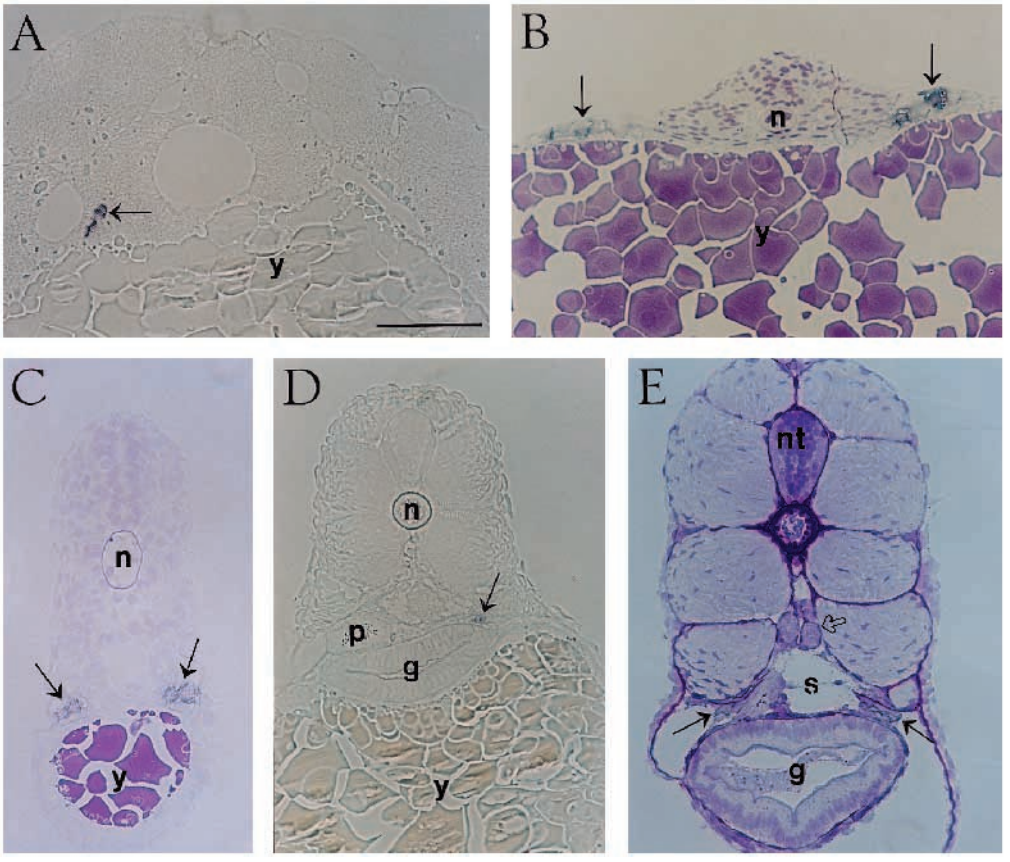


**Fig. 5.** Histological analysis of in situ hybridized embryos and larvae. Transverse sections (2 μm) were prepared following in situ hybridization. *vas*-expressing cells are indicated with a black arrow. (A) 32-cell, (B) 6-somite, (C) 24 hours, (D) 4 days, (E) 10 days. Note that, in A, at the 32-cell stage, the clump of *vas* RNA is considerably smaller than the size of a single cell, whereas in the older stages (B-E), the RNA is present throughout the cytoplasm. The position of the *vas*-expressing cells dorsolateral to the gut (g) and ventral to the pronephric tubules (outlined arrow) in 10 day larvae (E) suggests that these cells are the zebrafish PGCs. Other abbreviations: n, notochord; nt, neural tube; p, pancreas; s, swim bladder; y, yolk. Scale bar, 50 μm.

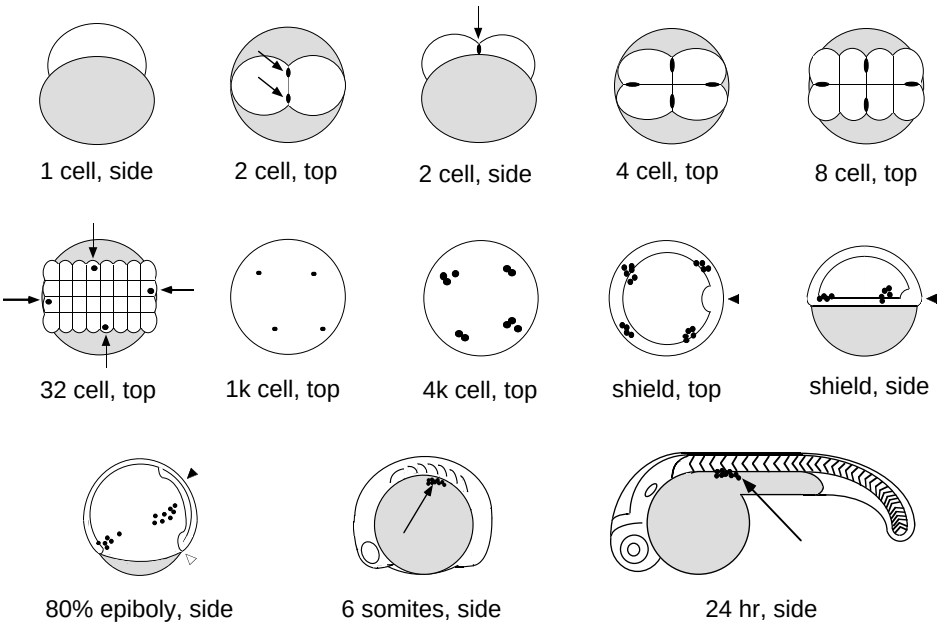


**Fig. 6.** Schematic summary of zebrafish germ-line development during embryogenesis. Schematic drawings of *vas* RNA expression in zebrafish embryos as determined by whole-mount in situ hybridization (Figs 3, 4), indicates that *vas* RNA is expressed in the zebrafish primordial germ cells (PGCs). The stages and views are indicated below each drawing and the yolk is shaded. Arrows point to the *vas*-expressing cells in selected stages. In shield-stage embryos, the arrowhead indicates the dorsal shield. In the 80% epiboly embryo, the black arrowhead indicates the developing notochord whereas the white arrowhead indicates the germ ring. *vas* RNA is first detected along the first two cleavage planes (arrows), then condenses into four subcellular clumps by the 32-cell stage (arrows). These 4 clumps are segregated to four cells through the 1000-cell stage. By the 4000-cell stage, the cells that inherited *vas* RNA, the PGCs, have begun to divide and *vas* RNA is located throughout the cytoplasm. Mitoses continue during early gastrulation to generate approximately 30 PGCs. *vas* RNA detected up to the 1000-cell stage is presumably maternal RNA, while after that it is presumed to be derived by zygotic transcription. During epiboly until early somitogenesis, the PGCs migrate towards the dorsal side of the embryo, forming two clusters of cells to the right and left of the notochord, adjacent to the third to fifth somite. They remain in this position through early larval stages, extending posteriorly for a variable distance to form two bilateral rows of cells in the gonadal anlagen and later resume mitosis (not shown).

They can be recognized with certainty after they have migrated to the gonadal anlagen and then, by working backwards in time using morphology as the criterion, in some species their location can be followed as far back as somitogenesis (Hamaguchi, 1982; Timmermans and Taverne, 1989). By early larval stages, zebrafish *vas*-expressing cells are seen at the site of the gonadal anlagen, dorsolateral to the intestine and ventral to the pronephric tubules (Figs 4O-Q, 5D,E), similar to that of PGCs described in rosy barb (Timmermans and Taverne, 1989), medaka (Hamaguchi, 1982) and carp (Parmentier and Timmermans, 1985). Furthermore, during somitogenesis (Fig. 4I-L), the *vas*-expressing cells are located in a position similar to the position of the PGCs in rosy barb (Timmermans and Taverne, 1989) and medaka (Hamaguchi, 1982). Although the PGCs are clustered adjacent to a more anterior somite (third through fifth somites) in zebrafish than in the rosy barb (near the tenth somite), the PGCs remain in their respective positions relative to the somites throughout somitogenesis in both organisms. Moreover, the positioning of the *vas*-expressing cells in the periphery, above the periblast during somitogenesis (Fig. 5B) is analogous to the position of the PGCs in medaka at a similar stage (Hamaguchi, 1982). The similarities in the position of the PGCs in other fish with the *vas*-expressing cells in zebrafish form the basis for our conclusion that the *vas*-expressing cells during and after the somite-stages are the zebrafish PGCs.

Since the DEAD-box family of RNA helicases shares a highly homologous RNA-binding domain, it is possible that our *vas* cDNA probe may cross-hybridize to other RNA helicases. We do not believe that this is the case, however. Although PCR using degenerate primers amplified the zebrafish *vas* homologue and *p68*, which both encode DEAD-box proteins (see Results), it was subsequently shown that our *vas* cDNA PCR product recognized a single band on a Southern blot (data not shown). Furthermore, using a *vas* cDNA fragment as a probe for northern blot hybridization, we detected a single band of the same size both before the mid-blastula transition (Kane and Kimmel, 1993) and also after zygotic transcription has begun (Fig. 2).

As for the earlier time points, we cannot be certain from our data that the *vas*-expressing cells that we detect before somitogenesis are the presumptive PGCs. Further experiments, such as ablation or transplantation of these cells, are necessary to confirm that the cells expressing *vas* RNA during cleavage, blastula and gastrula stages are indeed the precursors of the germ-cell lineage. Nonetheless, the consistent and continuous pattern of replication and migration of *vas*-expressing cells that we detected by whole-mount in situ hybridization is most simply interpreted as revealing a single population of cells from early cleavage (Fig. 3) through late larval stages (Fig. 4). Our data suggest, therefore, that we are observing the expression of a single gene, *vas*, in a single cell lineage, the PGCs.

### Number of PGCs

Studies by Walker and Streisinger (1983) led to an estimate of the number of germ cells in the early zebrafish embryo. By irradiating cleavage-stage embryos and subsequently analyzing mutant clone size at a pigmentation locus, *gol-1*, they estimated that the average number of PGCs in the zebrafish embryo is about 5 up until the 2000- to 4000-cell stages. Our observation that there are 4 *vas*-expressing cells during this time period is in striking agreement with their conclusions. Interestingly, when we made chimeras in our laboratory by transplanting about 50 cells from genetically pigmented embryos to albino

embryos when both were at the 1000- to 2000-cell stages, we never obtained more than 20% germ-line chimeras among the recipients (Lin et al., 1992). If we assume that the 4 cells containing subcellularly localized *vas* RNA at the 1000-cell stage are predetermined germ cells, then one in 250 transplanted cells would be a predetermined germ cell, resulting in an expected frequency of obtaining one germ-line chimeras for every 5 chimeras generated, the frequency that we observed. Further experiments would be needed, however, to learn whether the fate of *vas*-expressing cells is determined at the time of transplantation.

It is also interesting to note that the segregation of *vas* RNA to exactly 4 cells (Fig. 3E) during early cleavages is strikingly similar to the segregation of germ plasm in *Xenopus laevis* to exactly 4 presumptive PGCs until the 32-cell stage (Whitington and Dixon, 1975). The process by which this occurs in *Xenopus* embryos is somewhat different from the distribution of *vas* transcripts in early zebrafish embryos, however. *Xenopus* germ plasm is localized to the vegetal pole of *Xenopus* oocytes and is partitioned between each of the first 4 blastomeres by the first two cleavage planes. During subsequent cleavages, the germ plasm becomes positioned to one of the mitotic spindles and, as a consequence of its asymmetric positioning within these cells, it becomes segregated to one daughter cell at each of the early cleavages, maintaining 4 presumptive PGCs at the 32-cell stage. 'Nuage,' the electron-dense granulofibrillar component of the germ plasm (reviewed by Eddy, 1975), has been identified in zebrafish oogonia and developing oocytes (Selman et al., 1993). However, we do not know whether nuage is present in early embryos and whether it plays a role in the development of the zebrafish PGCs. It will be informative to determine whether *vas* RNA is localized to nuage in zebrafish.

It appears that the 4 zebrafish presumptive PGCs from the 1000-cell stage (Fig. 3E) undergo at most 3 mitoses during gastrulation to generate about 30 PGCs that migrate to the gonads (Fig. 4). The number of PGCs that are generated by the initial mitoses is similar to the number of PGCs in other fish during this period of development. In rosy barb (Timmermans and Taverne, 1989), medaka (Hamaguchi, 1982), and carp (Parmentier and Timmermans, 1985), the number of PGCs is between 30 and 50 per embryo during somitogenesis and does not increase greatly during the migratory period. The generation of a small founding population of PGCs and an absence of mitoses during their migration to the gonads is a general characteristic of germ cells in most species (Wei and Mahowald, 1994). As in other organisms, the zebrafish PGCs apparently resume mitosis once they have populated the gonad.

### Maternally supplied *vas* transcripts are specifically localized to cleavage planes at the 2- and 4-cell stages

The fact that, in zebrafish, zygotic transcription does not begin until the midblastula transition, which occurs at the 1000-cell stage (Kane and Kimmel, 1993), together with our result that *vas* RNA can be detected in a northern blot of RNA from freshly fertilized eggs (Fig. 2), argues that *vas* transcript is supplied maternally. As for the RNA that is detected by in situ hybridization from the 2-cell stage (Fig. 3A,B), it is probable that this is maternal RNA that aggregates into visible clumps along the cleavage plane, since the first cleavage occurs only about 45 minutes after fertilization. In fact, in unfertilized eggs that are incubated at 28.5˚C for 2.5 hours before fixation, *vas* RNA aggregates, apparently at random, into small blobs (data not shown). The fact that these blobs that form in unfertilized eggs seem to be randomly distributed throughout the cytoplasm, without a consistent number or size, suggests that cleavages are not necessary for the aggregation of *vas* RNA per se, but may play a role in correctly positioning *vas* RNA during the first two cleavages.

Given that *vasa* transcript is distributed throughout the *Drosophila* egg, the highly restricted, subcellular localization of *vas* transcripts in cleavage-stage zebrafish embryos was a surprise. However, localization of maternally supplied RNAs to germ plasm and then to embryonic germ cells is not in itself a new finding (reviewed by St. Johnston, 1995). What is novel in our studies is the actual site of localization of *vas* transcript: four short regions along the first two cleavage planes (Fig. 3A-D). This unusual localization pattern results in four subcellular clumps of *vas* RNA that persist within the embryo as cells continue to cleave around them and it provides a way for *vas* RNA to be segregated to exactly four cells (Fig. 3E). The molecular mechanism regulating the attachment of *vas* transcripts to the cleavage planes has not yet been determined. Cytoskeletal components or membrane-bound proteins might be expected to be involved (St. Johnston, 1995).

The function of the protein encoded by *Drosophila vasa* and its vertebrate homologues is not fully understood yet. Since they contain a DEAD-box protein sequence, it is suggested that their function in germ-cell determination is related to their ability to bind to and unwind RNA. These activities have been demonstrated for the *Drosophila* vasa protein in vitro (Liang et al., 1994). No biochemical studies of the vertebrate *vasa* homologues have been performed. A possible role for the vasa protein could be to bind to RNAs required for germ-cell determination and control their translation. Interestingly, it has been shown in *Drosophila* that, while vasa protein is necessary for pole cell development, it is not sufficient (Hay et al., 1990; Lasko and Ashburner, 1990). Further studies of the zebrafish vasa protein, such as its localization, will lead to a better understanding of its role in germ-line development.

The finding that *vas* transcripts are highly localized to cytoplasm that is apparently distributed to the future germ cells suggests ways in which one may be able to label and then purify this population of cells. At the minimum, these findings provide an assay for germ cells and might help us to determine culture conditions under which to propagate this important cell type. Whether this can be done and whether the cells could retain totipotency and be returned to the animal to participate in normal development will be important lines of investigation in the future.

We thank Brett Hayward, Jennifer Mugg and Dean Thompson for fish care, Robert Riggleman and Kathryn Helde for the cDNA library, David Grosshans for assistance with sequencing and Pat Reilly for assistance with sectioning. We also thank Miguel Allende, Carlos Semino, Matthias Hammerschmidt, and members of the Hopkins laboratory for reagents, technical advice and helpful discussions during the course of this work. We are grateful to Rudolf Jaenisch, Hazel Sive, Judy Plesset and Janni Nusslein-Volhard for their interest. We are especially grateful to Ruth Lehmann for her valuable encouragement, advice, and critical reading of the manuscript. C.Y. was supported by an NSF pre-doctoral fellowship and K.K. was supported by fellowships from Yamada Science Foundation and Toyobo

Biotechnology Foundation. This work was supported by Core Grant (CA14051) and grants from NSF and Amgen (to N.H.).

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(Accepted 10 June 1997)

## Note added in proof

L. C. Olsen, R. Aasland and A. Fojose have also cloned a zebrafish *vasa* homologue and studied expression of this gene during embryonic development (Mechanism of Development, in press).