Identification of the Zebrafish Primordial Germ Cells

by

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B.S., Molecular Biophysics and Biochemistry
Yale College, 1992

Submitted to the Department of Biology
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

at the

MASSACHUSETTS INSTITUTE OF TECHNOLOGY

September, 1997

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ABSTRACT

Despite the recent intensive interest in studying the zebrafish as a model system for vertebrate embryonic development, the identification of the germ cells in this organism had been elusive. The answers to the basic questions of where and when the germ cells arise during embryonic development remained unexplored largely because of the absence of a germ cell marker.

Here I describe two approaches I took to study the development of the zebrafish germ line, one of which was successful in identifying the primordial germ cells of the fish. First, to map the origin of the germ line, we used cell transplantation. When donor cells were placed at the margin of the recipient embryo, they were more likely to contribute to the germ line than when they were placed at the animal pole. This suggested that the germ line might originate from a specific location around the margin of the blastoderm. But in fact, we were unable to map this location more precisely.

The second area of research involved identifying a marker for the zebrafish primordial germ cells. I cloned the zebrafish homologue of the *Drosophila vasa* gene (zebrafish *vas* homologue) and analyzed the *vas* RNA expression pattern. I find that the *vas* transcript is specifically expressed in the primordial germ cells and has allowed their identification for the first time. Moreover, the RNA is localized to the cleavage planes at the 2- and 4-cell stages of development. During subsequent cleavages, the RNA is segregated as subcellular clumps to a small number of cells that may be the future germ cells.

I have found a novel mechanism of maternal RNA localization that appears to mark the germ line at an extremely early stage of development. These results now suggest new ways in which to develop techniques for the genetic manipulation of the zebrafish.

Thesis Supervisor: Nancy Hopkins

Title: Professor of Biology

ACKNOWLEDGEMENTS

I would like to acknowledge and thank those who worked with me on various aspects of this thesis: Miguel Allende, for spending long hours sorting the chimeras and cleaning the fish boxes with me; David Gerber, for guiding me throughout the monoclonal antibody production; Vivek Nadkarni, Cecilia Song, and Regina Burris, for their assistance with the antibodies; Noriyoshi Sakai, for helping me with the initial stages of the *vasa* cloning and with identifying the cells in the testis; and Koichi Kawakami, for helping with various molecular aspects of the *vasa* project. I also thank the past and present members of the Hopkins lab for technical advice and making the lab a pleasant place to work.

I am grateful to those people who guided my scientific career: Nigel Grindley, who gave me my first lab job; Dieter Soll, my undergraduate major advisor who mentored me throughout my college years; Bruce Demple, in whose lab I had my first publication; Bob Sauer for career advice; and Frank Solomon, for his caring and patience during many long conversations.

I also thank the members of my thesis committee: Rudolf Jaenisch, Ruth Lehmann and Hazel Sive for their interest, guidance and helpful discussions throughout the course of this work.

Also, I thank those who helped me survive graduate school: Nick Gaiano, Koichi Kawakami, Shuo Lin, and Noriyoshi Sakai for their friendship in the lab; my classmates Joe Delaney, Doug Fenger, Ken Poss, and Irena Royzman who made me realize I wasn't alone; Dindial Ramotar, for his encouraging e-mails; Jeffrey Botman, for the great advice and insights about my life; and the past and present members of my Bible study, for their prayers and support. Thank you!

Even though all the thanks in the world wouldn't be enough, I would like to thank my family: my parents, for loving and supporting me, for listening to me and trying to understand me during the tough times; my sister Laura, my best and most caring friend; Ee-mo and Ee-mo-bu, for their e-mails and phone calls; my in-laws, for encouraging me in my faith; and my husband, Sang, for his love and devotion, from the day we met, and for all that he endured last year--you really do deserve the "best supporting husband" award!

My thesis advisor, Nancy Hopkins, deserves special thanks. I am deeply indebted to her for her support and guidance over the past 4 years. In particular, I will never forget her concern for me during the difficult times. I am very thankful for helping me make it to the finish line. For this I will be forever grateful.

And finally, I thank God because none of this would have been possible without His grace and perfect love for me.

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CHAPTER 1

INTRODUCTION

Primordial germ cells and the origin of the germ line

Germ cells have the unique role of transmitting genetic information to the next generation, and although sperm and eggs are highly differentiated, when joined together at fertilization, they form a single totipotent cell, the zygote. Prior to sex-specific differentiation, these cells are referred to as primordial germ cells (PGCs). PGCs are often set aside early in development, originate extra-gonadally, and undergo extensive migrations to the gonad. They have been shown to be transcriptionally inactive in flies and worms, which may serve to prevent differentiation of PGCs along somatic pathways (Zalokar, 1976; Seydoux and Fire, 1994). There is a relatively small founding population of PGCs that colonize the gonad where they either mitotically proliferate to produce more gonial stem cells or undergo meiosis and sex differentiation (for review, see Wei and Mahowald, 1994).

In the 1930s, Bounoure discovered the existence of germ plasm in the egg of *Rana temporaria* and studied its segregation to the germ cells during embryogenesis (Bounoure, 1939). He noticed small subcortical patches of specialized cytoplasm, which he called "germinal plasm," present in the vegetal pole of the oocyte. During the first 2 cleavages, it was equally partitioned to the 4 blastomeres, but during subsequent cleavages, germ plasm was segregated to only one of the 2 daughter cells upon division, thereby restricting the number of germ plasm-containing cells to 4. During the late blastula, the position of the germ plasm within the cell changed, resulting in equal segregation of germ plasm to both daughter cells upon division, thereby increasing the number of germ plasm-containing cells. Because these germ plasm-containing cells migrated out of the endoderm and into the genital ridges where they colonized the gonad, they were called PGCs. This observation led to the idea of an uninterrupted germ line from the fertilized egg to the adult organism. The same pattern of germ plasm segregation was later shown to hold true for another anuran, *Xenopus laevis*, although in *Xenopus*, the clonal divisions did not take place until early gastrula stages (Whitington and Dixon, 1975).

The presence of an uninterrupted germ line can also be found in *Drosophila* and *C. elegans* and is also characterized by the presence of specialized cytoplasm. In *Drosophila*, germ plasm is referred to as pole plasm (or polar granules) as it is found at the posterior pole of the oocyte (Mahowald, 1962). After fertilization, the fly embryo develops through a syncytial stage, in which nuclei divide without cytokinesis. The first cells to cellularize in the embryo are the germ cells (pole cells), which form from nuclei that migrate to the posterior pole (Huettner, 1923; Sonnenblick, 1941). Pole cells contain pole plasm throughout their development (Mahowald, 1971).

The germ plasm equivalent found in *C. elegans* oocytes is referred to as P granules and their segregation pattern is similar to that of germ plasm segregation in *Xenopus* (Strome and Wood, 1982). Initially randomly located throughout the oocyte cytoplasm, P granules become localized in the posterior cytoplasm following fertilization so that at the first cleavage, they are exclusively segregated to the P1 cell. This posterior prelocalization of the P granules occurs prior to each of the next 3 cleavages, resulting in the progressive segregation of P granules to P2, P3, and finally P4, the exclusive precursor of the germ line (Deppe et al., 1978; Strome and Wood, 1982). Subsequent divisions of the P4 cell result in equal segregation of P granules to its daughters. P granules persist in germ cells throughout embryonic, larval and adult stages (Strome and Wood, 1982).

There are organisms in which no germ plasm equivalent exists in the early embryo, which include mouse (Everett, 1943), chick (Swift, 1914) and the teleosts, rosy barb (Timmermans and Taverne, 1989) and medaka (Hamaguchi, 1982). It is not until late gastrula or somite stages that germ cells have been identified on the basis of morphology in these organisms, possessing such characteristics as large size compared to surrounding cells, oval or round shape, large nucleus, dense and granular cytoplasm and pseudopodia. Moreover, there is little evidence that germ cell determination in these organisms depends on maternal factors localized in the oocyte.

In the mouse, germ cells have been shown to express high levels of alkaline phosphatase (ALP) which can be detected by staining for ALP activity (Chiquoine, 1954). In addition to confirming the morphological identification of PGCs (Ozdzenski, 1967), ALP staining was used to detect PGCs at an even earlier stage than had been seen previously. At 7 days post coitum (dpc), the beginning of gastrulation, a small cluster of ALP-positive cells was detected posterior to the primitive streak, in a bulge of extraembryonic mesoderm (Ginsburg et al., 1990). In an effort to learn more about the origin of the germ line before gastrulation, single epiblast cells were injected with a lineage tracer dye just before the onset of gastrulation and embryos were cultured, then analyzed for the presence of labeled PGCs. The results of this experiment suggested that PGCs are located in the proximal epiblast adjacent to the extraembryonic ectoderm before gastrulation and become lineage restricted early in gastrulation (Lawson and Hage, 1994).

The question of the origin of the avian germ line led researchers to pursue further morphological and experimental studies on the PGCs in the chick. Previous observations in chick embryos first identified PGCs at early somite stages in the "germinal crescent," the anterior, extra-embryonic crescent-shaped area at the boundary between the area pellucida and the area opaca (Swift, 1914). At this stage of development, the PGCs can be stained for their high glycogen content using the periodic acid-Schiff (PAS) reaction (Meyer, 1960; Meyer, 1964). Later in vitro experiments suggested, however, that the PGCs actually derive from the epiblast at an earlier stage (Eyal-Giladi et al., 1981; Sutasurya et al., 1983; Ginsburg and Eyal-Giladi, 1986), and these suggestions were confirmed using antibodies that specifically label PGCs (Urven et al., 1988) and further morphological studies of the blastula stages (Muniesa and Dominguez, 1990). And finally, by culturing isolated fragments from various positions of the early embryo and incubating them till somite stages when PGCs can be unambiguously identified using the PAS reaction, the origin of the PGCs was mapped to the most central part of the blastodisc (Ginsburg, 1994).

In rosy barb, the PGCs were first identified from 12 hr post fertilization, during early somitogenesis, located between mesoderm and the yolk syncytial layer and to the right and left of the notochord, at the level of the tenth somite (Timmermans and Taverne, 1989). Evidence that these cells are PGCs came from the discovery that they contain nuage, an electron dense germ plasm component (Eddy, 1975; Timmermans and Taverne, 1989). To study the origin of the PGCs, single cells were injected at the 64-cell stage and labeled PGCs were identified at 12 hr post fertilization (Gevers et al., 1992). Because the location of the injected blastomeres that gave rise to labeled PGCs varied with respect to the embryonic axis, they were unable to identify germ cell progenitors from this stage. They did, however, see a difference between injecting an "upper layer cell" versus a "lower layer cell." Cells in the upper layer never gave rise to PGCs and tended to give rise to embryos with more ectodermal labeling, whereas injecting cells in the lower layer gave rise to labeled PGCs about 25% of the time and tended to yield more endodermal labeling.

In summary, an uninterrupted germ line can be found in invertebrates, such as *Drosophila* and *C. elegans*, and in anuran amphibians, in which germ plasm present in the oocyte is segregated to the PGCs during embryonic development and remains associated with the germ line throughout the life of the organism. For the other vertebrates, such as birds, mammals and fish, PGCs have been identified in gastrula or somite stages by morphology. Further investigations on the origin of the germ line were accomplished using various staining techniques and fate mapping experiments, where for mouse and chick, PGCs seem to arise from embryonic ectoderm.

The role of germ plasm in germ cell determination

The observation that germ plasm is present in germ cells of frogs, flies and worms throughout the life of the organism raises the question of the significance of germ plasm for germ cell determination and differentiation. To address this question, researchers have

tried removing or destroying germ plasm and transplanting germ plasm to an egg lacking functional germ plasm, particularly focusing on anurans and *Drosophila.*

Evidence that germ plasm is required for germ cell development in anurans came from physically removing germ plasm (Buehr and Blackler, 1970) or destroying germ plasm by u.v. irradiation (for review, Smith and Williams, 1979), resulting in the reduction or absence of germ cells and a delay in their migration. It was further shown that u.v.-irradiated embryos could be rescued by injecting unirradiated vegetal cytoplasm (Smith, 1966) and injection of extra vegetal pole cytoplasm into unirradiated eggs could increase the numbers of germ cells (Wakahara, 1978). The idea therefore developed that the inheritance of a germ cell determinant present in the vegetal cytoplasm of the anuran embryo would cause that cell to become a germ cell. However, experiments in *Xenopus* showed that although transplantation of single blastomeres containing germ plasm can populate genital ridges of host embryos (Ikenishi, 1987), they are not irreversibly committed to contributing to the germ line and may adopt other cell fates (Wylie et al., 1985). It is possible that germ plasm may still be a germ cell determinant, but at a later stage than was tested (blastula and early swimming tadpoles; Wylie et al., 1985), or it may have the role of being required for the migration or targeting of the PGCs (Smith et al., 1983).

In *Drosophila*, similar experiments demonstrating that pole plasm contains a germ cell determinant have been performed. Pole plasm taken from oocytes can induce ectopic pole cell formation in host embryos at the site of injection (Illmensee and Mahowald, 1974, 1976), and it is also able to restore fertility to u.v.-irradiated sterile embryos (Okada et al., 1974). Newly formed pole cells have been transplanted and were shown to exclusively populate the gonad, indicating that they are determined from the time of pole cell formation (Underwood et al., 1980; Technau and Campos-Ortega, 1986). Furthermore, genetic screens have been done in *Drosophila* and have identified maternal effect sterility mutants (Schupbach and Wieschaus, 1986). It was found that in mutants that disrupt pole plasm assembly, pole cells do not form (reviewed in Rongo and Lehmann, 1996), again

suggesting that pole plasm segregation is critical to the determination of *Drosophila* pole cells.

There is recent evidence that suggests that inheritance of P granules in *C. elegans*, as in *Xenopus* and *Drosophila*, is a key factor in the determination of germ cells. *glh-1* and *glh-2* are RNA helicases that have been identified as P granule components (Roussell and Bennett, 1993; Gruidl et al., 1996). When antisense RNA was injected into wild type hermaphrodite gonads, about 10% of the offspring were sterile, possessing underproliferated germ cells with abnormal morphology that failed to stain with monoclonal antibodies against P granules (Gruidl et al., 1996). This suggests that inactivating *glh-1* or *glh-2* function prevents the assembly of P granules, thereby preventing germ line development.

For organisms that do not have an uninterrupted germ line, there is little evidence that their germ cells are determined early in development. Transplantation studies in the mouse have shown that cells of the early gastrula are pluripotent and are even able to contribute to the germ line. This argues against the notion that cells destined for the germ line are set aside early in development, as is true in organisms with an uninterrupted germ line, but instead are determined by inductive interactions later in embryogenesis (Gardner, 1978; Gardner et al., 1985). A theory that there are 2 modes of germ cell determination in vertebrates has thus emerged: preformationist (as in *Xenopus*) and epigenetic (as in mice) (Nieuwkoop and Sutasurya, 1979).

Cytological descriptions of germ plasm

Cytological studies indicate that germ plasm is similar between organisms. Using the electron microscope germ plasm can be identified in the *Xenopus* oocyte as granulo-fibrillar material (GFM) and also contains pigment granules and mitochondria (Czolowska, 1969, 1972). It originates from the mitochondrial cloud, a maternal source of mitochondria for the embryo (Heasman et al., 1984). The mitochondrial cloud increases in quantity during

previtellogenic stages, but is then dispersed into hundreds of small islands containing both GFM and mitochondria and remains localized to the future vegetal pole of the oocyte (Czolowska, 1969, 1972; Heasman et al., 1984). In *Rana pipiens*, by the time the PGCs reach the genital ridges, the germ plasm appears as amorphous bodies of a fibrous nature (Mahowald and Hennen, 1971). Referred to as "nuage," it is positioned close to the nucleus, sometimes even attached to the nuclear envelope and to the surface of mitochondria.

In *Drosophila*, pole plasm consists of spherical electron-dense masses called polar granules (Mahowald, 1962). Polar granules undergo various ultrastructural changes during development. They are first recognizable as small dense bodies which become attached to mitochondria in large clusters during early vitellogenesis. This mitochondrial association is lost at fertilization, and they become fragmented and associate with polysomes (Mahowald, 1968). Prior to pole cell formation, they again fragment and are replaced by perinuclear dense bodies, also known as nuage, and nuclear bodies, electron-dense structures in the nuclei (Mahowald, 1971). Nuclear bodies are no longer visible by mid-embryogenesis; however, nuage remains associated with *Drosophila* germ cells throughout the life of the organism (Mahowald, 1971).

The P granules that are segregated to the germ line founder cell in *C. elegans* are also electron-dense structures and have been likened to *Drosophila* polar granules and *Xenopus* germ plasm (Krieg et al., 1978). Using P granule antibodies, the segregation of P granules during early cleavages has been observed (Strome and Wood, 1982; Hird et al., 1996). In P0 and P1, P granules are numerous and small, and appear to migrate through the cytoplasm towards site of the posterior daughter, probably using actin microfilaments (Strome and Wood, 1982; Strome and Wood, 1983; Hill and Strome, 1988; Hird et al., 1996). In P2 and P3, however, P granules are segregated to the germ line daughter cell by a different mechanism. The small granules appear to have coalesced to form 3 to 5 large granules around the nucleus which are localized to the posterior cell by nuclear migration

and localized cytoplasmic degradation (Strome and Wood, 1982; Hird et al., 1996). This perinuclear localization persists in adult male germ cells, but in contrast, the granules disperse during oogenesis in hermaphrodites (Strome and Wood, 1982).

The presence of nuage has also been observed in the germ cells and oocytes of several other organisms, including mammals and teleosts (Eddy, 1974; Spiegelman and Bennett, 1973; Timmermans and Taverne, 1989; Selman et al., 1993). Because nuage in these vertebrates has the characteristic structure of nuage found in invertebrates and anurans, it is tempting to speculate that nuage might function as a germ cell determinant in mammals and teleosts as well. However, it remains to be identified in the early embryos of these organisms, and in the absence of being able to recognize PGCs at early stages of development, it can not be determined whether the nuage that is visible in PGCs is derived from the oocyte.

Identification of germ cell determinants

Xenopus

With the strong indication that germ plasm contains germ cell determinants, researchers have been intent on identifying germ plasm components. It has long been known that germ plasm contains both RNA and proteins (for review, see Beams and Kessel, 1974), however until recently, the identity of these components had been a mystery. In *Xenopus*, molecular and biochemical techniques have identified five vegetally localized RNAs. *Vg1* and *Xwnt11* mRNAs are broadly distributed in the vegetal cortex (Melton, 1987; Ku and Melton, 1993), whereas *Xcat-2, Xcat-3* and *Xlsirt* RNAs are localized with the germ plasm to a small cortical region at the vegetal pole (Elinson et al., 1993; Kloc et al., 1993; Mosquera et al., 1993; Zhou and King, 1996). *Xcat-2* is closely related to *nanos*, a gene required for germ cell development and abdomen formation in *Drosophila* and its RNA colocalizes with germ plasm during early cleavage stages, suggesting that it could be important for germ cell development in *Xenopus* (Mosquera et al., 1993; Forristall et al.,

1995). *Xcat-3* appears to be an RNA helicase, but its function remains unknown (Elinson et al., 1993). The noncoding *Xlsirt* RNA is localized to the germ plasm in oocytes only and is part of the cytoskeletal network that anchors *Vg1* mRNA in the vegetal cortex (Kloc and Etkin, 1994). A germ cell determinant localized to the germ plasm in *Xenopus* remains to be identified.

Drosophila

Molecular and biochemical approaches have also been used to identify polar granule components in *Drosophila*, however, the role of some of these components in pole cell formation is unclear. A 95 kDa protein that is localized to pole cells was biochemically isolated, but no functional studies have yet been performed and so its role in pole cell determination remains unknown (Waring et al., 1978). The mitochondrial large ribosomal RNA (mtlrRNA) was identified on the basis of its ability to restore pole cells to u.v.-irradiated embryos (Kobayashi and Okada, 1989), and has been shown to be tightly associated with polar granules (Kobayashi et al., 1993). However, the mtlrRNA-induced pole cells did not give rise to gametes, and injection of mtlrRNA was not able to induce pole cells at ectopic sites (Kobayashi and Okada, 1989). It has not been resolved whether mtlrRNA is required for pole cell formation (Ding et al., 1994; Kobayashi et al., 1995).

The *germ cell-less* (*gcl*) gene was identified on the basis of its posteriorly localized RNA (Jongens et al., 1992). It encodes a novel protein that is associated with the nuclear pores of pole cell nuclei (Jongens et al., 1994). Decreasing the levels of *gcl* inhibits pole cell formation (Jongens et al., 1992), while overexpression of *gcl* results in the formation of additional pole cells (Jongens et al., 1994), but like mtlrRNA, it was unable to induce ectopic pole cells when localized to the anterior pole of the embryo (Jongens et al., 1994). So, although *gcl* is able to initiate the events for pole cell formation, there is an additional, as yet unidentified, posteriorly localized factor required to complete this process.

The nontranslatable *Pgc* (for *polar granule component*) RNA was isolated in a mRNA differential display screen between wild type and mutant embryos lacking pole cells, and it, too, was shown to be a polar granule component (Nakamura et al., 1996). Reduction of *Pgc* function in transgenic flies expressing antisense *Pgc* RNA resulted in a pole cell migration and proliferation defect and often produced females with agametic ovaries, assigning a role for the *Pgc* RNA in the later stages of germ cell development.

Several other *Drosophila* pole plasm components have been identified in maternal effect genetic screens. Belonging to the "posterior group" or "grandchildless class" of mutants, these genes have functions required for the formation of both abdomen and pole cells (for review, see Rongo and Lehmann, 1996). This group of genes includes: *oskar, vasa, valois, tudor, cappuccino, spire, staufen* and *mago nashi.* Females homozygous for mutations in any one of these genes give rise to progeny that do not form pole cells due to an absence of pole plasm and polar granules as well. The progeny are not only sterile, but also lacking an abdomen, resulting from a failure to properly localize *nanos* RNA, the abdominal determinant, to the posterior pole (Wang and Lehmann, 1991).

Molecular cloning and analysis of the gene products has shown that the protein products of *oskar*, *vasa*, *tudor* and *staufen* are components of polar granules (Hay et al, 1988a; St. Johnston et al., 1991; Bardsley et al., 1993; Breitwieser et al., 1996). *oskar* RNA is also localized to the posterior pole (Ephrussi et al., 1991; Kim-Ha et al., 1991). The appearance of *oskar* RNA and protein foretells the appearance of polar granules (Markussen et al., 1995; Rongo et al., 1995), and the amount of *oskar* expression is directly correlated with the number of germ cells that form (Ephrussi and Lehmann, 1992; Smith et al., 1992). Importantly, *oskar* RNA is able to direct the formation of ectopic pole cells and a second abdomen when misexpressed to the anterior pole, indicating that *oskar* directs germ plasm assembly (Ephrussi and Lehmann, 1992). However, the process of ectopic pole cell formation also requires the function of both *vasa* and *tudor* (Ephrussi and Lehmann, 1992). *vasa* encodes an ATP-dependent RNA helicase in the DEAD-box family

(Hay et al., 1988b; Lasko and Ashburner, 1988), and although Vasa protein is required for pole cell formation, it is not sufficient (Lasko and Ashburner, 1990). As for *tudor*, the levels of protein in the pole plasm are also correlated with the number of pole cells that form, but it is also expressed in somatic cell nuclei, related to its function in segmental patterning of the abdomen (Bardsley et al., 1993). The other genes, *cappuccino, spire, mago nashi* and *staufen* are not required for ectopic germ cell formation, but instead are required for transporting pole plasm components to the posterior pole. Therefore, genes that are required for pole cell determination (*oskar, vasa, tudor*) have been identified, but it remains to be determined whether there are any genes solely required and sufficient for pole cell determination.

C. elegans

In *C. elegans*, a genetic approach has also been used in an effort to identify P granule components. By screening for mutations in genes that confer a maternal-effect sterile, or grandchildless, phenotype, six loci were identified and called the *mes* genes, for maternal-effect sterile (Capowski et al., 1991). These genes were found to encode maternal factors required for germ cell survival and proliferation, and none were found to disrupt the structural integrity of P granules. Another maternal-effect screen identified the *partitioning*, or *par*, genes which are required for the cytoplasmic localization of P granules (Kemphues et al., 1988). When these genes are mutated, P granules can be found throughout embryos from homozygous mutant females, abnormal cleavages occur and germ cells do not form. These genes probably play a role in the general reorganization of cytoplasm in the early embryo, including the asymmetric redistribution of cortical microfilaments (Kirby et al., 1990).

mex-1, mex-3, and *pie-1* are three genes identified by mutational analysis, whose protein products have recently been shown to be P granule components (Draper et al., 1996; Mello et al., 1996; Guedes and Priess, 1997). MEX-3 encodes a putative RNA

binding protein, however its function remains unknown (Draper et al., 1996). PIE-1, predominantly a nuclear protein, is required for the transcriptional repression within the germ cell lineage which probably acts to preserve the pluripotency of germ cells (Mello et al., 1996; Seydoux et al., 1996). PIE-1 expression and activity was found to be restricted to the germ line blastomeres during early cleavages by cytoplasmically localized MEX-1 (Guedes and Priess, 1997).

And finally, molecular cloning and analysis of expression patterns of the DEAD-box RNA helicases, *glh-1* and *glh-2*, has revealed that the proteins encoded by these genes are P granule components at all stages of germ line development (Roussell and Bennett, 1993; Gruidl et al., 1996). It is exciting to speculate that they are required for the assembly of P granules based on injection of antisense *glh-1* or *-2* RNA into hermaphrodite gonads which results in sterile progeny that lack detectable P granules (Gruidl et al., 1996).

Genetic manipulation of the germ line

Besides intrinsic biological interest, knowledge of germ cells can have practical implications as well, changing the way one approaches studies on development. In the mouse, embryonic stem (ES) cells, derived from mouse blastocysts, can be cultured in vitro, then injected into host embryos where they are able to colonize the germ line of the resulting chimeric mice (Evans and Kaufman, 1981; Martin, 1981; Bradley et al., 1984). This technique, combined with the inactivation of genes in ES cells by homologous recombination, has been used extensively to generate knockout mice and study the role of these genes in development (for review, see Capecchi, 1989).

Interestingly, pregonadal mouse PGCs cultured with a specific combination of growth factors take on the morphology of ES cells in culture (Matsui et al, 1992; Resnick et al., 1992), and like ES cells, are capable of contributing to the germ line when transplanted into host embryos (Labosky et al., 1994; Stewart et al., 1994). This result is in striking contrast to the observation that transplanted PGCs are normally not able to contribute to any

somatic or even the germ cell lineages (Stewart et al., 1994). Considering that EG cells appear and behave like ES cells, perhaps EG cells represent PGCs that have reverted to an ES-like stage. In support of this hypothesis are the observations that characteristics of normal PGCs, such as DNA methylation and gene expression profiles, tend to resemble those of early blastocyst cells, rather than somatic cells of the same stage (Monk et al., 1987; Kafri et al., 1992; Urven et al., 1993).

Although there have been intensive efforts, ES cells have not been established in any other organisms. Because PGCs possess similar characteristics in different organisms, it seems possible that PGCs in other organisms might be able to form ES cell-like colonies in vitro if cultured under conditions similar to those that allow mouse PGCs to become ES cells. This exciting possibility awaits investigation.

Zebrafish as a model system for vertebrate development

In recent decades, the model systems primarily used for the study of vertebrate development have been the mouse, frog, chicken, and more recently, fish. Each system is particularly well-suited to certain types of analysis (reviewed in Slack, 1991). For example, genetic studies have been performed extensively in the mouse. Genes mutated spontaneously or by radiation have been cloned based on their position on the genetic linkage map for the mouse (Copeland and Jenkins, 1991; Reith and Bernstein, 1991) and knockout mice have been created by homologous recombination in ES cells, enabling researchers to analyze the role of dozens of genes in development using this reverse genetic approach (Capecchi, 1989). Forward genetic experiments in this organism are not practical, though for most phenotypes, because of the small litter sizes and the enormous cost in housing and maintaining mouse colonies. Moreover, a disadvantage of using the mouse in developmental studies is the inaccessibility of the early embryo because it develops inside the mother, making it difficult to observe and manipulate at early stages of development.

On the other hand, the strength of using the frog and chicken for studying vertebrate development is the large size and external development of the embryos. They have been used extensively for studies on cell-cell interactions, cell lineage, and cell movement. But although a wealth of information has been obtained using an embryological approach to the study of vertebrate development, genetic experiments in these organisms are not possible due to ploidy, the size of the organism, and generation time. This limitation impedes the identification of novel genes involved in developmental processes.

The zebrafish is unique among vertebrate model systems in that it is well-suited to both embryological and genetic studies (Kimmel, 1989). The embryos are large, transparent, and develop outside the mother. Together, these features allow one to observe all developmental processes from the one-cell stage to a swimming larva and to perform embryological experiments throughout this time period. Moreover, the zebrafish is also well-suited for developmental studies because early development occurs so rapidly--within 24 hr, most of the organ primordia have formed. Extensive cell lineage analysis has been performed (Kimmel and Warga, 1988; Woo and Fraser, 1995), and in vitro assays have been devised recently for the study of inductive interactions in the early zebrafish embryo (Sagerstrom et al., 1996), indicating that embryological experiments traditionally performed in the frog and chick can be adapted for the fish.

The features of the zebrafish that make it amenable for genetic analysis are that the generation time is relatively short (2-3 months), adults are small and relatively easy to maintain, and single pair matings can yield hundreds of embryos. Additionally, haploid and gynogenetic embyros can be obtained which can be useful for screening purposes (Driever et al., 1994) and transgenic embryos can be generated by injection of plasmid DNA into fertilized eggs (Stuart et al., 1988, 1990; Culp et al., 1991) or by injection of pseudotyped retrovirus into blastula-stage embryos (Lin et al., 1994). That zebrafish is useful for genetic analysis has long been known and several interesting mutants have been

isolated in small-scale screens. But more recently, it was shown that large-scale genetic screens are possible either using ENU to create point mutations (for review, see Eisen, 1996) or by using a pseudotyped retroviral vector to create insertional mutations (Allende et al., 1996; Gaiano et al., 1996).

Despite this major progress in recent years, the development of the zebrafish germ line has not been extensively studied. Germ line chimeras have been generated by transplanting donor cells from blastula-stage embryos to the blastoderm of host embryos at an equivalent stage, demonstrating that cells contributing to the germ line reside among the blastomeres (Lin et al., 1992). However, since the cells were taken randomly from the donor embryos and transplanted to random locations, no conclusion could be made about the origin of the germ cells at this stage. There is one other previous study on the zebrafish germ line (Walker and Streisinger, 1983). In this experiment, by irradiating cleavage-stage embryos and subsequently analyzing mutant clone sizes at a pigmentation locus, *gol-1*, they estimated that the number of PGCs in the early zebrafish embryo is very small--about 5 up until the 2000-4000-cell stages.

Approaches to identifying the zebrafish germ line

The identification of the zebrafish PGCs would be of interest for several reasons. First, if the fish is to be a useful model system, the identification of the zebrafish PGCs is long overdue. Second, there are advantages to studying germ line development in this organism. The combination of the accessibility and optical clarity of the embryo and the ability to generate transgenic fish will be particularly useful in the study of the proliferation and migration of the zebrafish PGCs. And finally, identification of the PGCs would allow one to explore the possibility of creating knockout zebrafish using ES/EG cell technology. The ability to perform both forward and reverse genetics in a single organism that is also amenable to embryological studies would truly revolutionize the study of vertebrate development.

Stains and antibodies that label germ cells in other organisms

There are several stains and antibodies specific for germ cells in different organisms, some of which have been shown to label PGCs in more than one species. My initial attempts focused on identifying those that would cross-react with the PGCs in zebrafish. Alkaline phosphatase (ALP) has been shown to be strongly expressed in mouse PGCs (Chiquoine, 1954), however ALP staining of 3 d zebrafish larvae was detected in the pronephric tubules and not the germ cells (data not shown).

The monoclonal antibody, EMA-1, was raised against mouse embryonic carcinoma cells and labels migratory PGCs in the mouse (Hahnel and Eddy, 1986). Since it recognizes a cell-surface epitope, it is possible to isolate EMA-1-positive cells via fluorescence-activated cell sorting (FACS) (McCarrey et al., 1987). Furthermore, it has been shown that EMA-1 cross-reacts with PGCs in the chick embryo (Urven et al., 1988), but I was unable to detect EMA-1 staining of the zebrafish PGCs in gastrulating or somite-stage embryos (data not shown).

I also tried staining zebrafish embryos with a polyclonal antibody to the germ cell-specific *Drosophila* Vasa protein (Hay et al., 1988a), but did not obtain any specific staining. And finally, I tested WCS29, a monoclonal antibody that was raised against mature carp sperm and labels carp PGCs and PGCs in other fish as well (Parmentier et al., 1984; Parmentier and Timmermans, 1985). I observed staining in the trigeminal ganglia of 24 hr embryos, but not in the PGCs (data not shown). I do not discuss these failed attempts in this thesis as they yielded inconclusive results at best.

Monoclonal antibodies raised against mature zebrafish sperm

In the carp, of 11 monoclonal antibodies raised against mature carp sperm, 4 were found to be useful for marking the germ cells in developing larvae (Parmentier et al., 1984; Parmentier and Timmermans, 1985). Since WCS29 did not cross-react with zebrafish

PGCs, I decided to raise monoclonal antibodies against mature zebrafish sperm to see if any would recognize epitopes present on zebrafish PGCs as well. As will be discussed in the Appendix, I did successfully raise monoclonal antibodies that recognized mature sperm, however, none of these were specific for PGCs.

Fate mapping

As I was testing the stains and antibodies described above, I was also using cell transplantation to map the origin of the germ line. Germ line chimeras had been obtained previously in our lab using this approach (Lin et al., 1992). In these experiments, I first tested whether placement of donor cells in the blastoderm of the recipient had any effect on germ line transmission frequency. As is discussed in Chapter 2, my results indicated that the germ line might originate from a specific location around the margin of the blastoderm, however, I was unable to map this location more precisely.

Molecular markers

As had been previously discussed, many genes have now been identified that are expressed in PGCs. In *Drosophila*, the Vasa protein is specifically localized to the pole cells and was found to be a polar granule component (Hay et al., 1988a, b; Lasko and Ashburner, 1988). *vasa* encodes an RNA helicase, containing a highly conserved DEAD-box region (Hay et al., 1988b; Lasko and Ashburner, 1988), and it is required for pole cell formation (Ephrussi and Lehmann, 1992; Lasko and Ashburner, 1990). Of particular interest to me was that vertebrate homologs of the *vasa* gene had been cloned in frog (Komiya et al., 1994), mouse (Fujiwara et al., 1994), and rat (Komiya and Tanigawa, 1995), and the protein products were shown to be specifically expressed in germ cells in these organisms. Unfortunately, the antibody against the frog Vasa protein did not cross-react with zebrafish on a Western blot (T. Komiya, personal communication).

However, because of the identification of *vasa* homologues in 3 different vertebrates, it seemed likely that a *vasa* homologue could also be identified in zebrafish. In Chapter 3, I describe the cloning of the zebrafish *vasa* homologue (*vas*) and the analysis of its RNA expression pattern as determined by whole mount in situ hybridization. *vas* RNA is specifically expressed in the germ line and has allowed for the identification of the zebrafish PGCs for the first time. Furthermore, the RNA is localized in a novel pattern during early cleavages, and its segregation pattern bears some striking similarities to germ plasm segregation in *Xenopus*.

In Chapter 4, I will discuss the future experiments that could be performed to learn more about the role of *vas* in zebrafish germ line development and the ways in which this marker could be useful for technology development.

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CHAPTER 2

FATE MAPPING THE ZEBRAFISH GERM LINE

ABSTRACT

The ability to genetically manipulate an organism's germ line is critical to the mutational analysis of development. The zebrafish is an organism that is well-suited for the study of early vertebrate development, however, little is known about the origin of the zebrafish germ cells. In an attempt to fate map the zebrafish germ line, cells were transplanted to host embryos and the effect of transplant position on germ line transmission frequency was analyzed. First, we found that cells transplanted to the marginal region of host blastulae were more likely to contribute to the germ line than those placed at the animal pole. In a second set of experiments, in which we attempted to define a preferred position of the margin, we scored the position of the transplants at the shield stage and again analyzed the germ line transmission frequencies, but we were unable to map the germ line to a more localized region. The possible explanations for these results are discussed.

INTRODUCTION

The zebrafish (*Danio rerio*) presents the developmental biologist with a unique opportunity to combine genetic analysis with embryological studies in a vertebrate organism (Kimmel, 1989). Features of zebrafish that are well-suited for genetic analysis are that the generation time is relatively short (2-3 months), adults are small and relatively easy to maintain, and hundreds of embryos can be obtained from a single pair mating. Embryological studies benefit from the large and transparent embryos; moreover, they are easily accessible as development occurs outside the mother. Furthermore, the organism is well-suited for developmental studies because early development occurs so rapidly: cleavage starts within one hour of fertilization; at 6 hr (shield stage) gastrulation begins and dorsal-ventral polarity is established; somites begin to form at 10 hr; by 24 hr, most organ primordia have formed. Also, because eyes and melanocytes are heavily pigmented by 48 hr, in wild type embryos, pigmentation is a useful genetic marker.

Our lab has been developing insertional mutagenesis strategies to identify genes involved in early development of the zebrafish. In one potential approach, DNA would be introduced into cultured cells and those cells harboring mutations would be selected, then transplanted to recipient embryos and bred to homozygosity. A crucial step in this strategy is the ability to produce germ line chimeras efficiently. It has already been shown that germ line chimeras can be made in zebrafish by transplanting primary cells from donor embryos into genetically distinct recipients at the blastula stage (Lin, et al., 1992). Germ line transmission frequencies of randomly placed donor cells were typically about 20% in which one out of five chimeric fish were germ line chimeras. Targeting donor cells to the germ line at a high frequency would be a key step in making this strategy more efficient. This could be achieved by placing donor cells directly in the location from which the germ line originates.

Extensive fate mapping studies have been performed in the zebrafish. Injection of lineage tracer dyes into single cells in the blastula and gastrula, followed by analysis of its clonal progeny have shown that clones derived from a single cell of the gastrula are usually of a single cell type. This is in striking contrast to the scattering, among multiple tissues, of clonal progeny derived from single blastomere injection (Kimmel and Law, 1985; Kimmel and Warga, 1986). Using this approach, a fate map was constructed for the zebrafish from the shield stage, the beginning of gastrulation (Kimmel et al., 1990). However, despite numerous cell lineage studies, there has been no investigation of the origin of the zebrafish germ line.

We performed cell transplantations in order to answer the following question: Is there a region of the blastoderm that is most likely to give rise to the germ line and can we target donor cells to this region to increase germ line transmission frequencies? As is presented and discussed below, we show that donor cell transplantation location does affect germ line transmission frequency, however the exact location that gives rise to the germ line could not be mapped using our approach.

RESULTS

Donor cell transplantation location affects germ line transmission frequency

We wished to determine whether transplant location in the blastula is correlated with the contribution of donor cells to the recipient's germ line. If we assume that the none of the cells of the blastula are committed to a particular fate, then cells transplanted directly to the region that gives rise to the germ line would result in a higher germ line transmission frequency. As shown in Figure 1, wild type donor embryos were dissociated at the 2000-cell stage (3.5 hr post-fertilization at 28.5°C), and approximately 50 cells were transplanted to the margin, animal pole, or middle of the blastoderm of 2000-cell-stage albino embryos. To determine whether donor cells contributed to the germ line of the recipient, chimeras were raised to adulthood and mated to an albino fish. If a fish was a germ line chimera, a fraction of its progeny were pigmented at 48 hr.

The results of this experiment suggest that germ line transmission frequency is indeed affected by the placement of donor cells (Table 1). Statistical analysis indicates that cells at the margin (18% germ line transmission frequency) are more likely to give rise to the germ line than cells at the animal pole (0%). However, no statistically significant comparisons can be made with the middle placement germ line transmission frequency (10%). The range of contribution of the donor cells to the germ line of a single chimera was found to vary from 2% to 40%. Interestingly, there was no increase in germ line transmission frequency when cells were placed in 2 or 3 locations at the margin within a single recipient embryo (18%), although a higher contribution of donor cells to the germ line was obtained in some of the fish (up to 84% pigmented progeny).

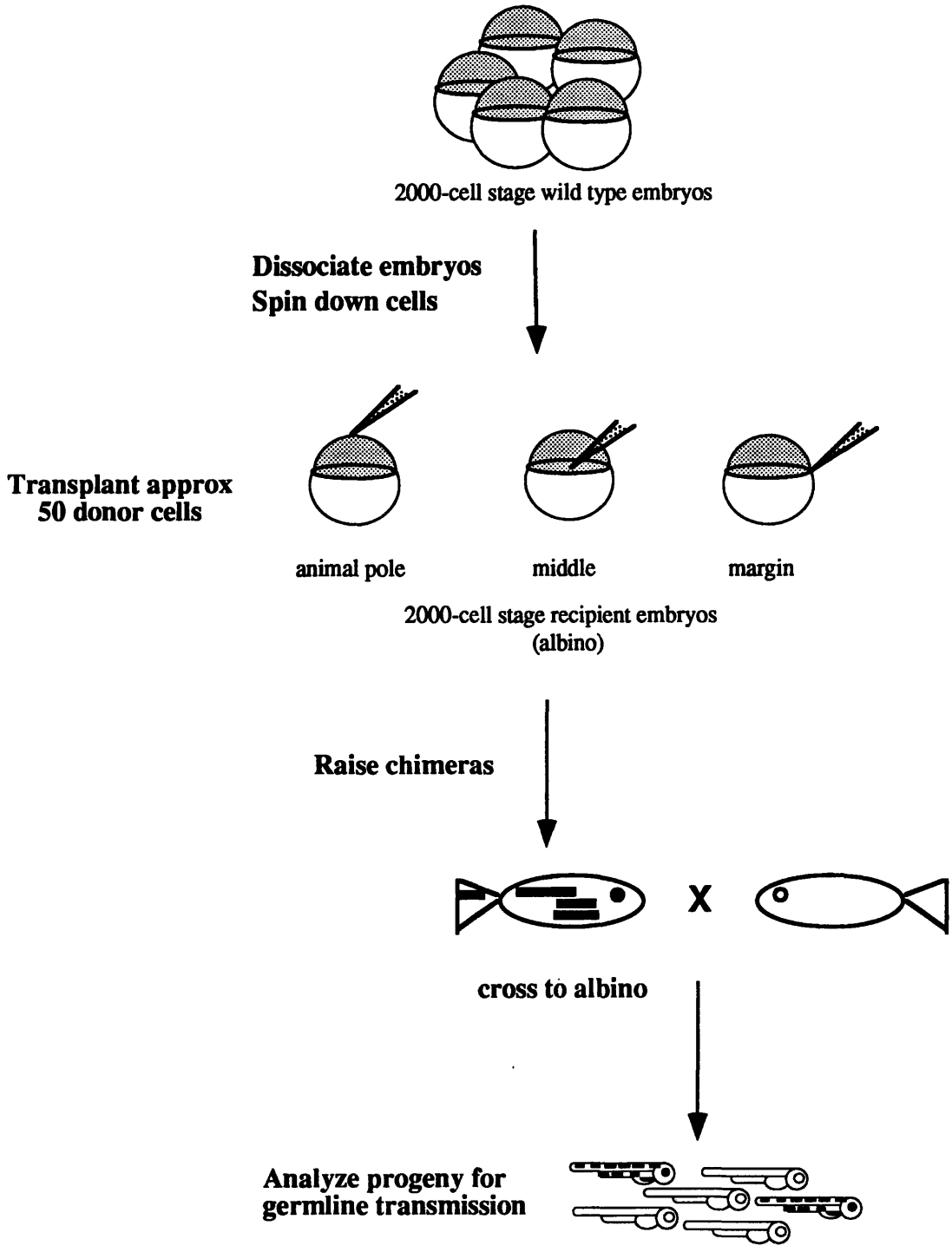
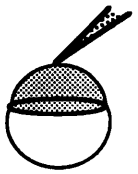
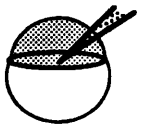
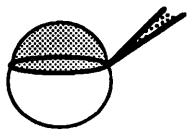


Figure 1. Experimental design to determine the effect of donor cell placement on germline transmission

Table 1. Donor cell placement affects germ line transmission frequency.

Transplant position	# Chimeras tested	# Germline chimeras	Germ line trasmission frequency†	Range††
 animal pole	20	0	0%	-
 middle	40	4	10%	2-8%
 margin	44	8	18%	2-40%
double/triple margin*	33	6	18%	4-84%

Chimeras were generated by cell transplantation to the indicated positions, then raised an tested for germ line transmission of the transplanted cells by mating. At least 50 progeny were analyzed per chimera.
† The percent of chimeras tested that were germ line chimeras.
†† The range in percent of pigmented offspring obtained for a single chimera tested.
* These chimeras had 2 or 3 donor cell placements at the margin of a single recipient embryo.

Mapping the germ line at the shield stage

The previous result that the cells transplanted to the margin are more likely to give rise to the germ line suggested that the exact location around the margin could be mapped. Again, the chimera approach was taken to map the germ line more precisely. At the time when the transplants are performed (3.5 hr), however, there is no landmark on the embryo. It is not until the shield stage (6 hr) when the dorsal shield appears that the transplant position is able to be scored. Thus, the experimental procedure was modified as follows.

Wild type donor embryos were labeled with fluorescein-dextran and cells were transplanted to the margin of unlabeled albino hosts (Figure 2). When the chimeras reached the shield stage (6 hr), the transplant position was scored relative to the dorsal shield by viewing the embryos from the animal pole. The position was recorded as a "time" relative to the shield at "12:00". For example, as shown in Figure 2, the transplant position in the representative embryo is "5:30-6:00". These chimeras were also raised to adulthood and tested for germ line transmission by mating.

The results of this experiment are shown in Figure 3. Of 70 chimeras tested, only 4 germ line chimeras were obtained. The positions of the transplanted cells in the 4 germ line chimeras were 6:30-7:30 (35% pigmented offspring), 8:00-11:00 (13%), 9:30-11:00 (32%), and 9:30-11:00 (58%). Based on these results, we are unable to map the origin of the germ line to a precise region around the margin of the embryo.

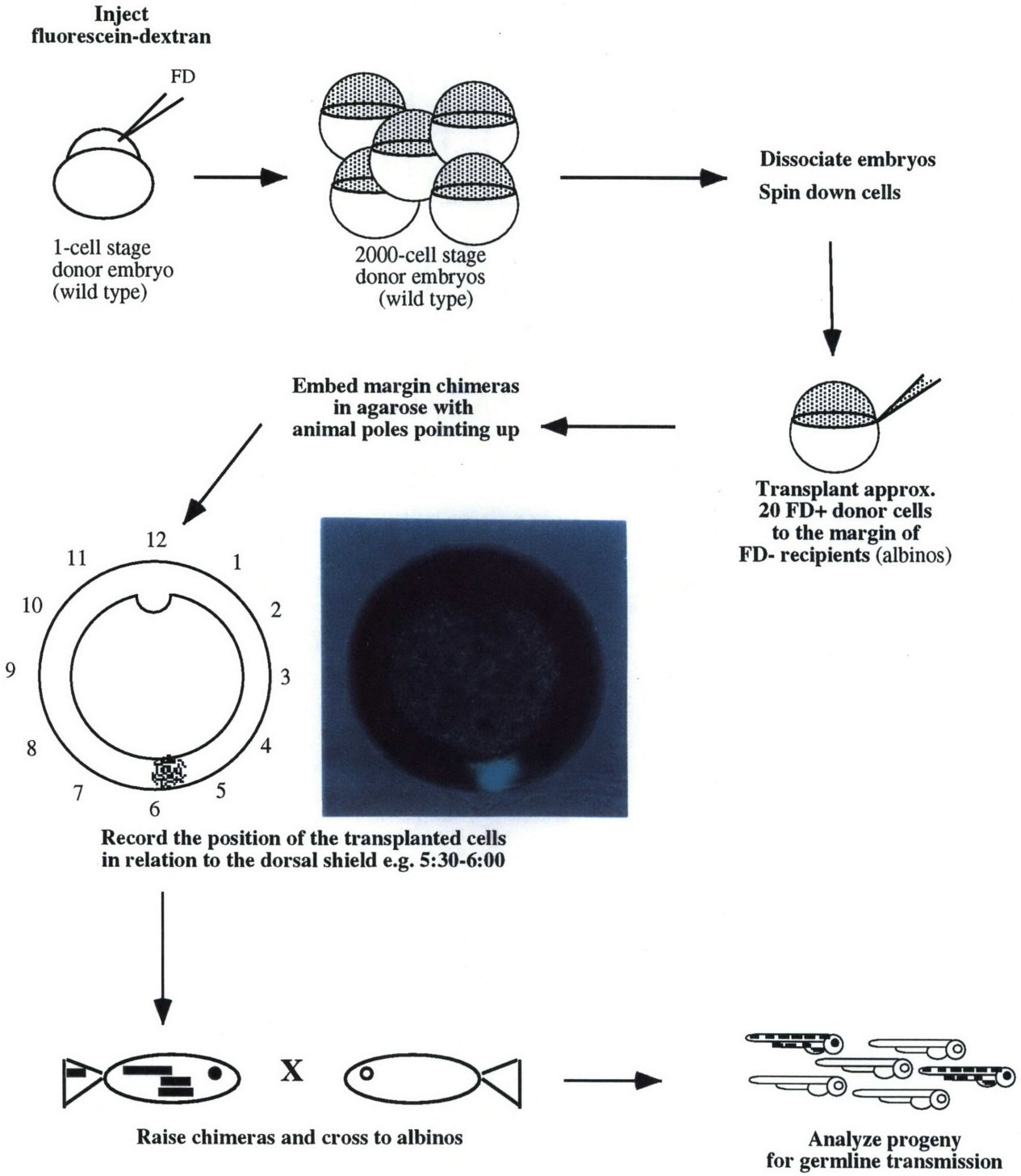


Figure 2. Experimental design to fate map the germline at the shield stage.

A representative chimera is included to show how we scored the transplant position relative to the dorsal shield.

A.

205	scored chimeras
149	pigmented at day 2
123	adult fish
29	non-maters
70	yielded over 50 progeny
4	germline chimeras

B.

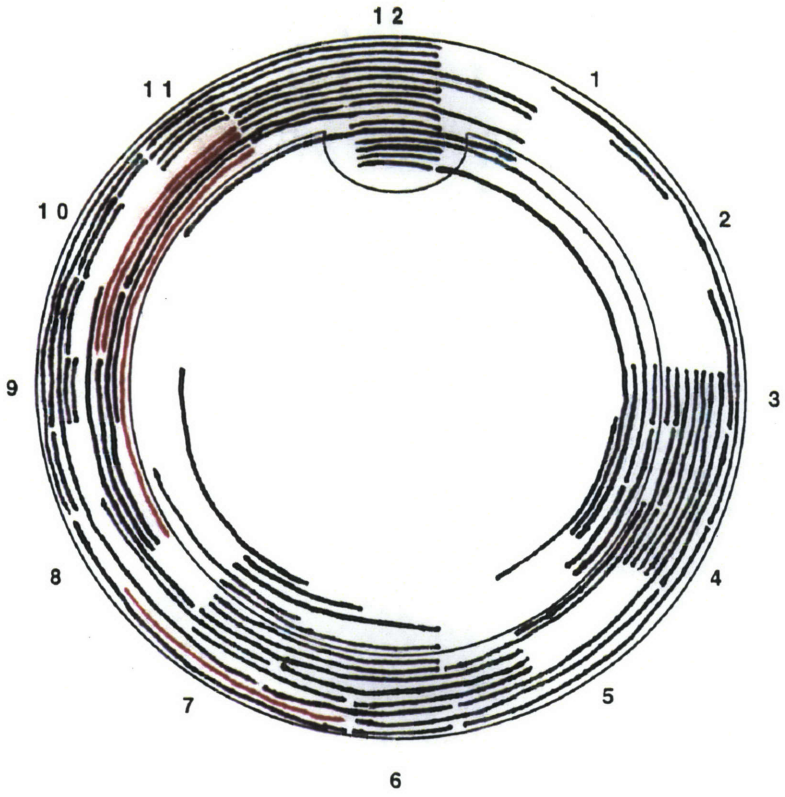


Figure 3. Summary of results for shield stage fate mapping experiment.

(A) Tabulation of numbers of fish generated in the shield stage fate mapping experiment. Scored chimeras are those embryos for which we were able to record the transplant position. Chimeras were recorded as being pigmented at day 2 of development if there was at least one melanocyte on its body, as seen in the dissecting microscope. Adult fish are those chimeras that survived to sexual maturity. Non-maters were those chimeras that did not mate even after being set-up for matings at least 5 times. Only those fish that yielded over 50 progeny were analyzed for germline transmission.

(B) Animal pole view of a shield stage embryo. Black lines show the transplant locations of non-germline chimeras; red lines signify germline transmission of the transplanted cells. The percent pigmented offspring for the germline chimeras were 35% (transplant position 6:30-7:30), 13% (8:00-11:00), 32% (9:30-11:00), and 58% (9:30-11:00).

DISCUSSION

The first set of experiments indicated that donor cell placement does have an effect on germ line transmission frequency. Cells were able to contribute to the germ line when placed at the margin or middle of the recipient blastoderm, but not when placed at the animal pole (Table 1). These results are consistent with germ line fate mapping studies in a closely related fish, the rosy barb, in which single cells were injected at the 64-cell stage and labeled PGCs were identified at 12 hr post fertilization (Gevers et al., 1992). Because the location of the injected blastomeres that gave rise to labeled PGCs varied with respect to the embryonic axis, they were unable to identify germ cell progenitors from this stage. They did, however, see a difference between injecting an "upper layer cell" versus a "lower layer cell." Cells in the upper layer never gave rise to PGCs, whereas cells in the lower layer gave rise to labeled PGCs about 25% of the time. That we did not observe germ line transmission of cells transplanted to the animal pole (derived from "upper layer" cells), but did when placed in the middle or at the margin (derived from "lower layer" cells), could be due to similarities in the origin of the germ line in the rosy barb and zebrafish.

Although the first set of experiments suggested that the germ line can originate from cells at the margin of the blastoderm, we were unable to map the origin of the germ line to a localized position (Fig 3). Our data are consistent with the explanation that there is not a single location around the blastoderm margin from which the germ cells originate. In fact, the germ line could arise from marginal locations that vary from embryo to embryo. In the mouse, lineage analysis of the origin of the PGCs indicated that they arise from a large region of the proximal epiblast, adjacent to the extraembryonic ectoderm (Lawson and Hage, 1994). The zebrafish PGCs might also originate from a large region.

A concern, however, is that the number of chimeras tested in this experiment was small and consequently, the data is not conclusive (Fig 3). 3 of the 4 germ line chimeras had donor cells placed within the region from 9:30-11:00, whereas the cells were placed at

6:30-7:30 in the fourth germ line chimera. It is possible that the position of the transplanted cells in the single 6:30-7:30 germ line chimera was mixed up with another chimera during the raising and mating to test for germ line transmission. Therefore, the possibility remains that the region from 9:30-11:00 could actually be the location of the origin of the zebrafish germ line and that testing more fish could support this conclusion.

Another issue to consider is that germ line transmission may actually have been obtained by transplanting cells already committed to the germ cell lineage. We do not know the origin of the donor cells since they are transplanted as a mixed population. In designing these experiments, we were acting without a knowledge of whether restriction of the germ line occurs only after the mid-gastrula, as found by single cell transplants for other lineages (Ho and Kimmel, 1993). In studies by Kimmel and colleagues, it was shown that marginal cells, that usually form hypoblast derivatives, retained pluripotency and were uncommitted from late blastula through early gastrula stages when transplanted as single cells to host embryos. These cells did not become committed until mid-gastrulation. Although the rest of the embryo may be uncommitted at the time of our transplantations, however, it is possible that germ line precursor cells become committed to the germ line earlier in development.

That primordial germ cells are committed to germ cell fate when transplanted to a new environment has been demonstrated in *Drosophila*. Transplanted pole cells were found to only form germ cells (Underwood et al., 1980; Technau and Campos-Ortega, 1986), and those cells that did not properly migrate to the gonad, did not differentiate into other cell types. The possibility that we are transplanting predetermined germ cells is also consistent with the results we obtained in the experiments described in this chapter. If it is true that the germ line arises from the margin of the blastoderm, then when predetermined cells are placed at any marginal region of the host blastoderm, they should be able to contribute to the germ line, as we observed; however when placed at the animal pole, they may not be able to properly migrate and therefore would not contribute to the germ line

(Table 1). By transplanting about 50 cells per host embryo, we obtained about 20% germ line transmission, which would yield an estimate of about 8 predetermined germ cells at the 2000-cell stage. Our calculation falls well within the range suggested by Walker and Streisinger in 1983: they estimated that the number of PGCs in the 1000-2000 cell stage zebrafish embryo is no more than 10 cells, but probably about 5.

As has been discussed, the results from these chimera experiments are difficult to interpret and will need to be clarified by further experimentation. Short-term fate mapping experiments or orthotopic grafting experiments would be greatly aided by the identification of the PGCs during embryogenesis or larval stages. Then difficulties such as the time-consuming and error-prone raising and mating of chimeras to test for germ line transmission, or not knowing the origin of the donor cells, could be avoided.

MATERIALS AND METHODS

Fish

Zebrafish (*Danio rerio*) were kept and raised essentially according to standard conditions (Westerfield, 1995) and using practices established in our laboratory (Culp, et al., 1991). Fertilization was achieved by natural spawning and embryos were raised at 28°C and staged according to Kimmel, et al. (1995).

Microinjection and cell transplantation

These procedures were performed according to Lin et al., 1992, using wild type and *alb-1/alb-1* fish, with the following modifications. All cell transplants were performed at the 2000-cell stage. Donor embryos were dissociated in Ca^{+2}-free Holtfreter's solution and about 50 wild type donor cells were transplanted to the margin, middle, or animal pole of an albino recipient blastoderm at the same stage. When the position of the margin transplant was recorded relative to the shield, the embryos were allowed to develop to the

shield stage (6 hr post fertilization) following transplantation, then embedded in 0.1% agarose in Holtfreter's solution and viewed from the animal pole. The position was recorded as a "time" relative to the shield at "12:00". Embryos were subsequently removed from the agarose and incubated in Holtfreter's solution.

Testing for germ line transmission

Chimeras were checked at 24 hr and 48 hr using a dissecting microscope for abnormal development. Only those that appeared normal were raised to adulthood. They were mated to a single albino fish and at least 50 progeny were obtained for each chimera. For germ line chimeras, in which the donor cells contributed to the germ line, a fraction of its progeny were pigmented. Germ line transmission frequency was measured as the number of germ line chimeras of the total number tested.

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CHAPTER 3

CLONING AND EXPRESSION OF ZEBRAFISH *VASA* HOMOLOGUE

ABSTRACT

Identification and manipulation of the germ line are essential 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 that in the fly, encodes a germ cell-specific protein. Using whole mount in situ hybridization, we investigated the expression pattern of zebrafish *vasa* homologue (*vas*) RNA in zebrafish embryos. Here we show that *vas* RNA is a germ cell-specific marker, allowing for 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 not only open the door to the development of techniques for the genetic manipulation of zebrafish, but also provide the basis for further studies on RNA localization and germ line development in general.

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; Illmensee and Mahowald, 1976; Okada et al., 1974). The application of genetics in *Drosophila* has made it possible to identify a number of genes essential for the determination of the germ line, and these include 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 stunning 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. During early cleavages *vas* RNA condenses into subcellular clumps that are subsequently segregated to a small number of cells. Later in development, the number and position of the *vas*-expressing cells suggests that they are the zebrafish primordial germ cells. Although it remains to be determined whether the cells that initially inherit the maternally expressed *vas* RNA are indeed the future zebrafish germ cells, we have succeeded in identifying the zebrafish germ line from somitogenesis onwards.

Moreover, we have discovered a novel mechanism of maternal RNA localization that potentially serves to mark the future germ line at an extremely early stage of development.

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.

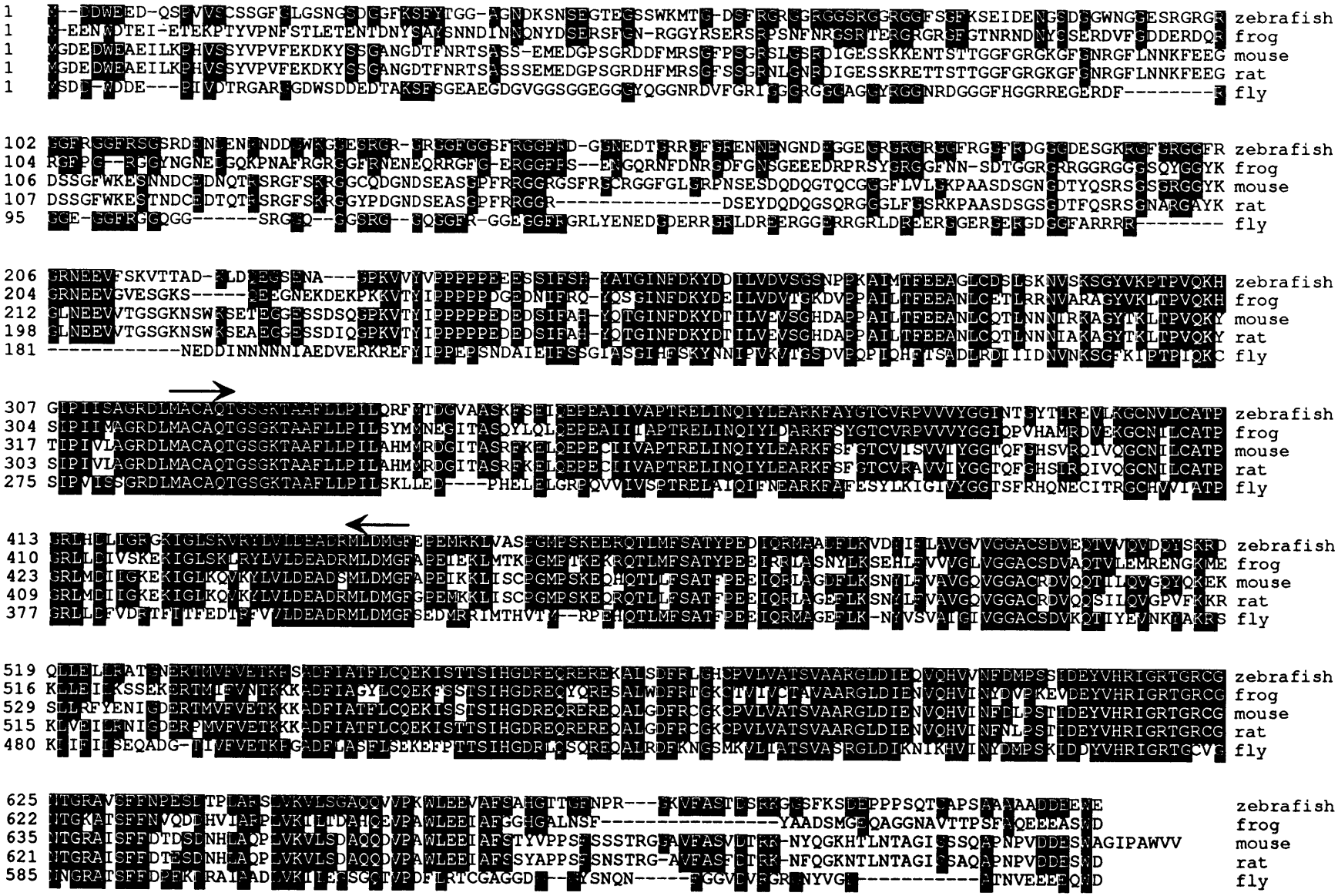

Figure 1. Alignment of the zebrafish, frog, mouse, rat and fly vasa proteins.

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.

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 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 lead us to conclude that the gene 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.

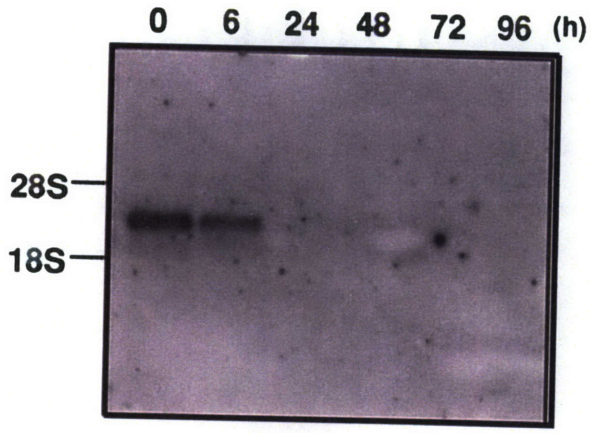

Fig. 2. Northern Blot Analysis Shows That *vas* is Maternally Supplied.

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 post fertilization 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 2 cleavages, *vas* expression along the first and second cleavage planes persists and remains strong. The expression that began as 4 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 4 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.

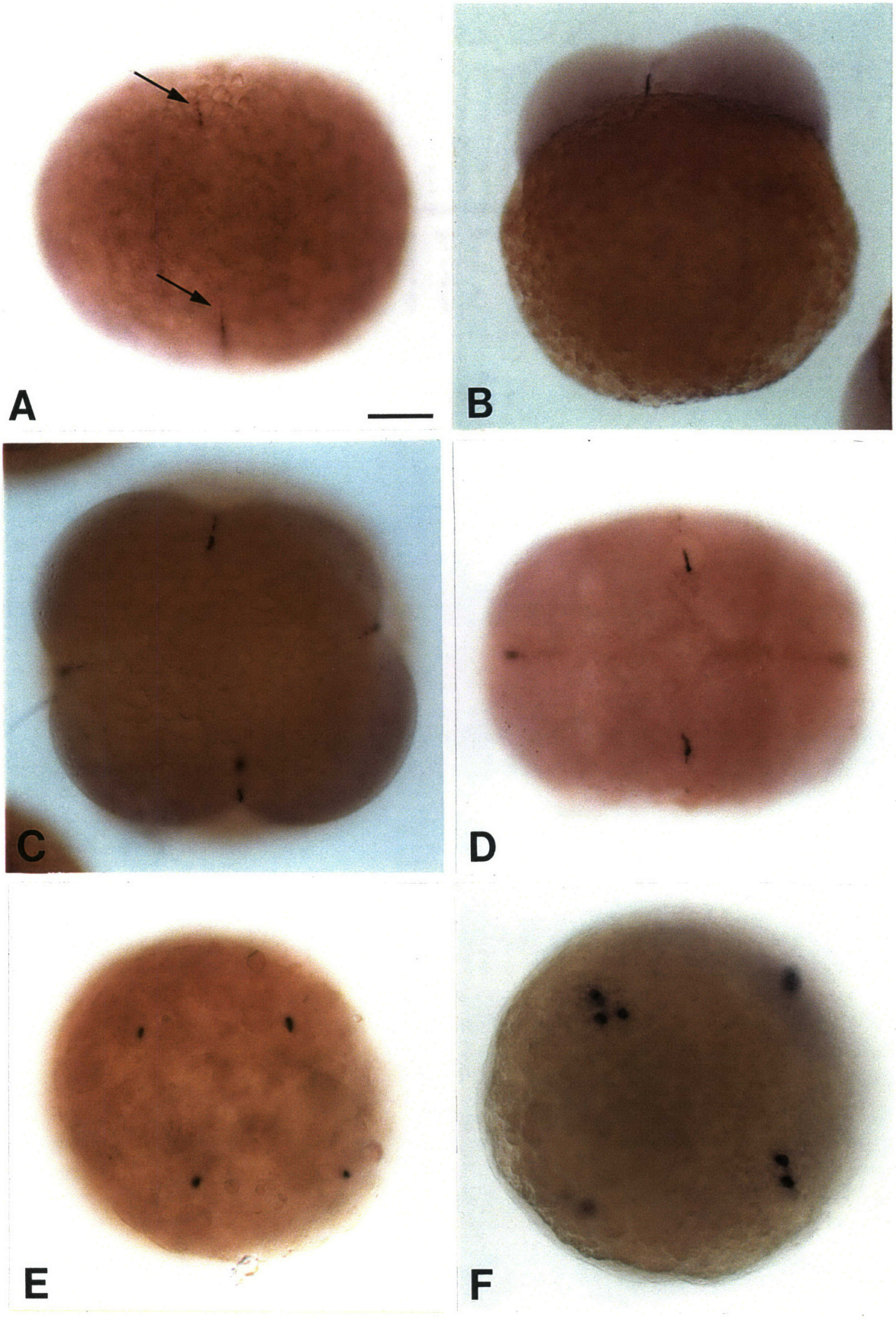
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Figure 3. Whole Mount In Situ Hybridization on Early Cleavage to Dome Stage Embryos.

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 and 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.

vas RNA remains localized to exactly 4 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, and 4A,B). The midblastula transition, when zygotic transcription begins, occurs at approximately 3 hours post fertilization 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 4 separate groups, spaced around the embryo, and generally near the margin. It appears that the 4 cells that inherited maternal *vas* RNA have undergone at most 3 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.

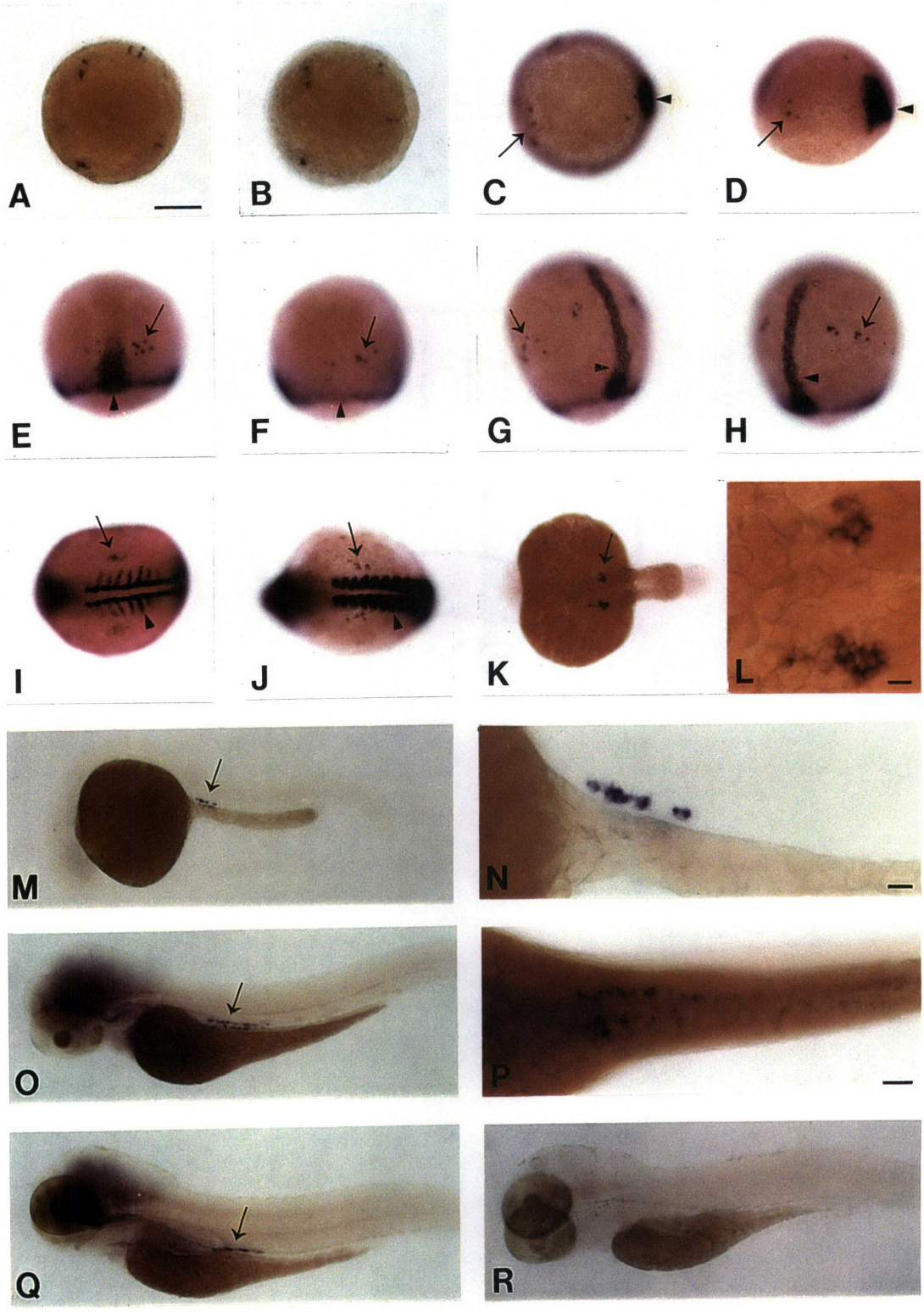
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Figure 4. Whole Mount In Situ Hybridization on Older Embryos and Larvae.
Embryos were hybridized with a *vas* riboprobe. The cells expressing *vas* are indicated with an arrow. In embryos that were simultaneously hybridized with *vas* and a second probe to mark the location of particular structures, the cells expressing the second gene are indicated with an arrowhead. In (I) through (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.
(P) Side view of a representative sense control (4 days).
Scale bars, 200 μm except 20 μm in L, N and 50 μm in P.

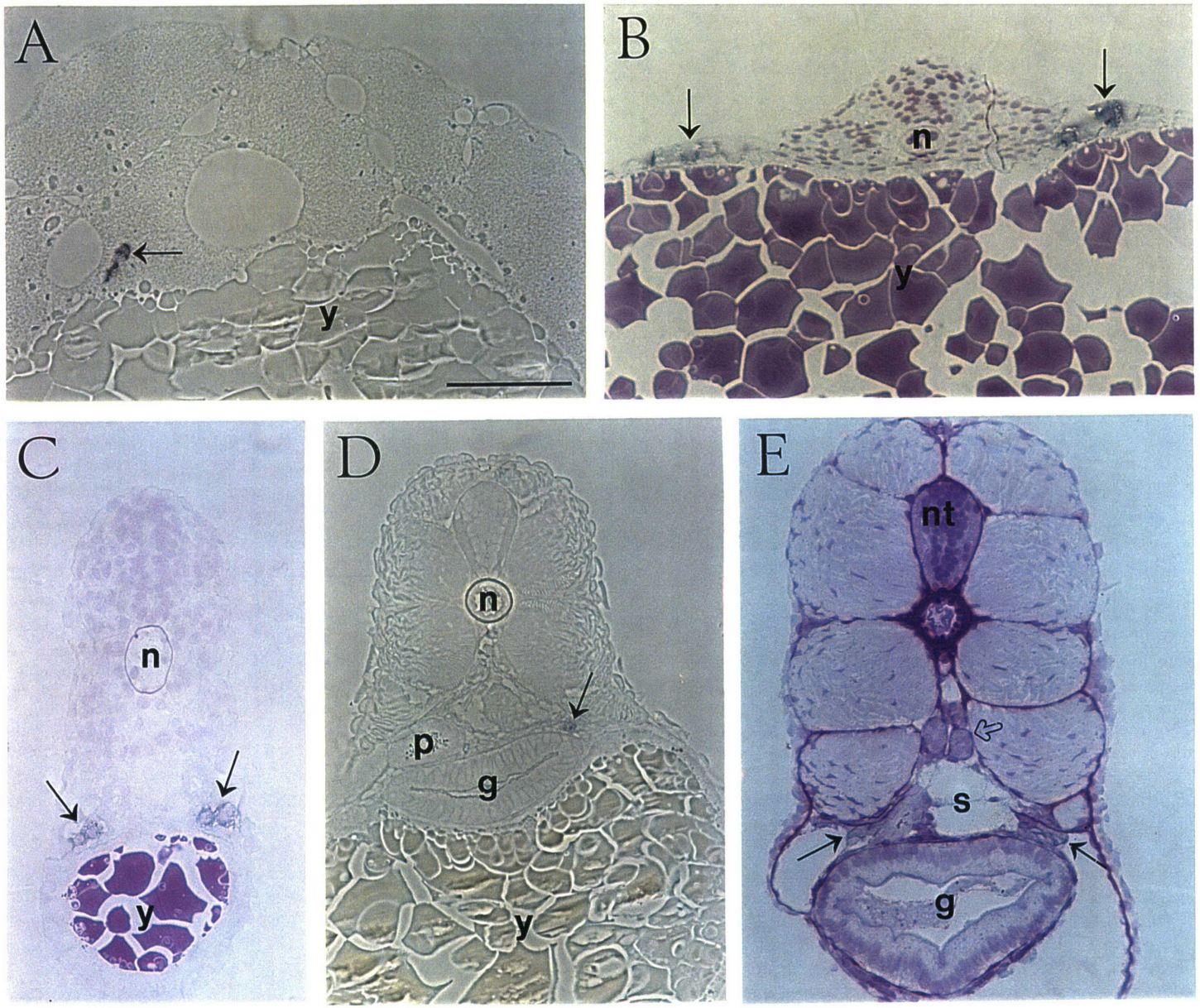
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Figure 5. Histological Analysis of In Situ Hybridized Embryos and Larvae.

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.

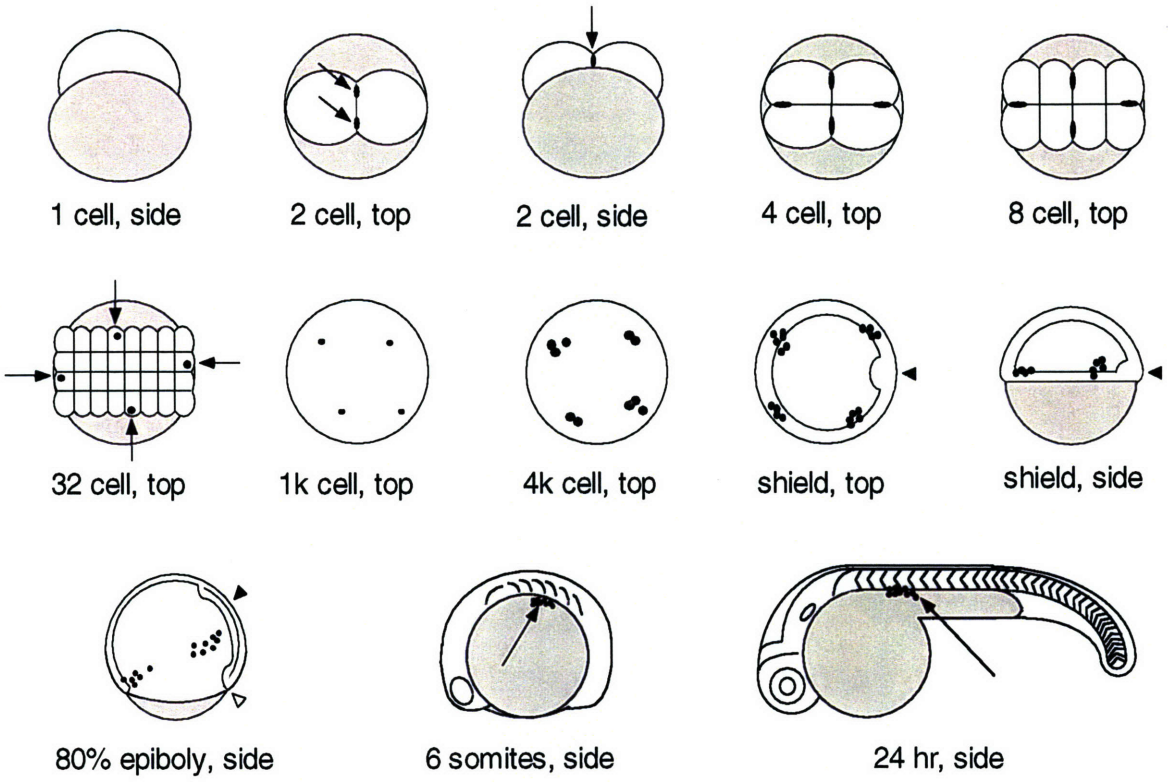

Fig. 6. Schematic Summary of Zebrafish Germline Development During Embryogenesis.

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 2 cleavage planes (arrows), then condenses into 4 subcellular clumps by the 32-cell stage (arrows). These 4 clumps are segregated to 4 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 2 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 2 bilateral rows of cells in the gonadal anlagen and later resume mitosis (not shown).

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 4 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 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 and 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 and 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 2 bilateral rows of cells that appear to have coalesced

into a gonad. The 2 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 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). 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 4 O,P,Q and 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 midblastula 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 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 suggests to us 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 lab 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 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 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 primordial germ cells 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 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 30-50 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 (Fig. 7). 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.

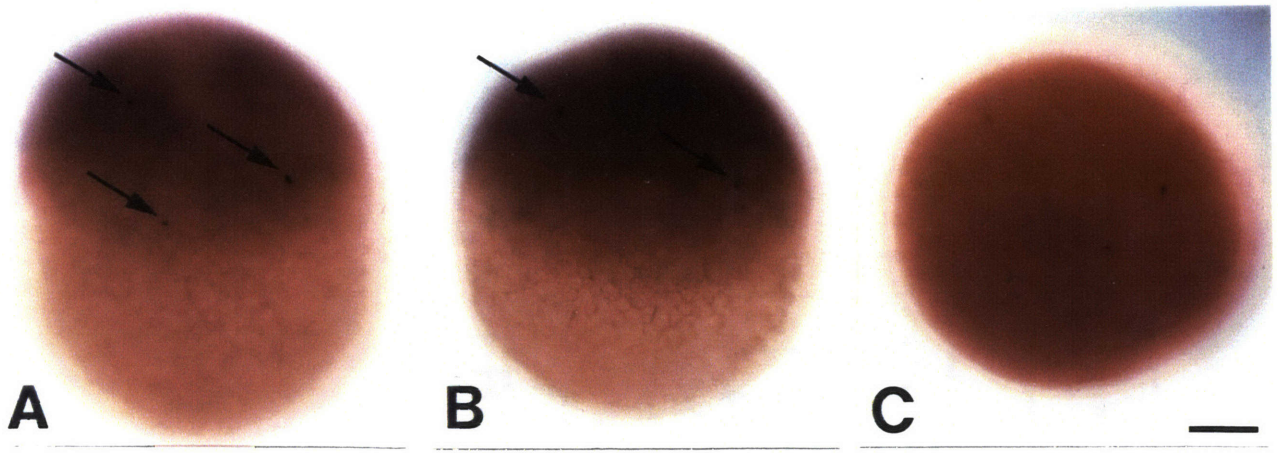

Figure 7. vas RNA expression in 2.5 hour unfertilized eggs.

Unfertilized eggs were incubated at 28.5^{o}C for 2.5 hr before fixation, followed by whole mount RNA in situ hybridization. In contrast to the lack of detectable expression in 1-cell-stage embryos (data not shown), we detect small clumps of *vas* RNA in 2.5 hr unfertilized eggs. These clumps appear to vary in number and location within the cytoplasm. Three different eggs are shown for comparison. (A, B) are side views while (C) is a top view.

Scale bar, 100 μm.

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 appear to remain relatively fixed in position within the embryo as cells continue to cleave around them and 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 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). Pole cell determination in *Drosophila* requires the activities of at least 2 other genes, *tudor* and *valois*. 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.

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 BglII and HindIII (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.

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CHAPTER 4

DISCUSSION:
FUTURE DIRECTIONS

With the cloning of the zebrafish *vasa* homologue, the question of where are the zebrafish primordial germ cells has finally been answered. As described in Chapter 3, *vas* RNA is localized to the zebrafish germ cells and serves as a molecular marker for the germ line from the 2-cell stage. This exciting result raises many questions about the mechanism of *vas* RNA localization and the role of *vas* in zebrafish germ line development. It also suggests new ways in which to develop techniques for the genetic manipulation of the zebrafish germ line. And finally, having this molecular marker in hand allows one to develop screens for mutants in germ line development.

Mechanisms of regional specification

In the analysis of early development, an issue of central importance concerns how different regions of the embryo become committed to different developmental programs (for review, see Slack, 1991). For example, how does the ventral marginal zone of the *Xenopus* blastula know that it should form erythrocytes and mesenchyme, instead of notochord, formed from the dorsal marginal zone? There are two mechanisms for specifying different regions in the early embryo. The first is the segregation of asymmetrically localized cytoplasmic determinants and the second is induction, which involves the communication between cells. Extensive studies have revealed that in many invertebrates and nonmammalian vertebrates that have been analyzed, the process of regional specification occurs stepwise, in a hierarchy of developmental decisions, with the initial establishment of larger, more general regions being specified by cytoplasmic localization, and the subsequent refinement depending on inductive interactions between these regions.

Cytoplasmic localization

The localization of determinants within the cytoplasm of a cell results in their unequal segregation to the daughters upon cell division. Different identities and therefore different fates are specified for the daughter cells based on the inheritance of these determinants.

The existence of localized cytoplasmic determinants had been suggested based on differences in the appearance of cytoplasm within the eggs, or based on the invariant cell lineage of some embryos. For example, the presence of specially staining cytoplasm in the vegetal pole of the amphibian egg, inherited by a small number of blastomeres which then go on to form germ cells, suggests that the vegetal pole cytoplasm contains a substance required for the formation of germ cells (Bounoure, 1939). However, proving the existence of a vegetally localized germ cell determinant required the transfer of vegetal pole cytoplasm to irradiated embryos and showing the rescue of germ cell formation (Smith, 1966).

Studies on the nature of cytoplasmic determinants has revealed that they may be localized RNAs, localized proteins or localized states of protein modification (for review, see Slack, 1991), however, I will only focus on localized mRNAs. The mechanism by which mRNAs become localized has been investigated in several systems (for review, see St. Johnston, 1995). The localization of mRNA can limit protein synthesis to the particular region of the cell or embryo where the protein is translated and required. In yeast, it has been found that mating type switching is controlled by the asymmetric localization of *ASH1* mRNA, leading to the unequal accumulation of ASH1 protein in the daughter cell (Long et al., 1997). Because Ash1p is a repressor of *HO* endonuclease transcription, a protein required for the initiation of mating type switching, a consequence of the localization of *ASH1* mRNA to daughter cells is the inhibition of mating type switching in daughter cells by Ash1p.

RNA localization is also used to establish a protein gradient emanating from this localized source of mRNA. An example of this is the diffusion of bicoid protein translated from localized *bicoid* RNA at the anterior pole, which gives rise to a gradient of bicoid protein with highest concentrations at the anterior of the *Drosophila* embryo (Berleth et al., 1988; Driever and Nusslein-Volhard, 1988). Trans-acting machinery required for RNA localization include proteins that bind to cis-acting localization signals and mediate

interactions with components of the cytoskeleton (Elisha et al., 1995) and organelles such as endoplasmic reticulum (Deshler et al., 1997).

Induction

Induction refers to the intercellular interactions in which a cell or tissue signals to another cell or tissue to assume a new fate (for review, see Slack, 1991). Inducing factors may be protein cytokines, small molecules or even nuclear proteins. One of the best studied examples is mesoderm induction in the early *Xenopus* embryo. It has been shown that the mesoderm forms from cells at the animal pole in response to signals emanating from the vegetal region of the embryo. This interaction has been extensively investigated in vitro using the induction assay and several inducer molecules involved in the process have been identified (Smith, 1989).

Understanding the mechanism of *vas* RNA localization

Localized maternal *vas* RNA expression, initially detected along the first 2 cleavage planes by whole mount in situ hybridization, and its subsequent segregation to the PGCs, could be the mechanism by which germ cell fate is determined in zebrafish. One of the questions raised by this observation is how does *vas* RNA get localized to the cleavage planes at this early stage. Based on what is known about RNA localization in other systems, it is likely that *vas* RNA attachment to cleavage planes is mediated by cytoskeletal components, such as microfilaments and microtubules. Inhibitors of microtubules and microfilaments have been widely used to determine whether either of these molecules is required for the localization of RNAs (Raff et al., 1990; Yisraeli et al., 1990; Pokrywka and Stephenson, 1991; Kloc and Etkin, 1995). For example, it has been shown that the initial localization of *Vg1* RNA (Weeks and Melton, 1987) to the vegetal hemisphere of *Xenopus* oocytes involves microtubules, while the anchoring of the message at the cortex requires microfilament function (Yisraeli et al., 1990). Similar cytoskeleton inhibition experiments

could be performed on early zebrafish embryos to determine which of these components is involved in *vas* RNA localization to the cleavage planes at the 2- and 4-cell stages.

The localization signals for several localized RNAs have been identified and are found to reside in the 3' untranslated region (3'UTR) of the mRNA (Macdonald and Struhl, 1988; Mowry and Melton, 1992; Dalby and Glover, 1993; Kim-Ha et al., 1993; Kislauskis et al., 1994; Gavis et al., 1996). This is likely to hold true for zebrafish *vas* homologue RNA as well. A possible approach for identifying the cis-acting sequences responsible for *vas* localization to the cleavage planes would involve first establishing an RNA injection system in which exogenous *vas* RNA is injected into 1-cell stage zebrafish embryos and properly localized to the cleavage planes. Then, one could test various *vas* 3'UTR deletion mutant RNAs to determine the minimal region required for proper *vas* RNA localization.

In some cases, localization signals are quite large, being several hundred nucleotides in length (Macdonald and Struhl, 1988; Mowry and Melton, 1992; Gavis et al., 1996). It has been suggested that this is due to the existence of multiple protein-binding sites within this region for different trans-acting factors thought to be required for localization (St. Johnston, 1992). Identification of the proteins that bind to localization sequences has been approached using biochemical means. In the case of *Vg1* RNA in *Xenopus*, several oocyte proteins have been shown to bind to its 340-nt localization element (Schwartz et al., 1992; Mowry, 1996; Deshler et al., 1997) and the functions of these proteins in RNA localization is just beginning to be studied (Elisha et al., 1995; Deshler et al., 1997). Interestingly, one of these proteins was found to mediate the interaction of the *Vg1* mRNA with the endoplasmic reticulum, uncovering a novel mechanism for RNA localization (Deshler et al., 1997). Identifying proteins that mediate *vas* mRNA localization in zebrafish would help build a general understanding of mechanisms of RNA localization.

As an aside, it would be interesting to see if the numbers of germ cells that form is dependent on the amount of *vas* RNA. The RNA injection system, as described above, could be used to analyze the effect of injecting different amounts of *vas* RNA on the number of germ cells. A similar experiment has been done in *Drosophila*: increasing the *oskar* gene dosage increased the amount of *oskar* RNA and protein, resulting in an increase in the number of germ cells formed (Ephrussi and Lehmann, 1992; Smith et al., 1992).

Localization of Vas protein

As described in Chapter 3, the expression of the zebrafish *vas* homologue has only been analyzed at the level of RNA expression. It is interesting to note that the specific localization of maternal zebrafish *vas* transcripts is in striking contrast to the uniform expression of maternal *Drosophila vas* RNA in oocyte and preblastoderm embryo (Hay et al., 1988b; Lasko and Ashburner, 1988). Localization of *Drosophila vasa* occurs at the level of Vasa protein expression and zygotic transcription which are both limited to pole cells (Hay et al., 1988a, b; Lasko and Ashburner, 1988). In mouse and frog, the *vasa* homologue RNA expression in early embryos was not reported, but the protein expression of the *vasa* homologues in these organisms was first detected in gonadal PGCs (Fujiwara et al., 1994; Komiya et al., 1994).

In understanding the role of zebrafish *vasa* homologue in zebrafish germ line development, it will be necessary to know the localization of Vasa protein. Following the guidelines given in Harlow and Lane, 1988, polyclonal antibodies are being generated in rabbits against a mixture of 3 12-amino acid peptides based on the deduced protein sequence (Figure 1). These peptides were selected based on their high degree of hydrophilicity, as determined using the Hopp and Woods hydrophilicity scale which was developed to locate antigenic segments (Figure 2) (Hopp and Woods, 1981). It has been

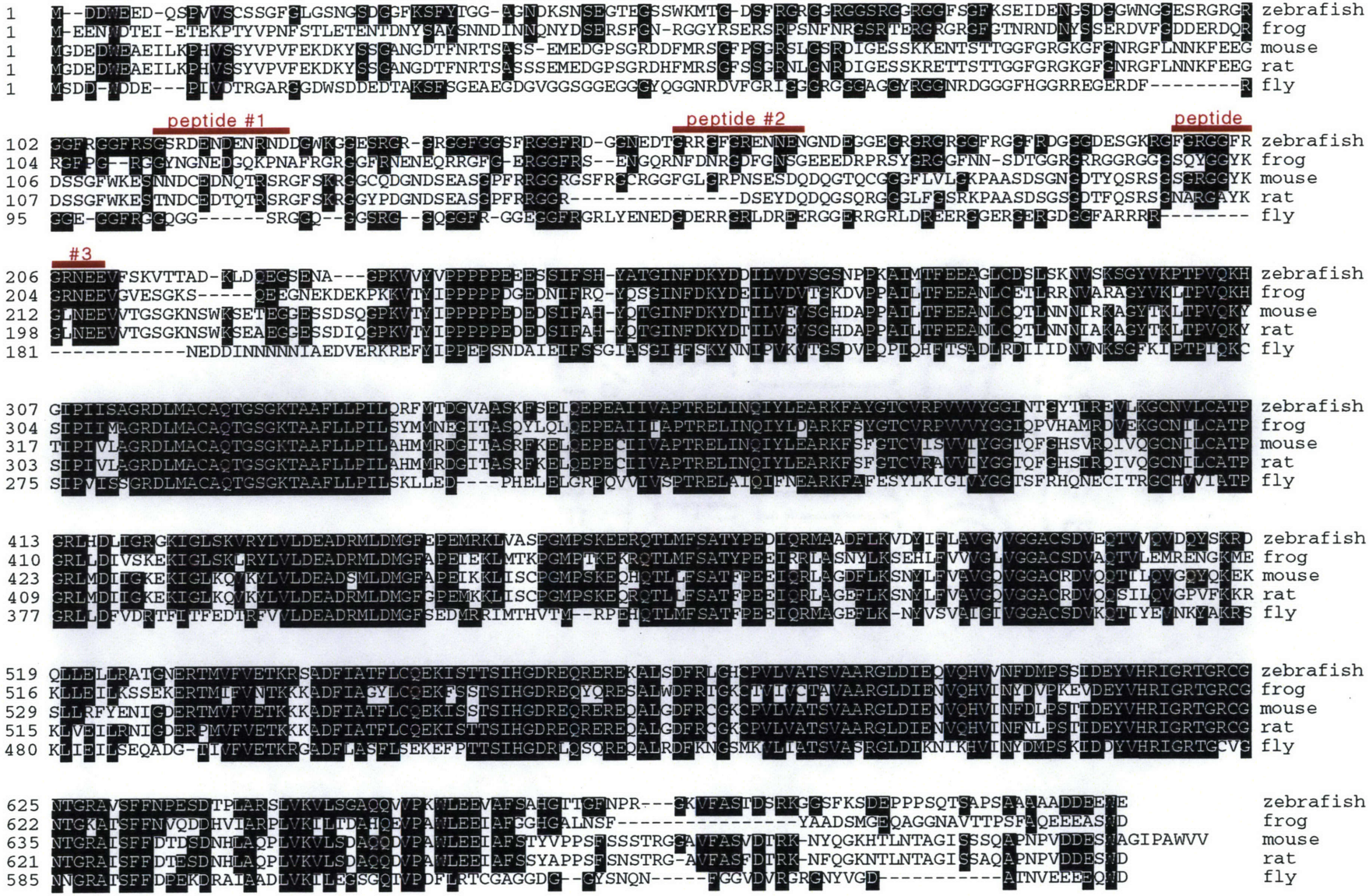
peptide #1
peptide #2
peptide
#3
zebrafish
frog
mouse
rat
fly

Figure 1. Peptides used for Vas polyclonal antibody production.

The alignment of 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., 1988b; Lasko and Ashburner, 1988) vasa protein sequences is shown, with the amino acids that are identical to the zebrafish sequence highlighted in black. Red lines are drawn above the zebrafish sequence for the 3 peptides used in the immunization.

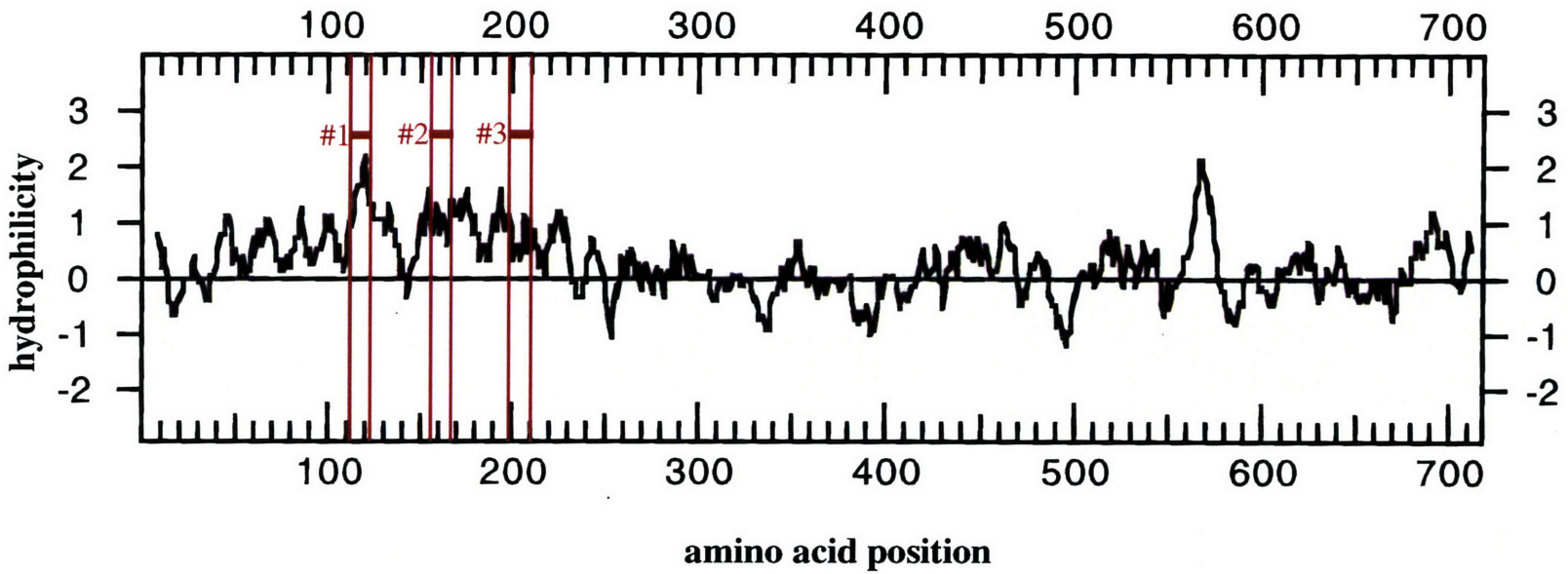

Figure 2. Hydrophilicity plot of the deduced zebrafish Vas protein sequence.

The hydrophilicity plot was generated using the Hopp and Woods hydrophilicity scale (DNA Strider computer program; Hopp and Woods, 1981) for the deduced amino acid sequence of the zebrafish *vas* homologue. The 3 peptides selected for the immunization for the polyclonal antibody were chosen based on their high degree of hydrophilicity and are indicated with red bars. They correspond to amino acids 111-122 (peptide #1), 155-166 (peptide #2) and 199-210 (peptide #3).

observed that hydrophilic regions tend to be exposed on the surface of folded proteins (Hopp and Woods, 1981, 1983; Kyle and Doolittle, 1982) and therefore are regions of the native protein that are more likely to be recognized by an antibody. Moreover, these peptides are located in the region of the protein that contains multiple Arg-Gly-Gly repeats, thought to be involved in binding RNA (Kiledjian and Dreyfuss, 1992) and are likely to be exposed on the surface of the native protein. And finally, raising the antibody against a mixture of the 3 peptides instead of single peptides could help if any of them proves not to be antigenic (Harlow and Lane, 1988). Once the antibody is generated, it should be tested on Western blots and in whole mount antibody staining of embryos.

Isolation and culture of primordial germ cells

Mouse embryonic stem (ES) cells have revolutionized the study of mouse development. Derived from mouse blastocysts, they can be genetically manipulated, then injected into host embryos where they are able to colonize the germ line of the resulting chimeric mice (Evans and Kaufman, 1981; Martin, 1981; Bradley et al., 1984). After inactivating genes in ES cells by homologous recombination, hundreds of knockout mice have been generated and their role in development has been extensively analyzed (Capecchi, 1989). Recognizing the power of this reverse genetic technology, researchers have attempted to establish ES cell equivalents in other organisms, but without success. The problem seems to be the immortalization of the cells and maintaining them in an undifferentiated state in vitro (Rossant, 1993). The discovery that mouse PGCs, cultured with specific growth factors, behave just like ES cells gives researchers new hope (Matsui et al, 1992; Resnick et al., 1992; Labosky et al., 1994; Stewart et al., 1994). Perhaps, by virtue of being PGCs, PGCs in other organisms might possess the characteristics that allow mouse PGCs to become ES cells.

In testing whether zebrafish PGCs could be cultured to form ES cell equivalents, the first issue is the isolation of the PGCs. One way in which this could be accomplished

could be the generation of a transgenic fish that with germ line-specific reporter gene expression that can be visualized fluorescently. Then fluorescence activated cell sorting (FACS) could be used on dissociated embryos to select for fluorescent PGCs. The most convenient reporter gene to use would be green fluorescent protein (GFP) because the protein product would be naturally fluorescent, without the need for additional procedures to visualize the PGCs. Transgenic fish that express GFP have been successfully generated (Amsterdam et al., 1994). A second possibility is *lacZ*, in which case one would have to stain the embryos with fluorescein-di-ß-D-galactopyranoside (FDG) first to detect *lacZ* expression (Lin et al., 1994).

Regardless of which reporter gene is used, it will be necessary to determine the sequences required for proper expression of these proteins in the PGCs. Such questions as what promoter sequences are necessary for germ line-specific expression, and whether the maternal *vas* RNA localization signals be necessary for Vasa protein expression in the germ cells will need to be addressed. Additionally, sequences required for translation of *vas* RNA may reside outside the 3'UTR as has been shown for *oskar* translational regulation in *Drosophila* (Rongo et al., 1995).

There may also be other ways in which PGCs can be isolated. Since PGCs tend to be larger in size than somatic cells (Nieuwkoop and Sutasurya, 1979), one possibility would be to try to sort embryonic cells based on size and to select the fraction containing the largest cells. Assaying for PGC enrichment in this fraction could be done using RNA in situ hybridization to the *vas* gene.

Once PGCs are isolated, culture conditions that promote in vitro germ cell survival will need to be established. Factors that promoted the immortalization of mouse PGCs, namely basic fibroblast growth factor, soluble leukemia inhibitory factor and feeder layers expressing the transmembrane form of steel factor, should be tested (Matsui et al, 1992; Resnick et al., 1992). Whether zebrafish versions of each of these factors, or other factors

altogether, will be necessary remains to be determined. PGC proliferation could be assayed using RNA in situ hybridization to the *vas* gene.

Screening for mutants in germ line development

Because the zebrafish *vas* homologue is a reliable marker for the primordial germ cells from early cleavage through their migration to and colonization of the gonad, it should be possible to use the *vas* probe in a genetic screen for mutants in zebrafish germ line development. Mutants would be identified by screening for aberrant *vas* RNA expression by whole mount in situ hybridization. Probing for *vas* expression would be even easier using the polyclonal antibody discussed above if it proves to reliably mark the zebrafish PGCs. And the simplest method of screening would be possible if mutations were generated in a transgenic line that has germ line-specific reporter gene expression (as discussed in the previous section), allowing for aberrant germ cell development to be visualized in vivo.

The identification of a zebrafish germ cell marker that can be used in a mutant screen comes at an opportune time. Our lab has recently shown that an insertional mutagenesis screen using a retrovirus is possible in the zebrafish (Allende et al., 1996; Gaiano et al., 1996), and currently is planning to conduct a large-scale insertional mutagenesis screen. It should be possible to incorporate an in situ hybridization screen for germ line development mutants along with the screen for mutants with visible morphological defects that is already planned. The types of germ line mutants we might expect to identify would be defective in germ cell formation, proliferation and migration. Because the mutated genes will be tagged with the proviral insertion, it should be relatively straightforward to clone the disrupted genes (Allende et al., 1996; Gaiano et al., 1996), which would allow one to begin to form a more complete understanding of zebrafish germ line development.

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APPENDIX

MONOCLONAL ANTIBODIES RAISED AGAINST MATURE ZEBRAFISH SPERM

ABSTRACT

In this appendix, I describe monoclonal antibodies raised against mature zebrafish sperm. I wished to identify a marker for the zebrafish primordial germ cells (PGCs) and chose this approach because it had been shown previously that mature sperm share antigenic membrane determinants with PGCs in another fish species (Parmentier et al., 1984). An antibody recognizing a spermatozoan membrane antigen that is also present on PGCs in zebrafish would be useful in marking the germ cells for studies on germ line development. Furthermore, it could possibly be used as a means of isolating the primordial germ cells for culturing. I obtained four types of antibodies, some of which labeled germ cells in the adult testes, but none of which labeled the primordial germ cells.

INTRODUCTION

Since the late 1800s, the development of the germ line has been intensively studied in several organisms, with much research devoted to the initial identification of the primordial germ cells (PGCs). It has been found that PGCs typically originate extra-gonadally and migrate through various tissues to the gonadal anlagen during development (Nieuwkoop and Sutasurya, 1979). In *C. elegans*, *Drosophila*, and anurans, germ cells contain specialized cytoplasm, called germ plasm (or pole plasm in *Drosophila*). Because germ plasm is segregated to and present in the germ cells throughout the development of the germ line, it has been a useful germ cell marker in these organisms (Eddy, 1975). In organisms for which no germ plasm equivalent exists, however, PGCs were traditionally identified morphologically, on the basis of a combination of the following characteristics: large size compared to surrounding cells, oval or round shape, large nucleus, dense and granular cytoplasm, and pseudopodia (Gamo, 1961; Spiegelman and Bennett, 1973; Timmermans and Taverne, 1989).

Having a means to identify the PGCs has greatly aided the analysis of germ line development. The presence of germ plasm makes germ cells easy to recognize in worms, flies and frogs. Genes that are specifically expressed in germ cells have been identified and some have even been shown to be localized to germ plasm (Guedes and Priess, 1997; Rongo and Lehmann, 1996; Zhou and King, 1996). Expression of these genes can then be used to track germ cells during development using RNA in situ hybridization or antibody staining. In other organisms, antibodies against molecules on the surface of PGCs have been particularly useful. For example, EMA-1, an antibody raised against mouse embryonic carcinoma cells, labels migrating murine PGCs (Hahnel and Eddy, 1986) and has been used to isolate murine PGCs via fluorescence-activated cell sorting (FACS) (McCarrey et al., 1987). Furthermore, EMA-1 has been shown to cross-react with the PGCs in chick (Urven et al., 1988).

In the teleost, carp, monoclonal antibodies were raised against mature carp spermatozoa (Parmentier et al., 1984). Of 11 sperm-specific antibodies obtained, 4 were shown to label primordial germ cells (Parmentier and Timmermans, 1985). This indicated that some antigens present on mature sperm first appeared on pre-germ cells and were continuously expressed during their development into spermatozoa. These antibodies have been useful in studying the migration and differentiation of germ cells in the carp (Parmentier and Timmermans, 1985; vanWinkoop and Timmermans, 1990).

We were interested in first identifying the PGCs and obtaining a PGC marker for the zebrafish. Initial attempts at examining the sections of 3 d zebrafish larvae for cells identifiable by morphology were unsuccessful. In addition, we tested EMA-1 (Hahnel and Eddy, 1982), a polyclonal antibody to Vasa, a *Drosophila* germ cell component (Hay et al., 1988), and WCS29, one of the PGC-specific carp sperm antibodies (Parmentier et al., 1984), in zebrafish by whole-mount immunostaining but all three antibodies failed to cross-react with the zebrafish PGCs (not shown). We therefore chose the same approach that was successful in the carp. We raised monoclonal antibodies to mature zebrafish sperm

and screened for antibodies that also labeled zebrafish PGCs. As will be discussed, although we succeeded in isolating sperm-specific antibodies, none of these labeled the PGCs.

RESULTS and DISCUSSION

Morphology of the adult testis

The zebrafish testis is a paired organ, located at the dorsal side of the coelomic cavity, ventrolateral to the swim bladder (Fig 1A). The structure of the testis is of the unrestricted spermatogonaial testis-type (Grier, 1981) (Fig 1B), meaning that spermatogonia can be found along the entire length of the tubule. Spermatocysts, containing germ cells surrounded by Sertoli cells, form when spermatogenesis begins. The germ cells within a cyst are linked by cytoplasmic bridges and develop synchronously. Upon spermiation, Sertoli cells separate, making cyst and tubule lumens continuous which consequently releases sperm into the lumen of the tubule. Spermatogenesis proceeds quasi-continuously throughout the life of the fish (Takashima and Hibaya, 1995).

Immunohistochemistry

In an effort to isolate a zebrafish PGC-specific antibody, we decided to use the same approach that was used in the carp to generate WCS29: we raised monoclonal antibodies against zebrafish sperm, then screened for those that also labeled zebrafish PGCs. The results from this experiment are summarized in Figure 2. We fused SP2/0 myeloma cells with spleen cells from mice immunized with mature zebrafish sperm and obtained 523 stable hybridoma clones. We screened all 523 hybridoma supernatants for reactivity to sperm using whole sperm cell ELISAs and identified 70 positive clones.

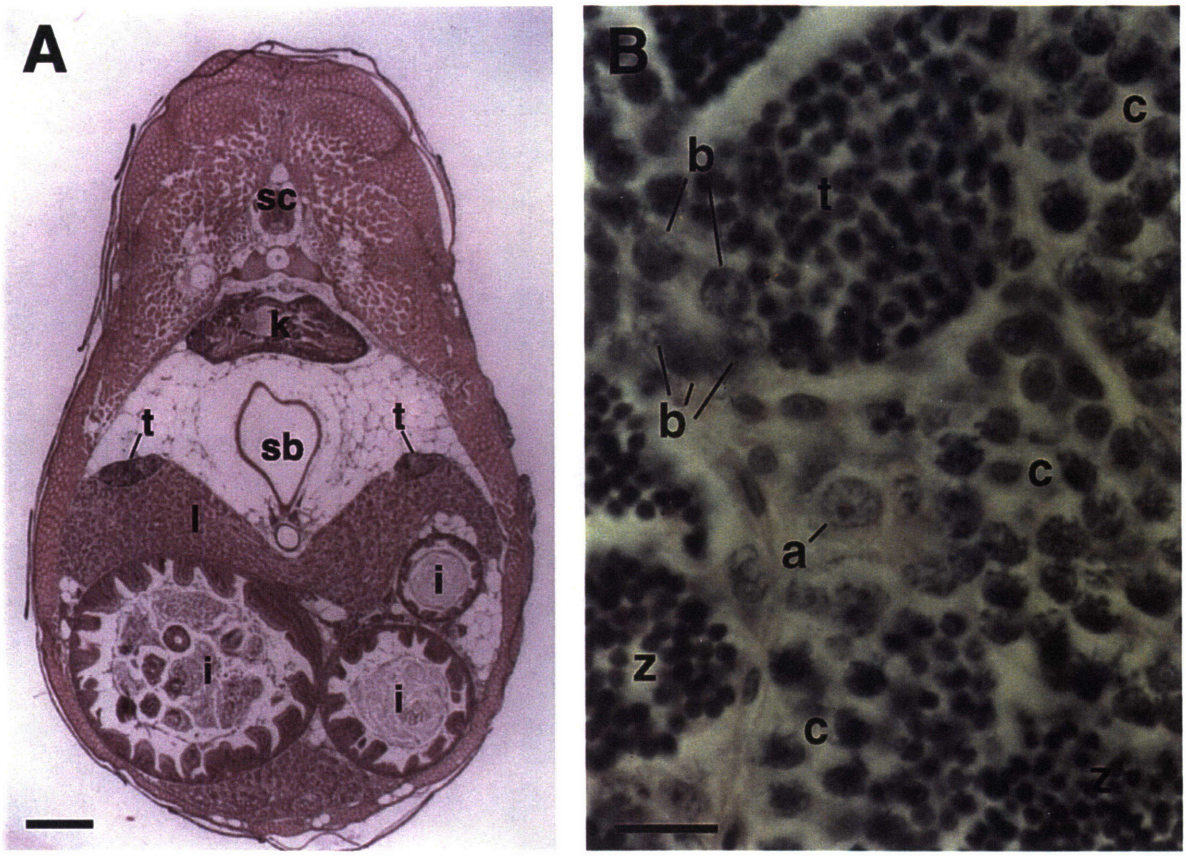

Figure 1. The position and structure of the adult zebrafish testis.

Transverse sections (4 μm) were prepared from Bouin's-fixed adult male zebrafish and stained with hematoxylin and eosin.

(A) A transverse section of an adult male zebrafish shows the position of the testes (t) relative to the other organs. Abbreviations: i, intestine; k, kidney; l, liver; sb, swim bladder; sc, spinal cord.
(B) A higher magnification view of the testis shows the structure this organ. Spermatocysts containing synchronously developing germ cells at all stages of spermatogenesis can be clearly seen. Abbreviations: a, type A spermatogonia; b, type B spermatogonia; c, spermatocytes; t, spermatids; z, spermatozoa.

Scale bars, 200 μm in A, 10 μm in B.

A.	Number of hybridomas screened		523
	Total positive clones selected in primary screen		70
B.	Isotypes:	IgM	55 clones
		IgG	18
		(mixed	8)
C.	Adult male fish staining patterns:		
		spermatozoa only	13 clones
		sperm, spinal cord	12
		nonspecific	34
		no staining	11
D.	15 day fish staining patterns:		
		spinal cord and muscle	7 clones
		no staining	3
		not tested	2

Figure 2. Results obtained in zebrafish sperm monoclonal antibody generation and screening.

(A) The results of the fusion and primary screen of hybridoma supernatants by whole sperm cell ELISA.
(B) The numbers of positive hybridomas obtained of each isotype. 8 clones were mixed and contained 2 or 3 different isotypes within the same hybridoma clone.
(C) The results from staining sections of paraformaldehyde-fixed adult male fish with the supernatants of positive clones.
(D) The results from staining sections of paraformaldehyde-fixed 15 d fish with the supernatants of the 12 clones that stained sperm and spinal cord.

The positive supernatants were subsequently tested on paraformaldehyde-fixed adult male sections, and we identified 4 staining patterns (Fig 3). 34 monoclonal antibodies stained the sections non specifically (Fig 3F), while 11 did not stain at all (not shown). Of the 2 specific types of antibodies obtained, one stained only mature spermatozoa (13 clones) (Fig 3A, B). The second specific type stained all stages of spermatogensis along with the spinal cord (12 clones) (Fig 3C, D). The significance of the shared antigen between sperm and the spinal cord is not known.

Given these four classes of antibodies, it was possible that the group that stained all stages of spermatogenesis and the spinal cord could also label the PGCs. Perhaps the antigen recognized by these antibodies appears early in germ cell development and is maintained throughout spermatogenesis. We therefore tested this class of antibodies on sections of paraformaldehyde-fixed 15 d fish at which time the gonads could be clearly identified. 7 antibodies in this class labeled the spinal cord and muscle cells, but not the PGCs (Fig 3E). Of the remaining 5, 3 antibodies did not stain the 15 d fish sections at all (not shown) and the other 2 have not been tested yet.

In summary, we did successfully raise monoclonal antibodies against mature zebrafish sperm and obtained a collection of antibodies that recognize different antigens. However, none of these antigens were shown to also be present on the zebrafish PGCs. The number of different antigens represented within each group of antibodies is unknown but could be determined by Western blotting. Furthermore, the antigens recognized by the class of antibodies that did not stain sections of paraformaldehyde-fixed adult male fish may be masked by the paraformaldehyde fixation. It would be interesting to test this class of antibodies on unfixed frozen sections. Any antibodies able to label unfixed PGCs would likely also label live PGCs and could be potentially useful in fluorescence-activated cell sorting of live PGCs for culturing.

Figure 3

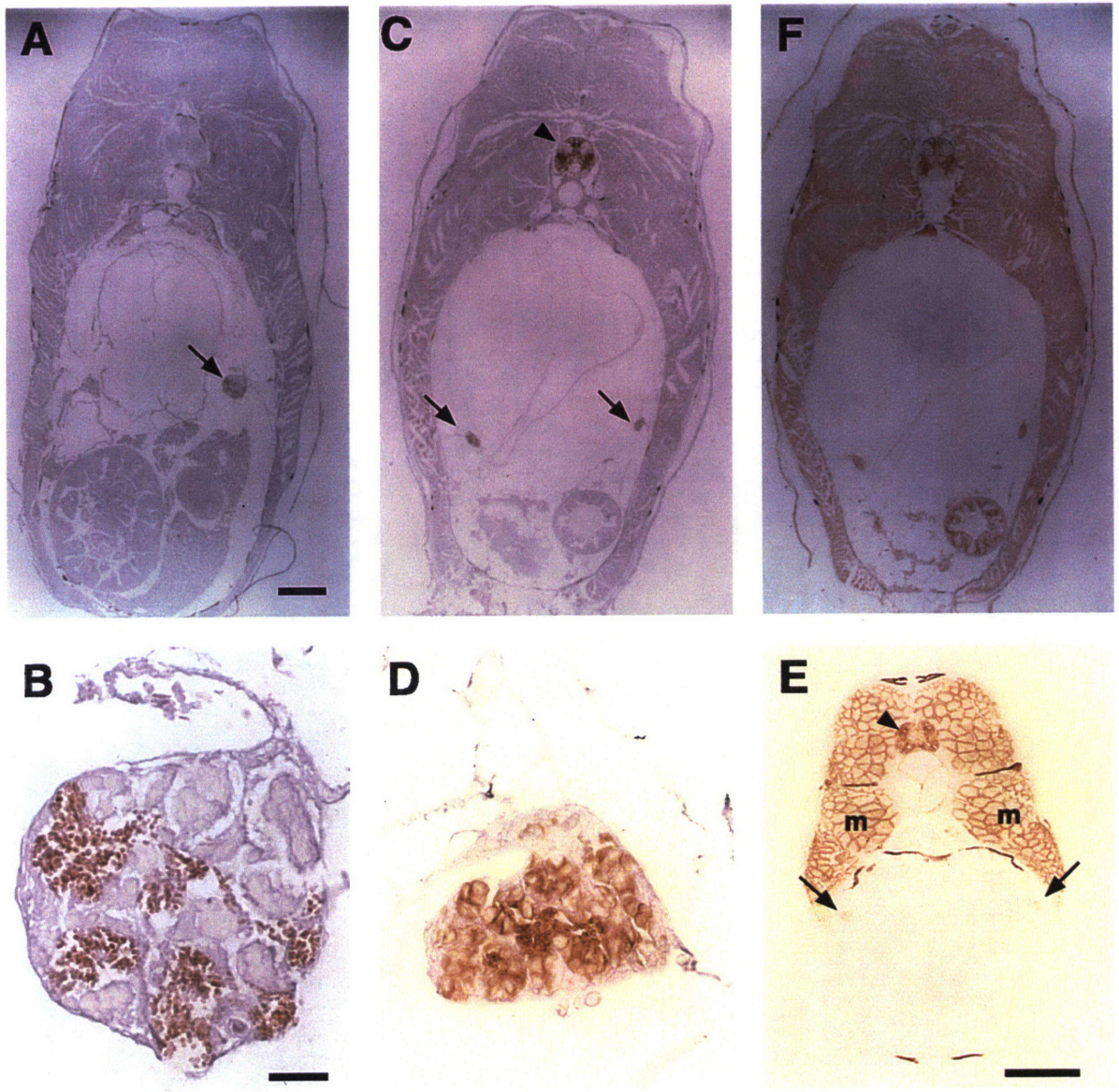

Figure 3. Staining patterns on sections of adult male fish.

Staining of sections (4 μm) of paraformaldehyde-fixed fish with hybridoma supernatants was detected using horseradish peroxidase-conjugated secondary antibodies followed by staining with diaminobenzidine (brown). Some sections were counterstained with hematoxylin (blue). Representative stainings are shown for three of the four different staining patterns on adult male fish that were obtained (A-D, F). A representative staining of a 15 d fish section is also included (E). Arrows point to testes and arrowheads indicate the spinal cord.

(A) Representative staining using an antibody that stains only spermatozoa (arrow).

(B) Higher magnification view of the testes in (A) to show that only spermatozoa are stained with this antibody.

(C) Representative staining of an adult male section using an antibody that stains germ cells in all stages of spermatogenesis (arrow) and the spinal cord (arrowhead).

(D) Higher magnification view of the testes in (C) to show that all sperm cell stages are labeled with this antibody.

(E) Representative staining of a 15 d fish section using the same class of antibody as in (C, D) that stains the spinal cord (arrowhead) and muscle cells (m). Note the absence of staining in the gonads (arrows).

(F) Representative staining of an adult male section using an antibody that gives a non-specific staining pattern.

Scale bars, 200 μm except 20 μm in B, D and 100 μm in E.

MATERIALS AND METHODS

Sperm collection and immunization of mice

Adult male zebrafish (*Danio rerio*) were anesthetized in 0.04% 3-aminobenzoic acid ethyl ester in fish water (0.05 g/l Instant Ocean). By squeezing fish gently with forceps on either side of its belly, sperm-containing fluid was obtained using a 10 µl capillary and collected in phosphate-buffered saline (PBS), pH 7.2. The sperm was washed three times and resuspended in PBS. Adult female Balb/c mice obtained from The Jackson Laboratory (Bar Harbor, Maine) were injected intraperitoneally with 4-5 x 10^7 sperm cells in 0.2 mls PBS. The mice were boosted twice with an intraperitoneal injection of 0.5-1 x 10^7 sperm in 0.2 mls PBS at 4 week intervals following the first injection. The final boost was administered another 6 weeks later and consisted of both an intraperitoneal and intravenous injection. Each injection contained 3 x 10^7 sperm cells in 0.2 mls PBS. Three days later the mice were euthanized, their spleens removed, and cell suspensions made by gentle teasing with forceps.

Generation and primary screening of monoclonal antibodies

Monoclonal antibodies were produced as described by Pereira et al., 1995, but modified for mouse cells. Hybridomas were obtained from a fusion between cells of the mouse myeloma SP2/0 and splenocytes from Balb/c mice immunized with zebrafish spermatozoa (see above). Supernatants from hybridoma clones were tested for reactivity to zebrafish spermatozoa using indirect cellular ELISA, essentially as described (Coligan et al., 1992). 1-5 x 10^6 sperm cells were plated per well, using a 96-well U-bottom plate, blocked with 3% BSA in PBS. Horseradish peroxidase-conjugated secondary antibodies (Cappel) were used and binding was detected with tetramethylbenzidine. Controls included no sperm and no antibody supernatant. Positive supernatants were isotyped using an antibody capture on anti-immunoglobulin antibodies assay as described (Harlow and Lane, 1988). Some

hybridomas were subcloned by plating limiting dilutions according to Harlow and Lane, 1988.

Preparation of tissue for sections

For the morphological study, adult male zebrafish were fixed in Bouin's solution. In preparation for antibody staining, adult male and 15 day fish were fixed in 4% paraformaldehyde in PBS. In both cases, the tissue was dehydrated then embedded in paraffin. 4 μm sections were cut on a microtome and collected on Superfrost slides (Fisher). Paraffin was removed using xylene. Sections were stained with hematoxylin and eosin or used in antibody staining.

Immunohistochemistry

Sections were rehydrated then blocked in PBS containing 2% BSA, 1% DMSO and 0.1% Triton X-100. After removing the blocking solution, slides were incubated with hybridoma supernatant in a humid chamber overnight at 4°C. Slides were washed with PBS then incubated with goat-anti-mouse heavy chain-specific immunoglobulin antiserum coupled to horseradish peroxidase (Cappel), diluted 1:200 in blocking solution, for 1 hr at room temperature. After washing with PBS, sections were stained with diaminobenzidine for 5-15 minutes, followed by rinsing in PBS then dH_2O. In some cases, sections were then counterstained lightly with hematoxylin. Following dehydration and clearing in xylene, slides were mounted in Permount (Fisher). Slides were viewed and photographed with a Nikon Microphot SA microscope.

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