Characterization of an Oocyte Specific Clone, KSZf5, in Zebrafish (*Danio rerio*)

By

Katrin Stedronsky

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CHARACTERIZATION OF AN OOCYTE SPECIFIC CLONE, KSZf5, IN ZEBRAFISH (Danio rerio)

BY

KATRIN STEDRONSKY

A Thesis/Practicum submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

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ABSTRACT

Localized maternal determinants have been recognized as having a significant role in establishing normal development in both *Drosophila* and *Xenopus*. Maternal determinants not only establish the embryo's axis for proper development, but they also determine cell fate. However in zebrafish, although this species is becoming increasingly popular as a model for vertebrate studies, the role of maternal determinants in normal development in the zebrafish is unclear. To gain a better understanding of what role maternal determinants have in defining normal development in zebrafish, detailed knowledge of the make up of a mature oocyte is needed. Working towards this goal, I have cloned and characterized an oocyte-specific transcript, KSZf5, from the zebrafish *Danio rerio*. *In situ* hybridization experiments demonstrate that KSZf5 transcripts are present throughout various stages of oocyte development, identifying them as maternally derived, the transcripts are also present at significant levels in the mature oocyte. Other RNA hybridization analyses demonstrate that KSZf5 transcripts remain present throughout various stages of post-fertilization embryos, suggesting the continued importance of the KSZf5 gene product in development. Although its role in the developing embryos is unknown, the expression pattern of KSZf5 suggests that it may be a maternal determinant that becomes localized to the zebrafish germ plasm, and that it may have a role in germ cell differentiation. Sequence analysis of KSZf5 identifies homology with several receptor proteins, suggesting that it may function as a receptor whose function is presently unknown.

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ABBREVIATIONS

BϕB	bromophenol blue
BSA	bovine serum albumin
CSPD	disodium 3-(4_methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate
C:I:A	chloroform:isoamyl alcohol
DEPC	diethylpyrocarbonate
DIG	digoxigenin
DMSO	dimethylsulfoxide
EDTA	ethylenediaminetetracetic acid
EtOH	ethanol
MBP	myelin basic protein
PBS	phosphate buffered saline
P:C:I	phenol:chloroform:isoamyl alcohol
PCR	polymerase chain reaction
RFLP	restriction fragment length polymorphism
SDS	sodium dodecyl sulfate
Tween 20	polyoxyethylenesorbitan monolaurate
UTR	untranslated region
vol	volume
X	times

INTRODUCTION

How a fertilized egg comes to develop into a fully functional organism represents one ot the most fundamental questions in biology. Researchers such as C. Nüsslein-Volhard directed their efforts in trying to understand the fundamentals of developmental biology towards examining the development of *Drosophila*. The insights that have been gained from examining *Drosophila* development are remarkable, particularly in relation to the role the mother plays in preparing the embryo for normal development. We now have an understanding of how significant maternal determinants are in the establishment of the body axis in the *Drosophila* embryo. The continued efforts of many investigators have pieced together the intricate interactive systems of maternal effect genes that act in concert to establish the body axis. This information gained from *Drosophila* has also been employed in trying to unravel the developmental program of other organisms, particularly the vertebrates. However, the research done in *Drosophila* development allows only for speculation into the control systems of higher vertebrates because there are significant differences between the two in how the embryo develops. This is particularly true in the very early stages, which begin in *Drosophila* with 13 nuclear divisions before actual cell formation, unlike any vertebrate organism. Since the developmental program of the vertebrate embryo is significantly different from that of *Drosophila*, there is a need to examine a number of vertebrate systems in order to determine how closely the developmental controls parallel those found in *Drosophila.* Development of the mouse has been examined but the research in this organism has been hindered by a low reproduction rate as well as the fact that the embryo develops internally and slowly. *Xenopus*, with external and rapid development is becoming a more popular model for research in the field of development, particularly in the attempt to identify the role of maternal determinants in establishing normal development in vertebrates. Research into the understanding of how development is controlled in this organism has met with some success. Researchers have been able to parallel a number of the interactive systems that control normal development in both *Drosophila* and *Xenopus*, particularly with respect to maternal effect genes.

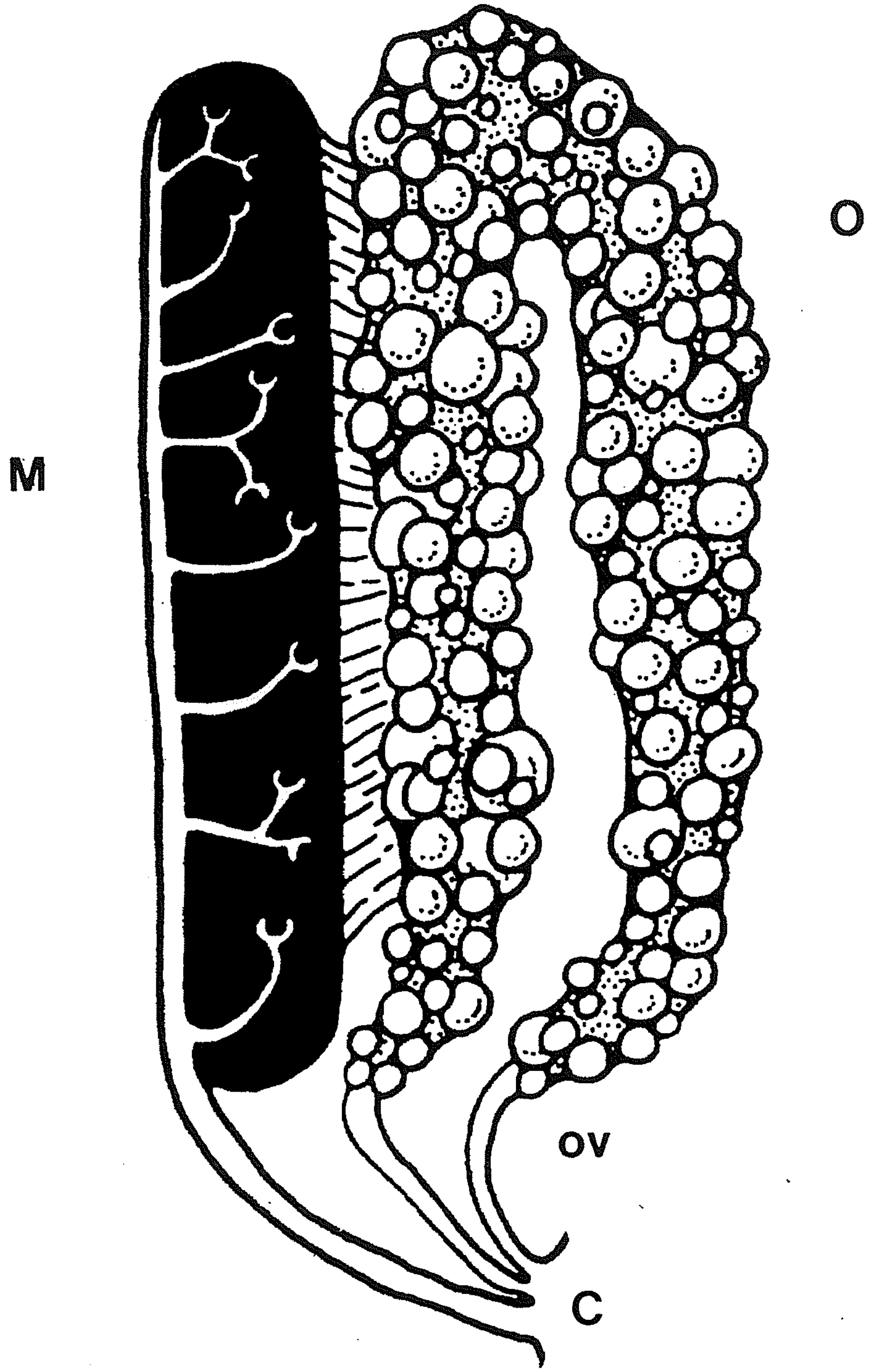
More recently the zebrafish has also demonstrated the potential to be an appropriate model system for these analyses. The zebrafish has become a more popular experimental organism for vertebrate developmental studies for several reasons. It has a high reproductive potential, often spawning 100 eggs at a time. The rate of development of the embryo is rapid: 72 hours after fertilization functionally competent fry hatch from their chorions. The developing embryos are transparent which makes it easy to follow the development of each organ system in the embryo. All of these features make the zebrafish a more suitable organism for examination.

The Teleost Ovary and Oocyte

During teleost development, the ovary forms from the fusion of gonadal ridges producing a coelomic cavity that is lined with peritoneal epithelium (Dodd, 1977). This ovary is a paired structure that is attached to the body cavity on either side of the dorsal mesentery (Hoar, 1969; Redding and Patiño, 1993), and runs parallel to the mesonephric system (Hoar, 1969). In most teleosts, the ovary persists as a cystovarian ovary (Figure 1) that has extensions towards the posterior forming oviducts that open into the cloaca (Dodd, 1977; Redding and Patiño, 1993). In other teleosts these extensions degenerate and eggs are released into the body cavity. The zebrafish *Danio rerio*, however, represents the former type in which oviducts are present and eggs are transported into the cloaca after ovulation (Dodd, 1977).

Ovarian development in teleosts, as in other non-mammalian vertebrates, is characterized by a proliferation of cells inward from the epithelial lining of the ovary (Chieffi and Pierantoni, 1987). Three patterns of ovarian development exist in fishes: synchronous, group synchronous and asynchronous. Synchronous development is characterized by the development of all oocytes in unison that leads to one ovulatory event. Group synchronous development is characterized by two or more clutches of oocytes, each clutch representing a different developmental stage. Asynchronous development is characterized by an ovary in which all oocytes are at different stages of development (Redding and Patiño, 1993), from germ cells to eggs. Asynchronous development is characteristic of zebrafish, and

Figure 1: Schematic diagram of the cystovarian ovary in teleosts, indicating the ovary (O) with oviducts (ov) extending towards the cloaca (C). The ovary runs parallel to the mesonephric system (M) (Hoar, 1969).


O
M
ov
C

the various stages of oocyte development can be seen in a cross-section through the zebrafish ovary (Figure 8A). Oogonial and early meiotic oocytes are embedded within the ovarian stroma (Selman *et al.*, 1993) which is a structural support system for the ovary and is composed of connective tissue, fibers and fibroblasts (Dodd, 1977). Within the ovary the oocytes grow and are incorporated into ovarian follicles. Dodd (1977) describes ovarian follicles as oocytes that have become arrested at the prophase stage of meiosis and have acquired a surrounding layer of epithelial cells called follicle cells. This layer of follicle cells, also known as the granulosa cell layer, consists of a single layer composed of flat cells (Hoar, 1969; Chieffi and Pierantoni, 1987) and has been implicated in steroid synthesis and transport necessary for oocyte maturation (Redding and Patiño, 1993).

Between the oocyte cell membrane and the granulosa layer lies a region that contains a perivitelline space equivalent to the zona pellucida in mammals (Dodd, 1977). Across the perivitelline space, microvilli extend from the granulosa cells and penetrate the oocyte cell membrane to form the zona radiata (Dettlaff, 1988). Microvilli from the surface of the oocyte membrane also extend into the zona radiata (Dodd, 1977; Dettlaff, 1988; Redding and Patiño, 1993) in the direction of the granulosa cells, forming pore canals. Situated above the granulosa layer, a layer of cells is present called the theca cells. The theca cell layer can be subdivided into the theca externa and the theca interna. In teleosts, the theca layer is a fibroblastic layer that is heavily supplied with capillaries important for transport of substances across the follicular wall to the oocyte (Chieffi and Pierantoni, 1987). The theca layer has also been implicated in steroid synthesis and transport, much like the granulosa layer (Redding and Patiño, 1993).

Zebrafish Oocyte Development

Selman *et al.* (1993) outline a series of five stages of oocyte development in zebrafish. Within the zebrafish ovary, germ cells can be distinguished from somatic cells on the basis of the size ratio between the nucleus and cytoplasm. Generally the primary germ cells have nuclei that take up at least 50% of the total volume of the cell, whereas in the somatic cells the proportion of the total volume taken up by the nucleus is much smaller. The cytoplasm within the primary germ cells contains mitochondria and an electron-dense nuage (Selman *et al.* 1993). This electron dense nuage has been implicated in localizing maternal transcripts in developing *Xenopus* oocytes to the vegetal cortex (Elinson *et al.*, 1993; Forristall *et al.*, 1995; Kloc and Etkin, 1995), as well as localizing germinal granules important in determining *Xenopus* germ plasm (Heasman et al., 1984). In zebrafish the role of this nuage is not well understood; however since zebrafish development is very similar to that of *Xenopus* it is reasonable to assume that this nuage plays a role in zebrafish germ plasm determination.

In the primary growth stage (stage I), oocytes progress through the early stages of prophase and become arrested in diplotene of the first meiotic division (Selman *et al.*, 1993). The primary growth stage can be further subdivided into two phases. In the pre-follicle phase, the developing oocyte lies in a nest of oocytes that at the same stage of meiotic prophase. In the follicle phase, the oocyte becomes surrounded by a layer of follicle cells. At this stage the oocyte grows in size and accumulates intercellular organelles such as mitochondria, Golgi complexes, and endoplasmic reticulum. The electron-dense nuage is associated with clusters of mitochondria, and the nucleoli of the oocyte nucleus (germinal vesicle) move to the periphery.

In stage II of oocyte development, cortical alveoli begin to develop. Cortical alveoli are membrane-bound vesicles that synthesize their own protein and carbohydrate contents (Selman *et al.*, 1993). During stage II the germinal vesicle enlarges and becomes irregular in shape, and the nucleoli within the germinal vesicle continue to increase in number. Mitochondria replicate within the mitochondrial cloud (Heasman *et al.*, 1984) and become associated with the cortex. Annulate lamellae increase in size and lysosome-like vesicles become visible. The three-layered vitelline envelope develops, producing a zona radiata externa, a zona radiata interna 1 and a zona radiata interna 2 (Selman *et al.*, 1993). The follicle cells become cuboidal and they continue to surround the developing oocyte; within the theca layer ovarian interstitial cells develop (Selman *et al.*, 1993). It has been suggested that these cells synthesize testosterone, and that the hormone is transferred to the granulosa layer where it is aromatized to estradiol (Chieffi and Pierantoni, 1987).

Vitellogenesis marks stage III of oocyte development. This stage is the major growth stage of the oocyte and is a result of the uptake of the yolk-precursor protein vitellogenin (Redding and Patiño, 1993; Selman *et al.*, 1993). The yolk proteins accumalate as the ovarian follicle enlarges. Once the ovarian follicle reaches a diameter of 0.34 mm, the yolk proteins begin to form yolk bodies within the oocyte. The accumulation of yolk bodies causes displacement of the oocyte cytoplasm, and all its constituents to the periphery of the egg (Selman *et al.*, 1993).

Oocyte maturation marks stage IV of oocyte development. Dettlaff (1988) defined oocyte maturity as the stage when an oocyte, enveloped by follicle cells, has the capacity to respond to a stimulating agent for further development. Maturation is completed when the developing oocyte nucleus becomes arrested in another meiotic phase and the cytoplasm of the oocyte contains all necessary components for normal development after fertilization. In zebrafish, oocyte maturation is dependent on ovarian follicle size as well as hormones. Once ovarian follicles reach a diameter of 0.52 mm, the oocyte gains competency to respond to a maturation-inducing hormone (Selman *et al.*, 1993). Selman *et al.* (1994) have identified DHP (17α,20β-dihydroxy-4-pregnen-3-one) as the hormone responsible for inducing zebrafish oocyte maturation, and it is synthesized in the granulosa layer of ovarian follicles (Selman *et al.* 1994). After stimulation by DHP, the maturation phase commences and the germinal vesicle migrates to the periphery of the oocyte and undergoes germinal vesicle breakdown (GVBD). As GVBD continues, the first meiotic division is completed and the oocyte becomes arrested at the second meiotic metaphase (Selman *et al.*, 1994).

Progesterone has also been implicated in oocyte maturation (Dettlaff, 1988), and unlike DHP, it acts by stimulating a release of Ca^{++} stores from the oocyte cell membrane, which ultimately results in increased intracellular concentrations of cAMP (Dettlaff, 1988). After GVBD, progesterone also stimulates an increase in RNA synthesis in nucleoli, suggesting that at this time the maturing oocyte is preparing stores of transcripts that will be used during development.

Prior to ovulation, follicle cells pull away from the oocyte by withdrawing their microvilli from the zona radiata. The eggs, still maintaining their vitelline envelope, are released into the lumen of the ovary and transported via oviducts to the cloaca. A layer of cortical alveoli is associated with the egg cortex. Upon fertilization, the alveoli release their contents into the perivitelline space in response to the cortical reaction (Selman *et al.*, 1993), which causes elevation of the fertilization envelope important in the prevention of polyspermy.

Maternal Effect Genes That Establish Antero-Posterior and Dorso-Ventral Axis During Development

The eggs of many organisms contain regions of cytoplasm that can direct the formation of a specific embryonic pattern. These regions within the egg cytoplasm contain localized mRNA's that function in organizing the embryonic axis in an organism, as well as establishing polarity. *Drosophila* has been the choice organism of choice in following the localization and function of various maternal mRNA's because the embryonic axis is established during oogenesis. In *Xenopus* and *C. elegans* the characterization of maternal mRNA's is being pursued, and although the information from these organisms is limited, researchers are finding sequence homologies as well as functional homologies between similar transcripts of these organisms and of *Drosophila*. Looking at *Drosophila* and *Xenopus*, generalized statements can be made about maternal mRNA's as to function, mode of regulation, mode of localization and effect on embryonic development. Maternal transcripts are known to be specifically localized, and they play the key role in development of defining the polarity of both the antero-posterior and dorso-ventral axes in *Drosophila*.

There are at least four genes that are important in defining the anterior end of *Drosophila*. All four gene products play some role in specifying head and thoracic structures in the developing embryo, but only *bicoid* (*bcd*) proves to be fundamentally important. *bcd* mRNA is synthesized by nurse cells and transported into the growing oocyte (MacDonald and Struhl, 1988). Once inside the oocyte, the transcripts become localized in the anterior region by several factors.

Correct localization of *bcd* transcripts requires an intact microtubule network as well as three maternal effect genes, *exuperantia (exu)*, *swallow* and *staufen*. Disruption of the microtubule network at the anterior end causes delocalization of *bcd* transcripts and prevents localization of newly translocated transcripts from nurse cells (Pokrywka and Stephenson, 1991). Of the three maternal effect genes, Frohnhöfer and Nüsslein-Volhard (1987) showed that embryos lacking maternally derived *exu* do not localize *bcd* to the anterior pole after transport from nurse cells, suggesting that *exu* is important in the initial localization of *bcd*. Following

Frohnhöfer and Nüsslein-Volhard (1987), St. Johnston *et al.* (1989) showed that the *swallow* mutation causes delocalization of *bcd* transcripts at the anterior pole well after the initial localization by *exu*. St. Johnston *et al.* (1989) also showed that in embryos lacking maternal *staufen*, *bcd* transcripts are delocalized to form an anterior-posterior gradient resulting in a weak anterior phenotype, suggesting that not only are *exu* and *swallow* important in *bcd* localization but that *staufen* is important as well. Work done by Ferrandon *et al.* (1994) showed that *staufen* interacts with the secondary structures formed in the 3' untranslated region (UTR) of *bcd* transcripts, further identifying *staufen* as the final localizer of *bcd*.

Normal development of the anterior region is dependent on the presence of *bcd* in the anterior end. Embryos derived from *bcd*- mothers lack a head and thorax and their abdominal segments are defective. Frigerio *et al.* (1986) localized the mRNA of *bcd* to 15% of the anterior region of the early cleaving embryo by *in situ* hybridization studies. After fertilization, *bcd* mRNA is translated into a functional protein that diffuses from the anterior end forming a gradient (Frohnhöfer and Nüsslein-Volhard, 1986). Frohnhöfer and Nüsslein-Volhard (1986) demonstrated, using rescue experiments on embryos derived from *bcd*- mothers, that anterior structures can be induced at any position along the antero-posterior axis. This induction is reduced as the distance from the anterior pole is increased, supporting the suggestion of the protein gradient.

The *bcd* protein is a DNA-binding protein that acts on the promoter region of the *hunchback* gene and activates its transcription. *Hunchback* is required for development of the thorax and part of the head, but it also prevents normal abdominal development (Wharton and Struhl, 1991). The *hunchback* gene contains a number of bicoid-binding sites 300 base pairs upstream from the start site, suggesting that expression of *hunchback* is regulated by *bcd* (St. Johnston and Nüsslein-Volhard, 1992).

Like the anterior end of the *Drosophila* embryo, the posterior region contains maternal determinants however, these control development of abdominal segments and the formation of pole cells important in germ cell differentiation (St. Johnston and Nüsslein-Volhard, 1992). *Nanos (nos)* and *pumilio (pum)* have been shown to be specifically involved in abdomen formation (Lehmann and Nüsslein-Volhard, 1987; Lehmann and Nüsslein-Volhard, 1991). Lehmann and Nüsslein-Volhard (1991) show that no posterior determinant activity is present in eggs that are derived from mothers that carry the *nanos* mutation, thereby implicating *nanos* as the sole posterior determinant.

Localization of *nanos* has been linked to at least seven maternal gene mutations in *cappucino, oskar, spire, staufen, tudor, valois* and *vasa* (Gavis and Lehmann, 1992). Ephrussi and Lehmann (1992) show that localization of *oskar* transcripts to the posterior pole is the key step in the localization of *nanos*, and that the *oskar* gene product also localizes *vasa* to the posterior end. Mothers that carry both the transgene *osk-bcd*3'UTR and the mutation for *vasa* produce embryos that do not form pole cells or abdominal segments. It is the binding of *vasa* protein to the secondary structures formed in the 3'UTR of *nanos* that directs its localization to the posterior pole (Ephrussi and Lehmann, 1992). Without proper localization of *oskar*, localization of *vasa* and subsequent *nanos* localization would not occur, thereby eliminating the formation of abdominal segments within the *Drosophila* embryo. *oskar* has also been identified as the crucial gene product in germ cell differentiation in *Drosophila* (Ephrussi *et al.*, 1991)

Previous work done by Irish *et al.* (1989) indicates that *nanos* directly regulates *hunchback* by suppressing translation of maternal *hunchback* transcripts at the posterior end of the embryo. In the developing embryo, *hunchback* mRNA is uniformly distributed throughout the egg at the time of fertilization, and after 8 nuclear divisions a gradient is formed running anterior to posterior (Wharton and Struhl, 1991). Work done by Wang and Lehmann (1991) on rescue assays support the earlier findings of Lehmann and Nüsslein-Volhard (1991). Injection of mutant embryos with *nanos* mRNA into the abdominal region restores normal development of the abdomen. *Nanos* can down-regulate *hunchback* expression by suppressing *bcd* expression (Wharton and Struhl, 1991). This suggests that the mode of action of *nanos* in blocking translation of *bcd* and *hunchback* transcripts must be the same. To prove this, Wharton and Struhl (1991) identified *nos* response elements (NRE) in both *hunchback* and *bcd* transcripts. *nanos* recognizes these sequences, binds to them and blocks translation of both *hunchback* and *bcd* transcripts.

Another class of maternal genes that function along the antero-posterior axis are called the terminal genes. These maternal genes are essential for the development of anterior and posterior terminal structures within the *Drosophila* embryo, and they utilize a signal transduction pathway to regulate zygotic genes. The pathway initially begins in the follicle cells surrounding the oocyte and results in activation of transcriptional factors within the oocyte that are important in regulating development of terminal structures (Wharton, 1993).

Torso (tor) has been identified as the key component of the above-mentioned terminal organizer system (Klinger *et al.*, 1988); embryos lacking maternally derived *torso* develop abnormally at terminal regions of the antero-posterior axis. In the head region the labrum is not formed and the head skeleton is reduced in size. At the posterior end, all abdominal segments posterior to segment 7 are absent. Klinger *et al.* (1988) suggest that *torso* is activated by a ligand, and in turn initiates a signal transduction pathway that results in spatial expression of zygotic gap genes like *tailless* and *huckebein* (Weigel *et al.*, 1990).

Using *in situ* hybridization techniques, Sprenger *et al.* (1989) examined the distribution of *torso* transcripts in ovaries and early embryos. *torso* transcripts were initially detected in nurse cells until oogenic stage 10, after which the transcripts were translocated out of the nurse cells and into the developing oocyte. Translation of *torso* transcripts occurs within the oocyte and produces a protein that has significant homology with the tyrosine kinase domains in a variety of proteins (Sprenger *et al.*, 1989). Comparing structures of the *torso* protein and growth factor receptor tyrosine kinases, Sprenger *et al.* (1989) suggest that *torso* is a transmembrane protein embedded in the oocyte cell membrane and that it has two functions, as a receptor for a ligand as well as a tyrosine kinase.

Sprenger *et al.* (1989) suggest *torso-like (tsl)* as the likely ligand for *torso*. Martin *et al.* (1994) showed that embryos derived from mothers carrying the mutation for *torso-like* do not develop abdominal segments, and that the transcripts are localized to follicle cells at each pole. To date it is not conclusively known whether *torso-like* is the ligand that binds to *torso*; however St. Johnston and Nüsslein-Volhard (1992) have suggested that the *torso-like* gene may produce an inactive form of the ligand for *torso*. It is anchored in the vitelline membrane, and after fertilization it is released to bind to *torso*, stimulating a signal transduction pathway that ultimately regulates expression of zygotic genes.

The fourth system for determining polarity in *Drosophila* is the dorso-ventral system. The components of the dorso-ventral system can be subdivided into two classes. The first class contains maternal effect genes required for eggshell and embryonic polarity. The second class contains maternal effect genes required for embryonic polarity only (Wilkins, 1993). The latter class is of particular interest because the basic features of regulating zygotic genes resemble those of the terminal group.

dorsal (dl) falls into the second class of maternal effect genes defining the dorso-posterior axis, and has been determined as one of the important molecules in establishing this axis (Wilkins, 1993). *dorsal* is a sequence-specific DNA-binding protein that regulates transcription of genes important in establishing germ layers and their derivatives, such as *twist*, *snail*, and *zen*. Jiang *et al.* (1991) showed both the *twist* and *zen* promoters to contain *dorsal*-binding sites, confirming *dorsal* as a regulator of these genes. Both *dorsal* transcripts and dorsal protein are synthesized during oogenesis and are distributed uniformly throughout the cytoplasm of the oocyte. After nuclear division, *dorsal* becomes concentrated on the ventral side of the embryo, forming a gradient along the dorso-ventral axis (Jiang *et al.*, 1991). The ventral signal that directs nuclear translocation of *dorsal* is transmitted from the perivitelline space across the oocyte membrane via the *toll* protein (Steward, 1987).

Toll is a transmembrane protein that acts as a receptor for an external ligand (St. Johnston and Nüsslein-Volhard, 1992) much like *torso*. It is distributed uniformly around the embryo, but its activity is spatially restricted to the ventral side as a result of ventral localization of its ligand. Morisato and Anderson (1994) identify *spätzle* as the protein that binds and activates *toll*. *Spätzle* is produced in ventrally located follicle cells and is secreted into the perivitelline space upon fertilization (Stein and Nüsslein-Volhard, 1992; Morisato and Anderson, 1994). Once released into the perivitelline space, spätzle binds to *toll* and activates it. Once activated, *toll* releases *dorsal* from a cytoplasmic bound state, thereby allowing *dorsal* to activate several zygotic regulatory genes (Davidson, 1994).

Although the research done on maternal determinants in *Xenopus* is not as extensive as that in *Drosophila*, researchers are finding similarities between the two organisms. Much as in *Drosophila*, *Xenopus* oocytes contain maternal determinants that are important in establishing the proper embryonic axis, as well as interactions with other determinants for normal development. In *Xenopus*, normal development is dependent upon the correct organization of these maternal determinants to either the vegetal or animal regions. Segregation of the maternal determinants to specific blastomeres determines the fate of the blastomeres (Elinson et al., 1993) In *Xenopus*, the animal-vegetal axis is determined during oogenesis by selective transport of maternal transcripts to the vegetal cortex. It is the localization of maternal transcripts to the vegetal cortex that not only defines the dorsal-ventral axis, but also the *Xenopus* germ plasm. To date (Micklem, 1995) there are a few maternal transcripts that have been identified that are localized to the vegetal cortex: *Vg1* (Melton, 1987), *Xcat2* (Elinson *et al.*, 1993), and *Xwnt11* (Mosquera *et al.*, 1993).

Vg1 protein codes for a growth factor that is related to a family of proteins called Transformation Growth Factors-β (TGF-β), suggesting that the *Vg1* protein acts as an intercellular signal (Weeks and Melton, 1987). Although *Vg1* has yet to be assigned a definitive function, Thomsen and Melton (1993) showed that *Vg1* induces dorsal mesoderm formation by possibly acting on *goosecoid* and *noggin*, these being dorso-anterior mesoderm markers, during development. *Vg1* transcripts are synthesized in the oocyte prior to vitellogenesis, and are uniformly distributed in the cytoplasm (Melton, 1987). Localization of Vg1 transcripts to the vegetal cortex is a result of an interaction between the 3'UTR and the microtubule network within the cell, and it occurs during vitellogenesis. Mowry and Melton (1992) first demonstrated that a 340nt sequence within the 3'UTR of *Vg1* transcripts is required for localizing the transcripts to the vegetal cortex. By inhibiting microtubule polymerization, Yisraeli *et al.* (1990) were able to disrupt the localization of *Vg1* transcripts to the vegetal cortex, thereby implicating the microtubule network as the chief localizer of *Vg1* transcripts. Further work done by Kloc *et al.* (1993) showed that another factor involved in localizing *Vg1* transcripts to the vegetal cortex is *Xlsirt* transcripts. *Xlsirt* transcripts are synthesized during oogenesis and contain short (79-810) interspersed nucleotide sequences that are repeated 3-13 times within the transcript. These *Xlsirts* have been shown by Kloc *et al.* (1993) to be localized to the vegetal cortex via the mitochondrial cloud in *Xenopus* oocytes. Destruction of *Xlsirts* causes delocalization of *Vg1* from the vegetal cortex (Kloc and Etkin, 1994), suggesting that *Xlsirts* play a role in localization of *Vg1* transcripts to the vegetal cortex. From the work done by Yisreali *et al.* (1990) and from their own research, Kloc and Etkin (1994) suggest that *Xlsirts* organize the cytoskeletal network at the vegetal cortex. Once organized, the cytoskeletal network then localizes *Vg1* to the vegetal cortex. After fertilization, Weeks and Melton (1987) showed that *Vg1* transcripts are partitioned into the blastomeres of the vegetal hemisphere during cleavage.

Xcat2 is a cytoskeletal-associated transcript that, when translated, produces a protein that belongs to a family of zinc-finger proteins (Mosquera *et al.*, 1993). More specifically, it contains a zinc-finger motif that is homologous to a motif found in *nanos*, suggesting that its RNA binding capacity is similar to that of *nanos*. Mosquera *et al.* (1993) show that *Xcat2* transcripts become localized to the vegetal cortex during oogenesis, which has been confirmed by Elinson *et al.* (1993), and that they are associated with the microtubule network of the oocyte. Work done by

Forristall *et al.* (1995) showed that *Xcat2* transcripts are associated with the mitochondrial cloud at the same time that *Vg1* transcripts are evenly distributed throughout the oocyte cytoplasm, and are localized to the vegetal cortex Vg1. Unlike *Vg1*, *Xcat2* remains associated with the vegetal cortex until the 4-cell embryo stagein which the transcripts become condensed into islands of germ plasm much like germinal granules. Since germinal granules are known to be involved in germ cell determination, Forristall *et al.* (1995) suggested that *Xcat2* may also play a role in germ cell determination. The researchers go as far as to suggest that *Xcat2* transcripts may in fact be a component of germinal granules.

Ku and Melton (1993) further characterized another maternally expressed RNA, *Xwnt11*, that is important for dorsal-ventral axis formation. *In situ* hybridization shows that *Xwnt11* transcripts are present in early oocytes, and are evenly distributed throughout the oocyte cytoplasm. During vitellogenesis, *Xwnt11* transcripts become localized to the vegetal pole and are associated with the vegetal cortex. After oocyte maturation, the transcripts become associated with the vegetal cytoplasm. Rescue experiments done by Ku and Melton (1993) show that *Xwnt11* transcripts dorsalize pre-existing mesoderm in UV-ventralized embryos, suggesting that *Xwnt11* is important in establishing the dorsal-ventral axis of the embryo.

Identification of a Zebrafish Maternal Determinant

Localized maternal determinants have been recognized as having major importance in early development in *Drosophila*. There is also evidence that

Xenopus employs maternally derived determinants in order to make crucial decisions in the early embryo, such as polarity, germ layer formation, and germ cell differentiation. However, very little is known about the role of maternal control of early developmental events in other organisms. This is despite the fact that other organisms such as zebrafish are becoming increasingly popular as a model for vertebrate development.

Zebrafish are an excellent model for understanding vertebrate development for several reasons. Eggs are externally fertilized and therefore require all necessary maternal determinants for proper development to be `packaged' in the oocyte prior to ovulation. Significant amounts of stored transcripts need to be readily accessible to the developing embryo prior to activation of the zygotic genome. Development of the organism is rapid compared to other vertebrates: hatched fry develop within 72 hours with relatively developed immune, circulatory, nervous, and nephric systems. The rate at which zebrafish develop is a great advantage for developmental studies, particularly for gaining information on how maternal determinants affect normal development. This information can be analyzed within a few days, so that subsequent research becomes more efficient. Also, the developing larvae are transparent, making whole mount *in situ* hybridization an ideal technique in following maternal transcripts throughout various stages of development. Almost nothing is known about the role of maternal determinants in zebrafish development. Helde and Grunwald (1993) identified a zebrafish homologue of *Xenopus Vg1*, *zDVR-1*, suggesting it may be a maternal determinant.

Dohrmann *et al.* (1996) confirmed that *Vg1* is a maternal determinant, and that it induces axial mesoderm formation in the zebrafish embryo. However, in the zebrafish *zDVR-1* displays a different pattern of localization as compared to *Vg1* in *Xenopus*. The transcripts are distributed evenly throughout the cytoplasm of developing oocytes and are equally distributed to all blastomeres at the animal pole, unlike in *Xenopus*.

Since all maternally derived determinants must be packaged within the mature oocyte prior to ovulation, an understanding of their role in development can be gained by first examining mature oocytes for the presence of these determinants, followed by an examination of their role in the development of fertilized embryos. Because of the asynchronous development of the ovary in zebrafish, the ovary allows for examination of not only mature oocytes, but also of the various stages of oocyte development which can provide important information on when maternal determinants are synthesized during oogenesis.

The initial object of this research was to isolate the zebrafish homolgue of the myelin basic protein gene in order to precisely define the regions controlling expression of the gene. While screening an adult zebrafish cDNA library for the myelin basic protein sequence, several clones were isolated and analyzed for tissue-specificity. Hybridization of specific sequences from the various clones on frozen sections of whole zebrafish, identified one clone as having tissue specificity. The tissue was determined to be either mesonephric or gonadal. Both tissues have a strong biological role in the zebrafish; the mesonephros has a role in both immunology and osmoregulation and the gonads in reproduction. Due to my interest, the prospective developmental implications of a gonad-specific transcript was intriguing. Consequently, various analytical techniques were used to characterize the expression patterns of this specific sequence, KSZf5, in zebrafish. *In situ* hybridization experiments demonstrate that KSZf5 transcripts are detected at various stages of oocyte development, identifying it as a maternally derived transcript. However, the distribution of KSZf5 transcripts throughout the cytoplasm of the developing oocytes vary. In germ cells about to enter the primary growth stage, the transcripts are uniformly distributed throughout the cytoplasm. Once an oocyte undergoes vitellogenesis, the transcripts become localized to the cortex of the egg by cytoplasmic displacement as a result of yolk body accumulation. KSZf5 transcripts have also been detected throughout various stages of post-fertilization embryos, suggesting that the maternally derived transcripts are not utilized by the developing embryo prior to activation of its zygotic genome. Although its role in the developing embryo is questionable, it is suggested that KSZf5 may be a cytoplasmic determinant that becomes localized to the zebrafish germ plasm, and that it may play a role in either germ cell differentiation or differentiation of the female reproductive system. Sequence analysis of KSZf5 identifies homology among several receptor proteins, as well as membrane proteins, suggesting the KSZf5 may act as a membrane bound receptor protein whose function is presently unknown.

MATERIALS AND METHODS

Zebrafish Maintenance

A lab stock of wildtype *Danio rerio* was supplied by Dr. Hans Laale (Department of Zoology, University of Manitoba) and supplemented periodically with wildtypes, long tails, and leopard danios (*Danio rerio frankei*) from local pet stores. The zebrafish were housed in 10 gallon aquaria at 28°C on a 14h light/10h dark cycle. The fish were fed three times a day with flake food (Nutrafin), except for a few days before intended breeding when the fish were fed frozen brine shrimp three times a day. To permit the collection of fertilized eggs, a tank containing a trap made of Plexiglas and a meshcloth bottom was used to prevent spawning adults from eating the fertilized eggs. On a breeding day, a single male and a single female were placed in a breeding tank to spawn. The eggs were collected 2-3 hours after spawning and rinsed with distilled water to remove debris. The embryos were maintained in beakers of distilled water and checked every hour for progression of development. Stages of development were identified on the basis of the morphological staging outlined by Westerfield (1993).

Amplification of Genomic Zebrafish DNA

Universal primers for myelin basic protein (MBP), designed by Warren Spivack at the Institute for Basic Research in Developmental Disabilities, were used to amplify a portion of the zebrafish genome utilizing polymerase chain reaction (PCR) amplification. The forward primer, designated as MBP38S, reads 5'tgcctccgcaagtaccttggaccat3' and begins in exon 1 of the MBP gene in elasmobranchs (W. Spivack pers. comm.). The underlined nucleotides are the last two bases of the ATG start codon of the MBP gene. The reverse primer, designated as MBP262A, reads 5'tgttcttaaagaaatggac3' and terminates at the end of exon 3 of the MBP gene in elasmobranchs. Optimal PCR conditions were determined empirically beginning with the conditions outlined by Innis and Gelfand (1990). Adult zebrafish genomic DNA (0.67µg) was added to 1X PCR buffer [10 mM Tris-Cl (pH 8.4), 25 mM KCl], 100 µM dNTP's (dATP, dCTP, dGTP, dTTP), 3.5 mM $MgCl_2$, 0.25 µM MBP38S, 0.25 µM MBP262A and 2.5 U *Taq* polymerase. A Thermolyne Temp-Tronic thermocycler was used for the PCR process. Double stranded DNA (dsDNA) was denatured at 94°C for 1 minute. The primers were allowed to anneal to the single stranded DNA (ssDNA) for 1 minute at 55°C and extension of the primers by *Taq* polymerase continued for 1.5 minutes at 72°C. This was repeated for 30 cycles with a final dwell at 72°C for 5 minutes to allow all polymerizing reactions to go to completion.

Purification of PCR-Amplified DNA

The PCR-amplified DNA was electrophoresed at 120 volts, in a 0.7% agarose gel with HindIII digested lambda DNA as size markers. The DNA fragments were visualized by soaking in a solution of 0.5 µg/µl ethidium bromide in TAE buffer [40 mM Tris-Acetate, 1 mM EDTA (pH 8.0)] and viewed on an ultraviolet light transilluminator. I used a transilluminator to cut a slit before and just after the desired band of DNA. A strip of pre-wetted DEAE ion-exchange membrane (Schleicher and Schuell NA-45) was used to recover the 240bp fragment. The DNA was eluted off the membrane with a high salt elution buffer [50 mM Tris-Cl (pH 8.0), 1 M NaCl, 10 mM EDTA (pH 8.0), 50 mM arginine] at 68°C for 2 hours (Sambrook *et al.* 1989). After elution, the membrane was removed and the aqueous solution was extracted twice with 1 vol P:C:I, and once with 1 vol C:I:A. 3 vol of isopropyl alcohol was used to precipitate the DNA. The precipitated DNA was washed in 75% EtOH, and 100% EtOH and then dried at 65°C for 10 minutes. The pellet was resuspended in TE buffer [10 mM Tris-Cl (pH 8.0), 1 mM EDTA], and the DNA concentration was determined using a spectrophotometer (Spectronic 601) set to read optical density at 260 nm.

Preparation of Probe for Hybridization

In order to screen an adult zebrafish cDNA library, the PCR-amplified DNA was radioactively labelled with α-^{32}PdCTP using a random primer labeling system (Gibco BRL). 50 ng of DNA was boiled in the presence of 10 pmol of random primers for 5 minutes to denature the dsDNA and allow annealing of the primers to the DNA. After cooling on ice, 48 µM dNTP mix [dGTP, dTTP, dATP], 50 µCi α-^{32}PdCTP, and 1X Large fragment DNA polymerase I reaction buffer (Klenow buffer) [50 mM Tris-Cl (pH 7.6), 10 mM $MgCl_2$] was added to the denatured DNA. Large fragment DNA polymerase I (Klenow enzyme) was added to the final reaction mix for 1 hour at 37°C to extend the primer and incorporate α-^{32}PdCTP. The reaction mix was placed on ice and the radio-labeled DNA was precipitated with 3.5 mM spermine and 80 ng non-denatured salmon sperm DNA for 20 minutes. The precipitated DNA was centrifuged for 10 minutes at 13000rpm, washed twice with TE^{-4} and 0.1% spermine and resuspended in TE^{-4} and 0.5M NaCl. The radioactively labeled DNA was boiled for 6 minutes and place on ice immediately. To determine the amount of incorporated α-^{32}PdCTP, an aliquot of 2 µl was placed in 1 ml of Biofluor (NEN) and counted in a scintillation counter (Chicago Nuclear 720 series). The specific activity was calculated to be approximately 1.25×10^9 cpm/µg.

Screening a Zebrafish cDNA Library

A lambda ZapII library (approximate titre 2.5×10^{10} pfu/ml) containing cDNA's from adult zebrafish was obtained from Dr. D. Grunwald at the University of Utah School of Medicine. This cDNA library was hybridized with the PCR-generated DNA as described by Sambrook *et al.* (1989). A stock of LB-media, supplemented with 0.2% maltose and 50 mg/ml of tetracycline, was inoculated with a single colony of XL1-Blue cells and grown overnight at 37°C in a shaker waterbath. The cells were harvested by centrifugation at 4,000 rpm for 10 minutes. The supernatant was discarded and the cells resuspended in 10 mM $MgSO_4$, $OD_{600}=2.0$. The cells were stored at 4°C until needed.

To determine the correct titre of the cDNA library, a dilution series of the original library was made in SM buffer [100 mM NaCl, 10 mM $MgSO_4$, 50 mM Tris-Cl (pH 7.5), 0.01% gelatin]. The XL-1 Blue cells were infected by adding 100 µl of each phage dilution to 100 µl of XL-1 Blue cells and incubating at 37°C for 20 minutes. After infection, 3 ml of molten agar (0.7% Bacto-Agar in LB-media at 47°C) supplemented with 100µg/µl tetracycline was added to the infected cells and poured immediately onto the plates (100 x 50 mm) containing solidified bottom agar and 100µg/µl tetracycline. These plates were left to solidify and then incubated overnight at 37°C. The phage dilutions were stored at 4°C with 1 drop of chloroform added to the SM buffer. The plates were scored for the number of plaques per plate. From the above dilution series, 150,000 plaques was estimated for an adequate screening of the library.

Once the correct phage dilution had been determined, infection of XL-1 Blue cells for plating of the library was done by adding 150 µl of the appropriate phage dilution to 1ml of XL-1 Blue cells. Infection was carried out for 20 minutes at 37°C. After infection, 30 ml of molten agar supplemented with 100 µg/µl tetracycline was added to the infected cells and immediately poured over plates (20 x 20 cm) containing bottom agar and 100 µg/µl tetracycline. The plates were allowed to solidify and incubated overnight at 37°C. The plates were cooled at 4°C for 2 days before proceeding with plaque lifts.

Plaque lifts were done using a positively charged nylon membrane (Micron Separation Inc.) that had been cut to the size of the plates. The nylon membrane was placed on top of the cooled (4°C) plates and asymmetrically marked with dots of India Ink (Osmiroid) such that orientation of the lifts could be identified. The membrane was carefully lifted off the plate and placed in 1X Southern denaturant [0.6 M NaCl, 0.2 M NaOH] for 5 minutes. Following denaturation, the membrane was placed in 1X Southern neutralizer [0.25 M Tris-Cl (pH 7.4), 0.6 M NaCl] for 5 minutes and washed in 2X SSC [0.3 M NaCl, 30 mM sodium citrate] for 5 minutes. To immobilize the DNA, the membrane was baked at 80°C for 2 hours and then prewashed for 30 minutes in 0.1X wash solution [0.1X SSC, 0.1% SDS] at 65°C. The membranes were placed in a Seal-a-Meal bag with prehybridization solution [6X SSC, 10X Denhardt's (0.2% Ficoll, 0.2% BSA, 0.2% polyvinylpyrrolidone), 500μg/ml denatured salmon sperm DNA, 0.5% SDS] and prehybridized at 42°C overnight.

After prehybridization, the screens were hybridized with radioactively labeled DNA (prepared as described in preparation of probe for hybridization) in a hybridization solution [50% deionized formamide, 4X SET (0.6 M NaCl, 0.12 M Tris-Cl, 8 mM EDTA) 1X Denhardt's (0.2% Ficoll, 0.2% BSA, 0.2% polyvinylpyrrolidone), 100 μg/ml denatured salmon sperm DNA, 0.5% SDS] at 42°C overnight. To remove any unbound probe, the membranes were first washed in 0.1X wash solution [0.1X SSC, 0.1% SDS] for 30 minutes at room temperature. All subsequent washes in 0.1X wash solution were done at 65°C for 30 minutes. After each wash, the membranes were checked for background levels of radioactivity using a Geiger counter. Once background levels were no longer detectable, the membranes were removed from the wash solution, blotted dry and wrapped in cellophane. The wrapped membranes were placed on X-ray film (Kodak X-AR) and exposed at -80°C overnight. The autoradiograph was developed using Kodak GBD Developer and Kodak Rapid Fixer.

Second Screening of the Adult Zebrafish cDNA Library

Potentially positive plaques were isolated by aligning the autoradiographs from the first screen with the marked membranes and the plates. Plaque plugs were taken out of the agar using the wide end of a pasteur pipette. Each agar plug was then placed in a 1.5 ml Eppendorf tube with 1 ml of SM buffer and 1 drop of chloroform. The agar plugs were incubated for 2 hours at room temperature to allow for the bacteriophage to diffuse into the SM buffer. After incubation, 100 µl of each bacteriophage solution was added collectively to one Eppendorf tube and mixed. The mixture of bacteriophage was serially diluted 10-fold, and used to infect XL-1 Blue cells to do a second plaque hybridization in order to confirm the positive plaques from the initial screening. Plaque lifts were done in the same manner as that described in the initial screening of the adult cDNA library. Prehybridization and hybridization was also done in the same manner as the initial screening.

Excision of Positive clones from Bacteriophage

Prior to excising the phagemid Bluescript from the Lambda ZapII vector, 50ml of LB broth supplemented with 0.2% maltose and 10 mM $MgSO_4$ was inoculated with a single colony of XL1-Blue MRF' cells. In addition, 50 ml of LB broth was inoculated with a single colony of SOLR cells. Both cultures were grown overnight at 37°C in a shaker waterbath. The cells were harvested by centrifugation at 4,000 rpm at 4°C for 10 minutes. The supernatant was discarded and the cells were resuspended in 10 mM $MgSO_4$ such that their absorbance was $OD_{600}=1.0$.

Excision of the phagemid from the Lambda ZapII vector was done by co-infecting XL1-Blue MRF' cells with ExAssit interference-resistant helper phage (Stratagene). From each of the bacteriophage stocks, 250 µl was added to 200 µl of XL1-Blue MRF' cells and 1 µl of ExAssit helper phage ($>1x10^6$ pfu/ml). The cells were co-infected for 15 minutes at 37°C. After co-infection, 3 ml of LB broth was added and the cells were incubated for 2.5 hours at 37°C in a shaker waterbath. The infected cells were heat-shocked at 70°C for 15 minutes and then centrifuged at 4,000 rpm for 15 minutes. The supernatant containing the excised phagemid was placed into a sterile tube. 200 µl of SOLR cells were added to eppendorf tubes in duplicate for each excised phagemid in order to package the excised phagemid. To one set of tubes 100 µl of the phagemid supernatant was added and 10 µl of the phagemid supernatant was added to the other set. The tubes were incubated at 37°C for 15 minutes and 200 µl of each tube was plated onto plates (100 x 50 mm) containing LB agar and 50 µg/µl ampicillin. The plates were incubated at 37°C overnight.

Analysis of Positive Phagemids

Phagemids were analyzed for cDNA inserts by digesting with restriction endonuclease Pvu II, and isolating those that contained an insert larger than 400 bp. To analyze the phagemids, 2ml cultures of LB media and 50µg/µl of ampicillin were inoculated by a single colony from each of the 15 plates previously prepared and incubated overnight in a 37°C shaker waterbath. Cells were harvested by centrifugation for 1 minute at 13,000 rpm, and the supernatant was discarded. The pellet was resuspended in 100 µl of Alkaline Lysis Solution I [50 mM glucose, 25 mM Tris-Cl (pH 8.0), 10 mM EDTA]. To lyse the cells in order to extract the phagemid DNA, 200 µl of Alkaline Lysis Solution II [0.2 M NaOH, 1% SDS] was added to the cell suspension. This was followed by addition of 150 µl of Alkaline Lysis Solution III [60 mM potassium acetate, 11.5 ml glacial acetic acid, 28.5 ml distilled water]. The cell suspension was incubated on ice for 5 minutes. The solution was centrifuged for 2 minutes to pellet the cellular debris, and the supernatant was removed to a sterile Eppendorf tube. The supernatant was extracted twice with P:C:I, and once with C:I:A, and precipitated with 3 vol of isopropyl alcohol. To pellet the DNA, the tube was centrifuged at 13,000 rpm for 10 minutes and washed in 75% EtOH, followed by 100% EtOH. The pellet was dried at 65°C for 10 minutes and resuspended in 50µl of TE^{-4} buffer. An aliquot of each sample was treated with 1ug/ul RNase A and restriction endonuclease PvuII for 1 hour at 37°C. The DNA fragments were separated by gel electrophoresis in a 0.7% agarose gel, using HindIII-digested lambda DNA as size markers, and stained by soaking in a solution of 0.5 µg/µl ethidium bromide in distilled water.

Random Primer Labeling of DNA

To identify which of the inserts contained a unique sequence of genomic zebrafish DNA, the clones were initially digested with two separate restriction endonuclease in order to isolate the inserts. The clones were first digested with restriction endonuclease BamHI for 1 hour at 37°C. The DNA was precipitated for 20 minutes with 3 vol isopropyl alcohol at -20°C, and pelleted by centrifugation. The pellet was washed in 75% EtOH, and 95% EtOH, dried at 65°C and resuspended in TE buffer. The DNA was digested with restriction endonuclease KpnI for 1 hour at 37°C. The DNA fragments were separated by gel electrophoresis in a 0.7% agarose gel, and stained by soaking in a solution of 0.5 µg/µl ethidium bromide in TAE buffer. The DNA fragment corresponding to that of the insert was gel-purified using the same method as that described for purification of PCR-amplified DNA fragments. Each insert was radioactively labeled with α-^{32}PdCTP using the same method as previously described for labeling of PCR-generated DNA.

Southern Hybridization

Zebrafish genomic DNA samples (20µg) from four different fish were digested with the restriction endonucleases HindIII, EcoRI, PstI and HpaII for 1 hour at 37°C. To determine if complete digestion of the genomic DNA had occurred, pUC19 was used as a control. An aliquot of the experimental digest was added to Eppendorf tubes containing the control plasmid digested with the same restriction endonuclease and the samples were incubated at 37°C for 1 hour. The control samples were separated by gel electrophoresis in a 0.7% agarose gel and the restriction digests were considered to be complete if the control plasmid with the experimental digest showed an identical digestion pattern to that of the digested control plasmid.

The DNA in the experimental samples was precipitated with 3 vol of isopropyl alcohol at -20°C overnight. The DNA was centrifuged at 13,000 rpm for 10 minutes, and the pellet was washed in 75% EtOH followed by 95% EtOH, dried at 65°C for 10 minutes and resuspended in TE buffer. The genomic DNA fragments were separated in a 0.7% agarose gel at 25 volts overnight. HindIII-digested lambda DNA was used for size markers. Following electrophoresis, the gel was stained in 10µg/µl ethidium bromide for 30 minutes and destained in distilled water for 30 minutes. A Polaroid photograph (Polaroid 667) of the gel was taken for future reference. Prior to transferring the DNA to a nylon membrane (Micron Separation Inc.), the genomic DNA was denatured in 1X Southern denaturant [0.6 M NaCl, 0.2 M NaOH] for 30 minutes.

Transfer of the genomic DNA was done overnight as described by Sambrook *et al.* 1989. Immobilization of the DNA to the nylon membrane was done by baking the membrane at 80°C for 2 hours, followed by prewashing the membrane in 0.1X wash solution [0.1X SSC, 0.1% SDS] at 65°C for 30 minutes. Prehybridization and hybridization of the Southern blots was performed that as previously described. All inserts showing sequence homologies to genomic zebrafish DNA were further analyzed.

Sequence Analysis of DNA

DNA sequencing was done using the dsDNA Cycle Sequencing System (Gibco BRL). The phagemid was digested with restriction endonuclease PvuII at 37°C for 1 hour in order to maintain the sequencing primer sites adjacent to the insert.

Both primers (1pmol) (Gibco BRL) were radioactively labeled with γ^{33}PdCTP in separate reactions using an end-labeling system. Labeling conditions were modified from the dsDNA Cycle Sequencing System (Gibco BRL) such that incorporation of the labelled nucleotide would occur at 37°C for 45 minutes in the presence of 1U of T4 polynucleotide kinase and kinase buffer [60 mM Tris-Cl (pH 7.8), 10 mM $MgCl_2$, 200 mM KCl]. To inactivate the T4 polynucleotide kinase, the reaction mix was incubated at 55°C for 5 minutes. Template DNA (30 fmol of previously digested DNA), *Taq* sequencing buffer [30 mM Tris-Cl (pH 9.0), 5 mM $MgCl_2$, 30 mM KCl, 0.05% (w/v) W-1], and 1.25 U *Taq* polymerase were added to both the forward and reverse reaction mixes, and brought up to 36 µl with distilled water.

Tubes containing termination mixes were prepared by adding 2 µl of each termination mix -A [2 mM ddATP and 100 µM each of dATP, dCTP, 7-deaza-dGTP and dTTP], -C [1 mM dCTP and 100 µM each of dATP, dCTP, 7-deaza-dGTP and dTTP], -G [0.2 mM ddGTP and 100 µM each of dATP, dCTP, 7-deaza-dGTP and dTTP], -T [2 mM ddTTP and 100 µM each of dATP, dCTP, 7-deaza-dGTP and dTTP] (Gibco BRL) into separate tubes for both the forward and reverse reactions. From each reaction mix, 8 µl was aliquoted to each termination tube. One drop of silicone oil was added to each of the termination mix tubes to prevent evaporation of tube contents. The sequencing reaction began with denaturation of the dsDNA for 30 seconds at 95°C, followed by annealing of the primers to the single stranded DNA for 30 seconds at 55°C. Extension of the primers by *Taq* polymerase was done for 1 minute at 70°C. This was repeated for 20 cycles, followed by denaturation for 30 sec at 95°C and extension for 1 minute at 70°C for 10 cycles. Samples were removed to ice and 5 µl of stop buffer [95% formamide, 10 mM EDTA (pH 8.0), 0.1% BϕB, 0.1% w/v xylene cyanol] was added to each tube.

Products of the sequencing reaction were separated by gel electrophoresis on a 5% denaturing polyacrylamide gel (Sambrook *et al.* 1989) over a period of 6 hours. The gel was run at 45°C [1,700 volts, 53 amps, 80 watts], using 1X TBE buffer [90 mM Tris-borate (pH 8.0), 1 mM EDTA] as the running buffer. Three loadings of both the forward and reverse reaction were spaced 2 hours apart. The gel was transferred to Whatman No.1 filter paper, wrapped in cellophane and dried at 80°C for 45 minutes in a gel dryer (Model 583 Gel Dryer-BioRAD). When dry, the cellophane was removed and the gel was placed on BioMax film (Kodak) and exposed at -80°C for up to 5 days. The resulting autoradiograph was developed and the sequence was read for both the forward and reverse reactions.

Due to limitations of the sequencing system, a maximum of 400 bp of sequence can be read at one time. To sequence the rest of the KSZf5 insert, primers were generated from the previously determined sequence for a total of 4 sets of primers (Appendix I).

Northern Hybridization

Zebrafish RNA (40µg) was precipitated with isopropyl alcohol for 20 minutes at -80°C. Samples were centrifuged at 13,000 rpm for 10 minutes. The pellets were washed in 75% EtOH and 95% EtOH, and dried at 65°C for 10 minutes. The pellets were resuspended in 10 µl of DEPC-treated distilled water. Samples were treated with 20 µl of RNA sample buffer [15 mM $NaPO_4$, 75% DMSO, 6.8% deionized glyoxal, 0.15% SDS], and incubated at 50°C for 30 minutes (Thomas, 1980). The samples were removed to ice and 2 µl of BφB loading dye [0.25% BφB, 40% w/v sucrose in water] was added to each sample. The RNA's were separated by gel electrophoresis on a 1.5% agarose gel made with 10 mM $NaPO_4$. To prevent pH changes in the running buffer (10 mM $NaPO_4$), the buffer was circulated using a peristaltic pump (Manostat Varistaltic Pump). RNA size markers (Gibco-BRL) ranging from 0.24-9.5 kb were used to determine the size of the transcript. The portions of the gel containing markers were removed, stained with 1 µg/µl ethidium bromide in 0.5 M ammonium acetate, and photographed together with a ruler (Gibco BRL) that flouresces in the presence of UV light. Direct transfer of the RNA to a nylon membrane (Micron Separations Inc.) was done overnight as described by Sambrook *et al.* (1989). The membrane was rinsed in distilled water and baked at 80°C for 2 hours to immobilize the RNA to the membrane. The membrane was prewashed in 0.1X wash solution for 30 minutes at 60°C, and prehybridized in a solution of 50% deionized formamide, 5X SSC, 0.1% N-lauroylsarcosine, 0.02% SDS, and 2% blocking reagent (Boehringer mannheim) overnight at 42°C. Radioactively labeled DNA, prepared as previously described in random primer labeling of phagemid inserts, was added to prehybridization solution and hybridized overnight at 42°C. Post-hybridization washes were done at 60°C in 0.1X wash solution as described for Southern blotting. The Northern blot was placed on X-ray film (Kodak X-AR) overnight at -80°C.

Non-radioactive Labeling Using DIG-11-dUTP

DNA was labelled with DIG-11-dUTP using the Genius Nonradioactive Nucleic Acid Labelling and Detection System (Boehringer Mannheim). Using the random primer labeling method, 500 ng of DNA was denatured by boiling for 10 minutes and immediately placing on ice. 2 µl of 10X hexanucleotide mix [156 µg/µl random hexanucleotides in 500 mM Tris-HCl (pH 7.2), 100 mM $MgCl_2$, 1 mM DTE, 2 µg/µl BSA], 2 µl of 10X dNTP labeling mix [1 mM dATP, 1 mM dCTP, 1 mM dGTP, 100 mM dTTP, 0.35 mM alkali-labeled DIG-dUTP (pH 6.5)], and 2 U of Large fragement DNA polymerase I were added to the denatured DNA and mixed. The sample was incubated overnight at 37°C. The DIG-labeled DNA was precipitated with 3 vol of isopropyl alcohol for 30 minutes at -20°C. The DNA was collected by centrifugation at 13,000 rpm for 15 minutes. The pellet was washed in 75% EtOH, followed by a wash with 95% EtOH. The pellet was dried at 65°C for 10 minutes and resuspended in TE/SDS buffer [10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1% SDS].

To estimate the yield of DIG-labeled DNA, 10-fold serial dilutions of the labeled DNA were done in DNA dilution buffer [50 ng/µl herring sperm DNA, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA]. Serial dilutions were also done for DIG-labeled control DNA [5 ng/µl DIG-labeled pBR328 DNA]. From each dilution, of both the experimental and control DNA, 1 µl was spotted onto a nylon membrane (Micron Separations Inc.). The DNA was cross-linked to the membrane by exposure to UV light for 3 minutes. The membrane was placed directly into washing buffer [100 mM maleic acid, 150 mM NaCl, 0.3% Tween 20 (pH 7.5)]. The membrane was placed in 1% blocking solution [1% w/v blocking reagent (Boehringer Mannheim), 100 mM maleic acid, 150 mM NaCl (pH 7.5)] for 5 minutes at room temperature with agitation. The blocking solution was removed and the membrane was incubated for 10 minutes at room temperature with 0.75 U of antibody [Anti-DIG (Fab) conjugated to alkaline phosphatase] that had been diluted 1:5000 in 1% blocking solution. The membrane was washed twice in washing buffer, 15 minutes per wash, and placed in filtered detection buffer [100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 50 mM $MgCl_2$] to activate the alkaline phosphatase that is conjugated to the antibody. The membrane was removed from detection buffer and placed in a Seal-a-Meal bag. Chemiluminescent detection of the DIG-labeled DNA was done by diluting 25 mM CSPD® (Boehringer Mannheim) 1:100 in detection buffer and adding it to the membrane. The membrane was incubated at 37°C for 15 minutes. Exposure to X-ray film (Kodak X-AR) was done for 5 minutes at room temperature. By comparing spot intensities of the experimental and control dilutions, the concentration of the DIG-labeled DNA was estimated to be 10 ng/µl, yielding a total of 500 ng of labeled DNA.

Frozen Sections of Whole Zebrafish, Ovary and Testis

Adult zebrafish were sectioned using a cryostat (CRYO-CUT II Microtome; American Optical). Slides were coated with 0.1% poly-L-lysine so that the frozen sections would adhere to the slides. Adult zebrafish were anesthetized with 2-phenoxyethanol prior to being frozen in 2-*n*-methylbutane cooled in a dry ice/ethanol bath. The frozen fish were mounted in O.C.T Compound (Tissue-TekII), and thick (10 µm) sagittal and transverse sections were cut at -20°C. Each section was removed from the knife by placing the coated slides on top of the sections, and were allowed to dry at room temperature for 20 minutes. Sections were fixed in freshly prepared 4% paraformaldehyde/PBS [130 mM NaCl; 7 mM Na_2HPO_4; 3 mM NaH_2PO_4] for 20 minutes and washed for 5 minutes in 3X PBS (Vacca, 1985). The sections were washed twice in 1X PBS, 5 minutes per wash, and dehydrated in 5 minute changes of 30%, 60%, 80%, 95%, and 100% ethanol. Sections were air-dried and stored at -20°C for up to one month in a dessicator.

Female and male zebrafish were anesthetized with 2-phenoxyethanol and dissected for ovaries and testes respectively. The ovaries and testes were immediately placed in 2-*n*-methylbutanol cooled in a dry ice/ethanol bath. The frozen tissues were mounted and sectioned in the same manner as described for a whole fish.

Paraffin Sections of Zebrafish Ovary

Ovarian tissue was embedded in paraffin and serial-sectioned for *in situ* hybridization with a DIG-labeled probe. Females were anesthetized with 2-phenoxyethanol and dissected for ovarian tissue. The tissue was immediately placed in freshly prepared Ammerman's fixative as described in Humason (1962a), for a maximum of 2 hours. The tissue was washed under running tap water overnight and then dehydrated (Humason, 1962b) in an ethanol series. Changes of 75% EtOH and 95% EtOH were done for 1 hour each, and two 30 minute changes of 100% EtOH were done. The tissue was placed in 2 changes of toluene for 30 minutes each to ensure complete removal of any water that might still have been left in the tissue. The tissue was then infiltrated with 2 changes of molten (56°C) paraffin for 30 minutes each. Once infiltration was complete the tissue was embedded in paraffin using the technique described by Humason (1962c).

The paraffin block was then sectioned on a Leitz Wetzlar microtome to produce serial sections (7 µm thick) of the ovary. The sections were placed in a warm water (37°C) bath to allow the paraffin to expand, evening out the sections. Sections were lifted from the water bath by albumin-coated slides and were allowed to dry on a slide warmer. Sections were then prepared for *in situ* hybridization with a DIG-labeled probe.

In situ Hybridization of Frozen and Paraffin Sections

In situ hybridization of a DIG-labeled probe was optimized for both frozen sections and paraffin sections based on techniques outlined by Hillan (1992), Leitch *et al.* (1994), MacPhee *et al.* (1995) and Wilkinson (1992). All steps were performed at room temperature unless otherwise stated. Prior to hybridization of a DIG-labeled DNA previously prepared, paraffin was removed from sections by clearing in toluene for 3 minutes. Both paraffin and frozen sections were run through an ethanol rehydration series of 100% EtOH, 95% EtOH, 70% EtOH for 3 minutes each (Humason, 1962b) prior to treatment with 0.2M HCl for 20 minutes.

The sections were rinsed in distilled water for 5 minutes and washed in 2X SSC for 15 minutes followed by a second rinse with distilled water. The sections were then treated for 5 minutes at 37°C with 0.25 μg/μl of pronase in 50 mM Tris-HCl (pH 7.5) and 5 mM EDTA. The pronase solution was pipetted off and the slides were immediately washed in 1X PBS and 2 μg/ml glycine for 45 seconds (with agitation). Two washes in 1X PBS for 45 seconds with agitation preceded a second fixation in 4% paraformaldehyde for 20 minutes. The sections were washed once in 3X PBS for 5 minutes, followed by two 5 minute washes in 1X PBS. Sections were treated for 10 minutes with freshly prepared 0.25% acetic anhydride in 100 mM triethanolamine, followed by three 5 minute washes of 1X PBS. 50μl of prehybridization solution (as outlined for Northern blotting) was added to each slide and a coverslip was placed on the sections to ensure that the solution remained restricted to the area of the sections. The slides were placed in a humid chamber for 1 hour at 45°C.

Prior to hybridization, 150 ng of DIG-labeled insert was denatured by boiling for 5 minutes, cooled on ice and added to freshly prepared prehybridization solution. 50µl of hybridization solution was added to each slide and coverslips were placed on top to ensure that the solution remained restricted to the area of the sections. The slides were submerged in preheated mineral oil (45°C) and hybridized overnight at 45°C.

Slides were removed from the mineral oil and rinsed in three 5 minute changes of chloroform to remove any residual mineral oil. The slides were air-dried and placed into 2X SSC where the coverslips were removed. The sections were washed twice in 2X SSC for 15 minutes each, once in 0.1X SSC at 45°C for 20 minutes, followed by a final wash in 0.1X SSC at room temperature for 10 minutes to remove any unbound probe.

In order to confirm that hybridization of the DIG-labeled insert was specific, control slides were run at the same time. The slides were treated in the same manner as described above with the exception that prior to hybridization with the DIG-labeled probe the sections were hybridized with unlabeled probe (500 ng) in order to mask the message. Sections were also hybridized with a DIG-labeled plasmid pUC19 (150 ng) as a second control.

Colorimetric Detection of DIG-labeled Probe

Following post-hybridization washes, the sections were equilibrated for 1 minute in wash buffer, placed in 1% blocking solution and agitated for 30 minutes at room temperature. The slides were drained and placed in a humid chamber. Anti-DIG-alkaline phosphatase (Boehringer Mannheim) was diluted 1:2000 in 1% blocking solution and added to the sections. The sections were incubated with the antibody for 30 minutes without agitation in a humid chamber. After incubation in the antibody, sections were washed in two 15 minute changes of washing buffer and placed in detection buffer for 2 minutes to activate the alkaline phosphatase. The color substrate solution was prepared by adding 22.5 µl of NBT [75 mg/ml nitroblue tetrazolium salt, 70% dimethylformamide], and 17.5 µl of X-phosphate [50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt, 100% dimethylformamide] to 5 ml of detection buffer. Slides were removed from the detection buffer, drained and placed in a humid chamber. The color substrate solution was added to the slides and coverslips were placed on top to ensure that the substrate solution remained restricted to the area of the sections. The slides were then placed in a humid chamber in drawer overnight to allow for color development. Once color had developed, the sections were rinsed in TE buffer and fixed in 4% paraformaldehyde/PBS for 10 minutes to prevent further color development. The sections were rinsed in 1X PBS for 5 minutes, dehydrated in an ethanol series and mounted in glycerol. Sections were examined for localized color development using light microscopy. Photographs were taken at various magnifications using Ektachrome Tungsten 64 slide film (Kodak).

Hematoxylin Staining of Frozen and Paraffin Sections

To be able to histologically identify tissues that showed positive results in the *in situ* hybridization experiment, a modified version of Delafield's hematoxylin staining (Humason, 1962d) was used. Paraffin was removed from paraffin sections by clearing in two changes of xylene for 3 minutes. Both frozen and paraffin sections were rehydrated in 3 minute changes of 100%, 95%, and 70% ethanol, and placed in Lugol's solution [1.0 gm iodine crystals, 2.0 gm potassium iodide, 12.0 ml distilled water] for 3 minutes. Sections were rinsed for 3 minutes in running tap water and placed in 5% sodium thiosulfate for 3 minutes. The sections were rinsed in running tap water for 3 minutes and placed in hematoxylin for 3 minutes. The sections were rinsed in running tap water for 3 minutes and then placed in Scott's solution [2.0 gm sodium bicarbonate, 20.0 gm magnesium, 100.0 ml distilled water] for an additional 3 minutes. The sections were rinsed in running tap water for 3 minutes and counterstained with eosin for 2 minutes. Sections were dehydrated in 3 minute changes of 70%, 95%, and 100% ethanol, with a final step in xylene for 3 minutes. Sections were mounted with glycerol and examined under light microscopy, and photographs at various magnifications were taken using Ektachrome Tungsten 64 slide film (Kodak).

RNase Protection Assay

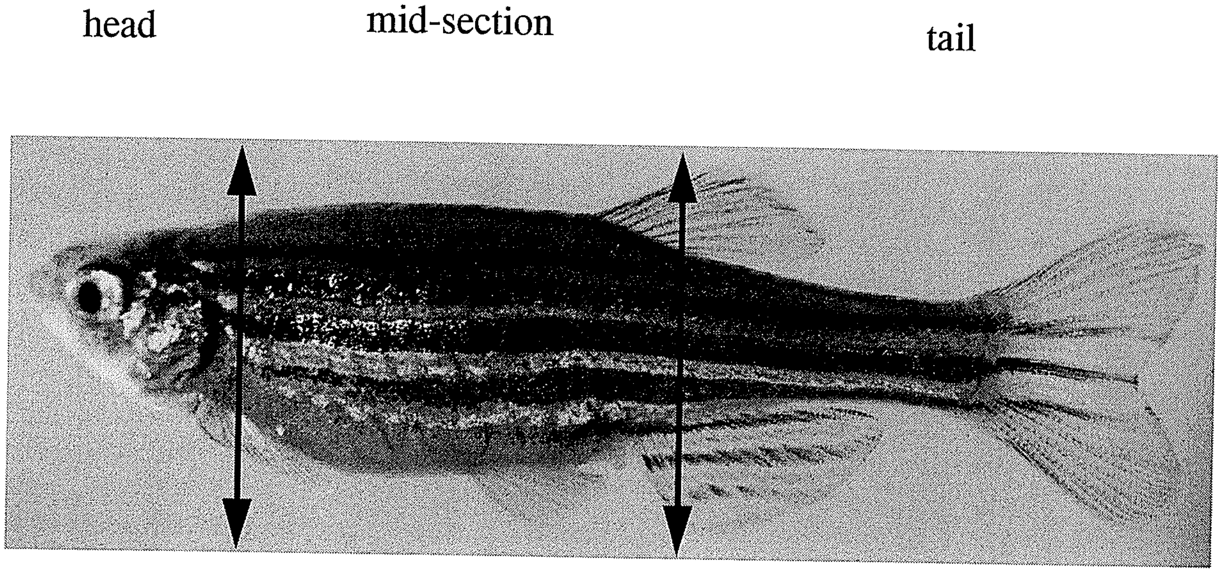
Embryos (66) were collected at various developmental stages and placed in 1.5ml Eppendorf tubes. The medium was removed and 300µl of lysis buffer [4M guanidine thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl] was added to the embryos. Using a 22 gauge needle, the embryos were drawn through the needle and expelled 3 times to shear the tissue. The tubes were vortexed and stored at -20°C until needed. The RNase protection assay outlined by Kelly and Moon (1993) was modified in order to accommodate use of a DNA probe for hybridization in place of an RNA probe.

One of the primers used for sequencing [5'tggtgccataatgagagtctggt3'] was end-labeled using γ^{33}P-dCTP. The ability of the primer to hybridize to the mRNA from the embryo lysates determined the orientation of the clone, thereby identifying the coding and non-coding strands. The above-mentioned primer was identified as having the ability to hybridize to the mRNA through a preliminary RNase protection assay that included 2 sets of sequencing primers. 150 pmol of the oligonucleotide was added to 10 µCi γ^{33}P-dCTP, kinase buffer and T_4 kinase. Labeling was done at 37°C for 1 hour, followed by precipitation with 3 vol isopropyl alcohol. The sample was washed in 75% and 95% ethanol, and dried at 65°C for 10 minutes. The radioactive sample was resuspended in lysis buffer. Oligonucleotide β-actin [5'gtggggcgccccaggcacca3'] (Gibco BRL) was also end-labeled (100 pmol) in order to identify differences in quantity of RNA within each sample.

Embryo lysates were centrifuged to pellet chorion and cellular debris, and 45 µl of each sample lysate was transferred to a 1.5 ml Eppendorf tube. To each sample, 5 µl (2.0×10^6 cpm) of each labeled probe was added, and each sample was boiled for 5 minutes before hybridization overnight at 68°C. After hybridization, 500 µl of RNase cocktail [200 µg RNase A previously boiled for 20 minutes, 500 U RNase T_1, 200 U DNaseI in 10 mM Tris-HCl (pH 7.5), 300 mM NaCl, 5 mM EDTA] was added to each sample and incubated at 37°C for 1 hour. The samples were digested with 20µl of 10% SDS and 100µg of proteinase K at 37°C for 45 minutes. Each sample was extracted twice with P:C:I and once with C:I:A, and precipitated with 1 vol isopropyl alcohol and 3 ng *torula* *t*-RNA for 20 minutes at -20°C. The samples were centrifuged and washed in 75% EtOH and 95% EtOH, and dried at 65°C for 5 minutes. The pellets were resuspended in 10 µl of loading buffer [88% deionized formamide, 10 mM EDTA, 1 mg/ml xylene, 1 mg/ml cyanol, 1 mg/ml BφB]. Samples were denatured at 75°C for 4 minutes and placed on ice before loading on a 5% polyacrylamide denaturing gel. 5 µl of each sample was loaded onto the denaturing gel. The gel was run at 1,000 volts for 1 hour and did not exceed 40°C in temperature. The gel was transferred to Whatman No.1 filter paper, wrapped in cellophane and dried at 80°C for 45 minutes in a gel dryer. The gel was placed on Biomax film (Kodak) and exposed at -80°C overnight.

Control samples were run along with the experimental samples. 10ug of *torula* *t*-RNA in lysis buffer was used to ensure that the RNase A would degrade unhybridized RNA, purified whole fish RNA was used as a positive control, purified tail RNA was used as a negative control and undigested probe was used as a size marker. Several zebrafish were subdivided into head, mid-section and tail as outlined in Figure 2. Crude lysates of adult zebrafish head, mid-section, and tail were used as controls for efficiency of the assay on crude samples. Lysates of brain, ovary, and testis were run to identify tissue specificity.

Figure 2: Scanned image of an adult zebrafish indicating head, mid-section and tail regions used in the RNase protection assay (Photographic image courtesy of Dr. H. Laale, Department of Zoology, University of Manitoba).


head
mid-section
tail

RESULTS

PCR Amplification

The universal primers MBP38S and MBP262A should give rise to a DNA fragment that holds seqeuence homology to a portion of the MBP gene (size of fragment should be approximately 232-244 bp) when genomic DNA from a number of organisms is used in PCR amplification reactions (W. Spivack pers. comm.). These primers were used in PCR amplification of zebrafish genomic DNA to generate a DNA fragment that held sequence homology to the MBP gene in zebrafish. After PCR amplification, the products were separated by electrophoresis in an agarose gel as outlined in Methods. After visualization of the DNA by ethidium bromide staining, a band of approximately 240bp was identified within the expected range for the PCR product as suggested by W. Spivack. The DNA fragment was eluted from the gel and used as a probe in subsequent analyses.

Screening of an Adult Zebrafish cDNA Library

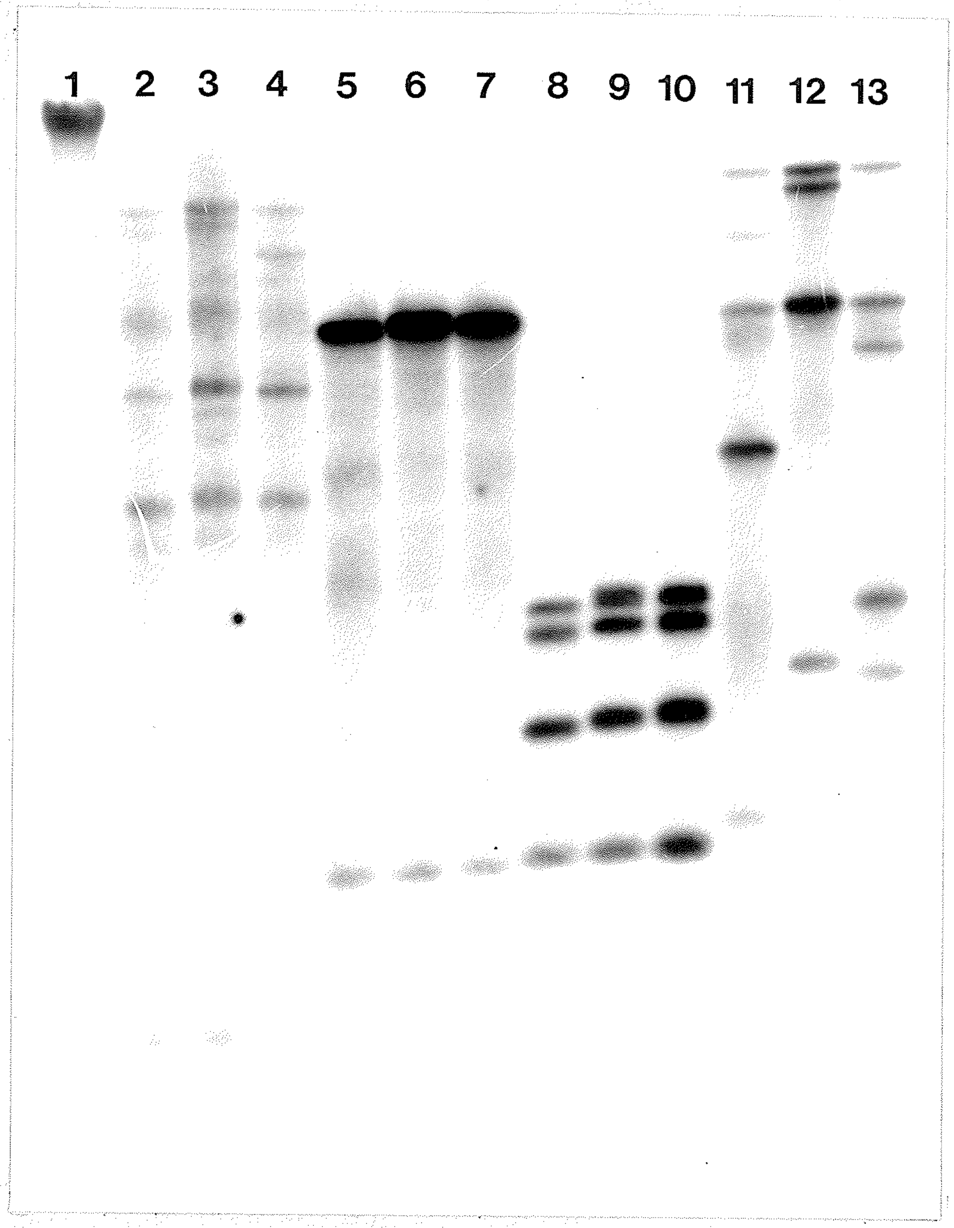
An adult zebrafish cDNA library was hybridized with the PCR-amplified DNA and 8 potential positives were observed. These positives were collected and pooled for a second screening with the same probe. Fifteen potential positive plaques were selected from the second screening. Phagemids from each of the 15 positive plaques were excised from the Lambda Zap II vector and analyzed by restriction endonuclease digestion with PvuII. This enzyme will cleave the phagemid DNA at two sites, each on opposite sides of the multiple cloning site, producing a two fragments of phagmid DNA; one being about 400 bp and the other about 2500 bp. Phagemids containing DNA sequences that have been inserted into their multiple cloning sites will generate fragments greater than 400 bp when digested with PvuII. Four clones were identified as having inserts greater than 400 bp; each of these four clones was digested with BamHI and KpnI and the inserted DNA could be isolated.

Southern Blot Analysis

The DNA sequences inserted into each of the selected phagemids were labeled with α-^{32}PdCTP using a random primer labeling system, and sequentially hybridized to the same Southern blot using DNA from three different varieties of *Danios* (wildtype, leopard, and longtail) that had been digested with HindIII, EcoRI, PstI, and HpaII. Hybridization of the inserts isolated from clones KSZf8, KSZf14 and KSZf7 resulted in no distinct bands on the Southern blot of the zebrafish genomic DNA's. This suggests that the three inserts did not have significant sequence homology to zebrafish genomic DNA and investigation of these clones was abandoned.

Hybridization of the insert isolated from KSZf5 to the same Southern blot resulted in distinct bands (Figure 3) throughout all three varieties of *Danios*. Because hybridization was done under relatively stringent conditions, this suggests

Figure 3: Southern blot analysis of DNA (20 μg in each lane) from wildtype (lanes 2, 5, 8, 11), leopard (lanes 3, 6, 9, 12), and longtail (4, 7, 10, 13) *Danios*. The genomic DNA was digested with restriction endonucleases HindIII (lanes 2-4), EcoRI (lanes 5-7), PstI (lanes 8-10) and HpaII (lanes 11-13), and hybridized with radioactively labeled KSZf5 insert at 42°C overnight as indicated in Methods. Lane 1 contains HindIII lambda DNA as size markers. Autoradiographic exposure was done overnight at -80°C.


1
2
3
4
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6
7
8
9
10
11
12
13

that the insert possesses a unique sequence that has a high degree of homology to sequences within the zebrafish genome. Distinct RFLP's were also observed between the different zebrafish strains (lanes 11-13 of Figure 3) indicating that genotypic variation exists at this locus.

Sequencing Analysis of DNA

Due to the limitations of the nucleotide sequencing system, nucleotide sequence analysis of the KSZf5 insert was done using successive primers to generate consecutive sequences. By matching areas of overlap within these sequences, a continuous sequence for the KSZf5 insert was generated. The complete sequence for the KSZf5 insert is shown in Figure 4, and is 1.89 kb in length. Sequence data was forwarded to BLAST Network Service (Blaster) (Altschul *et al.* 1990) to identify whether KSZf5 showed significant sequence homology to any previously cloned sequences. Comparison of our sequence to databanks of other cloned sequences by the Blast program revealed no obvious identity with any other gene. However a number of sequences did display some sequence homology to KSZf5 to selected portions of the sequence, generally 50-80 bp (see Appendix II). These tended to be gene sequences encoding receptor proteins such as immunoglobulin receptors, androgen receptors, T-cell receptors and progesterone receptors. The service also found homologies with K^+ channel RNA's, and Na^+/H^+ exchanger isoforms. The forward sequencing reaction determines the 3' end of the cDNA. There is no obvious homology to other proteins within this region suggesting that either the

Figure 4: Complete DNA sequence of KSZf5. Underlined nucleotides represent sequences that hold homology to the 4 sets of primers used to completely sequence the 1.89kb insert. Forward (f) and reverse (r) primers are labeled accordingly. Autoradiographic exposure was done at -80°C for 5 days. An unknown base is represented by n and can be any one of the four bases: a, c, g, or t.

```
           10          20          30          40          50          60
5'  caattcnggg  gcnctcggca  atgcaccgtc  gagcaacagc  gcccattctt  ccnnccgcna

    gcaacagggg  agccgggggt  gcagccgggg  ggatcaaccg  nngcgcgggg  aattcgaacc
                            r1
    ctnagttcgg  gccggngaac  ggccggaggc  accgagtctg  cggtgagata  gcacgtcgag

    ctgctgctac  acaaccctcc  tcggcagacg  gtcggccaga  gcngagcctc  ttaggcgcta

    gcgtcaaaca  tgacggcgac  atcttcatca  tcatcatcct  cctcctcatc  ctcatccgcg
                            r2
    tctaccgcat  cctcctccgt  cgatgcgtcc  gccgccgccg  ccgtctctcc  gcttcccagc

    agcccggctt  cggttttngt  ttatcgagag  ccggtctaac  aactggcaag  cnacaaaaag

    tacagtnaaa  ganagatttg  ctctcgctgt  gggaagtgcc  gtgtttgatg  ccatgttcaa

    cggtggcatg  gcaacaacgt  cgaccgaaat  tgaactgcca  gatgttgaac  cggctgcatt
                r3
    tctagccctt  ctcaaatttt  tatactcaga  tgangtgcaa  attggaccag  agaccgtnat

    gaccacatta  tacacagcca  aaaagtatgc  agtacctgcn  ttgnccactg  ntgaattctg

    angaaaaacc  tacgcgctga  caacgatttn  tgttgcttac  ccaggcacgg  ctttttgacg

    agccccagct  ggcaagtntc  tgcttagaaa  atatcgacaa  gaacacagct  gatgcactcg
                                                    r4
    ctgctgaggg  cttcacggat  gttgaccttg  atacctcgtg  gcagtccggg  gcacactggt

    cagtcctgga  gagagacaca  cttggtgtac  gggaagtgcg  tctcttcgga  gctgctgttc

    gttgggcaga  agctgaggcc  caaaggcaac  aattacagcc  tacaccggag  aacaagcgaa

    gagtgttggg  aaaggcactt  cccttattcg  ctttccactc  atgacaattg  aagagtttgc

    agcaggtccg  gctcnntctg  gtatactcac  agaccgggag  gtggtcagtc  tgttcctgca

    ttttaccgtc  atccgaaacc  acatgtggag  tttattgacc  ggccccgctg  ttgcctccgg

    gggaaagagt  gcagcatcac  gcgtntcagt  caggtggaga  gccgctgggg  gtacagtgga

    accagtgacc  gcatccggtt  ttctgtgaac  cgcacaatat  ttgtgggggg  atttggactt
          f3
    nntggctcta  tacatggtcc  aactgactat  naggtcaaca  tccagatnat  acanacagac

    agtaacacag  ttctcggaca  gaacgataca  ggcttncttg  tgacggaaca  gccagcacan

    nnncagtgat  gttnnaggag  ccagtggaaa  ttcttccatg  caattacacc  gcctgcgcca

    cctgaaggac  cagactctca  ttatggcacc  aagggatgcg  taaagtcacc  catgaagcnc
                      f2
    ccgcanctgg  tactaagcct  gcttnacatc  tgctatgctg  caggcaacaa  caacggcact

    tctgtntaga  tggacaaacg  cncaggtcat  cttctacact  aatgaccgag  cagagtcgtc
```

```
            1630         1640        1650         1660         1670         1680
5'  aaacaatcga   ggactcttag  attgcatttt   gtggccannc   gccagtcttc   ggtgtgtaga

    ctggtgacca   aatactgatc  ttcatttcta   aaggctncat   tgttaagnta   tgagnattca
                                             f1
    gttctntcaa   agtgtactta  gtgtaatntc   agattttgtt   tctacccaat   gagcnccttg

    tagtacaacc   tttaagcaca  accngccaat   gtctagttgc   tcagtaatga   acgtactnta

    cagagctatc   aatcaggtac  agcacagaga   a3'
```

KSZf5 protein is unique, or that it may contain a portion of its 3'UTR. This is of specific interest because several maternal effect transcripts such as *bcd* (MacDonald and Struhl, 1988) and *Vg1* (Kloc and Etkin, 1995) are localized to regions of activity based on recognition of secondary structures formed in their 3'UTR.

The nucleotide sequence was translated to the primary amino acid sequence for further analysis. Translation products were derived from three reading frames, and based on the minimal length of amino acid residues for an open reading frame, a number of open reading frames were determined. Two open reading frames were identified in frame shift 3, when a minimal amino acid length of 150 residues was chosen. The reading frames ranged from position 3 to 542 nucleotides, and position 21 to 542 (see Appendix III and IV).

One of the negative clones, KSZf14, was also examined in order to determine the nature of the false positives. Sequencing of the clone revealed that a small fragment of the *E. coli* genome had been inserted into the cloning site rather than the desired zebrafish DNA (sequence in Appendix IV). This sequence had a trinucleotide repeat similar to that identified in the PCR-generated probe, which would explain the identification of the false positives from the library screens.

Northern Blot Analysis

RNA hybridization analyzes were performed on total RNA from whole zebrafish to determine if the isolated insert from KSZf5 held homology to a transcriptional product. RNA hybridization analysis using radioactively-labeled KSZf5 produced a strong signal in whole zebrafish total RNA (Figure 5), identifying a transcript that holds homology to KSZf5. Comparison with RNA size markers showed that the transcript corresponding to the KSZf5 clone was approximately 5 kb. Further investigation of tissue specificity was done using the RNase protection assay and *in situ* hybridization.

In Situ Hybridization

Whole zebrafish were frozen in 2-*n*-methylbutane, embedded in O.C.T compound and sectioned on a cryostat. Initial *in situ* hybridization of frozen sagittal sections (10 µm) with DIG-labeled KSZf5 revealed strong hybridization in tissue located in the dorsal portion of the abdominal cavity, more specifically in the region of the mesonephros and gonads, as compared to the adjacent gut (Figure 6). The signal appeared to be more localized more posteriorly, suggesting that there may be differential expression of KSZf5 within one of these tissues. From Northern blot analysis and *in situ* hybridization experiments, it is clear that KSZF5 holds homology to a transcriptional product specific for a tissue within the zebrafish abdominal cavity. However, to determine if KSZf5 transcripts are specific for the mesonephros or the gonads, male and female zebrafish were dissected for kidney tissue and their respective gonads. The tissues were sectioned on a cryostat and *in situ* hybridization of DIG-labeled KSZf5 was performed on all tissues. After an overnight incubation in alkaline phosphatase substrate, signal was detected only in the ovary

Figure 5: Northern blot analysis of total RNA (40µg) from whole fish (lane 2). Total RNA were hybridized with radioactively labeled KSZf5 insert at 42°C overnight as described in Methods. Lane 1 contains 0.24-9.5 kb RNA ladder. Autoradiographic exposure was done overnight at -80°C.

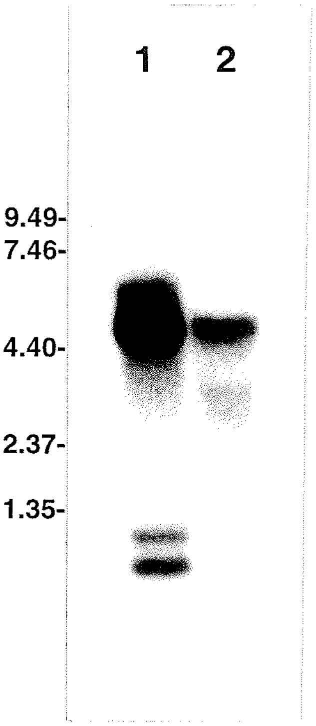
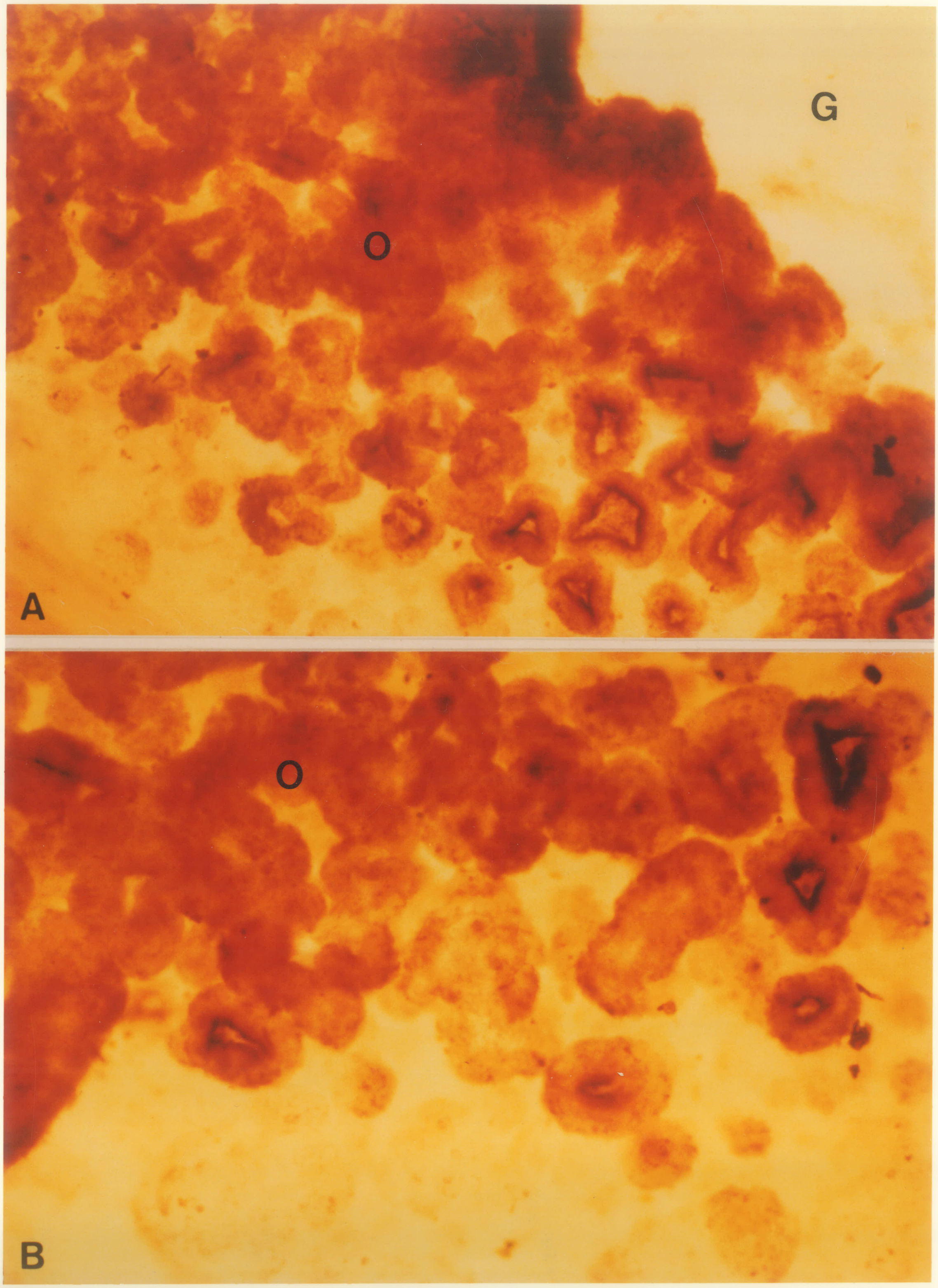

1
2
9.49-
7.46-
4.40-
2.37-
1.35-

Figure 6: Light micrographs showing *in situ* hybridization of adult zebrafish ovary at low (figure 6A, x23) and high (figure 6B, x38) magnifications. Frozen sagittal sections were hybridized with DIG-labeled KSZf5 insert at 45°C overnight as described in methods. KSZf5 transcripts were detected using alkaline phosphatase-linked Anti-DIG Fab-fragments, within the zebrafish ovary (O) only as compared to the adjacent gut (G).


G
O
A
O
B

(Figure 7B). No signal was detected within the testis or the kidneys (data not shown) identifying that KSZf5 transcripts are specific for ovarian tissue. The identity of this tissue was verified by hematoxylin staining of frozen ovarian sections (Figure 8). Sections hybridized with DIG-labeled pUC19 showed no signal, suggesting that the hybridization of DIG-labeled KSZf5 was specific (Figure 7A). Further analysis of the ovarian sections by light microscopy indicated that expression is localized to the periphery of the developing oocytes themselves (Figure 7C and D). Due to the poor morphological representation of the frozen sections, identification of signal localization was difficult. The signal could be localized in either the oocyte or the follicle cells.

To better understand the localization of the KSZf5 transcripts within the developing oocytes, paraffin sections were hybridized as well. Ovarian tissue was fixed in Ammermann's fixative and embedded in paraffin to preserve morphology of the developing oocytes. Hematoxylin staining of serial sections of the ovary revealed that cellular morphology had been retained in the sections, although there was evidence of minor tissue shrinkage. Figure 9A shows the asynchronously developing ovary, where cells of all stages, from germ cells to mature oocytes, are randomly interspersed throughout the stroma of the ovary. Figure 9B, C and D shows oocytes at various stages throughout development.

Sections were hybridized with DIG-labeled KSZf5 insert, and after an overnight exposure to alkaline phophatase substrate, the sections were examined for localization of the KSZf5 transcripts. *In situ* hybridization results indicate that KSZf5

Figure 7: Light micrographs of *in situ* hybridization of frozen sections of adult zebrafish ovary. **A.** Control sections were hybridized with DIG-labeled pUC19 at 45°C overnight as described in methods and no signal was detected in the sections. Magnification x200. **B, C and D.** Sections were hybridized with DIG-labeled KSZf5 insert and signal was detected in the developing oocytes (oc). **B** shows signal in oocytes at various stages of development. Magnification x40. **C** shows a mature oocyte with signal localized to the cortex of the egg. Magnification x130. **D** shows an oocyte at stage III of development with signal more dispersed throughout the cytoplasm than in C. Magnification x290.

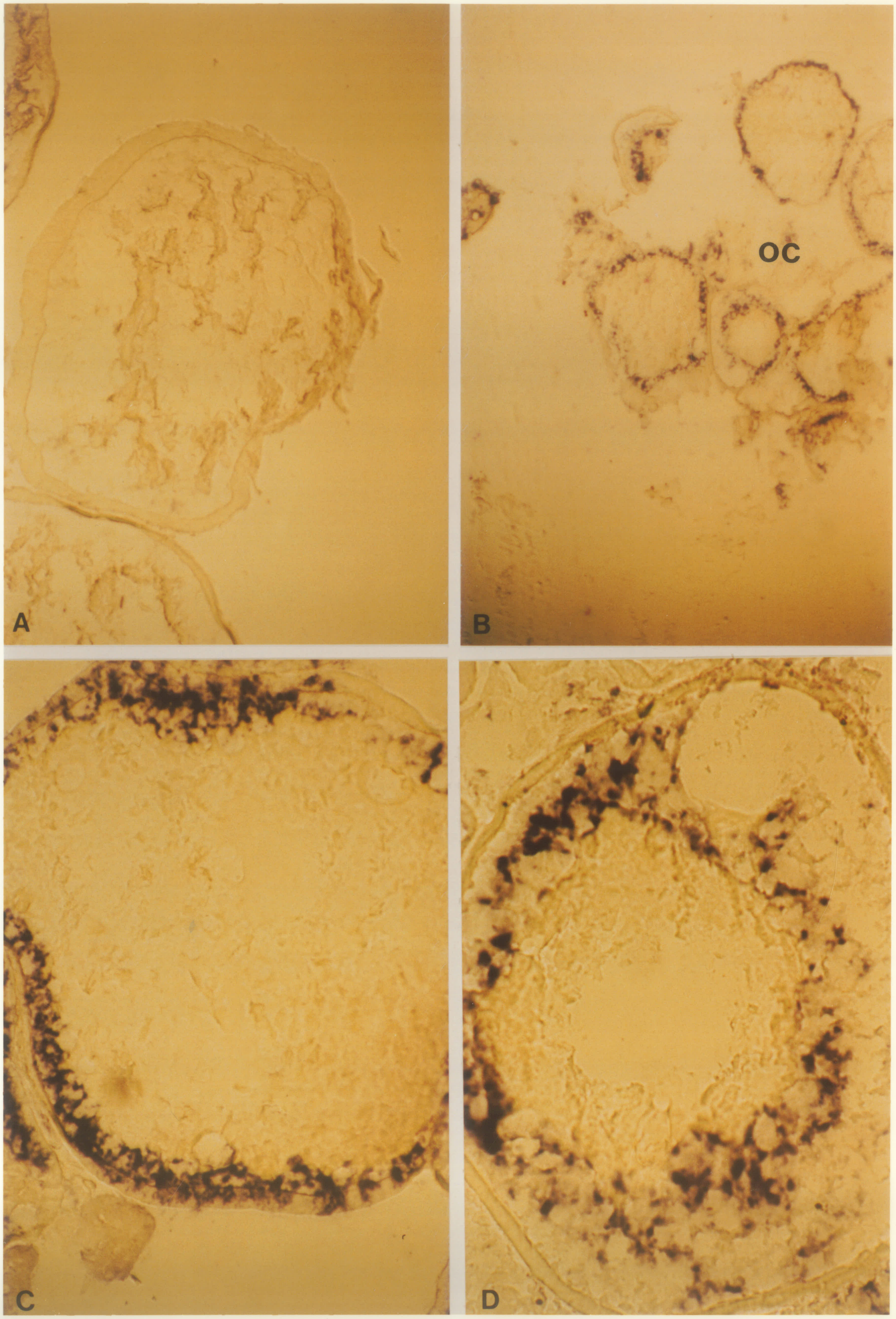

OC
A
B
C
D

Figure 8: Light micrographs of frozen sections of **A**) adult zebrafish ovary and **B**) mature oocyte (oc) stained with hematoxylin. Magnification x44, x180.

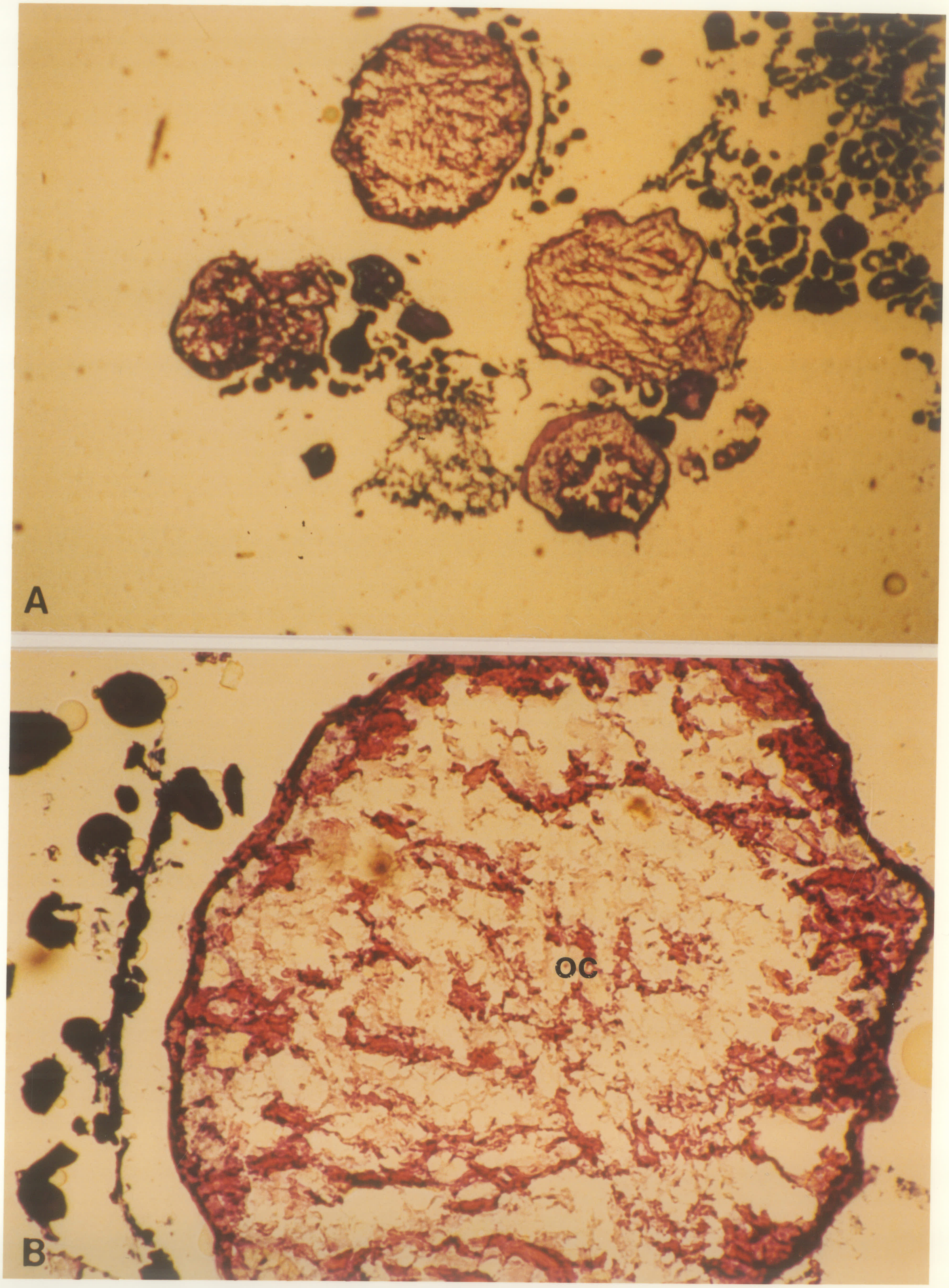
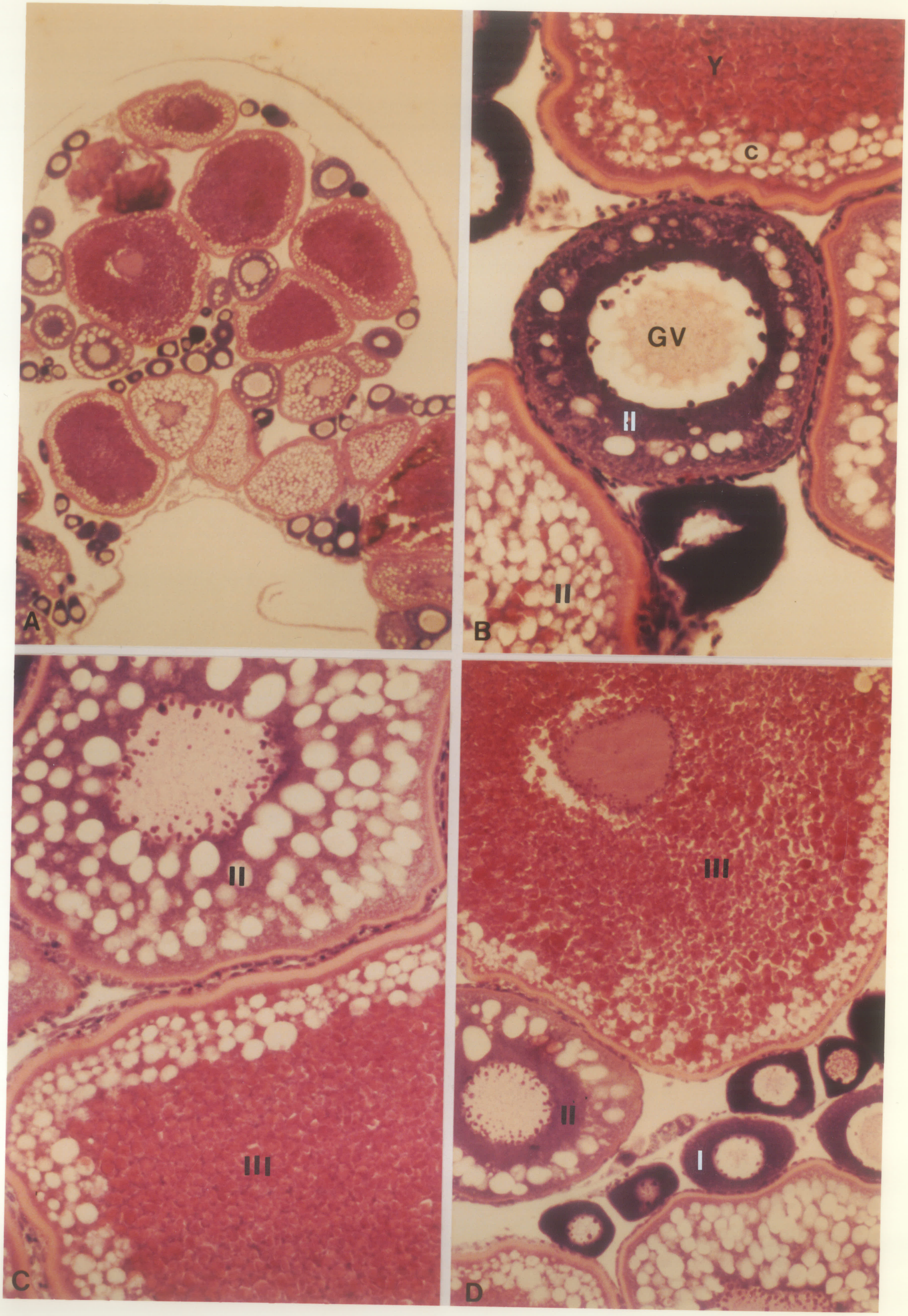

A
OC
B

Figure 9: Light micrographs of paraffin-embedded sections of adult zebrafish ovary stained with hematoxylin. **A** shows oocytes at various stages of development. Magnification x30. **B** shows an oocyte during the cortical alveolus (early) stage (II) of oocyte development, where the germinal vesicle (GV) makes up 50% of the total volume of the cell. Cortical alveoli (c) are dispersed throughout the oocyte cytoplasm in primary oocytes and become localized to the cortex as the oocyte accumulates yolk bodies (Y). Magnification x390. **C** shows one oocyte during the cortical alveolus stage. The number of cortical alveoli has increased and they are evenly dispersed throughout the oocyte cytoplasm. The adjacent oocyte has completed vittelogenesis (III) and is beginning to undergo maturation. The cortical alveoli have become displaced to the periphery of the oocyte due to yolk accumulation. Magnification x195. **D** shows a higher magnification of A x210. Oocytes in the primary growth stage (I), oocytes in the cortical alveolus stage (II), and oocytes during vitellogenesis (III).


A
Y
c
GV
II
II
B
II
III
C
III
II
I
D

transcripts are present at a significant level in oocytes that are undergoing vitellogenesis, and continue through the maturation stage of oocyte development (Figure 10B). The transcripts are interspersed throughout the cytoplasm of the vitellogenic oocyte (Figure 10D), and as yolk bodies accumulate, the transcript becomes localized to the cortex of the egg due to cytoplasmic displacement. Localization of the cortical alveoli also became restricted to the cortex of the egg; however, KSZf5 transcripts were not detected within these structures, but are limited to the cytoplasmic fraction of the developing oocytes (Figure 10B, C and D). Further analysis of paraffin sections indicate that a weak signal for KSZf5 transcripts is present in primary germ cells (Figure 10B). Comparison to control sections hybridized with DIG-labeled pUC19 showed that the signal was not a result of non-specific hybridization (Figure 10A), thereby suggesting that the transcripts are present within the primary germ cells.

RNase Protection Assay

Embryos were collected at various stages of development starting from high blastula stage (3.3 hrs after fertilization) to 2 week old fry. RNA within each sample was hybridized with radioactively-labeled antisense oligonucleotides (DNA); KSZf5 and β-actin. The radioactively-labeled β-actin was used to provide a measure of the relative RNA present within each sample. The samples were digested with RNase and the protected RNA's were separated on a polyacrylamide/urea denaturing gel (Figure 11). A comparison of the levels of β-actin across each sample, suggests

Figure 10: Light micrographs of *in situ* hybridizations of paraffin-embedded sections of adult zebrafish ovary. **A.** Control sections were hybridized with DIG-labeled pUC19 at 45°C overnight as described in Methods. No signal was detected in the developing oocytes (oc). Magnification x235. **B, C and D.** Paraffin sections were hybridized with DIG-labeled KSZf5 insert at 45°C overnight as described in methods, and signal was detected in the developing oocytes using alkaline phosphatase-linked Anti-DIG Fab-fragments. **B** shows signal in oocytes at various stages of development. In primary (I) oocytes the signal is evenly distributed throughout the oocyte cytoplasm. As the oocyte undergoes vitellogenesis and maturation the signal becomes localized to the cortex of the oocyte. Magnification x 210. **C** shows that the signal is limited to the oocyte cytoplasm that has been displaced to the cortex of the egg due to yolk accumulation. Magnification x250. **D** shows that signal is dispersed throughout the oocyte cytoplasm in oocytes that are beginning to undergo vitellogenesis and as they accumulate yolk the signal becomes localized to the cortex. Magnification x190.

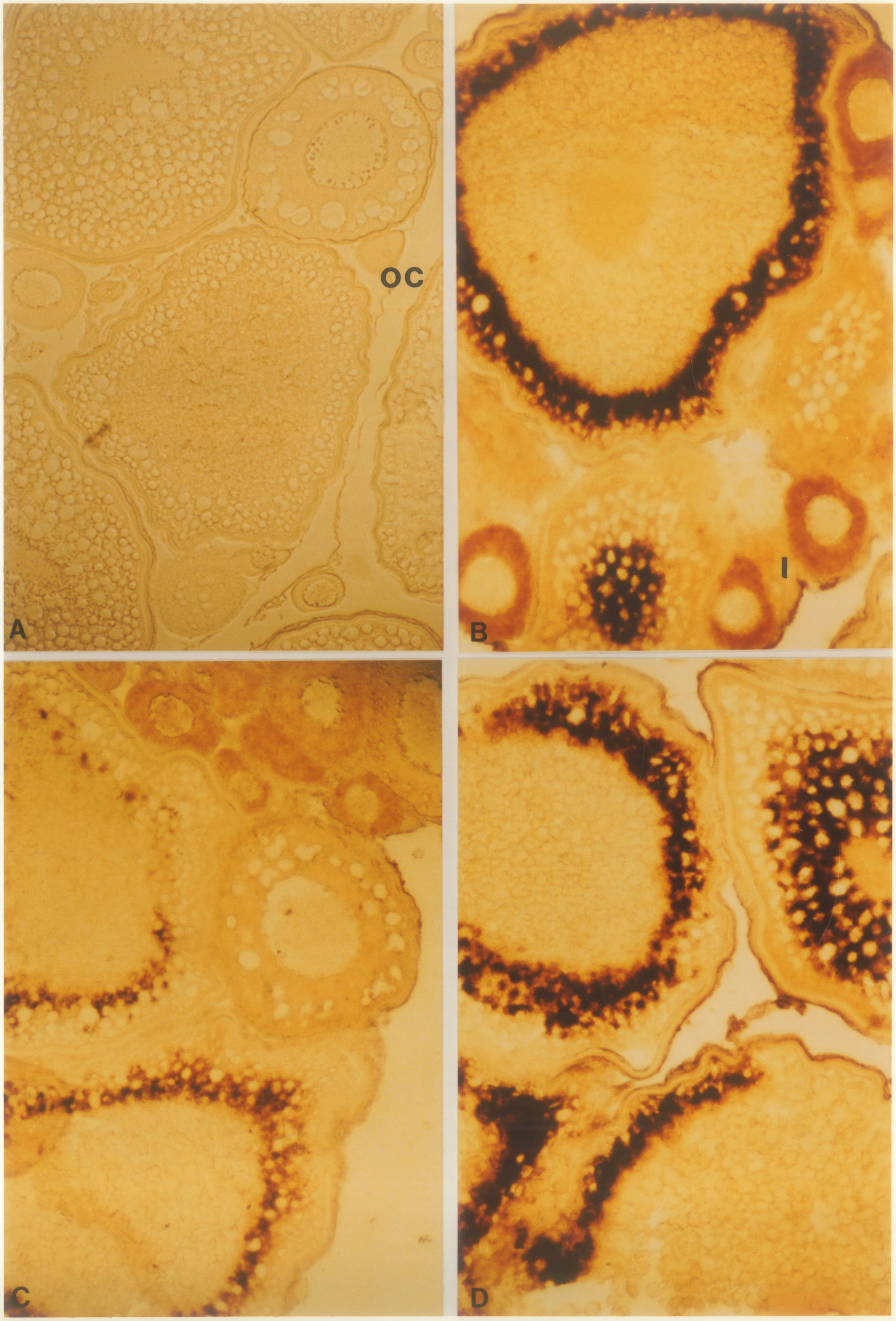
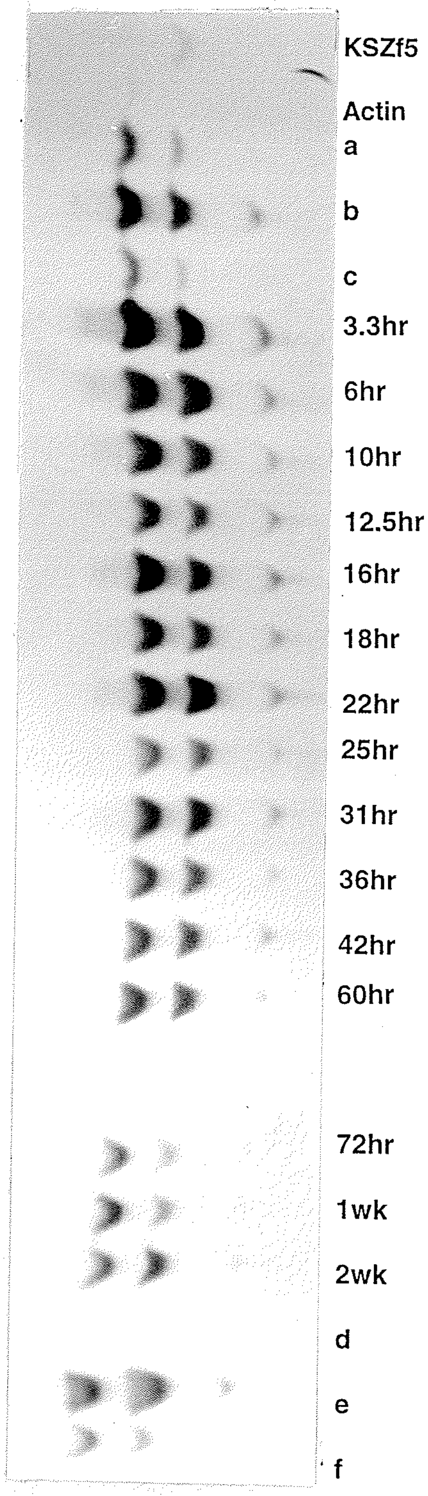

OC
A
B
I
C
D

Figure 11: RNase protection assay used to examine crude lysates of post-fertilization embryos at various stages of development, for the presence of KSZf5 transcripts. Stages of development ranged from 3.3 hours after fertilization to two week old fry. Each sample was hybridized with radioactively-labeled oligonucleotides KSZf5 (24-mer) and β-actin (20-mer) as described in Methods. The RNA protected by the antisense oligonucleotides in crude lysates of mid-section (b), tail (c), brain (d), ovary (e) and testis (f). Purified RNA's were also included in the assay. Radioactively-labeled oligonucleotides, KSZf5 and β-actin, were included to demonstrate that the faster migrating band represents β-actin protected RNA, and the slower migrating band represents the KSZf5 protected RNA. 10 µg of *torula* *t*-RNA (a) was used as a control for digestion of non-hybridized RNA. The autoradiographic exposure time was overnight at -80°C.

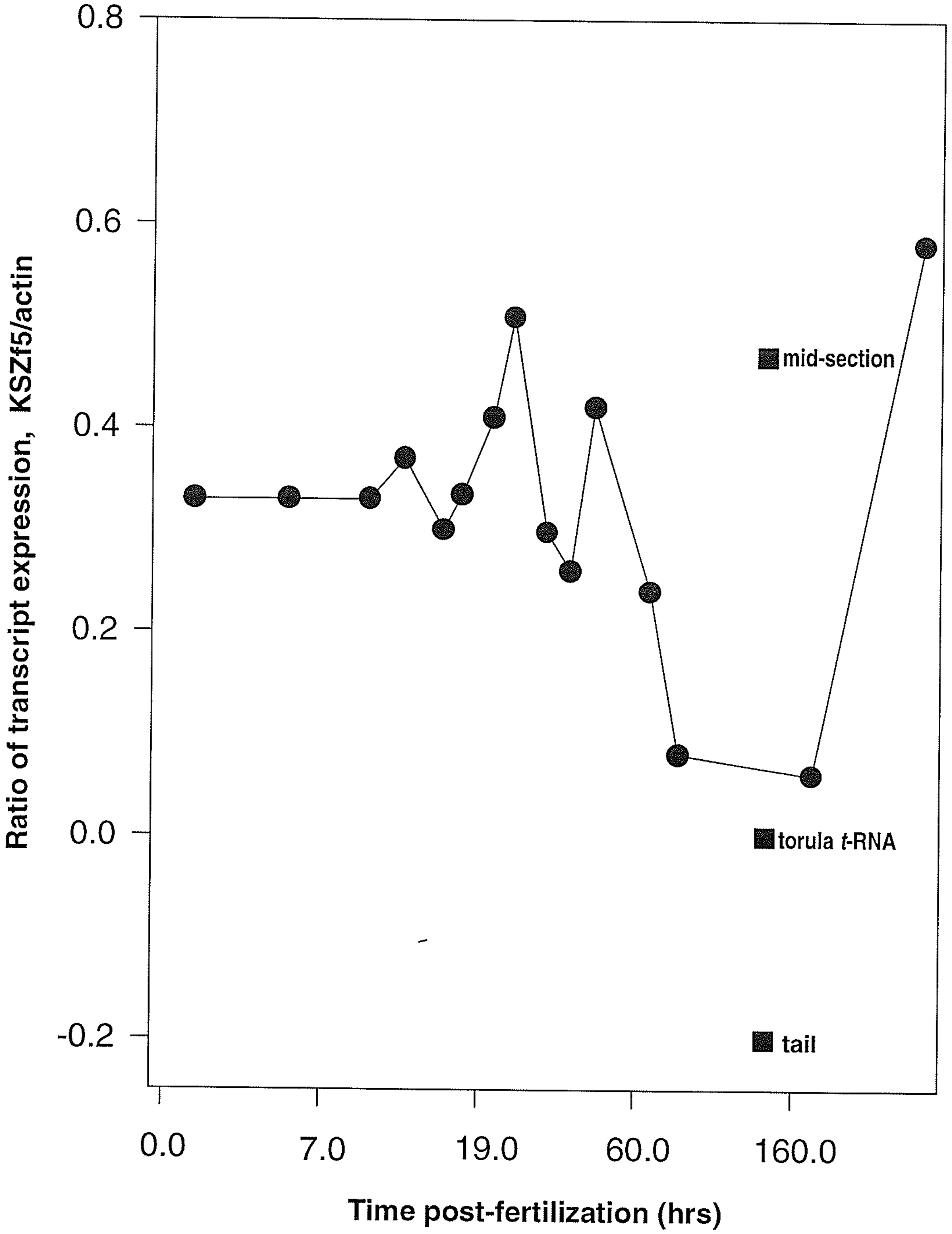

KSZf5
Actin
a
b
c
3.3hr
6hr
10hr
12.5hr
16hr
18hr
22hr
25hr
31hr
36hr
42hr
60hr
72hr
1wk
2wk
d
e
f

there are some small variations in the amount of RNA per lane. The KSZf5 protected RNA concentrations in each sample were equalized by comparing to β-actin protected RNA levels, and the resulting values were graphed in Figure 12. From the RNase protection assay results, it appears that the KSZf5 transcripts are already present at the high blastula stage (3.3 hrs), and do not diminish throughout development until hatching (72hrs) (Figure 11 and 12). However, there appears to be a decrease in expression at 72 hours of development. This continues until fry are two weeks old, at which time expression of KSZf5 increases.

The pre-hatching period, during which the levels of KSZf5 do not appear to change, was further examined by repeating the RNase protection assay on more developmental time points (Figure 13). A visual examination of the autoradiograph showed that the signal pattern throughout development was the same as in Figure 11, with no evidence of non-specific hybridization. This suggests that the signal detected throughout the various stages of development is due to specific hybridization of the labeled oligonucleotide for KSZf5 transcripts to a homologous RNA sequence.

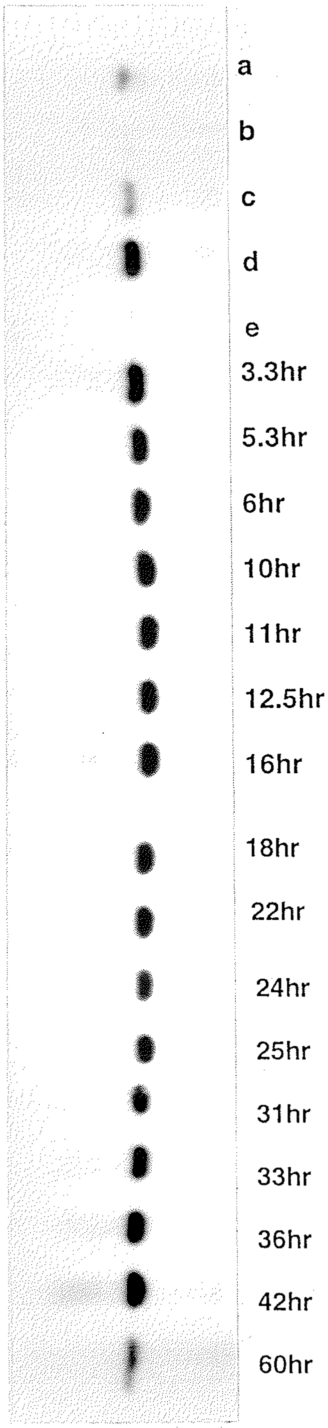
Analyses of various tissue RNAs (Figure 11) indicate that significant expression was found in the ovaries, confirming the results from the *in situ* hybridization experiments. Although a signal was also present in the testes, densitometric analyses indicated a measurably lower intensity as compared to the ovary. A signal was also detected in both the *torula t*-RNA and tail RNA control lanes suggesting that there was a degree of hybridization occuring that was not due to specific hybridization. Although the presence of KSZf5 transcripts in other tissues can not be ruled out, these results in combination with the results from *in situ* hybridization experiments suggests that the major expression tissue is the ovary.

Figure 12: Change in expression of KSZF5 transcripts in *Danio rerio* embryos over a developmental time survey. Values of transcript expression were determined by densitometric analysis of band intensities reflecting levels of RNA protected by oligonucleotides (DNA) for KSZf5 and β-actin. A conversion from a numeric to logrithmic scale was done. Mid-section and tail reflect the expected levels of KSZf5 protected RNA in tissues from adult zebrafish. Negative control is represented by *torula t*-RNA.



0.8
0.6
0.4
0.2
0.0
-0.2
Ratio of transcript expression, KSZf5/actin
■ mid-section
■ torula *t*-RNA
■ tail
0.0
7.0
19.0
60.0
160.0
Time post-fertilization (hrs)

Figure 13: RNase protection assay used to examine crude lysates of post-fertilization embryos at various stages of development, for the presence of KSZf5 transcripts. Stages of development ranged from 3.3 hours after fertilization to 60 hours. 20µg of purified RNA from whole fish (c), mid-section (d), and tail (e) regions of zebrafish were included in the assay. Radioactively-labeled oligonucleotide KSZf5 (a) was used as a size marker. 20µg of *torula t*-RNA (b) was used as a control for digestion of non-hybridized RNA. The autoradiographic exposure time was overnight at -80°C.


a
b
c
d
e
3.3hr
5.3hr
6hr
10hr
11hr
12.5hr
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25hr
31hr
33hr
36hr
42hr
60hr

DISCUSSION

In an organism where fertilization and development occur externally, such as the zebrafish *Danio rerio*, all maternal input into the embryo must be achieved before release of the unfertilized egg. This includes the nutritional input necessary to support the embryo during its growth, as well as the controls necessary to initiate and run its developmental program. Molecular studies of embryonic development have contributed a great deal to the understanding of how interactions between genes and gene products control the normal development of an organism. The identification of mutations that disrupt these gene interactions has been particularly informative regarding gene product function. Researchers often use embryos that are deficient in one gene function in order to identify the specific role of its gene product. It is this approach that has become a very valuable tool in identifying the function of maternal determinants in development, whether it is the establishment of the body axis or the induction of germ layers. Research done in both *Drosophila* and *Xenopus* has determined that the establishment of the dorso-ventral and the antero-posterior axis of a developing embryo relies heavily on the action of specific maternal transcripts that have been sequestered during oocyte development. It is the absence of these transcripts that has been shown to cause a disruption in the organization of axes in the early embryo, ultimately leading to abnormal development. Given the overwhelming role that maternal determinants play in correctly directing early development in some organisms, an examination of oocyte maturation and the role it plays in the early development of other organisms is important.

This is particularly true for the zebrafish because the organism is increasingly being used as a model system in an attempt to understand vertebrate development. In zebrafish, early developmental cell fate mapping has been undertaken to gain an understanding of how and when cells are set aside for development of specific tissue and organ systems in the embryo (Kimmel *et al.* 1990). Various zebrafish mutants have been identifed and characterized in regard to their effects on development (Felsenfeld *et al.* 1990; Eisen and Pike, 1991; Hatta *et al.* 1991; Ho and Kane, 1991; Halpern *et al.* 1993; Abdelilah *et al.* 1994; Shulte-Merker *et al.* 1994), thereby allowing researchers to gain a better understanding of the controls required for normal development. The zebrafish also provides an ideal system for use in trying to gain a better understanding of the important role that maternal determinants play in normal development. Fertilization of mature oocytes is external and, therefore, all maternal determinants must be present within the oocytes prior to ovulation for normal development to occur. Inherently, this provides an isolated system for studying interactions of maternal determinants and their various effects on normal development.

Most of the research done on zebrafish to date has been primarily focused on fin regeneration, neural development, and germ layer induction. Very little research has been done on oocyte development and its role in embryogenesis, particularly with respect to the role of maternal determinants on development. To understand the primary components of zebrafish development, a look into sequences that are tissue-specifically expressed in oocytes is needed. These sequences may represent either gene products important in oocyte formation and/or maturation, or maternal determinants that direct early developmental events in the embryo. Either of these alternatives are of significant interest in terms of how the germ line is sequestered and how early development is controlled.

Preliminary screening of an adult zebrafish cDNA library with a PCR-generated probe presumed to contain a portion of the MBP gene identified several clones that potentially held homology with the zebrafish MBP gene. Southern blot analysis identified only one clone, KSZf5, that showed significant sequence homology to zebrafish genomic DNA, however it did not represent the zebrafish homologue of the myelin basic protein gene. One explanation as to why it was identified in a library screen of adult zebrafish cDNA may be due to the presence of a trinucleotide repeat that was detected after sequence analysis. This trinucleotide repeat was also detected in the PCR-amplified DNA that was used to probe the adult zebrafish cDNA library (sequence shown in Appendix IV). After sequence analysis of at least one false positive, KSZf14, the presence of this trinucleotide repeat was also identified, suggesting that the PCR-amplified DNA was hybridizing to sequences that held homology to the trinucleotide repeats as opposed to the MBP nucleotide sequence. Nucleotide sequence analysis of the PCR-amplified DNA revealed that the DNA contained a high number of triple nucleotide repeates (sequence in Appendix VI), which would explain the number of flase positives that were detected on the library screens. From the sequence data that had been forwarded to the Blast Network Service, homology to several receptor proteins was also based on this trinucleotide repeat (Appendix II). However an examination of the regions of homology between KSZf5 and those sequences obtained through the Blast Network Service did not identify a consistent function for the trinucleotide repeats.

The clone KSZf5, identified as having sequence homology to zebrafish genomic DNA by Southern blot analysis, was further characterized for its expression patterns using both Northern blot analysis and *in situ* hybridization. From *in situ* hybridization experiments, KSZf5 was identified as an oocyte-specific transcript and thus its role in zebrafish development became intriguing. Since KSZf5 represents a gene that is expressed in oocytes, it provided a unique opportunity to investigate oocyte development and possibly its role in directing early development in zebrafish. The KSZf5 clone, therefore, was characterized in order to determine its DNA sequence and expression profile in an attempt to identify its potential function in zebrafish oocytes.

RNA analysis identified a 5 kb transcript that was present in mRNA isolated from whole fish and mid-section suggesting that the transcripts for KSZf5 were synthesized by a tissue or tissues in the abdominal cavity of the fish. Initial *in situ* hybridization of DIG-labeled KSZf5 insert on frozen sections of adult zebrafish identified the ovary as the site of KSZf5 transcript synthesis. Due to the variety of cell types within the ovarian tissue, *in situ* hybridization on frozen sections of an adult zebrafish ovary were done to identify which cell types specifically synthesized the transcripts. Transcripts were identified to be oocyte-specific, but due to the poor morphology of the frozen sections the identification of the site of localization of KSZf5 transcripts within the ooycte was difficult. For this reason *in situ* hybridizations were repeated with paraffin sections in the place of frozen sections. This resulted in a significant improvement in the morphology of the tissue sections, allowing for identification of the site of KSZf5 transcript localization.

From *in situ* hybridization experiments on paraffin sections of adult zebrafish ovary, the KSZf5 transcripts were found to be uniformly distributed in the cytoplasm of germ cells that are ready to enter the primary growth stage of oocyte development. At this stage the germ cells have not acquired a layer of follicle cells, so this observation identifies the oocyte as the site of KSZf5 transcript synthesis. At the onset of vitellogenesis, the oocyte cytoplasm begins to be displaced to the cortex of the developing oocyte taking with it the KSZf5 transcripts. By the time the oocyte has reached full maturity, KSZf5 transcripts became completely displaced to the cortex of the oocyte. The cytoplasmic displacement during vitellogenesis is due to yolk body accumulation.

The identification of significant levels of RNA transcripts specific to KSZf5 in the mature oocyte suggests the possibility that the gene product may play an important role in the development of the early embryo. Frequently maternal transcripts generated by the developing oocyte are utilized by the embryo to allow for development to begin before zygotic expression is activated (Anderson and Lengyel, 1979). This is particularly important in organisms that display rapid development such as *Drosophila* or zebrafish because the zygotic genome cannot be organized rapidly enough to direct initial cleavage after fertilization. The activation of the zygotic genome is usually marked by a decrease in detection of maternal transcripts in early embryogenesis (Anderson and Lengyel, 1979). However in some organisms like *Xenopus* and *Drosophila*, portions of the zygotic genome are activated well before maternal transcripts are completely utilized. In order to examine the loss of maternal transcripts during embryogenesis, an RNase protection assay was used to examine post-fertilization embryos for the presence of KSZf5 transcripts. Due to the small number of cells present in very young embryos, it was difficult to isolate adequate nucleic acids for analysis from pre-blastocyst stage embryos. However, at the blastocyst stage in *Xenopus*, maternal transcripts can still be detected. Therefore the blastocyst stage was chosen as a reasonable starting point for analysis. After 72 hrs of development, zebrafish hatch with developed organ systems that are functionally competent. In *Xenopus* it is known that maternal transcripts are completely utilized by the time the organ systems become functionally competent, and it is only the zygotic genome that remains active. Therefore, one- and two- week old fry were chosen as the end point for the RNase protection assay, on the assumption that at maternal transcripts would still be detected. Based on developmental time course experiments performed with maternal determinants from other organisms, the expression pattern for KSZf5 transcripts was expected to vary between the various developmental stages. Results from the RNase protection assay demonstrated that KSZf5 transcripts were present throughout developmental stages ranging from high blastula stage (3.3 hours after fertilization) to hatching stage (72 hours). The expression of KSZf5 transcripts appears to decrease at 72 hours and then increases after 2 weeks of development. This suggests that zygotic expression of KSZf5 transcripts may be activated before the blastocyst stage, and possibly before maternal transcripts are completely utilized. At present we have not found any obvious allelic variation in the KSZf5 transcripts and cannot, therefore, determine when the zygotic copy of the gene is activated.

KSZf5, a Candidate for Germ Cell Differentiation?

The determinants for germ cell differentiation in *Drosophila* and *Xenopus* are maternally derived and lie in the developing oocyte. P-granules have been linked to pole plasm assembly and germ cell differentiation in *Drosophila* (Ephrussi and Lehmann, 1992). There are a number of gene products associated with P-granules that play a role in organizing pole plasm, such as *oskar*, *vasa*, and *staufen*. *Germ cell-less* (*gcl*) has been identified as the main determinant in pole cell formation and consequently germ cell differentiation (Jongens *et al.*, 1992). The gene product for *gcl* has not been assigned a definitive function; however Jongens *et al.* (1992) demonstrated that the removal of *gcl* mRNA, and consequently of the gene product, results in a failure to establish functional germ cells in the adult.

Cyclin B mRNA has also been identified as a germ cell determinant in *Drosophila*. Although it has no immediate role in early development, the transcripts that are maternally derived are incorporated into pole cells and translated only after the pole cells have migrated to the developing gonads of the organism (Dalby and Glover, 1993). In *Xenopus*, mitochondrial clouds within the developing oocyte carry germinal granules that when localized to the vegetal cortex, determine germ plasm (Heasman *et al.*, 1984). In both organisms, the cytoplasmic components of the oocyte that define germ plasm become segregated into specific cells early on in development, and will eventually differentiate into primordial germ cells.

In the developing *Xenopus* embryo, small islands of germ plasm are initially found throughout the subcortex of the vegetal pole (Savage and Danilchick, 1993). The organization of the germ plasm during oocyte development is dependent on a microtubule framework (Ressom and Dixon, 1988), which directs the components of the germ plasm such as germinal granules (Heasman *et al.*, 1984), Xcat2 (Elinson *et al.* 1993), Xlsirts (Kloc *et al.* 1993), and Xwnt11 (Ku and Melton, 1993) to the vegetal cortex of the egg. At the time of fertilization, the germ plasm appears as small cortical islands distributed throughout the cortex of the vegetal pole (Czolowska, 1972). Since *Xenopus* is an organism that is more closely related to zebrafish than *Drosophila*, an examination of germ cell differentiation in this organism will aid in the understanding of germ cell differentiation in zebrafish.

During the first cleavage stage, the islands of germ plasm begin to aggregate, forming larger islands. It is during the second cleavage stage that one large island of germ plasm is included into each blastomere (Ressom and Dixon, 1988). The distribution of the germ plasm at this stage is the result of re-organization by microtubule networks within each blastomere (Savage and Danilchik, 1993) such that germ plasm migration is directed towards the most interior portion of the blastomere (Ressom and Dixon, 1988). As cleavage continues, each island of germ plasm is segregated into one daughter cell such that by the blastula stage the germ plasm remains restricted to only four cells (Dixon, 1981). Each of these cells contains all the necessary cytoplasmic determinants for germ cell differentiation, and are, therefore, referred to as the presumptive primordial germ cells. During gastrulation, as in *Drosophila*, the germ cells migrate to the gonadal ridges of the developing embryo where they give rise to germ line stem cells (Rongo and Lehmann, 1996). These stem cells proliferate from the lining of either the ovary or testes to produce oocytes and spermatocytes respectively.

In the zebrafish, Kimmel and Warga (1986) traced cell lineages during gastrulation by injecting blastomeres with lineage-tracer molecules during various stages of cleavage. They demonstrated that blastomeres as early as the 64 cell stage show commitment to a particular tissue type. Since *Xenopus* and zebrafish are both non-mammalian vertebrates with similar oocyte characteristics, it is reasonable to suggest that zebrafish germ plasm and germ cell differentiation follow a similar path to that described in *Xenopus*. Although research in the area of zebrafish germ plasm determinants is lacking, Kimmel and Warga's (1986) experiments suggest that the germ plasm in zebrafish may become restricted to certain blastomeres as early as the 64 cell stage (Kimmel et al., 1990), and that further cleavage would generate presumptive primordial germ cells. Following gastrulation these cells would migrate to the gonadal ridges of the developing embryos and subsequently produce germ-line stem cells.

The presence of KSZf5 transcripts in oocytes and throughout development makes it a reasonable candidate for being involved in germ cell differentiation. KSZf5 transcripts may represent a cytoplasmic determinant that becomes restricted to presumptive primordial germ cells during cleavage. Since KSZf5 transcripts are found in oocytes, this suggests that the gene product of KSZf5 may actually be important in the differentiation of female gonads or in the maintenance of stem-cell proliferation. Sequence analysis shows homologies between KSZf5 and several receptor proteins, suggesting that the gene product for KSZf5 may possibly function as a receptor for factors important in the differentiation of the female reproductive system.

Research done by several investigators on maternal transcripts has consistently shown that localization is a result of recognition of secondary structures that form within a transcript's 3'UTR (Ephrussi and Lehmann, 1992; Mowry and Melton, 1992; Ferrandon *et al.*, 1994), whether the recognition molecule is another protein or the microtubule network within the cytoplasm. Since KSZf5 appears to contain a portion of its 3'UTR, it may be possible to use the clone to determine whether the RNA is localized within the developing oocytes by a similar mechanism. If KSZf5 is a germ plasm determinant, localization of its transcripts may be the result of a mechanism similar to the one used to localize *Xcat2* (Forristall *et al.*, 1995) and *Xwnt11* (Ku and Melton, 1993) in *Xenopus*. The cytoskeletal network within a cell provides structural support for localizing cytoplasmic components within the oocyte. In Xenopus, *Xcat2* and *Xwnt11* are initially transported to the vegetal cortex by a mitochondrial cloud. After being transported, *Xcat2* (Forristall *et al.*, 1995) and *Xwnt11* (Ku and Melton, 1993) are localized by microfilaments to the vegetal cortex of the *Xenopus* embryo. It would be reasonable to suggest that KSZf5 transcripts may be localized via a similar microfilament system.

Future Prospects

I have identified and characterized an oocyte-specific sequence that is expressed in the zebrafish, *Danio rerio.* Its presence in mature oocytes implicates this sequence as a potential maternal determinant involved in the control of early development of the embryo. Its presence throughout embryonic development and in various oocyte stages suggests that it may be involved in germ cell determination; however there is no direct evidence that this is the case. Several obvious questions remain outstanding, the first concerning the possible localization of the KSZf5 transcripts within the developing embryo, and the second concerned with the expression and the function of the protein product.

Whole mount *in situ* hybridization of zebrafish embryos during the very early preblastocyst stages of development (from first cleavage stages) should be done in order to actively track the distribution of the transcripts during development. Since zebrafish embryos are transparent during the early stages of development, *in situ* hybridizations would be an ideal method for establishing the final destination of KSZf5 transcripts and whether or not they are germ cell determinants.

If KSZf5 is in fact a germ cell determinant, the identification of the specific mechanism for the localization of KSZf5 transcripts should be characterized. To determine if the cytoskeletal system of the oocyte cytoplasm is responsible for the localization of the transcripts, cytoskeletal inhibitors could be used. To determine if localization is based on the microtubule network system, inhibitors of microtubule polymerization such as nocodazole or colchicine could be used. If localization is based on the microtubule network system within the oocyte then inhibition of microtubule polymerization should cause an alteration in transcript localization. Inhibitors of microfilament polymerization, such as cytochalasin B, could also be used in a similar fashion to determine if in fact microfilaments are responsible for the localization of KSZf5 transcripts.

To identify whether KSZf5 codes for a protein, an attempt to translate KSZf5 *in vitro* is necessary. If KSZf5 codes for a protein then initial characterization of the gene product could be done. Antibodies could be generated against this gene product allowing for immunohistochemical identification of the region within the oocyte where translation of KSZf5 takes place, when translation is activated during development, and where the gene product is finally localized. Zebrafish mutants deficient for this gene could be generated to follow the effect of the KSZf5 gene product on zebrafish development. This would allow for direct identification of its function in zebrafish embryogenesis.

Since KSZf5 is a partial clone, there is a need to re-screen a zebrafish cDNA library in order to obtain the full-length cDNA. This would allow for identification of regions important in transcriptional and translational control, as well as identifying secondary structures in its 3'UTR that may be important in localization. Screening mouse cDNA libraries may identify clones with sequence homology and the identification of such clones would provide information on the role of KSZf5 gene products on mammalian development. Sequence comparisons between KSZf5 and homologous clones derived from different organisms would identify areas within the sequences that are highly conserved, aiding in the identification of active sites within the final gene product. An analysis of the chemical nature of the active sites would ultimately result in a better understanding of what role KSZf5 has in zebrafish development, and how it may interact with other cytoplasmic determinants in establishing normal development of the zebrafish embryo.

If KSZf5 is in fact a germ plasm determinant it will provide future researchers a key into the dynamics of the development of the reproductive system in zebrafish. This could provide an understanding of how germ cell determinants are important in organizing the germ cell line in higher vertebrates. It may also provide an understanding into the inductive effect that germ cell determinants may have on establishing the reproductive system, particularly in differentiating the male reproductive system from the female reproductive system. Further research directed at characterizing KSZf5 will provide a primary building block to the understanding of the importance of maternal determinants in directing the zebrafish embryo through normal development, and this would also provide another piece to the complete understanding of higher vertebrate development.

Appendix I: Sequencing primers

List of primers used for sequencing of the clone isolated from an adult zebrafish cDNA library.

Set 1:

Forward primer: 25-mer	5'cttaacaatgnagcctttagaaatg3'
Reverse primer: 24-mer	5'agccgggggtgcagccgggggatc3'

Set 2:

Forward primer: 24-mer	5'ttggtgccataatgagagtctggt3'
Reverse primer: 25-mer	5'gacggcgacatcttcatcatcatca3'

Set 3:

Forward primer: 25-mer	5'ccggaggcggtcactggttccactg3'
Reverse primer: 25-mer	5'caacggtggcatggcaacaacgtcg3'

Set 4:

Reverse primer: 22-mer	5'cgacaagaacacagctgatgca3'

Appendix II: Sequences that Display Some Homology to KSZf5

Once sequence analysis of KSZf5 was complete, the sequence was forwarded to the BLAST Network Service to identify whether KSZf5 showed significant homology to any previously cloned sequences. A number of previously cloned sequences did display some sequence homology to KSZf5. It should be noted that the matched sequences were not based on the entire nucleotide sequence for KSZf5, but rather a few sequences of 50-85 bases (Y). The high degree of homology within these small sequences is listed below. X=number of bases matched.

	X/Y
Human high mobility group 2 protein gene	82%
Rat polymeric immunoglobulin receptor	79%
Mus musculus GTP-binding protein	77%
Mouse Ig germline kappa-chain V region	75%
Mouse Na/K ATPase beta 2 subunit gene	74%
Mouse T-cell receptor alpha/delta chain	73%
sodium-hydrogen exchange protein	73%
Rattus norvegicus plasma membrane Ca^{++} ATPase	71%
Chicken progesterone receptor gene	70%
Human alpha-2 adrenergic receptor	70%
Rattus norvegicus K^{+} channel mRNA	70%
Rat gene for alpha-lactalbumin	68%

Appendix III: Amino acid sequences determined for KSZf5

After sequencing KSZf5, the entire sequence was translated using three open reading frames (ORF's). All three translated products are listed according to the frame that they were read in. ??? denotes unknown amino acid due to unknown (n) in a codon. MET represents a start codon, and TER represents a stop codon.

1 CAA TTC NGG GGC NCT CGG CAA TGC ACC GTC GAG CAA CAG CGC

Gln Phe ??? Gly ??? Arg Gln Cys Thr Val Glu Gln Gln Arg

Asn Ser Gly Ala Leu Gly Asn Ala Pro Ser Ser Asn Ser Ala

Ile ??? Gly ??? Ser Ala **MET** His Arg Arg Ala Thr Ala Pro

43 CCA TTC TTC CNN CCG CNA GCA ACA GGG GAG CCG GGG GTG CAG

Pro Phe Phe ??? Pro ??? Ala Thr Gly Glu Pro Gly Val Gln

His Ser Ser ??? Arg ??? Gln Gln Gly Ser Arg Gly Cys Ser

Ile Leu Pro ??? Ala Ser Asn Arg Gly Ala Gly Gly Ala Ala

85 CCG GGG GGA TCA ACC GNN GCG CGG GGA ATT CGA ACC CTN AGT

Pro Gly Gly Ser Thr ??? Ala Arg Gly Ile Arg Thr Leu Ser

Arg Gly Asp Gln Pro ??? Arg Gly Glu Phe Glu Pro ??? Val

Gly Gly Ile Asn Arg ??? Ala Gly Asn Ser Asn Pro ??? Phe

127 TCG GGC CGG NGA ACG GCC GGA GGC ACC GAG TCT GCG GTG AGA

Ser Gly Arg ??? Thr Ala Gly Gly Thr Glu Ser Ala Val Arg

Arg Ala Gly Glu Arg Pro Glu Ala Pro Ser Leu Arg **TER** Asp

Gly Pro ??? Asn Gly Arg Arg His Arg Val Cys Gly Glu Ile

169 TAG CAC GTC GAG CTG CTG CTA CAC AAC CCT CCT CGG CAG ACG

TER His Val Glu Leu Leu Leu His Asn Pro Pro Arg Gln Thr

Ser Thr Ser Ser Cys Cys Tyr Thr Thr Leu Leu Gly Arg Arg

Ala Arg Arg Ala Ala Ala Thr Gln Pro Ser Ser Ala Asp Gly

211 GTC GGC CAG AGC NGA GCC TCT TAG GCG CTA GCG TCA AAC ATG

Val Gly Gln Ser ??? Ala Ser **TER** Ala Leu Ala Ser Asn **MET**

Ser Ala Arg Ala Glu Pro Leu Arg Arg **TER** Arg Gln Thr **TER**

Arg Pro Glu ??? Ser Leu Leu Gly Ala Ser Val Lys His Asp

253 ACG GCG ACA TCT TCA TCA TCA TCA TCC TCC TCC TCA TCC TCA

Thr Ala Thr Ser Ser Ser Ser Ser Ser Ser Ser Ser Ser Ser

Arg Arg His Leu His His His His Pro Pro Pro His Pro His

Gly Asp Ile Phe Ile Ile Ile Ile Leu Leu Leu Ile Leu Ile

295 TCC GCG TCT ACC GCA TCC TCC TCC GTC GAT GCG TCC GCC GCC

Ser Ala Ser Thr Ala Ser Ser Ser Val Asp Ala Ser Ala Ala

Pro Arg Leu Pro His Pro Pro Pro Ser **MET** Arg Pro Pro Pro

Arg Val Tyr Arg Ile Leu Leu Arg Arg Cys Val Arg Arg Arg

337 GCC GCC GTC TCT CCG CTT CCC AGC AGC CCG GCT TCG GTT TTN

Ala Ala Val Ser Pro Leu Pro Ser Ser Pro Ala Ser Val ???

Pro Pro Ser Leu Arg Phe Pro Ala Ala Arg Leu Arg Phe ???

Arg Arg Leu Ser Ala Ser Gln Gln Pro Gly Phe Gly Phe ???

379 GTT TAT CGA GAG CCG GTC TAA CAA CTG GCA AGC NAC AAA AAG

Val Tyr Arg Glu Pro Val **TER** Gln Leu Ala Ser ??? Lys Lys

Phe Ile Glu Ser Arg Ser Asn Asn Trp Gln Ala Thr Lys Ser

Leu Ser Arg Ala Gly Leu Thr Thr Gly Lys ??? Gln Lys Val

421 TAC AGT NAA AGA NAG ATT TGC TCT CGC TGT GGG AAG TGC CGT

Tyr Ser ??? Arg ??? Ile Cys Ser Arg Cys Gly Lys Cys Arg

Thr Val Lys ??? Arg Phe Ala Leu Ala Val Gly Ser Ala Val

Gln ??? Lys ??? Asp Leu Leu Ser Leu Trp Glu Val Pro Cys

463 GTT TGA TGC CAT GTT CAA CGG TGG CAT GGC AAC AAC GTC GAC

Val **TER** Cys His Val Gln Arg Trp His Gly Asn Asn Val Asp

Phe Asp Ala **MET** Phe Asn Gly Gly **MET** Ala Thr Thr Ser Thr

Leu **MET** Pro Cys Ser Thr Val Ala Trp Gln Gln Arg Arg Pro

505 CGA AAT TGA ACT GCC AGA TGT TGA ACC GGC TGC ATT TCT AGC

Arg Asn **TER** Thr Ala Arg Cys **TER** Thr Gly Cys Ile Ser Ser

Glu Ile Glu Leu Pro Asp Val Glu Pro Ala Ala Phe Leu Ala

Lys Leu Asn Cys Gln **MET** Leu Asn Arg Leu His Phe **TER** Pro

547 CCT TCT CAA ATT TTT ATA CTC AGA TGA NGT GCA AAT TGG ACC

Pro Ser Gln Ile Phe Ile Leu Arg **TER** ??? Ala Asn Trp Thr

Leu Leu Lys Phe Leu Tyr Ser Asp ??? Val Gln Ile Gly Pro

Phe Ser Asn Phe Tyr Thr Gln **MET** ??? Cys Lys Leu Asp Gln

589 AGA GAC CGT NAT GAC CAC ATT ATA CAC AGC CAA AAA GTA TGC

Arg Asp Arg ??? Asp His Ile Ile His Ser Gln Lys Val Cys

Glu Thr Val **MET** Thr Thr Leu Tyr Thr Ala Lys Lys Tyr Ala

Arg Pro ??? **TER** Pro His Tyr Thr Gln Pro Lys Ser **MET** Gln

631 AGT ACC TGC NTT GNC CAC TGN TGA ATT CTG ANG AAA AAC CTA

Ser Thr Cys ??? ??? His ??? **TER** Ile Leu ??? Lys Asn Leu

Val Pro Ala Leu ??? Thr ??? Glu Phe **TER** ??? Lys Thr Tyr

Tyr Leu ??? ??? Pro Leu ??? Asn Ser ??? Glu Lys Pro Thr

673 CGC GCT GAC AAC GAT TTN TGT TGC TTA CCC AGG CAC GGC TTT

Arg Ala Asp Asn Asp ??? Cys Cys Leu Pro Arg His Gly Phe

Ala Leu Thr Thr Ile ??? Val Ala Tyr Pro Gly Thr Ala Phe

Arg **TER** Gln Arg Phe ??? Leu Leu Thr Gln Ala Arg Leu Phe

715 TTG ACG AGC CCC AGC TGG CAA GTN TCT GCT TAG AAA ATA TCG

Leu Thr Ser Pro Ser Trp Gln Val Ser Ala **TER** Lys Ile Ser

TER Arg Ala Pro Ala Gly Lys ??? Leu Leu Arg Lys Tyr Arg

Asp Glu Pro Gln Leu Ala Ser ??? Cys Leu Glu Asn Ile Asp

757 ACA AGA ACA CAG CTG ATG CAC TCG CTG CTG AGG GCT TCA CGG

Thr Arg Thr Gln Leu **MET** His Ser Leu Leu Arg Ala Ser Arg

Gln Glu His Ser **TER** Cys Thr Arg Cys **TER** Gly Leu His Gly

Lys Asn Thr Ala Asp Ala Leu Ala Ala Glu Gly Phe Thr Asp

799 ATG TTG ACC TTG ATA CCT CGT GGC AGT CCG GGG CAC ACT GGT

MET Leu Thr Leu Ile Pro Arg Gly Ser Pro Gly His Thr Gly

Cys **TER** Pro **TER** Tyr Leu Val Ala Val Arg Gly Thr Leu Val

Val Asp Leu Asp Thr Ser Trp Gln Ser Gly Ala His Trp Ser

841 CAG TCC TGG AGA GAG ACA CAC TTG GTG TAC GGG AAG TGC GTC

Gln Ser Trp Arg Glu Thr His Leu Val Tyr Gly Lys Cys Val

Ser Pro Gly Glu Arg His Thr Trp Cys Thr Gly Ser Ala Ser

Val Leu Glu Arg Asp Thr Leu Gly Val Arg Glu Val Arg Leu

883 TCT TCG GAG CTG CTG TTC GTT GGG CAG AAG CTG AGG CCC AAA

Ser Ser Glu Leu Leu Phe Val Gly Gln Lys Leu Arg Pro Lys

Leu Arg Ser Cys Cys Ser Leu Gly Arg Ser **TER** Gly Pro Lys

Phe Gly Ala Ala Val Arg Trp Ala Glu Ala Glu Ala Gln Arg

925 GGC AAC AAT TAC AGC CTA CAC CGG AGA ACA AGC GAA GAG TGT

Gly Asn Asn Tyr Ser Leu His Arg Arg Thr Ser Glu Glu Cys

Ala Thr Ile Thr Ala Tyr Thr Gly Glu Gln Ala Lys Ser Val

Gln Gln Leu Gln Pro Thr Pro Glu Asn Lys Arg Arg Val Leu

967 TGG GAA AGG CAC TTC CCT TAT TCG CTT TCC ACT CAT GAC AAT

Trp Glu Arg His Phe Pro Tyr Ser Leu Ser Thr His Asp Asn

Gly Lys Gly Thr Ser Leu Ile Arg Phe Pro Leu **MET** Thr Ile

Gly Lys Ala Leu Pro Leu Phe Ala Phe His Ser **TER** Gln Leu

1009 TGA AGA GTT TGC AGC AGG TCC GGC TCN NTC TGG TAT ACT CAC

TER Arg Val Cys Ser Arg Ser Gly Ser ??? Trp Tyr Thr His

Glu Glu Phe Ala Ala Gly Pro Ala ??? Ser Gly Ile Leu Thr

Lys Ser Leu Gln Gln Val Arg Leu ??? Leu Val Tyr Ser Gln

1051 AGA CCG GGA GGT GGT CAG TCT GTT CCT GCA TTT TAC CGT CAT

Arg Pro Gly Gly Gly Gln Ser Val Pro Ala Phe Tyr Arg His

Asp Arg Glu Val Val Ser Leu Phe Leu His Phe Thr Val Ile

Thr Gly Arg Trp Ser Val Cys Ser Cys Ile Leu Pro Ser Ser

1093 CCG AAA CCA CAT GTG GAG TTT ATT GAC CGG CCC CGC TGT TGC

Pro Lys Pro His Val Glu Phe Ile Asp Arg Pro Arg Cys Cys

Arg Asn His **MET** Trp Ser Leu Leu Thr Gly Pro Ala Val Ala

Glu Thr Thr Cys Gly Val Tyr **TER** Pro Ala Pro Leu Leu Pro

1135 CTC CGG GGG AAA GAG TGC AGC ATC ACG CGT NTC AGT CAG GTG

Leu Arg Gly Lys Glu Cys Ser Ile Thr Arg ??? Ser Gln Val

Ser Gly Gly Lys Ser Ala Ala Ser Arg Val Ser Val Arg Trp

Pro Gly Glu Arg Val Gln His His Ala ??? Gln Ser Gly Gly

1177 GAG AGC CGC TGG GGG TAC AGT GGA ACC AGT GAC CGC ATC CGG

Glu Ser Arg Trp Gly Tyr Ser Gly Thr Ser Asp Arg Ile Arg

Arg Ala Ala Gly Gly Thr Val Glu Pro Val Thr Ala Ser Gly

Glu Pro Leu Gly Val Gln Trp Asn Gln **TER** Pro His Pro Val

1219 TTT TCT GTG AAC CGC ACA ATA TTT GTG GGG GGA TTT GGA CTT

Phe Ser Val Asn Arg Thr Ile Phe Val Gly Gly Phe Gly Leu

Phe Leu **TER** Thr Ala Gln Tyr Leu Trp Gly Asp Leu Asp ???

Phe Cys Glu Pro His Asn Ile Cys Gly Gly Ile Trp Thr ???

1261 NNT GGC TCT ATA CAT GGT CCA ACT GAC TAT NAG GTC AAC ATC

??? Gly Ser Ile His Gly Pro Thr Asp Tyr ??? Val Asn Ile

??? Ala Leu Tyr **MET** Val Gln Leu Thr ??? Arg Ser Thr Ser

Trp Leu Tyr Thr Trp Ser Asn **TER** Leu ??? Gly Gln His Pro

1303 CAG ATN ATA CAN ACA GAC AGT AAC ACA GTT CTC GGA CAG AAC

Gln ??? Ile ??? Thr Asp Ser Asn Thr Val Leu Gly Gln Asn

Arg ??? Tyr ??? Gln Thr Val Thr Gln Phe Ser Asp Arg Thr

Asp ??? Thr ??? Arg Gln **TER** His Ser Ser Arg Thr Glu Arg

1345 GAT ACA GGC TTN CTT GTG ACG GAA CAG CCA GCA CAN NNN CAG

Asp Thr Gly ??? Leu Val Thr Glu Gln Pro Ala ??? ??? Gln

Ile Gln Ala ??? Leu **TER** Arg Asn Ser Gln His ??? ??? Ser

Tyr Arg Leu ??? Cys Asp Gly Thr Ala Ser Thr ??? ??? Val

1387 TGA TGT TNN AGG AGC CAG TGG AAA TTC TTC CAT GCA ATT ACA

TER Cys ??? Arg Ser Gln Trp Lys Phe Phe His Ala Ile Thr

Asp Val ??? Gly Ala Ser Gly Asn Ser Ser **MET** Gln Leu His

MET ??? ??? Glu Pro Val Glu Ile Leu Pro Cys Asn Tyr Thr

1429 CCG CCT GCG CCA CCT GAA GGA CCA GAC TCT CAT TAT GGC ACC

Pro Pro Ala Pro Pro Glu Gly Pro Asp Ser His Tyr Gly Thr

Arg Leu Arg His Leu Lys Asp Gln Thr Leu Ile MET Ala Pro

Ala Cys Ala Thr **TER** Arg Thr Arg Leu Ser Leu Trp His Gln

1471 AAG GGA TGC GTA AAG TCA CCC ATG AAG CNC CCG CAN CTG GTA

Lys Gly Cys Val Lys Ser Pro **MET** Lys ??? Pro ??? Leu Val

Arg Asp Ala **TER** Ser His Pro **TER** Ser ??? Arg ??? Trp Tyr

Gly **MET** Arg Lys Val Thr His Glu Ala Pro Ala ??? Gly Thr

1513 CTA AGC CTG CTT NAC ATC TGC TAT GCT GCA GGC AAC AAC AAC

Leu Ser Leu Leu ??? Ile Cys Tyr Ala Ala Gly Asn Asn Asn

TER Ala Cys ??? Thr Ser Ala **MET** Leu Gln Ala Thr Thr Thr

Lys Pro Ala ??? His Leu Leu Cys Cys Arg Gln Gln Gln Arg

1555 GGC ACT TCT GTN TAG ATG GAC AAA CGC NCA GGT CAT CTT CTA

Gly Thr Ser Val **TER MET** Asp Lys Arg ??? Gly His Leu Leu

Ala Leu Leu ??? Arg Trp Thr Asn Ala Gln Val Ile Phe Tyr

His Phe Cys ??? Asp Gly Gln Thr ??? Arg Ser Ser Ser Thr

1597 CAC TAA TGA CCG AGC AGA GTC GTC AAA CAA TCG AGG ACT CTT

His **TER TER** Pro Ser Arg Val Val Lys Gln Ser Arg Thr Leu

Thr Asn Asp Arg Ala Glu Ser Ser Asn Asn Arg Gly Leu Leu

Leu **MET** Thr Glu Gln Ser Arg Gln Thr Ile Glu Asp Ser **TER**

1639 AGA TTG CAT TTT GTG GCC ANN CGC CAG TCT TCG GTG TGT AGA

Arg Leu His Phe Val Ala ??? Arg Gln Ser Ser Val Cys Arg

Asp Cys Ile Leu Trp Pro ??? Ala Ser Leu Arg Cys Val Asp

Ile Ala Phe Cys Gly ??? ??? Pro Val Phe Gly Val **TER** Thr

1681 CTG GTG ACC AAA TAC TGA TCT TCA TTT CTA AAG GCT NCA TTG

Leu Val Thr Lys Tyr **TER** Ser Ser Phe Leu Lys Ala ??? Leu

Trp **TER** Pro Asn Thr Asp Leu His Phe **TER** Arg Leu His Cys

Gly Asp Gln Ile Leu Ile Phe Ile Ser Lys Gly ??? Ile Val

1723 TTA AGN TAT GAG NAT TCA GTT CTN TCA AAG TGT ACT TAG TGT

Leu ??? Tyr Glu ??? Ser Val Leu Ser Lys Cys Thr **TER** Cys

TER ??? **MET** ??? Ile Gln Phe ??? Gln Ser Val Leu Ser Val

Lys ??? **TER** ??? Phe Ser Ser ??? Lys Val Tyr Leu Val **TER**

1765 AAT NTC AGA TTT TGT TTC TAC CCA ATG AGC NCC TTG TAG TAC

Asn ??? Arg Phe Cys Phe Tyr Pro **MET** Ser ??? Leu **TER** Tyr

??? Ser Asp Phe Val Ser Thr Gln **TER** Ala Pro Cys Ser Thr

??? Gln Ile Leu Phe Leu Pro Asn Glu ??? Leu Val Val Gln

1807 AAC CTT TAA GCA CAA CCN GCC AAT GTC TAG TTG CTC AGT AAT

Asn Leu **TER** Ala Gln Pro Ala Asn Val **TER** Leu Leu Ser Asn

Thr Phe Lys His Asn ??? Pro **MET** Ser Ser Cys Ser Val **MET**

Pro Leu Ser Thr Thr ??? Gln Cys Leu Val Ala Gln **TER TER**

1849 GAA CGT ACT NTA CAG AGC TAT CAA TCA GGT ACA GCA CAG AGA

Glu Arg Thr ??? Gln Ser Tyr Gln Ser Gly Thr Ala Gln Arg

Asn Val Leu Tyr Arg Ala Ile Asn Gln Val Gln His Arg Glu

Thr Tyr ??? Thr Glu Leu Ser Ile Arg Tyr Ser Thr Glu

1891 A

Appendix IV: Analysis of open reading frames

An analysis of the amino acid sequences from all three reading frames was done to identify any significant open reading frames. The minimum open reading frame length used were 150 and 100 amino acids.

1. Minimum ORF length selected: 150 amino acids

```
                 Frame 1

------------------------------------------------------------

    Range      Nucl. Length   Polypeptide Length  Type of orf

------------------------------------------------------------

no 5'end-Ter, Met-Ter or Met-3'end orf's found longer than 150

no Ter-Ter, or Ter-3' orf's longer than 150
```

```
                 Frame 2

------------------------------------------------------------

    Range      Nucl. Length   Polypeptide Length  Type of orf

------------------------------------------------------------

no 5'end-Ter, Met-Ter or Met-3'end orf's found longer than 150

no Ter-Ter, or Ter-3' orf's longer than 150
```

```
              Frame 3

      --------------------------------------------------------------

        Range      Nucl. Length   Polypeptide Length  Type of orf

      --------------------------------------------------------------

        3  to  542     540            180           5'end-Ter

       21  to  542     522            174            Met-Ter

        3  to  542     540            180           Ter-Ter
```

2. Minimum ORF length selected: 100 amino acids

```
              Frame 1

      --------------------------------------------------------------

        Range      Nucl. Length   Polypeptide Length  Type of orf

      --------------------------------------------------------------

      no 5'end-Ter, Met-Ter or Met-3'end orf's found longer than 100

      no Ter-Ter, or Ter-3' orf's longer than 100
```

Frame 2

Range	Nucl. Length	Polypeptide Length	Type of orf
323 to 658	336	112	Met-Ter
254 to 658	405	135	Ter-Ter

Frame 3

Range	Nucl. Length	Polypeptide Length	Type of orf
3 to 542	540	180	5'end-Ter
21 to 542	522	174	Met-Ter
3 to 542	540	180	Ter-Ter

Appendix V: Partial Sequence of False Positive

From the adult zebrafish cDNA library screens, several false positives were identified. Partial sequencing was done on one of the false positives using both forward and reverse primers. The sequence was forwarded to BLAST Network Service and the service identified that the insert had a high degree of homology to the *E. coli* genome. An analysis of the sequence revealed that it contained a trinucleotide repeat that held homology with the trinucleotide repeat identified in the PCR amplified DNA.

Using the forward primer, the sequence generated was:

```
            10          20          30          40          50          60
5'  aaaattacat   ttatcgngtg  gttgcgattt  tgnaaaaata  attctggtgt  gtgttcatcg
    ngaggcgctg  aatacgcgca   tattgccctg  atggacattg  acccccaccc  gctggaagag
    tcgcatattg   nggtgcgtaa  gctatggatt  cagcagggca  gcgtaaatac  ctgccacacc
    caacagaaag  aagccttaga   ggatgccgtt  tg 3'
```

Using the reverse primers, the sequence generated was:

```
            10          20          30          40          50          60
5' ccgccgttcc  ctgcaccgaa  tggcacagcc  cgacctgttt  gatatgcgga  tagcgggcat
   acatcgccag  gtattcatcg  ccattgggtt  aacatagttg  agcatggtgg  catcggggca
   gacttccgtc  atgtcctcgc  aaatttgcca  cagatgcgga  atggtacgta  gcgcgcgcat
   aataccgccg  gcccaac 3'
```

Appendix VI: Partial Sequence for PCR Amplified DNA

The PCR amplified DNA generated for screening of adult zebrafish cDNA libraries was sequenced as outlined in methods. Partial sequence analysis revealed that the PCR amplified DNA contained a number of trinucleotide repeats, which were also identified in KSZf5 and a false positive.

```
             10          20          30          40          50          60
5' gctgtgggaa  gtgccgtgtt   tgatgccatg   ttcaacggtg  gcatggcaac  aacgtcgacc
   gatattgaac  tgccagatgt  ngaaccggct  gcatttctag  cccttctcaa  atttttatac
   tca  3'
```

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