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The Structure and Fate of Germinal Plasm in the Asiatic Fire-Bellied Frog, Bombina orientalis (Anura-Discoglossidae)

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THE STRUCTURE AND FATE OF GERMINAL PLASM IN THE ASIATIC FIRE-BELLIED FROG, BOMBINA ORIENTALIS (ANURA-DISCOGLOSSIDAE)

(TITLE)

BY

TED W. ODOM

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THE STRUCTURE AND FATE OF GERMINAL PLASM IN THE ASIATIC FIRE-BELLIED FROG, BOMBINA ORIENTALIS (ANURA-DISCOGLOSSIDAE).

Presented by

TED W. ODOM

A Candidate for the Degree of Master of Science

And Hereby Certify That, In Their Opinion, It Is Acceptable

(Signatures & Date)

Lehman's polychrome stain was used to follow the fate of germinal plasm in early development of Bombina orientalis embryos. Islets of germinal plasm approximately 6 um in diameter were observed in the vegetal pole region of the 2-cell embryo. By the 4-cell stage, some of these islets had coalesced to form larger ones so that two size classes of islets were present (approximately 7.5 and 19.5 um in diameter).

As cleavage continued to the 16-cell stage, the islets were moved further up the cleavage furrow and were always observed to remain in close association with the cleavage membrane. The islets at this time had reached a diameter of approximately 32 um. In the 32-cell embryo, the cells that contained germinal plasm and that were also adjacent to the developing blastocoel, exhibited a protuberance of cytoplasm consisting of pigment, yolk and germinal plasm.

At the mid-to late blastula stage, the germinal plasm was contained in a few cells located in the lower one-third of the embryo. The peripheral position of the germinal plasm was maintained in these cells. During early gastulation, the germinal plasm began to migrate from its peripheral location to a juxtanuclear position.

Examination of neurula and early tail bud stages did not reveal any cells containing germinal plasm. The implication was that the germinal plasm may have been utilized previously to these stages. At the time of opercular

closure (which is an equivalent stage to Shumway stage 25), there were approximately 61 primordial germ cells in the dorsal mesentery and genital ridges.

TABLE OF CONTENTS

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INTRODUCTION

The problem of anuran germ cell origin and determination has been a long standing one. Allen (1907) was first to demonstrate that the primordial germ cells in Rana pipiens arise from the dorsal endodermic crest. He described a process where by the primordial germ cells were pinched off and carried by the dorsally developing mesentery. Eventually these primodial germ cells came to rest at the base of the dorsal mesentery. Following from Allen's work, Witschi (1929) attempted to distinguish primordial germ cells from other endoderm cells in earlier stages of Rana sylvatica, but was unsuccessful. Bounoure in 1934 found that just after fertilization in Rana temporaria, many localized stainable cytoplasmic islets appear free of yolk and associated with clusters of mitochondria in the vegetal pole region of the zygote. These islets of cytoplasm were observed to move during the process of cleavage, up the cleavage furrow to be contained eventually in a few cells in the blastocoel floor. Bounoure believed that the cells containing these islets were specialized in that they would give rise to the future primordial germ cells. He gave this special islet containing cytoplasm the name of "germinal plasm," because he thought it functioned in some way in the germ cell determination process.

Blackler (1958) confirmed Bounoure's findings in Rana temporaria and extended them to Xenopus laevis, and Bufo bufo. Common to all of these species was the appearance of islets of germinal plasm in the vegetal pole after fertilization and the incorporation of this material into a few cells at the blastula stage. These islets were always observed to remain in close association with the plasma membrane throughout cleavage. By the time gastulation had begun, these islets of germinal plasm had moved from their peripheral association with the plasma membrane to a juxtanuclear position. The cells containing germinal plasm were then moved by morphogenic movements of gastulation to a position deep within the endoderm of the neurula. From this position the cells migrated to the dorsal region of the endoderm where the staining character of the germinal plasm completely diminishes.

Further studies by Di Berardino (1961) have extended Bounoure's (1934) and Blackler's (1958) findings to Rana pipiens. A quantitative approach to the study of germinal plasm was undertaken by Whitington and Dixon (1975). They determined the volume of germinal plasm and the number of cells containing it in many important developmental stages of Xenopus laevis. The details of their results are discussed elsewhere in this paper.

Since Blackler (1958) had shown that RNA was a rich constituent of the germinal plasm, Czolowska (1969) thought

that it may be produced during oogenesis when many important RNA's are being synthesized. She looked at Xenopus laevis mature oocytes which had been treated with hormone to induce germinal vesicle breakdown, as well as immature oocytes and fertilized eggs. With the use of the stain pyronine (Blackler, 1958), she was able to demonstrate patches of RNA rich cytoplasm in the cortex of the vegetal pole of the hormone treated oocytes. These patches however, were much smaller than the ones she observed in fertilized eggs. This suggested that some of the components of the germinal plasm were present even before fertilization. Attempts at demonstrating RNA rich cytoplasm in immature oocytes was negative.

The determinative function of the germinal plasm was realized from the studies of Bounoure, Aubrey, and Huck (1954). They were able to induce partial or complete sterility in Rana temporaria tadpoles that had been irradiated in the vegetal pole region during the first cleavage with ultraviolet light. The results suggested that the ultraviolet light destroyed or disrupted the normal state of the germinal plasm so that it could not function properly. Smith (1966) achieved complete sterility in Rana pipiens tadpoles by irradiating the vegetal pole regions of four cell embryos with ultraviolet light. He was also able to produce partial restoration of the germ cells by micropipeting vegetal pole cytoplasm from unirradiated embryos into the vegetal pole

regions of irradiated embryos. In addition, he found that the ultraviolet light wavelength that was the most effective in producing sterility was in the absorption region for nucleic acid. Buehr and Blackler (1970) induced sterility in a different manner. By pricking the vegetal pole of Xenopus laevis embryos, they produced tadpoles that displayed a large reduction in the number of primordial germ cells in the genital ridges. Whether the germinal plasm is destroyed by ultraviolet light or deleted, it is obvious that it is important in the determination of germ cells in anurans.

The ultrastructure of Rana pipiens germinal plasm was looked at by Mahowald and Hennen (1971) with the transmission electron microscope. They found that it was composed of clouds of mitochondria as was previously known. More important was the finding that scattered among these mitochondria were electron dense bodies about 0.2 micrometers in diameter which appeared to stain positively with the RNA stain, indium trichloride. Simultaneously, Williams and Smith (1971) also discovered the same electron dense bodies in Rana pipiens. Since they believed these dense bodies to be the main inducing factor, they gave them the name, germinal granules. Mahowald, Hennen, Williams and Smith believe that the RNA contained in the germinal granule is a messenger type which contains the determinative information (as was postulated by Smith, 1966). Czolowska (1972) has extended these observations to Xenopus laevis embryos.

Much of the work on germinal plasm in early development has been restricted to a few species anurans. Table 5 lists the seven species in which work has been reported. I feel that extending these earlier observations to other species, will contribute valuable information concerning the occurrence of this important substance, in the germ cell determination process of aunurans. Bombina orientalis was a suitable organism for this study, because its use in laboratories has been increasing and embryological material could be easily acquired through the use of hormones.

MATERIALS AND METHODS

The adult Bombina orientalis used in this study were purchased from the amphibian facility of the University of Michigan. Ovulation and amplexus were induced by injecting the female with 300 I.U. and the male with 500 I.U. of human chorionic gonadotropin. After a period of approximately 8 hours in the breeding tank, eggs were collected and transferred to clean petri dishes containing aerated tap water. Development of all of the embryos took place at room temperature.

The following developmental stages were employed: 2-cell, 4-cell, 8-cell, 16-cell, 32-cell, mid-blastula, late blastula, early gastula, neurula, early tail bud, and a stage which is comparable to Shumway's stage 25 for Rana pipiens. All material for this study was fixed and post-fixed according to Bounoure (1934). The method consisted of fixation in hot 10% formalin (made neutral by addition of marble chips) followed by post-fixation in a saturated solution of potassium dichromate. Potassium dichromate is a good preserver of mitochondria (Humason, 1972) and thus is important in maintaining the granularity of the germinal plasm. Dehydration was carried out in tertiary-butyl alcohol because it gives better results (Blackler, 1958). The schedule for fixation, dehydration and embedding appears in

Appendix IA.

After the embryos were embedded, they were serial sectioned at 10 micrometers on a Spencer lens microtome. The ribbons were then collected and mounted on standard 1 by 3 inch microscope slides. The mounting media consisted of four drops of Mayer's albumin (Humason, 1972) to 40 ml of boiled distilled water.

Lehman's polychrome stain (Lehman, 1965) was employed in this study. This particular stain was designed as a general screening stain. More specifically, Lehman states, "Celestine blue stains nuclei steel blue and cytoplasmic RNA lavender. Napthol yellows stains histones, hemoglobin, keratin, and other basic proteins or proteins rich in S-H groups yellow. Aniline blue stains all mucopolysacchrides various shades of clear blue. Chromotrope 2R stains acid and neutral proteins scarlet. When two of the above molecular components are concentrated in the same cellular region, the multiple dye binding will result in complementary colors. For example, mitotic chromosomes appear green by virtue of celestine blue staining of nucleic acids and napthol yellow S staining of chromosomal histones." The procedure used for Lehman's polychrome stain can be found in Appendix II.

Since some of the above results can be altered by using formalin (personal communication from W. S. James), a comparison was made against Kahle's fixative, which can be

used with Lehman's polychrome stain. The procedure is described in Appendix IB.

Measurements throughout this study were made using a Bausch and Lomb monocular microscope fitted with an optical micrometer lens which was calibrated using a standard calibration slide. Photo micrographs were taken on Panatomic X black and white film with a Pentax SP500 35mm camera attached to a Spencer binocular microscope. With each new role of film or a change in microscopic power, a photo micrograph was taken of the calibration slide to use as a reference.

RESULTS

2-cell to 8-cell embryo

The yolk platelets in the 2-cell embryo appear bright yellow due to the napthol yellow S stain. The embryo is surrounded by the vitelline membrane which is stained red by chromotrope 2R. Celestine blue stains the nuclei steel blue and the RNA rich islets of germinal plasm deep lavender.

The islets of germinal plasm are seen in areas free of yolk and are associated with mitochondrial aggregations. They are approximately 10 micrometers from the cortex in the vegetal pole region (Fig. 1), and extend up to 650 micrometers from the midpoint of the cleavage furrow. At this time the islets are about 6 micrometers in diameter (Table 3).

The 4-cell embryo exhibits the same staining characteristics as the 2-cell embryo does. The islets of germinal plasm are still located about 10 micrometers from the cortex, although some can be seen just below it (Fig. 2). Two different sizes of islets are apparent: about 7 micrometers and 19 micrometers in diameter (Table 3). This may suggest that the islets of germinal plasm coalesce to form larger islets. As the second cleavage furrow forms, these islets of germinal plasm are divided among the four

resulting blastomeres (Table 2). Figure 3 shows a group of these islets of germinal plasm in the vegetal region of one of these four blastomeres. Also the islets of germinal plasm are moved, probably passively by the forces of cleavage, closer to the midpoint of the cleavage furrows. Some islets of germinal plasm were observed to have moved as far as 30 micrometers up the cleavage furrow. Such islets were no more than 10 micrometers from the cleavage membrane.

During the third cleavage, the islets of germinal plasm probably continue to coalesce since their diameter is greater than in the 4-cell embryo (Table 3). As this third cleavage reaches its finish, the islets of germinal plasm are observed much further into the cleavage furrows. On completion of this cleavage, the embryo is divided into four animal pole blastomeres and four vegetal pole blastomeres. Each of the four vegetal pole blastomeres contains islets of germinal plasm (Table 2) that are located 10 micrometers below the cortex near the cleavage furrow to approximately 100 micrometers up the furrow in close proximity with the membrane (Fig. 4). The diameter of these islets is given in Table 3. As before, the staining appearance of the embryo is consistent with the 2-cell embryo.

16 cell-embryo to 32-cell embryo

The germinal plasm in the 16-cell stage embryo is a very dense lavender. The diameter of the islets of germinal plasm is given in Table 3. They are diversified in their

location at this time. Some are seen in the cortex, 35 micrometers from the cleavage furrow, others have moved by the action of cleavage to a position as deep as 300 micrometers from the surface of the vegetal pole region (Fig. 5).

Because the germinal plasm is located peripheraly toward one side of the cell membrane, it is distributed to only one of the daughter cells during mitosis. Thus as cleavage progresses and the 32-cell embryo is formed, the islets of germinal plasm are included in only four blastomeres (Table 2). There are usually two to three islets per cell and their size is given in Table 3. The granularity of the germinal plasm at this stage is prominent in the deep lavender islets (Fig. 6). Each islet is located in a peripheral position in the cell (Fig. 6). Two of the four 32-cell embryos examined exhibited an interesting phenomenon. Those cells that possessed germinal plasm and were next to the blastocoel developed protuberances of cytoplasm containing one large islet of germinal plasm along with pigment and yolk platelets (Fig. 7).

Mid-blastula to early-gastula

All through the blastula to early gastula stages, the staining appearance of the embryos was as before, with the exception that the nuclei of the early gastula varied in their morphology as well as in staining characteristics. The nuclei in the invaginating endodermal cells were oval

and stained steel blue, whereas the nuclei in the region of the blastocoel floor were lobulated and stained brownish to green.

The process of cleavage has greatly reduced the volume of cytoplasm in the cells of the mid-blastula, so that the germinal plasm in its peripheral position has indirectly moved closer to the nucleus (Fig. 8). The islets of germinal plasm at this time are spherical, surrounded by small yolk platelets, and very granular in appearance (Fig. 9). There were from four to seven cells (Table 2) in the lower one-third of the embryo that possessed one to two islets of germinal plasm. Their diameter was recorded in Table 3.

By the late blastula stage, the cell volume has been further reduced and the germinal plasm occupies a closer position with the nucleus (Fig. 10). The number of cells containing germinal plasm does not dramatically increase (Table 2), as a result of its passage to only one of the daughter cells during mitosis. Also, these germinal plasm containing cells are still located in the lower one-third of the vegetal hemisphere.

At the onset of gastulation, when there is a well developed dorsal lip, the germinal plasm begins to migrate from its peripheral association with the cell membrane (Fig. 11) to a juxtanuclear position (Fig. 12). All phases of this migration can be observed occurring in the same embryo. There

are about six cells containing germinal plasm at this stage (Table 2).

Embryos from later gastula stages were not examined and thus it can only be postulated as to the fate of these germinal plasm containing cells during late gastulation. As the movements of gastulation continue, the cells containing germinal plasm are passively moved from their position in the lower vegetal pole (Fig. 12) to a region that is midway between the archenteron floor and the ventral surface of the endoderm. Examination of this region in neurula and early tail bud embryos did not reveal any endodern cells containing germinal plasm. Figure 13 illustrates this theoretical region in an early tail bud embryo.

The results of the comparison in staining characteristics of Lehman's polychrome with Kanle's fixed and formalin (10%) fixed, potassium dichromate post-fixed tissue, are recorded in Table 4.

At Shumway stage 25 when closure of the operculum is complete, the primordial germ cells are lined up along the genital ridge (Fig. 14). Here they occupy positions at the dorsal root of the dorsal mesentery (Fig. 15, 16), or in the lateral and median ridges (Fig. 17). Since the nuclei of the primordial germ cells are very prominent during this period (Fig. 18), it was easy to determine the number of primordial germ cells (Table 2) occurring in this species.

DISCUSSION

Staining of embryonic tissue with Lehman's polychrome is influenced by the fixation employed. In Kahle's fixed tadpole muscle cells (which contain myosin and acid protein), the cytoplasm stains red due to chromatrope 2R. If neutral formalin and chromation are used (as Bournoure (1934) and others found necessary to preserve the germinal plasm), the muscle cells of the tadpole stain a reddish-purple (Table 4). Other staining abnormalities are apparent in Table 4 using neutral formalin fixation and chromation. Although these staining abnormalities did occur in muscle cells and nuclei of other cells, the staining of the germinal plasm with celestine blue was consistent with what Lehman (1965) reported for cytoplasmic RNA, that is a deep lavender color. These deep lavender islets were compared with slides that had been stained with pyronine y and proved to be identical with the deep red islets in these sections (Fig. 18).

The islets of germinal plasm in the 2-cell embryo were located about 10 micrometers below the cortex in the vegetal pole region. This is congruent with what Whitington and Dixon (1975) reported in Xenopus laevis. The fact that the islets of germinal plasm are spread out as far as 650 micrometers from the cleavage furrow in Bombina orientalis, may be to insure that at the blastula stage the cells

containing the germinal plasm will be situated in the lower one-third of the embryo. Xenopus laevis, in which the islets of germinal plasm are spread out at the 2-cell stage as far as several hundred micrometers from the cleavage furrow (Whitington and Dixon, 1975), also exhibit the cells containing germinal plasm in the lower one-third of the blastula. In contrast, the cells containing the germinal plasm in Rana pipiens are located in the blastocoel floor (Di Berardino, 1961) and at the 2-cell stage, the islets of germinal plasm are concentrated in areas much closer to the cleavage furrow (unpublished observations).

The observation that the islets of germinal plasm coalesce into larger islets and move by the action of cleavage, close to and up the cleavage furrow is in agreement with the findings of Blackler (1958) and Whitington and Dixon (1975). The germinal plasm always remains in close association with the cell membrane throughout cleavage and is usually passed to only one of the daughter cells during mitosis (also reported by Blackler, 1958; Whittington and Dixon, 1975). Thus by the 32-cell stage, the germinal plasm is contained in only a few cells.

Blackler (1958) first described the phenomena of extrusion of germinal plasm in cells that are located next to the forming blastocoel in the 32-cell embryo. He believed it to be some kind of elimination process peculiar only to Bufo bufo. This phenomenon has also been reported in Discoglossus (Blackler, 1966). Why it occurs in Bombina

orientalis and the above species is yet to be elucidated.

The number of cells containing germinal plasm from early cleavage up to gastulation, and the number of primordial germ cells in Bombina orientalis can be compared with other species listed in Table 5.

As cleavage continues, the number of cells containing germinal plasm or "the presumptive primordial (p.p.) germ cells" as Whitington and Dixon (1975) refer to them, does not appreciably increase. At the mid-to late blastula, the cells are concentrated into one region, and the germinal plasm is located much closer to the nucleus as a result of the reduction in cell volume. During the onset of gastulation, the germinal plasm begins to migrate to a juxtonuclear position (also reported by Blackler, 1958; Di Berardino, 1961; Whitington and Dixon, 1975).

Blackler (1958, 1966, and 1970) assumed that this juxtanuclear position of the germinal plasm prevented any further mitosis in these p.p. germ cells, and as a result the cells would remain in an embryonic state. This theory was later shown to be incorrect by Dziadek and Dixon (1975, 1977). They performed experiments that involved pulsing embryos of Xenopus laevis with tritiated thymidine and tritiated uridine. What they found, was that in cells of the gastula that exhibited germinal plasm in the juxtanuclear position, the nuclei showed a high level of incorporation of the tritiated thymidine. This demonstrated that

the cell nuclei were not quiescent, but were actively synthesizing DNA. Also cells in the process of mitosis were observed. The germinal plasm in this case was distributed to both daughter cells. The intracellular migration of the germinal plasm was found to be independent of RNA synthesis, as they were unable to achieve any tritiated uridine incorporation into the cytoplasm.

From estimates of rates of cell division and number of p.p. germ cells in the genital ridges of Xenopus laevis, Whitington and Dixon (1975) suggested that there were two cell divisions occurring in these cells between gastulation and the time of their migration from the endoderm. Because of the juxtanuclear position of the germinal plasm, both daughter cells receive a portion of the germinal plasm during mitosis. Thus, the number of p.p. germ cells can now be increased (Whitington and Dixon, 1975). These divisions have been termed "cloning divisions" by Whitington and Dixon (1975), because the number of cells increases without changing their cytological state. Dziadek and Dixon (1977) have recently found that there are actually three cloning divisions that occur instead of only two.

Germinal plasm was believed by Kerr and Dixon (1974) to initiate the migration of the p.p. germ cells from the endoderm. This seemed very logical since the staining ability of the germinal plasm disappears during this time (Blackler, 1958; Whitington and Dixon, 1975; Michiko, et al., 1976).

In the present study, no detectable substance resembling germinal plasm was seen in cells of the neurula or early tail bud embryos. It may be that in Bombina orientalis, there is a precocious disappearance of the germinal plasm, or the amount present in the p.p. germ cells could have been small and thus was overlooked. Blackler (1966) has mentioned that it is the staining characteristic of the germinal plasm which makes observation of germ cells in the endoderm easy. If it were not present, identification would be most difficult.

Most investigators (Blackler, 1958; Whitington and Dixon, 1975; and Michiko, et al., 1976) agree that the migration of the p.p. germ cells to the dorsal crest of the endoderm is an active one, possibly by some kind of ameboid movement. The first definite indication that p.p. germ cells may move by a type of ameboid movement came from a study by Michiko (et al., 1976). They employed the technique of embedding the embryos in epon and then sectioning them at one micrometer. As a result, the cellular membranes of the endoderm cells were much more distinct. Observation revealed that the p.p. germ cells that were in the process of migration, showed certain morphological characteristics. For example, they were very round in appearance compared to the polygonal shape of the other endodermal cells. Also, the intercellular space between the p.p. germ cells and the endoderm cells was greater and more irregular than the space

between adjacent endoderm cells. This suggested that the p.p. germ cells, were actually isolated from the other endoderm cells.

Once the p.p. germ cells have begun to migrate, there is the question of, how this migration is controlled. Gipouloux (1970, as described by Giorgi, 1974) has suggested that the migration of the p.p. germ cells in the endoderm takes place under the attraction of the dorsal mesenteric tissue. Evidence to support this came from grafting experiments by Giorgi (1974). He found that by grafting dorsal regions from one embryo of Bufo bufo to the ventral region of another, some of the p.p. germ cells from the endoderm migrated into the genital ridges of the ventral graft. It appears that dorsal mesenteric tissue does play an important role in controlling this migration.

There are conflicting opinions as to how the p.p. germ cells leave their position in the dorsal crest of the endoderm, and make their way to the genital ridges. Blackler (1958, 1966, 1970) believes that the migration of the p.p. germ cells out of the endoderm is both an active and passive process. Whitington and Dixon (1975) are in agreement with Blackler. They have described a process in Xenopus laevis whereby the lateral mesodermal sheets meet and fuse in the midline, pinching off the p.p. germ cells which are then carried with the developing mesentery. As this latter process takes place, the p.p. germ cells actively

move up the mesentery to its dorsal root. It has been shown (Wylie and Heasman, 1976) with the aid of the transmission electron microscope, that there are no cell junctions between the p.p. germ cells and the mesenteric cells in Xenopus laevis. Michiko (et al., 1976) have observed p.p. germ cells in the dorsal crest region of Xenopus laevis which appear to be actively pushing their way through the surrounding mesenchymal sheet into the mesentery. Due to the lack of any additional evidence, it seems that this active migration of the p.p. germ cells that was observed is not representative of the normal process.

From their position at the dorsal root of the mesentery, the primordial germ cells are believed to move actively under a chemo tactic attraction from somatic cells which constitute the lateral genital ridge (Wylie, et al., 1976). Time lapse photography of dissociated primordial germ cells (Wylie and Roos, 1976) from the genetal ridges of Xenopus laevis have shown, at least in vitro, that these cells are capable of active movement.

As was discussed in the introduction of this paper, RNA is a major constituent of the germinal plasm. It is this feature of the germinal plasm, that allows its ease of observation of sections stained with RNA specific stains such as, pyronin Y or stains like celestine blue which reacts with cytoplasmic RNA. Since this stain was employed in the present study, a comparison was made with slides that had been

stained previously with the RNA specific stain, pyronine Y. Those areas that reacted with pyronine (Fig. 18) were identical with the areas reacting with celestine blue. Thus areas reported to be islets of germinal plasm in the results of this paper, represent true areas rich in RNA.

From the ultrastructural studies of Mahowald and Hennen (1971), Williams and Smith (1971), and Czolowska (1972), it was revealed that the RNA staining characteristic of the germinal plasm was mainly due to a large aggregation of ribosomes in the germinal plasm rather than to any specific concentration of messenger RNA. The electron dense bodies or "germinal granules" as Williams and Smith (1971) refer to them, appear to stain with indium trichloride (Mahowald and Hennen, 1971) an RNA stain. This reaction could represent messenger RNA which is called up in some way inside the germinal granule, as was suggested by Mahowald and Hennen (1971), Williams and Smith (1971), and Smith and Williams (1975). This would explain why there are masses of ribosomes in the area. The germinal granule is thought to be composed of a fibrous network of protein (Mahowald and Hennen, 1971; Williams and Smith, 1971; and Smith and Williams, 1975). The function of the protein part of the granule could be to protect or mask the messenger RNA until the specified time that it is to be read by the ribosomes.

A substance analogous to germinal plasm occurs in many species of Drosophila (for review, see Mahowald, 1971).

This substance, pole plasm, induces pole cells in the vegetal pole region of the embryo. The pole cells subsequently give rise to the germ cells of the adult fly. Ultrastructually, pole plasm is very similar to germinal plasm. It consists of aggregates of mitochondria and electron dense bodies (polar granules) which are associated with large number of ribosomes.

Illmensee et al. (1976) have performed experiments involving a transfer of pole plasm (containing polar-granules) from posterior pole cells to anterior cells in Drosophila embryos. If the pole plasm contains the germ determinate, it should induce an anterior somatic cell to become a pole cell. This is exactly what occurred in the experimental embryos. The anterior cells showed a precocious mitosis, which separated them from the other cells in that region. In normal development, pole cells also become separated early from the other somatic cells. Further evidence, that these anterior cells were becoming pole cells came from transferal of these cells to the vegetal region of the early embryo. With the use of an enzyme deficiency as a marker, it was found through subsequent matings, that the transferred cells in fact were capable of becoming germ cells.

The presence of mitochondria in the germinal determinate has been a puzzling one. In both anurans (Mahowald and Hennen, 1971; Williams and Smith, 1971; and Czolowska,

1972) and Drosophila (Mahowald, 1962) the electron dense bodies appear to be fused to the outer membrane of the mitochondria at certain times in early development. Williams (personal communication) has noted that in highly magnified electron micrographs, the germinal granules are not as closely applied to the outer mitochondrial membrane as was believed. She has also mentioned that this aggregation of mitochondria could represent some high energy requirement in the germinal plasm region.

Dawid and Blackler (1958) have proposed a more convincing explanation. During early oogenesis, there is an amplification of a small group of mitochondria to form the Balbiani body. As the germinal vesicle breaks down, the mitochondria are dispersed. Those mitochondria that aggregate in the germinal plasm region are subsequently incorporated into the primordial germ cells and give rise to the next generation of mitochondria. Thus mitochondria are maternally inherited in this manner.

Bounoure (et al., 1954), has shown that irradiation of the germinal plasm with ultraviolet light at the time of first cleavage, causes sterility in Rana temporaria. This observation has been extended to Rana pipiens (Smith, 1966), Xenopus laevis (Inkeniski, et al., 1974), and Bombina orientalis (Odom and James, unpublished observations). Ijiri (1976) has reported ultraviolet light sensitivity in Xenopus laevis as early as ten minutes after fertilization. From the

studies of Tanabe and Kotani (1971), there appears to be a quantitative relationship between the amount of germinal plasm that is irradiated and the number of primordial germ cells in the genital ridges of Xenopus laevis. Irradiation of half of the germinal plasm, reduced the number of primordial germ cells by one-half. Ijiri and Egami (1976) have studied the effects of ultraviolet light irradiation on the germ cell determination process in Xenopus laevis. They have also proposed a mathematic model for this determination process.

There are several theories on how ultraviolet light irradiation induces sterility. Smith (1966) postulated from his studies on Rana pipiens, that there is destruction of some type of information molecule, such as a messenger RNA. This would explain the fact that the most effective wavelength used was in the region of absorbance for nucleic acid. Fragmentation of germinal granules in Xenopus laevis eggs as a result of their exposure to ultraviolet light, has been reported by Ikeniskhi (et al., 1974). Such a phenomenon would obviously prevent the germinal granules from functioning in a normal manner. On the other hand, Smith and Williams (1975) have never observed this phenomenon of germinal granule fragmentation in Xenopus laevis, and suggest that it could be attributed to a fixation problem.

Zust and Dixon (1975) have presented an altogether different explanation. They find that ultraviolet light

causes a delay in cleavage in the vegetal pole. A syncytium is formed as a result of inhibition of cytokinesis without disruption of karyokinesis. At about blastula, the syncytium breaks down. There is no noticeable difference in gross morphology of the cells in the vegetal pole from those in the normal embryos. It is postulated that this delay may modify the normal events of compartmentalizing off the germinal plasm, and thus primordial germ cells are never formed. This syncytium may be caused by the ultraviolet light effecting the mucopolysacchride adhesive substance between the blastomeres (Beal and Dixon, 1975).

As a final note, Williams (personal communication) recently has found that ultraviolet light may not actually cause sterility. The usual assay for sterility is to check the genital ridges when the primordial germ cells are present. If the gonads of the metamorphosized froglet are examined, they contain germ cells. It seems that the primordial germ cells are delayed in their migration from the endoderm, and arrive at a later time in the gonadal rudiments.

The origin of germinal plasm has been investigated by Czolowska (1969) in Xenopus laevis. She found RNA rich patches of cytoplasm in the vegetal pole cortex of hormonally treated oocytes. Similar areas have been found in Rana pipiens by Williams and Smith (1971). The ultrastructure of these areas consisted of mitochondria surrounded by electron dense bodies resembling germinal granules, but much smaller in size.

Thus some of the precursors of the germinal plasm are present even before fertilization. Autoradiographic studies by Williams (personal communication) in which she used [^{3}H] uridine and [^{3}H] leucine after hormone treatment of oocytes was negative. The components that compose the germinal granules must be synthesized earlier in oogenesis.

The presence of an intermitochondrial electron dense granular substance of "nuage," has been described in primordial germ cells of Rana pipiens (Mahowald and Hennen, 1971) and Xenopus laevis (Al-Muktar and Webb, 1971). Nuage material has also been reported in; oogonia and early meiotic oocytes of Rana pipiens (Eddy and Ito, 1971) and Xenopus laevis (Al-muktar and Webb, 1971) and primary spermatocytes and spermatogonia (Kerr and Dixon, 1974) of Xenopus laevis.

The nuage material in the primordial germ cells is located in a juxtanuclear position. This material could represent the remains of the protein component of the germinal granule (Mahowald and Hennen, 1971). As the primordial germ cells develop into oogenia (Al-Muktar and Webb, 1971) and spermatogenia (Kerr and Dixon, 1974), the nuage material keeps its close association with the nucleus. In primary spermatocytes, the nuage material degenerates (Kerr and Dixon, 1974), but in oogonia, it has been observed as late as the late lampbrush chromosome stage (Smith and Williams, 1975). The mitochondria which inhabit this juxtanuclear region are thought to give rise to the Balbiani body (Dawid and Blackler,

1970; and Al-Muktar and Webb, 1971). Long before the mitochondria in the Balbiani body are dispersed, the nuage material disappears, therefore no evidence exists that this nuage material is continuous with the germinal granules.

It is apparent from the foregoing discussion that germ cell determination is an active area of research and future investigations should prove very interesting, especially those dealing with isolation and determination of the composition of the dense germinal granules. Germ cell determination in the anuran also offers an ideal system in which to study the intricate interactions between the cytoplasm and the nucleus in embryonic cells.

To conclude, I believe that the information gathered from the present study contributes to the present knowledge of the understanding of germ cell development in the anuran. Further, it is evident that this process is fairly consistent among anurans thus studied, including Bombina orientalis used in this study. Finally, it was imperative that this study be conducted so that further research on germ cell development using Bombina orientalis could be possible.

SUMMARY

1. Lehman's polychrome stain was used to follow the fate of germinal plasm in early development of Bombina orientalis embryos. The results were consistent with earlier findings.

2. Islets of germinal plasm approximately 6 um in diameter were observed in the vegetal pole region of the 2-cell embryo. By the 4-cell stage, some of these islets had coalesced to form larger ones so that two size classes of islets were present.

3. As cleavage continued to the 16-cell stage, the islets were moved further up the cleavage furrow and they always remained in close association with the cleavage membrane. The islets had reached a diameter of approximately 32 um.

4. Because of their peripheral location on one side of the cells containing them in the 32-cell embryo, the islets of germinal plasm were passed to only one of the daughter cells during mitosis. Also those cells which contained germinal plasm and were adjacent to the developing blastocoel, exhibited a protuberance of cytoplasm consisting of pigment, yolk and germinal plasm.

5. At the mid-to late blastula stage, the germinal plasm was contained in a few cells located in the lower one-third of the embryo. The peripheral position of the germinal plasm was maintained in these cells.

6. During early gastulation, the germinal plasm began to migrate from its peripheral location to a juxtanuclear position. It was postulated that the movements of gastulation would deliver these cells containing germinal plasm to a mid-posterior region of the endoderm.

7. Examination of neurula and early tail bud stages did not reveal any cells containing germinal plasm. The implication was that the germinal plasm had been utilized previously to these stages.

8. Primordial germ cells were observed in the dorsal mesentery and genital ridges of stage 25 tadpoles.

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APPENDIX I

A. Embryos were collected at desired stages and dejellied with watchmaker forceps. They were then transferred to carrying tubes made out of small sections of glass tubing with lens paper fastened to one end. These carrying tubes allowed the material to be passed through solutions with relative ease. Fixation was in hot (approximately 60°C) 10% neutral formalin for 5 minutes, followed by post-fixation in a saturated solution of potassium dichromate for 48 hours at 45°C. After rinsing in running water over night, the embryos were dehydrated and embedded according to the following procedure:

Grad. No.	95% Ethanol	Tertiary butanol (TBA)	Distilled Water	Time
1'	5 ml	---	95 ml	15 minutes
1''	5 ml	---	95 ml	15 minutes
2	10 ml	---	90 ml	15 minutes
3	20 ml	---	80 ml	15 minutes
4	30 ml	---	70 ml	15 minutes
5	40 ml	10 ml	50 ml	15 minutes
6	50 ml	20 ml	30 ml	15 minutes
7	50 ml	35 ml	15 ml	15 minutes
8	40 ml	50 ml	10 ml	15 minutes
9	25 ml	75 ml	---	15 minutes
10'	---	100 ml	---	15 minutes
10''	---	100 ml	---	30 minutes
10'''	---	100 ml	---	30 minutes
11	50 ml TBA-50 ml paraffin oil			30 minutes
12	TBA-paraffin oil from 12 and solidified para last			until melted
13	Transfer tissue through 4 changes of paraplast			30 minutes each
14	Embed in paraplast			

Appendix A, cont'd.

B. Six tadpoles, morphologically equivalent to Shumway stage 25, were fixed in Kahle's fixative for comparison with the formalin potassium dichromate fixation method.

Composition of Kahle's fixative (Henley and Costello, 1957):

commercial formalin (40%)	16 c.c.
Ethanol (95%)	32 c.c.
Acetic Acid (glacial)	2 c.c.
Distilled water	60 c.c.

After fixation in Kahle's fixative for 12 hours, these six embryos were passed through 3 changes of 70% ethanol, then through 2 changes of 80% ethanol and stored in fresh 80% ethanol. Dehydration and embedding was carried out by bringing the material down to 70% and starting at grade number 6 in the TBA series.

APPENDIX II

The Lehman's polychrome stain was formulated after the developer's description (Lehman, 1965). All slides used in this study were treated in the following manner:

1.	Two changes of Xylene	2 min. (each)
2.	Two changes of absolute ethanol	2 min. (each)
3.	95% ethanol	2 min.
4.	80% ethanol	2 min.
5.	70% ethanol	2 min.
6.	Wash in two changes of distilled water	2 min. (each)
7.	0.5 acid celestine blue, pH 1.6	2 min.
8.	Wash in two changes of distilled water	Total of 1 min.
9.	Differentiate in 1% chromalum	30 sec.
10.	Wash in distilled water	1 min.
11.	0.5% napthol yellow s, pH 2.7	1 min.
12.	Differentiate in 1% acetic acid	1 min.
13.	Mordant in mixture of 1% phosphomolybdic acid and 1% phosphotunastic acid	30 sec.
14.	1% acid aniline blue and 1% chromotope 2R	90 sec.
15.	Rinse with one quick dip in 1% acetic acid	
16.	Differentiate, three to five dips acid ethanol and tertiary butanol	
17.	Dehydrate in 3 changes of absolute ethanol	2 min. (each)
18.	Two changes of Xylene	2 min.
19.	Mount in Eukitt	

TABLES AND FIGURES

Table 1

Stage of development	Number of embryos that were examined	Number of embryos containing germ plasm
2-cell	12	5
4-cell	5	5
8-cell	7	4
16-cell	8	7
32-cell	4	4
Mid-blastula	4	4
Late blastula	1	1
Early gastula	1	1
Neurula	2	0
Early tailbud	6	0
Stage 25 tadpole	4	4

Table 2

Stage of development	Number of cells containing germ plasm
2-cell	2
4-cell	4
8-cell	4
16-cell	4
32-cell	4
Mid-blastula	5 ± 1.4*
Late blastula	8
Early gastula	6
Neurula	none where found
Early tailbud	none where found
Stage 25 tadpole	60.8 ± 3.86*

* represents the mean ± the standard deviation

Table 3

Stage of development	n	Diameter of germinal islets in micrometers[1]
2-cell	15	6.30 ± 1.07
4-cell	9	a. 7.44 ± 2.60
	5	b. 19.50 ± 3.62
8-cell	7	24.77 ± 5.45
16-cell	7	31.57 ± 6.12
32-cell	5	22.10 ± 7.08
Mid-late blastula	5	13.03 ± 3.54
Gastula	5	8.50 ± 9.96

[1] Diameters are expressed as the mean ± standard deviation

n Number of observations

Table 4

Staining Characteristics of Lehman's Polychrome

Type of cell	Kahle's fixed	Formalin (10%) fixed Potassium dichromate post-fixed
Blood cell	green nuclei	deep purple nuclei
Nerve cell	lavender cytoplasm	light purple cytoplasm
	green nuclei	purple nuclei
Notochord cell	sky blue cytoplasm	same
	purple nuclei	
Cells containing yolk platelets	yellow	same
Muscle cell	red cytoplasm	reddish purple cytoplasm
	steel blue nuclei	steel blue nuclei

Table 5

Number of Germ Cells Recorded in Anuran Embryos
(Taken from Table 5, Whitington and Dixon, 1975)

Species	Stage of development			
	Cleavage	Blastula	Gastula	Genital Ridge
X. laevis	---	---	---	22.4±14.1
	---	---	---	29.4±11
	---	---	---	22.4±4.6
	4.5±0.9	5.3±1.9	8.7±3.0	13.9±4.8
R. temporaria	---	10-15	---	45.9±23.3
	---	5-7	11-23	39 ± 6.8
R. esculenta	---	---	---	65.7±30.9
	---	---	---	26*
R. pipiens	---	8.8±2.7	8.6±4.4	---
	---	---	---	61, 82*
B. bufo	---	17	---	15*
B. viridis	---	---	---	21.3±2.1
	---	---	---	120.4±31.8
D. pictus	---	---	---	24.6*

Numbers are expressed as the mean ± S.D.
* Represents only one or two measurements.

Figures 1-6

1 - Cross-section through the vegetal pole of a 2-cell stage embryo. An islet of germinal plasm (G) is visible approximately 10 micrometers below the vegetal cortex. Cleavage membrane (CM), yolk platelet (Y).

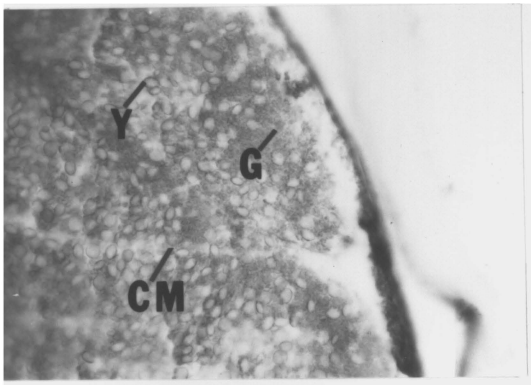
2 - Cross-section through a 4-cell stage embryo.

3 - Tangential section of a 4-cell embryo approximately 15 micrometers above the vegetal pole. Many islets of germinal plasm can be seen dispersed throughout the cytoplasm of one of the blastomeres.

4 - Cross-section through an 8-cell stage embryo. Large yolk free islets of germinal plasm are located near the cleavage membrane.

5 - Cross-section through a 16-cell stage embryo. An islet of germinal plasm is in close proximity with the cleavage membrane.

6 - High magnification of an islet of germinal plasm in a 32-cell stage embryo. The granularity of the germinal plasm is apparent at this stage. Plasma membrane (M).



1

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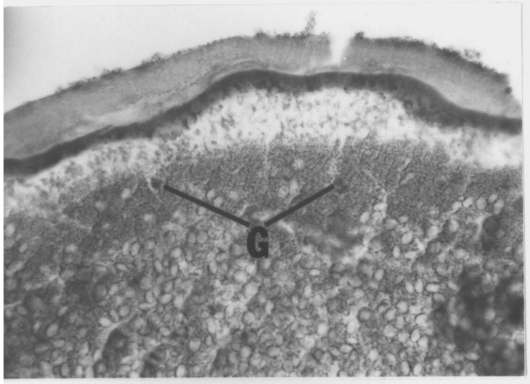

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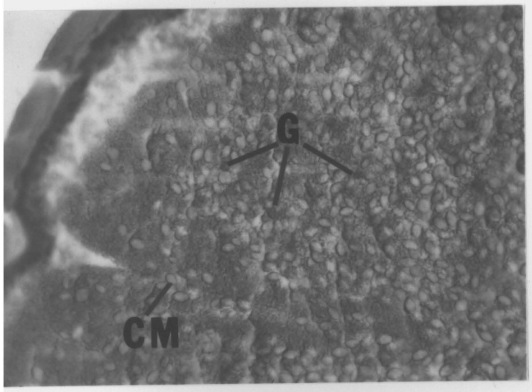

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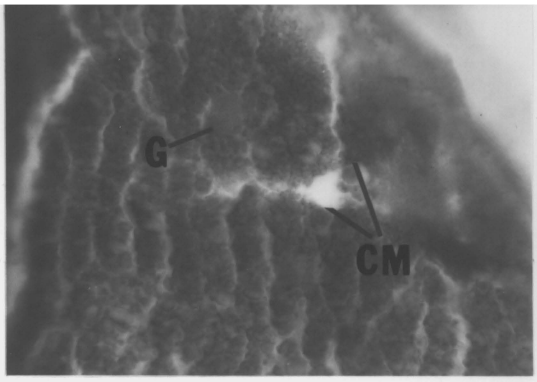

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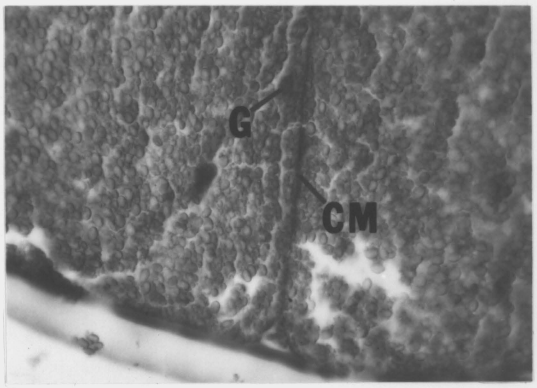

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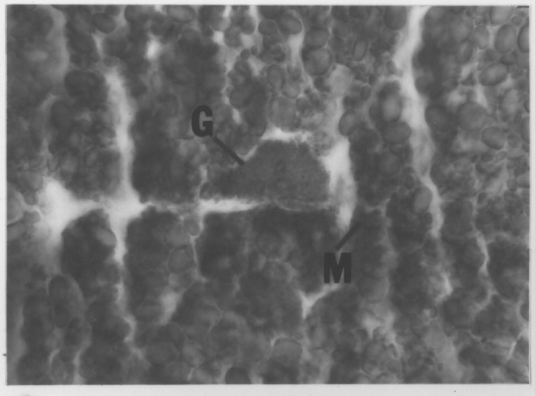


6

60 μm

Figures 7-12

7 - Blastomere of a 32-cell embryo exhibiting a cytoplasmic protuberance of germinal plasm and yolk granules. Forming blastocoel (B).

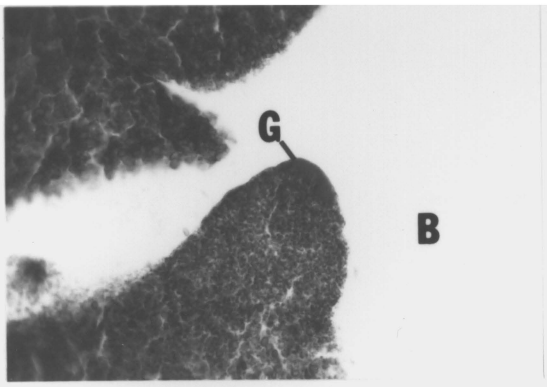
8 - Cross-section through mid-blastula embryo. An islet of germinal plasm can be seen lying in a position adjacent to the plasma membrane. Nucleus (N).

9 - High magnification of an islet of germinal plasm in cell of mid-blastula embryo. The granularity of the germinal plasm is apparent.

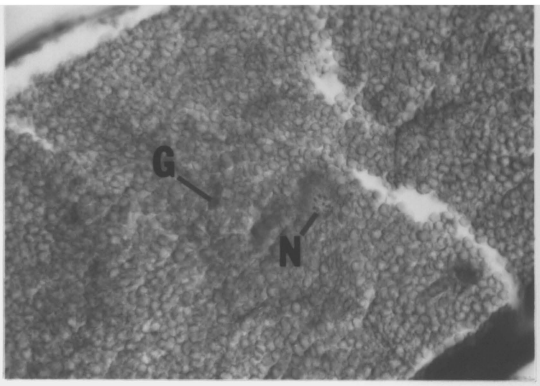
10 - Cross-section through late-blastula embryo. The germinal plasm is still in a position adjacent to the plasma membrane. The blastomere is undergoing mitosis.

11 - Cross-section through early-gastula embryo. A cell containing germinal plasm is visible in the vegetal pole region of the embryo.

12 - Same stage as in figure 11. The germinal plasm has moved from its pheripheral location to a juxtanuclear position.



7 140 μm



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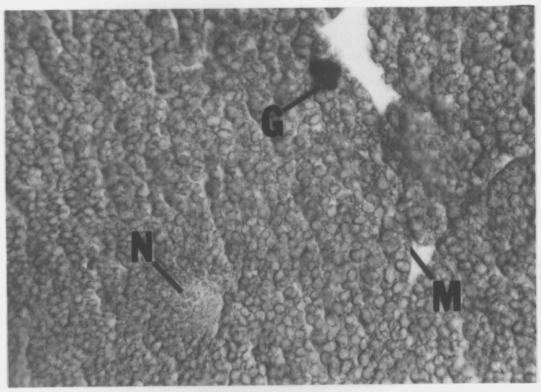

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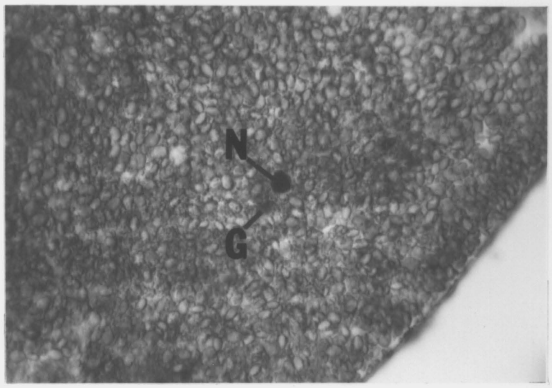
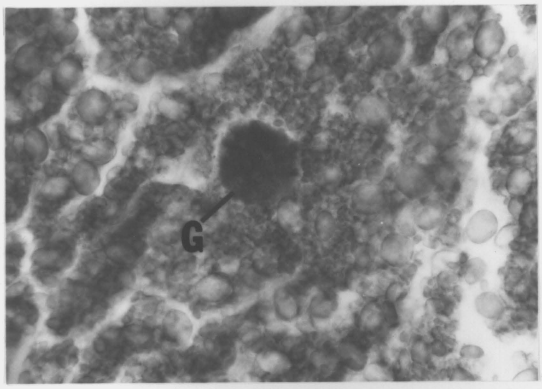
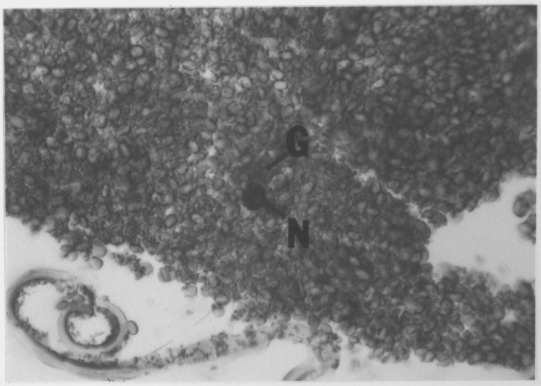


11 140 μm



9 60 μm



12 140 μm

Figures 13-18

13 - A sagittal section through an early tailbud embryo. The boxed area represents the region of the endoderm where cells containing germinal plasm theoretically should be located. Hindgut (H), midgut (MG).

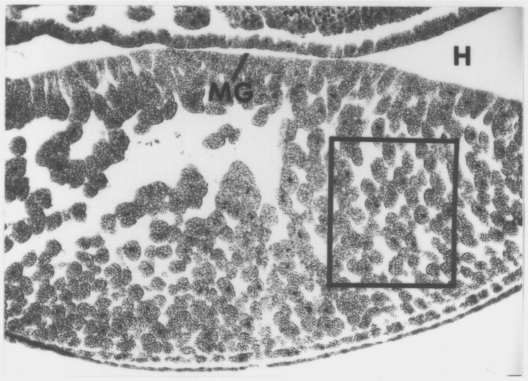
14 - A sagittal section through a stage 25 tadpole. The primordial germ cell (PGC) are seen lying in the genital ridge (GR). Notochord (NC), coelom (C), intestine (I).

15 - Cross-section through a stage 25 tadpole. Two primordial germ cells are located at the base of the dorsal mesentery (DM) in the genital ridge.

16 - High magnification of figure 15.

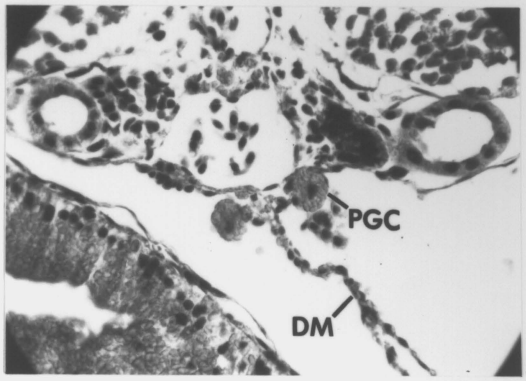
17 - Cross-section through a stage 25 tadpole showing primordial germ cells located in the median and lateral genital ridges. The primordial germ cells are surrounded by germinal epithelial cells (EC).

18 - Cross-section through a 2-cell embryo stained with methyl green and pyronine Y. RNA rich germinal plasm can be seen located in the cleavage furrow in the vegetal pole region.



13

630 μm



16

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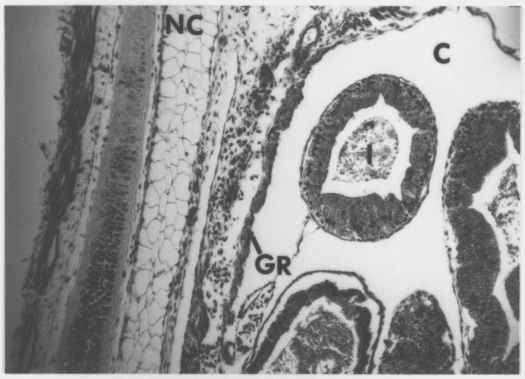

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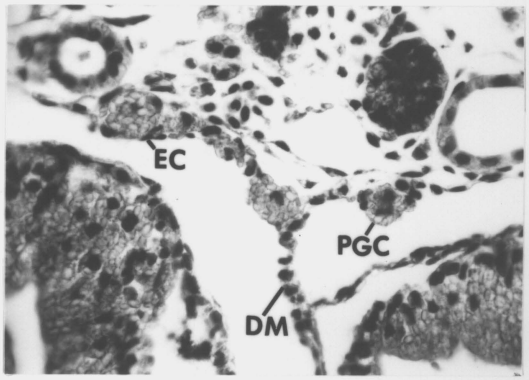
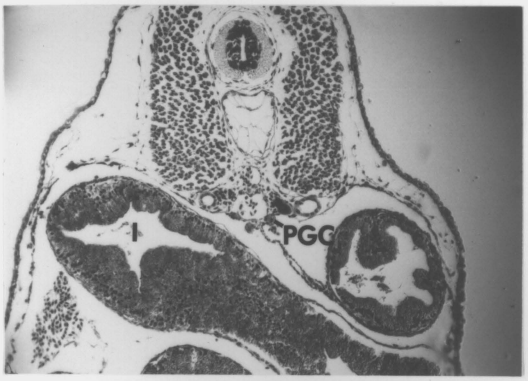


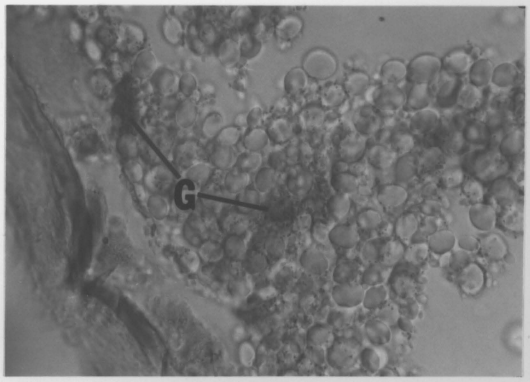
17

140 μm



15

630 μm



18

60 μm