The relationship between cleavage and blastocoel formation in *Xenopus laevis*

II. Electron microscopic observations

By MARVIN R. KALT

*From the Department of Anatomy, Case Western Reserve University*

---

SUMMARY

The development of the blastocoel in *Xenopus laevis* was studied by electron microscopy. Two major morphological specializations were associated both with blastocoel formation and with cleavage. The first specialization was the formation of close junctions at specific points along cleavage furrows, starting as early as the first division. The second was the secretion of material into the blastocoel and extracellular spaces. Secretion occurred by three processes, one merocrine, the other two apocrine. The main secretory product released was β-glycogen, although other products were also liberated. The membrane added to the cell surface during secretion contributed to the growth of both the blastocoel and the furrows. Filaments 80 Å in diameter were found to be present at some, but not all, furrow tips during embryogenesis, but the filaments apparently played no direct role in the formation of the blastocoel.

INTRODUCTION

In the preceding article (Kalt, 1971), light microscopic evidence was presented indicating that in *Xenopus laevis*, cytokinesis in the zygote was related to both division of the cell and to formation of the blastocoel. In the present study, electron microscopic examination of these processes revealed that morphological specializations associated with both blastocoel formation and cleavage mechanisms occur at the beginning of the first division of the zygote. The first specialization was the formation of close junctions at specific points along cleavage furrows. The second was the secretion of materials into extracellular spaces, including the blastocoel. Together, these processes play a significant role in determining the shape, growth, and location of the blastocoel during early embryogenesis.

MATERIALS AND METHODS

Early embryos of *X. laevis* (Daudin) were obtained as previously described (Kalt, 1971). All embryos were prepared for electron microscopic examination.
using acrolein and glutaraldehyde, or acrolein, glutaraldehyde, and formaldehyde in dimethyl sulphoxide, followed by postosmication (Kalt & Tandler, 1971). In some cases, ruthenium red (Brooks, 1969) was added to the fixative in order to demonstrate the glycocalyx. Alternate thick and thin sections were cut with glass or diamond knives on a Servall MT 1 or MT 2 ultramicrotome. Thick sections were stained with 1% toluidine blue for examination by light microscopy. Thin sections were serially stained with uranyl acetate (Stempak & Ward, 1964) followed by lead tartrate (Millonig, 1961). To demonstrate glycogen at the electron microscope level, some thin sections were stained by the periodic acid-lead technique (Perry, 1967). Sections were examined in either a Siemens Elmiskop Ia or an RCA EMU 3E electron microscope.

**OBSERVATIONS**

As described previously (Kalt, 1970, 1971; Kalt & Tandler, 1971), the newly fertilized egg of *Xenopus* contains three distinct cytoplasmic regions: the cortex, the subcortex, and the endoplasm. The cortex includes the plasmalemma and the immediately underlying cytoplasm, the latter merging into the subcortical ectoplasm. The cytoplasm of the cortex contains numerous pigment granules, mitochondria, vesicles, Golgi complexes, and isolated cisternae of rough endoplasmic reticulum. These membranous elements are surrounded by a densely packed matrix of glycogen and ribosomes. The cortical surface is smooth, except for the presence of microvilli in the immediate vicinity of the animal pole. The subcortical ectoplasm, except for the presence of vacuoles, is characterized by a paucity of membranous organelles, and an abundance of glycogen and ribosomes. The endoplasm is dominated by numerous yolk platelets of all sizes.

All figures except numbers 1 and 2 are electron micrographs. The direction of the animal pole is indicated by an arrow above the figure number. The primary fixative employed is indicated in each caption.

**Fig. 1.** This illustration and the following one are light micrographs of the major portion of a single advanced animal pole furrow in the first-cleavage zygote. The most external portions, which would lie above the area shown in Fig. 1, are omitted. The boxed area at the top of the micrograph represents the region of close membrane apposition. The fusiform cavity directly below is the nascent blastocoel. The boxed area below the blastocoel is that portion of the furrow where vacuole dehiscence occurs. Below this, the furrow (arrow) continues downward into the embryo. DMSO-trialdehyde. × 740.

**Fig. 2.** The portion of the furrow shown in this micrograph is in direct continuity with that shown at the bottom of Fig. 1. Its path is marked by a more or less linear arrangement of pigment granules, and is indicated by the series of arrows. The termination of the furrow lies within the box at the bottom of the micrograph. DMSO-trialdehyde. × 740.

**Fig. 3.** Micrograph of the region corresponding to the upper box in Fig. 1. At this level, the furrow is characterized by serried close junctions (see boxed area). Acrolein-glutaraldehyde. × 7600.
Electron microscope study of blastocoel formation

and large accumulations of lipid. Surrounding these inclusions is a cytoplasmic matrix similar in nature to that seen in the ectoplasm.

The first cleavage in *Xenopus* involves components of all three cytoplasmic regions, and leads to the formation of a highly complex furrow, which extends downward from the animal pole of the zygote. An advanced animal pole furrow has five distinct regions. Proceeding downward from the animal pole, the first region of the furrow is characterized by a region of serried close junctions between apposing sides of the invaginated furrow walls. There are two size ranges of these junctions: one consisting of a zone with a gap of 30–40 Å between membranes, the other consisting of a zone with a gap of 100–125 Å between membranes. Series of both zones occur intermittently along the length of this initial furrow region (Figs. 1, 3, 6, 7). The second region of the furrow occurs as a large filiform to pyriform cavity 20–50 μm in width. This region is the incipient blastocoel of the embryo (Figs. 1, 4). The third region of the furrow occurs immediately below the blastocoel cavity, and consists of several large interconnected vacuoles, which contain a large amount of glycogen (Figs. 1, 4, 8, 9). The fourth region of the animal pole furrow extends downward from the midline vacuole, and consists of an unusually long region of closely apposed membranes, up to 1 mm in length, which form close junctions similar to those in the first region of the furrow (Figs. 1, 2, 5). The fifth and last region of the furrow, its tip, occurs as a small expansion at the base of the fourth region (Figs. 2, 5, 11).

The development of this type of complex animal-pole furrow structure begins at the animal-pole cortex, and is marked by the appearance of a band of cortical filaments measuring 80 Å in diameter and running parallel to the cell surface.

Fig. 4. Micrograph of the same vacuole demarcated by the lower box in Fig. 1. The blastocoel (B), a small portion of which is seen at the top of the micrograph, opens into a large midline vacuole. This structure contains two large masses of glycogen (G), each continuous with the rest of the cytoplasm. Two smaller, empty vacuoles (V) flank the channel connecting the blastocoel with the midline vacuole. The furrow continues below the vacuole, initially with its walls obviously separated, but these rapidly converge to form an extended zone of close junctions (F). DMSO-trialdehyde. × 3000.

Fig. 5. This micrograph depicts an animal pole furrow tip similar to the boxed area in Fig. 2. At the top of the micrograph, the walls of the furrow are closely apposed (arrows). These gradually diverge and form an ovoid dilation which marks the terminus of the furrow. The dilated portion of the furrow (FT) contains a finely granular material. DMSO-trialdehyde. × 3000.

Fig. 6. Close junctions in an area comparable to that shown within the box in Fig. 3. Note the asymmetry of the plasma membranes. DMSO-trialdehyde. × 62500.

Fig. 7. Junctional region similar to that shown in Fig. 6, except that the specimen was treated with ruthenium red during fixation. This dye, which appears as the dense black surface layer, has penetrated for only a short distance into the furrow cleft (arrow). This indicates that these junctional zones act as diffusion barriers to larger molecules. DMSO-trialdehyde. × 28000.
These filaments are distinct from those seen in the microvilli of the animal pole region, and they remain restricted to the cortex of the forming furrow tip. At first, the incipient furrow is U-shaped, with a broad opening to the surface. As it progresses into the animal hemisphere, however, it becomes pyriform, until the sides near the surface are drawn together to form close junctions, creating the first region of the mature furrow. The junctions formed in this region are capable of excluding ruthenium red (Fig. 7), and result in a vacuole-like furrow cavity which is effectively sealed off from exchange of larger molecules with the external environment. This cavity is the incipient blastocoel.

At approximately the same time as the sealing process occurs, ultrastructural changes can be observed in the subcortical ectoplasm directly below the incipient blastocoel. The glycogen of the cytoplasmic matrix in this region becomes sequestered, resulting in the formation of several areas of essentially pure β-glycogen (Fig. 9). Invariably, especially large areas of this nature develop just below the blastocoel/furrow tip when the latter structure has reached the equatorial region of the zygote. At this point, the advance of the furrow is temporarily halted. Then, by a process which remains obscure, the area of glycogen is liberated into the furrow by apocrine release (Figs. 4, 8). In addition, other areas of glycogen, some of which may be bounded by a single limiting membrane, may serially fuse with the furrow. Together, these processes produce the third region of the mature animal furrow.

By the above fusion mechanism, new membrane is introduced along the furrow. In contrast to the original surface, no filaments are visible beneath the newly added membrane. However, filaments shortly develop, and the furrow proceeds downward through the zygote. At a point just beneath the lowest vacuole, a 30-40 Å close junction develops. As the furrow proceeds downward, formation of a series of continuous zones of close junctions occurs between apposing membranes, so that eventually a long area of close apposition develops.

---

Fig. 8. Enlargement of a glycogen area similar to that seen in Fig. 4. The luminal surface is bounded by a plasma membrane (arrows), but there is no membranous barrier separating the glycogen from the rest of the cytoplasm. Scattered glycogen particles within the lumen of the vacuole probably were released by rupture of another area of glycogen. DMSO-trialdehyde. ×11000.

Fig. 9. Micrograph of an area of glycogen sequestration occurring beneath the furrow region of a first cleavage zygote. While the general area of glycogen is readily apparent, its boundaries are indistinct, gradually blending into the surrounding cytoplasmic matrix. DMSO-trialdehyde. ×22500.

Fig. 10. Luminal surface of a 16-cell-stage animal blastomere bordering the blastocoel (B). Double membrane-bounded areas of glycogen (G) appear throughout the cytoplasm. Large empty vacuoles, which presumably represent areas that have lost their inner membrane-bounded contents, are also present. DMSO-trialdehyde. ×9600.
Electron microscope study of blastocoel formation
This is the fourth region of the mature animal furrow. As a result of the formation of these junctions, the furrow tip is restricted to a width of only a few micra (Figs. 2, 5, 11). Only this tip, and not the large blastocoel cavity above it, exhibits cortical filaments (Fig. 15). The tip is the fifth and final region of the mature animal furrow. As the tip continues down through the embryo, it usually comes into contact with remnants of the first mitotic midbody, as previously described (Selman & Perry, 1970) (Figs. 11, 12). Cortical filaments occasionally appear to be associated with the midbody, but this relationship is not consistent. A thin layer of subcortical ectoplasm precedes the tip as it continues to advance through the endoplasm toward the rising vegetal furrow, and a path free of yolk platelets is established.

The vegetal pole furrow, which starts to develop after the animal pole furrow is about two-thirds of the way through the zygote, has a much simpler structure than its animal pole counterpart. It remains open and U-shaped throughout its development, and little or no secretory activity is connected with its growth. Filaments may occasionally be present in the cortex of the furrow tip (Fig. 14), but more often only an increase in the density of the cortex immediately beneath the plasmalemma is observed (Fig. 13). No cavity analogous to the blastocoel is produced during the ingression of the vegetal furrow. Instead, the furrow proceeds straight upward surrounded by a narrow layer of subcortical cytoplasm, and eventually fuses with the animal furrow tip.

By the completion of the first cleavage, the blastocoel is of an appreciable size, and the cytoplasm surrounding it is highly vacuolated and contains segregated areas of both free and membrane-bounded β-glycogen. Granular and fibrillar material is present in the blastocoel cavity. The second cleavage occurs at right angles to both the first cleavage plane and to the equator of the blastomeres. The development of the second cleavage furrow is similar to the first in the morphologies of the furrow tip regions, but unlike the first division, the animal pole furrow does not form a blastocoel cavity. Instead, the furrow proceeds downward through the blastomere, and the newly created surfaces behind the advancing furrow tip rapidly form a zone of close junctions. Throughout the second division, an extensive ectoplasmic region remains localized near the

---

Fig. 11. A first-cleavage animal pole furrow tip approximately 1100 μm beneath the animal pole. Microvilli protrude into the moderately dense, granular material within the furrow cavity (FC). A large group of microtubules embedded in a dense matrix and representing the transversely sectioned midbody remnant (MR) is visible beneath the furrow. DMSO-trialdehyde. × 30000.

Fig. 12. A midbody remnant at higher magnification. DMSO-trialdehyde. × 134000.

Fig. 13. A first vegetal pole furrow tip approximately 200 μm above the vegetal pole. Ruthenium red lines the furrow surfaces, which are thrown into slender folds. A narrow, dense cytoplasmic band (DB) is faintly apparent beneath the plasmalemma. Acrolein, glutaraldehyde, and ruthenium red. × 8400.
blastocoel. This region is bisected by the second furrow, but the two areas thus formed continue to develop localized glycogen accumulations, and contain large numbers of smooth-membraned vesicles which contain a fine, flocculent material.

The third division, which occurs horizontally, is first indicated by migration of the ectoplasm surrounding the blastocoel toward the external surface of the embryo, as demonstrated previously (Kalt, 1971). At this point, actual furrowing of the plasmalemma has not occurred. Shortly thereafter, 80 Å filaments and an occasional 225 Å microtubule develop in a localized region of the blastocoel wall (Fig. 16), and furrowing is initiated. As this internal furrow proceeds outward, transient close junctions of both sizes develop at some distance from the blastocoel. The apposed membranes proximal to the region of the close junctions separate, thereby adding substantial quantities of surface to the blastocoel. At several points along the furrow, glycogen areas are observed (Fig. 10), and these may fuse with the cell surface. The external furrow which forms on the outer surface of the blastomere during this division proceeds inwardly in a manner similar to that described for the animal pole furrow of the second division, forming close junctions along its entire length. The external furrow is preceded by a less extensive ectoplasmic zone than is the internal furrow emerging from the opposite, blastocoel side of the cell, but this ectoplasm does contain some small glycogen areas and many smooth-surfaced vesicles. Completion of the third division results in the formation of animal and vegetal blastomeres.

The next several divisions continue to demonstrate that furrows forming on opposite sides of a given blastomere behave differently with respect to junction formation. As in the third division, close junctions within the furrows forming at the blastocoel occur deep to the point of furrow initiation, or may not develop at all. Furthermore, these internal furrows are surrounded by substantial ectoplasm. In such furrows, enlargement of the blastocoel and liberation of materials into it continue to take place. In contrast, external furrows form close junctions very near their point of inception, and are not surrounded by subcortical ectoplasm. In all furrows, filaments within the cortex of the furrow tip are observed less frequently than in previous divisions, or may be entirely absent. If the latter

---

Fig. 14. A portion of the cortex from the lateral wall of a vegetal pole furrow tip. Faint 80 Å filaments are present in a band (FB) parallel to the plasmalemma. A crystalline yolk platelet occupies the lower half of the micrograph. DMSO-trialdehyde. × 132000.

Fig. 15. A tangential section of a second-cleavage animal pole furrow tip. Interweaving 80 Å filaments form a band immediately beneath the surface. Acrolein-glutaraldehyde. × 53000.

Fig. 16. Blastocoel side of an animal blastomere in an eight-cell embryo. Note the microtubule extending parallel to the cell border (arrow). While only a small portion of this microtubule is shown, the structure actually measures 18 μm in length. DMSO-trialdehyde. × 37000.
is the case, a dense band devoid of particulate matter is usually observed in the cortex of the furrow tip.

As described previously (Kalt, 1971), in succeeding cleavages all furrows progressively undergo morphological changes until internal and external furrows become identical. As the blastocoel approaches its maximum size, junctions within internal furrows are formed closer and closer to the blastocoel, so expansion of furrows contributes progressively less surface to the blastocoel. Furrow tips no longer have obvious filaments, but retain the dense band described earlier. Finally, there is a reduction in the amount of glycogen and other materials liberated into the blastocoel and its tributary furrows, although some activity of this nature is retained into the early gastrula.

DISCUSSION

These ultrastructural observations support the contention that the first cleavage in *Xenopus* is directly involved in both the division of the zygote and the formation of the blastocoel (Kalt, 1969; 1971). The earliest manifestation of blastocoel formation is the distension of the animal pole furrow tip. Formation of two classes of close junctions at both the superior and inferior margins of this distension seals off the nascent blastocoel from the surrounding extracellular space. Furthermore, the dehiscence of cytoplasmic vacuoles at the cell surface provides additional membrane for both the elongation of the furrow and the enlargement of the blastocoel.

The developmental sequence and possible morphogenetic significance of intercellular junctions has been studied in many different species (Gustafson & Wolpert, 1961; Trelstad, Hay & Revel, 1967; Trinkaus & Lentz, 1967), but the present work is the first to suggest the importance of close junctions so early in development. In *Xenopus*, close junctions are involved in both the initial formation and the subsequent development of the blastocoel. As cleavage progresses, many of the furrows originating from the blastocoel surface totally lack close junctions. This permits the internal furrows to dilate, and their membranes to become part of the surface of the enlarging blastocoel. Concomitantly, close junctions are formed near the origin of each corresponding external furrow, thus maintaining the separation of the blastocoel from the external milieu. In this manner, close junctions, by their presence or absence, affect the size, shape, and location of the blastocoel in *Xenopus*. Similar close junctions have been illustrated, but not discussed, in the embryo of another amphibian, *Triturus* (Selman & Perry, 1970), and in the crustacean, *Artemia* (Anteunis, Fautrez-Firlefyn & Fautrez, 1961). Thus, it seems possible that close junctions may play an important role during cleavage in a number of different organisms.

The fusion of vacuoles with the cell surface during cleavage has a dual function: addition of new membrane to the surface, and liberation of material
Electron microscope study of blastocoel formation

into extracellular spaces. The role of vacuoles in expansion of the surface has been extensively discussed by Selman & Perry (1970) and by Bluemink (1970). These authors cite indirect morphological and physiological observations which suggest that new surface is added to the furrow region by fusion of vacuoles with the plasmalemma. The morphological evidence presented in the present study supports this view, but direct experimental confirmation of this process is still lacking.

Insofar as secretion is concerned, two major mechanisms appear to be involved, the first being a merocrine process and the second involving two distinct apocrine processes. The merocrine process involves fusion of single membrane-bounded vacuoles with the plasmalemma, with subsequent extrusion of their contents. Such vacuoles contain either glycogen or other materials. The first apocrine process involves rupture of the plasma membrane adjacent to glycogen areas, and a spilling of this material into the extracellular space. The second apocrine process is a highly modified one, and resembles that recently described in rat adrenal cortex by Rhodin (1971). The process involves packaging of glycogen into spherical structures delimited by two distinct membranes. The outer membrane then fuses with the plasmalemma, and a packet of glycogen, still delimited by the inner of the two membranes, is released into the external milieu. Once released, these packets may rupture, permitting the glycogen to mingle with other released extracellular substances. All the mechanisms of release may occur concurrently. Together, the operation of the secretory mechanisms is undoubtedly responsible for the presence of the large quantities of extracellular glycogen found in previous studies of Xenopus embryos (Van Gansen, 1966, 1967; Van Gansen & Schram, 1969).

Those single membrane-bounded secretory vacuoles which do not contain glycogen usually contain instead a flocculent, finely fibrillar material, and are similar in appearance to the 'light vesicle' reported to occur in Ambystoma by Bluemink (1970). In Xenopus, such vacuoles are occasionally noted in relation to Golgi complexes, and large numbers of them are seen in the juxtanuclear regions of the cell throughout the period of blastulation (Kalt & Tandler, 1971). The nature and function of these vacuoles is unknown, but preliminary histochemical evidence (Kalt, unpublished) indicates that some of them may contain mucosubstance. Bluemink (1970) speculated that similar vacuoles may have a role in calcium regulation. That this is possible is suggested by the work of Stableford (1967), who demonstrated that the amount of calcium in the blastocoel increases dramatically as cleavage progresses. In any event, secretion of materials into the blastocoel and other extra-cellular spaces is not restricted to Xenopus, but occurs in other organisms such as echinoderms (Monné & Hárde, 1951; Motomura, 1966), crustaceans (Fautrez-Firlefyn & Fautrez, 1967), and other amphibians (Motomura, 1960, 1967), and deserves further investigation.

While cytoplasmic filaments are abundant close to the surface of the forming animal pole furrow tip during the first division, they are never observed in
appreciable numbers in relation to the forming blastocoel. Those few filaments noted near the beginning blastocoel probably are vestiges of the filamentous network which existed when that area was still an undifferentiated furrow. Once the presumptive blastocoel is established, the cortical filaments disappear from the blastocoel wall, and seem to be restricted to the furrow tip lying below the blastocoel. During the third division, however, filaments again form in the cytoplasm that borders the blastocoel in the region where the internal portion of the third cleavage furrow will develop. This suggests that while filaments are not directly involved in the conformation of the blastocoel cavity, their precursors are probably present in the cortical regions surrounding the blastocoel.

The observation that not all furrows possess filaments raises some question as to their necessity for division. Experimental observations (Kubota, 1966, 1969; Sawai, Kubota & Kojima, 1969) suggest that real differences exist between the first animal and vegetal pole furrows in their capacities to recover from deformations. Furthermore, ultrastructural observations made during this and other investigations (Selman & Perry, 1970; Kalt & Tandler, 1971) suggest that the presence of filaments is neither a constant nor a uniform phenomenon in amphibian embryos. Instead, it appears that distinct filaments are localized only in scattered regions of a given furrow at various times during its progress through the cell, as opposed to the extensive uniform arrays reported in some invertebrate embryos (Schroeder, 1968; Tilney & Marsland, 1969; Szollosi, 1970). The possibility that the occasional absence of distinct filaments in some amphibian furrows, as reported in the present study, is only a fixation artifact has been ruled out on the basis of an investigation of fixation parameters by Kalt & Tandler (1971). These authors have demonstrated that real differences in filament orientation and occurrence do in fact exist within single amphibian embryos. This is true even though the extent and arrangement of the filaments vary with the type of preservation procedure employed. However, the fact that an inverse relationship between the density of cytoplasmic arrays and the preservation of cytoplasmic matrix was found to exist suggests that the apparent concentration and orientation of filaments around a furrow is dependent on the fixation procedure utilized. Since, as Bluemink (1970) has pointed out, filaments do not have to be absent, but merely need be randomly oriented to be non-functional in division, alternative filament configurations merit further investigation in amphibians.

In conclusion, it is now clear that in *Xenopus*, morphological specializations associated with blastocoel formation occur at the beginning of the first division of the zygote. In addition, the same specializations are intimately associated with mechanisms involved in the division of the cell, making the two processes inseparable during the early development of the embryo. The regular pattern of events concerned with blastocoel development demonstrates that the blastocoel undergoes a complex genesis, and is more than a haphazardly formed separation between blastomeres.
Electron microscope study of blastocoel formation

The author would like to thank Drs Bernard Tandler and Joseph Grasso for their continued guidance during the course of this investigation and in the preparation of this manuscript. This research was supported in part by N.I.H. grant AM 11896 awarded to Joseph Grasso, N.I.H. grant GM 00820-09 awarded to the Department of Anatomy, Case Western Reserve University, and an N.S.F. Predoctoral Fellowship Award to the author. Some preliminary aspects of this work were presented at the tenth annual meeting of The American Society for Cell Biology, 1970.

REFERENCES


*(Manuscript received 4 November 1970)*