Studies on the gastrulation of amphibian embryos: Light and electron microscopic observation of a urodele *Cynops pyrrhogaster*

By NORIO NAKATSUJI

From the Department of Zoology, University of Kyoto

SUMMARY

The course of gastrulation in embryos of a urodele, *Cynops pyrrhogaster*, was studied with 1 μm Epon sections and transmission and scanning electron microscopy. During the initial period of gastrulation, the bottle cells and a groove are formed in the dorsal part. The outer ends of the bottle cells have many microvilli, an electron-dense layer and many small vesicles. Microtubules are present parallel to the long axis of the bottle cells, and a yolk-platelet-free region is observed at the inner end. Thereafter, the archenteric roof makes contact with the inner surface of the blastocoelic wall. Cells of the archenteric roof form lobopodia, filopodia and lamellipodia. These cells make many focal contacts, having gaps of less than 20 nm, with the blastocoelic wall. Invaginating mesodermal cells of the lateral and ventral parts also form pseudopodia, and are in contact with the blastocoelic wall. Some of these cells appear to flatten against the wall. These observations suggest that, after the bottle cells and the blastoporal groove are formed, the invaginating cells actively migrate along the inner surface of the blastocoelic wall, and that these locomotive forces have an important role in the morphogenetic movements during gastrulation.

INTRODUCTION

The ‘sinking in’ activity of the bottle cells (Holtfreter, 1944; Baker, 1965) and the epibolic expansion of the ectodermal layer (Holtfreter, 1944) have been proposed as the forces that drive the morphogenetic movements during gastrulation in amphibian embryos. Besides these two forces, the present author has proposed active cell locomotion along the inner surface of the blastocoelic wall in anuran embryos, from the observation that cells of the foregut endoderm and mesoderm form pseudopodia coinciding with their movement toward the animal pole along the blastocoelic wall (Nakatsuji, 1974, 1975). Because the process of gastrulation in urodele embryos has many differences from that in anuran embryos, it is necessary to investigate whether the cells form pseudopodia and whether active cell locomotion is suggested also in urodele gastrulation.

Observations of urodele gastrulation reported hitherto are either those with...
paraffin sections (Vogt, 1929) or those with electron microscopy but restricted to a small part such as the bottle cells (Perry & Waddington, 1966). In this report, the course of gastrulation of a urodele, *Cynops pyrrhogaster*, is observed by use of semi-thin (about 1 μm) Epon sections and with transmission and scanning electron microscopy. It is shown that the invaginating cells form pseudopodia and it is suggested that active cell locomotion along the inner surface of the blastocoelic wall probably plays an important role in urodele gastrulation.

**Material and Methods**

**Embryos**

Fertilized eggs of *Cynops pyrrhogaster* (formerly named *Triturus pyrrhogaster*) were obtained from natural ovipositions by females collected at Kojoro Pond of Mt. Hira in Shiga Prefecture. The stages of the embryos were determined according to Okada & Ichikawa (1947). Embryos of stage 9–16 (early blastula to initial neurula) were divested of the jelly layer and chorion manually.

**Semi-thin and ultrathin sections**

Five or six embryos at each stage (total of 58 embryos) were fixed for half a day at 3–5 °C in a buffered (0.05 M phosphate buffer, pH 7.2) 2.5 % glutaraldehyde solution containing paraformaldehyde (2 %) and picric acid (0.1 %) (Ito & Karnovsky, 1968). Fixed blastulae were cut into halves with two razor blades, in the plane passing the animal and vegetal poles, using a binocular microscope. Fixed gastrulae and neurulae were cut in the mid-sagittal or equatorial plane. These halves were fixed in the same fresh fixative solution additionally for a day, rinsed in the same buffer that contained sucrose (4%), and were post-fixed for 2 h at 2 °C in a buffered (pH 7.2–7.4) 1 % osmium tetroxide solution that contained sucrose (2.25%). The samples were dehydrated in a graded series of

![Figure 1](image-url)
ethanol and transferred through propylene-oxide, propylene-oxide Epon mixtures and into Epon. Finally the samples were embedded into Epon moulds.

One μm sections were mounted on glass slides that had been previously coated with 0.5% collodion (Aoki & Gutierrez, 1967) and were stained with 1% toluidine blue (in 0.1 M phosphate buffer, pH 7.4) for 30 min at 90 °C or with 0.5% methylene blue and 0.5% azur II (in 0.5% sodium borate) (Richardson, Jarett & Finke, 1960) for 10 min at 90 °C. They were examined with a bright field microscope. After the 1 μm sections were cut and examined, blocks were trimmed for the desired region, and ultrathin sections were cut. The ultrathin sections were mounted on 100-mesh Formvar-film-coated copper grids, double stained with 1% uranyl acetate (in 10% methanol) and lead citrate (Reynolds, 1963), and examined with a Hitachi electron microscope (Model HU-11D-1) operated at 75 kV.

Scanning electron microscopy

Embryos for scanning electron microscopy were fixed for a day in the same pre-fixation solution described above. They were rinsed in the phosphate buffer (0.05 M, pH 7.2) that contained sucrose (4%), and were dehydrated in a graded series of ethanol. Samples were transferred to iso-amyl acetate and allowed to dry in dust-free air. The dried embryos were fractured carefully with fine forceps using a binocular microscope. Pieces consisting of the blastocoelic wall and the invaginating cells attached to it were picked up and placed so that the invaginating cells were uppermost. Some of the invaginating cells were removed with a hair loop until the optimal number remained on the inner surface of the blastocoelic wall. In some embryos, fracturing was done before the dehydration. In this case, pieces of the embryos were dehydrated and dried as described above. Dried pieces for scanning electron microscopy were mounted on specimen holders using an electrically conductive paint, Dotite paint D-550 (Fujikura Kasei Co. Ltd, Tokyo), and coated with carbon and gold (or gold:palladium = 60:40) in a vacuum evaporator. They were photographed with a JEOL JSM S1 scanning electron microscope.

Figure 2

Light micrographs of two embryos at stage 12b.

(A) A mid-sagittal section.

(B) An enlarged micrograph of the area shown by the small rectangle in (A).

(C) An equatorial section.

(D) An enlarged micrograph of the area shown by the rectangle (left) in (C). An invaginating cell sends a cell process (arrow) toward the blastocoelic wall.

(E) An enlarged micrograph of the rectangle (right) in (C). ar, archenteron.

(F) An enlarged micrograph of the rectangle in (E).
Microscopic observation of urodele gastrulation
RESULTS

Stage 9–10 (early to late blastula)

Some blastomeres are observed to form blebs or lobopodia (Fig. 1B). Electron micrographs show that these lobopodia are poor in yolk platelets and oil droplets (Fig. 1C).

Stage 11–12a (straight to crescent-shape blastopore)

The bottle cells and the blastoporal groove are formed in the dorsal lip region (Fig. 1D). At the inner ends of the bottle cells, yolk-platelet-free regions are observed to exist (Fig. 1E and F). The outer ends of these cells have many microvilli, an electron-dense layer and many small vesicles (Fig. 1G). Microtubules are present parallel to the long axis of the bottle cells (Fig. 1H).

Stage 12b (semi-circular-shape blastopore)

The archenteron is formed in the dorsal part, and its roof appears to be in contact with the inner surface of the blastocoelic wall (Fig. 2A, C). The archenteric roof is more than two cells thick at this stage (Fig. 2E). The invaginating cells are observed to form pseudopodia of various shapes, which are seen along the inner surface of the blastocoelic wall (Fig. 2B, D, F and Fig. 3). These pseudopodia are poor in yolk platelets. The invaginating cells make many focal contacts having gaps of less than 20 nm with the cells of the blastocoelic wall (Fig. 3A–E). Focal contacts were observed between the invaginating cells as well.

Stage 12c (horse-shoe-shape blastopore)

The archenteric roof elongates and thins to become almost one-cell thick (Fig. 4A, B). Cells of the archenteric roof are observed to form pseudopodia of various shapes (Fig. 4C), and make focal contacts having gaps of less than 20 nm with the blastocoelic wall (Fig. 4D).

**Figure 3**

Electron micrographs of the interface between the archenteric roof and the inner surface of the blastocoelic wall of the same embryos shown in Fig. 2.

(A) A portion of Fig. 2B. The blastocoelic wall is to the left.

(B) An enlarged micrograph of the area shown by the small rectangle in (A). The upper cell is part of the blastocoelic wall.

(C) A portion of Fig. 2B. An invaginating cell forms a long cell process (arrow). The blastocoelic wall is to the left.

(D) An enlarged micrograph of the area shown by the small rectangle in (C). Granular material is present in the intercellular space. The upper cell is part of the blastocoelic wall.

(E) A portion of Fig. 2F. A fine cell process is formed by an invaginating cell. Granular material is present in the intercellular space. Cross and oblique sections of the cell processes are present (arrows).
Stage 13-14 (large to small yolk plug)

The archenteron becomes large (Fig. 5 A, C). Its roof is one-cell thick (Fig. 5B, D). In the ventro-lateral part, the invaginating cells form pseudopodia, and appear to be in contact with the inner surface of the blastocoelic wall (Figs. 5E, F and 6A, B). These pseudopodia have the shapes of filopodia and of flattened pseudopodia or lamellipodia. An amorphous material is present in the intercellular space between the invaginating cells and the blastocoelic wall (Fig. 6C, D).

Stage 15-16 (final gastrula to initial neurula)

The neural plate becomes conspicuous, and presumptive notochordal cells become closely packed one on another (Fig. 7A, B, E and F). The intercellular space between the presumptive notochordal cells and the neural plate cells becomes much smaller than at earlier stages (Fig. 7C, D). In the dorso-lateral part, the presumptive somite and lateral plate cells form pseudopodia and appear to be in contact with the inner surface of the ectodermal layer (Fig. 7G, H).

Scanning electron microscopy

The invaginating cells of the archenteric roof were observed with a scanning electron microscope (Fig. 8A-F). These cells form filopodia and thin lamellipodia, and are apparently attached to the inner surface of the blastocoelic wall.

Discussion

From the blastula stage, some blastomeres were observed to protrude blebs or lobopodia toward the blastocoel or intercellular spaces. Lobopodium formation was observed from the initial gastrula stage and was rare at the blastula stage in the case of anuran embryos (Nakatsuji, 1974, 1975). In anuran blastulae, blastomeres are closely packed and the inner surface of the blastocoelic wall is relatively smooth. Formation of lobopodia or blebs was observed from the blastula stage in teleost embryos (Lentz & Trinkaus, 1967; Wourms, 1972a), in which the deep cells protrude blebs toward the segmentation cavity. In the case of echinoderm embryos, cells of the blastocoelic wall protrude bulbous pseudopodia at the blastula stage before the filopodia are formed by the mesenchyme cells (Gustafson & Wolpert, 1967; Gibbins, Tilney & Porter, 1969).

Electron micrographs showed that the outer ends of the bottle cells have many microvilli, an electron-dense layer and many small vesicles. Microtubules were observed to be present parallel to the long axis of the bottle cells. These features of the bottle cells had been already reported in Triturus (Perry & Waddington, 1966) and in Hyla (Baker, 1965), and probably show the contraction of the outer end and the elongation of the cell body. Yolk-platelet-free regions were
Fig. 4. Micrographs of an embryo at stage 12c. (A) A mid-sagittal section. ar, archenteron; bl, blastocoel. (B) An enlarged light micrograph of the area shown by the small rectangle in (A). ar, archenteron. (C) An electron micrograph of the interface between the archenteric roof (lower) and the blastocoelic wall (upper) shown in (B). (D) An enlarged electron micrograph of the area shown by the rectangle in (C).
observed at the inner ends of the bottle cells. These regions resemble the hyaline cap of the disaggregated blastomeres of vermiform or cylindrical shape (Holtfreter, 1946, 1948), which perform the peristaltic movement and uni-directional locomotion in vitro. Formation of the blastoporal groove was shown to occur in the isolated vegetal hemisphere of the urodele embryos (Stableford, 1948; Doucet-de Bruïne, 1973). This suggests that the formation of the bottle cells and the blastoporal groove are brought about autonomously by the cells in that region.

Electron micrographs of the lobopodia in the blastula and the yolk-platelet-free region of the bottle cells showed that these regions are poor in large organelles such as yolk platelets and oil droplets. Their appearances are similar to the lobopodia in echinoderm late blastulae (Gibbins et al. 1969) and those in teleost blastulae (Lentz & Trinkaus, 1967; Trinkaus & Lentz 1967).

At stage 12b, the archenteron is formed. Its roof is more than two-cells thick at this stage and becomes one-cell thick shortly after. Cells of the archenteric roof and the invaginating mesoderm of the lateral and ventral parts were observed to form pseudopodia and to be in contact with the inner surface of the blastocoelic wall. These pseudopodia have the shapes of lobopodia, filopodia and lamellipodia flattened against the blastocoelic wall, and are poor in yolk platelets and oil droplets. Their morphological appearances have similarity with the pseudopodia formed by deep cells of teleost embryos during epiboly (Lentz & Trinkaus, 1967; Trinkaus & Lentz, 1967; Wourms, 1972b; Trinkaus, 1973). The pseudopodia shown in Fig. 6 appear to be flattened against the inner surface of the blastocoelic wall. Their ends are raised up from the wall. Their appearances resemble the leading lamella and lamellipodium of the fibroblast in tissue culture (Abercrombie, Heaysman & Pegrum, 1971; Heaysman & Pegrum, 1973). Cells of the archenteric roof make many focal contacts having gaps of less than 20 nm with the inner surface of the blastocoelic wall and with the other invaginating cells. These focal contacts seem to have enough adhesive

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**FIGURE 5**

Light micrographs of two embryos at stage 13.

(A) A mid-sagittal section. *ar*, archenteron; *bl*, blastocoel.

(B) An enlarged micrograph of the area shown by the rectangle in (A).

(C) An equatorial section. *ar*, archenteron.

(D) An enlarged micrograph of the area shown by the upper rectangle in (C).

(E) An enlarged micrograph of the lower rectangle in (C).

(F) An enlarged micrograph of the rectangle in (E). An invaginating mesodermal cell takes a flattened shape against the inner surface of the blastocoelic wall. A fine cell process (arrow) is formed by another cell.
Fig. 6. Micrographs of an embryo at stage 14. (A) A light micrograph of the ventrolateral region. Invaginating mesodermal cells (left) form fine cell processes (arrows) along the blastocoelic wall (right). (B) A near region of (A). A part of a mesodermal cell appears to flatten against the inner surface of the blastocoelic wall. (C) An electron micrograph of a fine process shown in (A). (D) An electron micrograph of the same cell process shown in (C) in another section. An amorphous material is present in the intercellular space.
force for the locomotion of the cells along the wall (Curtis, 1967). In the case of chick embryos, the migrating mesoblasts make focal close 2.5–10 nm junctions and focal tight junctions with the epiblasts and hypoblasts (Trelstad, Hay & Revel, 1967; Revel, Yip & Chang, 1973). The deep cells in teleost embryos make 20 nm and more close contacts with the other cells during locomotion (Trinkaus & Lentz, 1967). Focal close contacts were observed between the mesodermal cells in anuran gastrulae (Johnson, 1970).

By use of scanning electron microscopy, invaginating cells were observed clearly to form filopodia and lamellipodia flattened against the inner surface of the blastocoelic wall. Filopodia and lamellipodia were observed, with scanning electron microscopy, to be formed by the migrating cells during corneal endothelium formation in the chick embryo (Bard, Hay & Meller, 1975; Nelson & Revel, 1975).

The formation of pseudopodia by the invaginating cells and their contact with the blastocoelic wall suggest that the cells migrate actively along the blastocoelic wall and that these locomotive forces have an important role in the morphogenetic movements during gastrulation. This concept was stated previously in the case of anurans (Nakatsuji, 1974, 1975) from a study of the formation of the pseudopodia by the invaginating cells of the foregut endoderm and mesoderm. The archenteric roof of anuran embryos is more than two-cells thick throughout gastrulation. Accordingly the suspected locomotive forces of the cells, which are attached to the blastocoelic wall, must be transported to the cells which line the archenteron, in order to bring about the elongation of archenteron. On the other hand, the archenteric roof of the Cynops embryo is almost one-cell thick. Accordingly the locomotive forces of these cells can work immediately as the elongation force of the archenteric roof.

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REFERENCES


Figure 7
Micrographs of two embryos at stage 16.

(A) A sagittal section near the median plane. ar, archenteron.

(B) An enlarged light micrograph of the area shown by the rectangle in (A).

(C) An electron micrograph of the interface between the presumptive notochord (lower) and the neural plate (upper) shown in (B).

(D) An electron micrograph of the interface at higher magnification.

(E) An equatorial section. ar, archenteron.

(F) An enlarged light micrograph of the area shown by the rectangle in (E).

(G) An enlarged light micrograph of the area shown by the upper small rectangle in (E). Presumptive somite cells (lower) form lobopodia and appear to be in contact with the ectodermal layer (upper).

(H) An enlarged light micrograph of the lower small rectangle in (E). A mesodermal cell (lower) appears to be in contact with the ectodermal layer (upper) with a pseudopodium.
Figure 8

Scanning electron micrographs of the archenteric roof cells on the inner surface of the blastocoelic wall at stage 12b. Cells are migrating toward the left-above direction.

(A) A micrograph showing four invaginating cells attached to the blastocoelic wall.

(B) An enlarged micrograph of the portion shown by the rectangle in (A).

(C) An enlarged micrograph of the rectangle in (B).

(D) A micrograph when the tilt angle was changed from that in (A). An invaginating cell at the centre of this figure corresponds to the uppermost cell in (A).

(E) An enlarged micrograph of the portion shown by the smaller rectangle in (D).

(F) An enlarged micrograph of the larger rectangle in (D).