SURFACE SPECIALIZATIONS OF

FUNDULUS CELLS AND THEIR RELATION TO

CELL MOVEMENTS DURING GASTRULATION

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ABSTRACT

Cell movements in *Fundulus* blastoderms during gastrulation were studied utilizing time-lapse cinemicrography and electron microscopy. Time-lapse films reveal that cells of the enveloping layer undulate and sometimes separate briefly but remain together in a cohesive layer. During epiboly, the marginal enveloping layer cells move over the periblast as it expands over the yolk sphere. Movement occurs as a result of ruffled membrane activity of the free borders of the marginal cells. Deep blastomeres become increasingly active during blastula and gastrula stages. Lobopodia project from the blastomeres in blastulae and adhere to other cells in gastrulae, giving the cells traction for movement. Contact specializations are formed by the lateral adjacent plasma membranes of enveloping layer cells. An apical junction is characterized by an intercellular gap of 60–75 A. Below this contact, the plasma membranes are separated by 120 A or more. In mid-gastrulae, cytoplasmic fibrils occur adjacent to some apical junctions, and small desmosomes appear below the apical junction. Septate desmosomes also appear at this time. A junction with an intercellular gap of 60 A occurs between marginal enveloping layer cells and periblast. Contacts between deep blastomeres become numerous in gastrulae and consist of contacts at the crests of surface undulations, short areas of contact in which the plasma membranes are 60 or 120 A apart, and long regions characterized by a 200-A intercellular gap. Lobopodia contact other blastomeres only in gastrulae. These junctions contain a 200-A intercellular space. Some deep blastomeres are in contact with the tips of periblast microvilli. The mechanism of epiboly in *Fundulus* is discussed and reevaluated in terms of these observations. The enveloping layer is adherent to the margin of the periblast and moves over it as a coherent cellular sheet. Periblast epiboly involves a controlled flow of cytoplasm from the thicker periblast into the thinner yolk cytoplasmic layer with which it is continuous. Deep cells move by adhering to each other, to the inner surface of the enveloping layer, and to the periblast.

INTRODUCTION

The mechanisms by which cells move and contact each other have attracted increasing attention (2, 28, 31). Of these movements, one of the most impressive is epiboly of teleost eggs. During blastula stages, a blastoderm consisting of a few thousand cells surmounts a yolk sphere many times its size. Then, as gastrulation begins, it flattens and spreads over the yolk until it finally encompasses it. There are three separate structures involved in this epibolic spreading: the enveloping layer of the blastoderm, a precocious, cohesive epithelium that forms the outer membrane of the blastoderm;

the deep cells of the blastoderm, which engage in extensive morphogenetic movements beneath the spreading enveloping layer; and the periblast, a syncytial layer at the surface of the yolk beneath the deep cells and the enveloping layer. The role of these parts in the mechanism of epiboly has been studied extensively in eggs of the trout and *Fundulus*, and some progress has been made in recent years toward defining their distinctive activities and interactions (11, 31).

In *Fundulus heteroclitus* the periblast spreads independently of the blastoderm (29). If a blastoderm is removed, the periblast can complete epiboly in its absence. The blastoderm spreads independently of its position on the periblast, but appears to require the periblast as its substratum. The cells of the blastoderm seem to have an intrinsic capacity to spread in epiboly, given the presence of a subjacent spreading periblast. Dissociated cells of early gastrulae tend to flatten on the substratum and spread in culture, in contrast to blastula cells, which remain spherical with protruding lobopodia (30). These observations indicate that changes in the surface properties of the cells, such as their adhesiveness, lie at the basis of epiboly.

Further progress in the analysis of epiboly depends on a knowledge of the surface behavior of the cells in vivo, within the blastoderm, in relation to each other and to the periblast. The transparent egg of *Fundulus* is ideal for such studies. Preliminary observations of cell activity in the blastoderm, before and during epiboly, with time-lapse cinemicrography show that the formation of cell processes and their adhesion to other cells and to the periblast is essential to the initiation and continuance of epiboly.[1] The studies described in this paper were undertaken to correlate the fine structural characteristics of intercellular relationships with changes in cell surface activity and adhesiveness in the living system preceding and during epiboly

MATERIALS AND METHODS

The procedures employed in obtaining blastoderms of *Fundulus heteroclitus* have been described (21). Fixation in glutaraldehyde and osmium tetroxide was carried out in the same manner. Most sections were stained with lead hydroxide. Additional sections were stained with a saturated solution of uranyl acetate prior to

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[1] Trinkaus, J. P., and R. Ebstein. Time-lapse cinematographic study of cell movements during epiboly of *Fundulus heteroclitus*. In preparation.

lead hydroxide staining. All sections were examined with an RCA EMU 3F electron microscope. Stages 3 (two-cell) through 12⅓ (mid-gastrula) were examined and particular attention was paid to cell contacts and other surface specializations.

Time-lapse films were taken of dechorionated whole eggs. The larger lipid droplets were removed with an orally controlled micropipette, in order to improve observation of cell activities. This procedure had no apparent effect on the behavior and development of the blastoderm. Throughout the period of observation the egg rested in double-strength Holtfreter's solution (unbuffered) at the bottom of a deep depression *Paramecium* slide. The depression containing the egg was uncovered throughout the period of observation, to allow for adequate gaseous exchange with the atmosphere. All observations were made at room temperature (18°–25°C) with bright-field optics (Zeiss GFL) and an Arriflex 16 camera with DOM animation, time-lapse motor model 109, Arriflex Master Intervalometer, and Kodak Plus-X Pan film. The magnification was either 63 or 100 and the interval between frames was 2 or 4 sec.

RESULTS

Surface Activity of Blastomeres

Time-lapse cinemicrography reveals a changing pattern of behavior on the part of the deep blastomeres (see reference 21, for histology of developing blastoderms). During early cleavage, blastomeres show no surface activity. Beginning around the 64-cell stage, however, gentle undulations of the cell surface become evident. As cleavage continues and a segmentation cavity is defined, deep blastomeres gradually protrude blunt, rounded lobopodia with increasing frequency (Fig. 1). By middle blastula, lobopodia form quite rapidly, every minute or so. They are rounded or elongated hyaline projections which are withdrawn soon after they form. A new one then forms from another part of the cell surface and it in turn is withdrawn. There is no evidence that these lobopodia adhere to other cells during early and middle blastulae. The rapid formation and withdrawal of lobopodia without accompanying adhesions causes the cells to continually change shape in situ, with no translocation. The films give a picture of a constantly jostling mass of surface-active cells.

During late blastula, just before the onset of epiboly, many lobopodia begin to adhere to the surfaces of other cells. When this occurs, the lobopodium is stretched into either an elongate filopodium or a spreading membranous fan (Figs. 2

and 3 b). In either case, the adhesions give the cells traction, so that, as the processes retract, the cells may be pulled in the direction of the adhesion. In this manner, translocation of deep cells begins. This locomotory behavior is augmented with the onset of epiboly, with increasing numbers of moving cells. At first, the movements appear to be largely random and cause the deep cells to occupy the expanding space beneath the spreading enveloping layer. In the dorsal and lateral marginal regions of the blastoderm, however, the movements of the deep cells soon take on a directional component causing them to converge dorsally and form the embryonic shield.

Cells of the enveloping layer also show surface activity. They contract and expand and show surface undulations. There is no cell displacement, however, and they remain together in a cohesive layer (Figs. 2, 3 a, and b). Occasionally, the surfaces of two adjacent cells appear to separate over a part of the area of contact (Fig. 3 b). Cells bordering the gap show ruffled membrane activity or send processes across the gap (Fig. 3 b). The gap is short-lived and closes again in several minutes. During epiboly, the marginal cells of the enveloping layer crawl over the expanding periblast (Fig. 3 a, and b). The free surfaces of marginal cells show extensive ruffled membrane activity. The marginal cells often retract and then, with associated ruffled membrane activity, move again toward the periblast margin. The enveloping layer spreads over the yolk sphere until the marginal cells contact each other, closing the blastopore.

Enveloping Layer Contacts

The external cells of the blastoderm contain contact specializations of the apical portions of lateral adjacent plasma membranes (Figs. 4 to 8). During cleavage and blastula stages (3–10), a single junction with a narrow intercellular gap occurs between adjacent cells. A widened intercellular space occurs below this junction. In the gastrula stages and especially in mid-gastrula (stage 12⅓), junctional complexes consisting of two or three components are present between adjacent cells (Figs. 4 and 5). In these complexes, there is an initial zone of close contact (Figs. 4 and 5). Below the apical junction, the plasma membranes diverge at a small angle to produce an intercellular gap of 120 A (Figs. 5 and 6). In some cases, a small desmosome is present between or below

these two regions (Fig. 5). Occasionally, a series of two or three close junctions are present, separated by regions in which the intercellular space is 120 A or larger (Fig. 5). Proximally, the plasma membranes diverge to produce a wider intercellular space.

At low magnification of the apical junction, the plasma membranes appear as dense lines which come into close approximation at the place where the surface membranes of adjacent cells turn inward (Figs. 4 and 5). The intercellular space is extremely narrow and in places appears obliterated. The zone of close contact extends for about 0.5 μ after which the membranes diverge. The apical junction was observed in all sections of adjacent external cells and probably forms a continuous bandlike attachment around the cells. At higher magnifications, where the section is normal to the entire junction, there is no fusion of the plasma membranes; the intercellular space is maintained throughout. The intercellular space is extremely narrow and of fairly constant width, approximately 60–75 A (Figs. 6 and 7). The space is occupied by material of low density. In other sections the intercellular gap is obliterated, but this image is probably the result of curving or bending of the plasma membranes that, consequently, are sectioned tangentially. The adjacent lateral membranes pursue a parallel straight or slightly curved course along most of the junction (Figs. 6 to 8). In some places, the two membranes diverge and then converge to form a local dilation of the intercellular space.

The plasma membranes of *Fundulus* are about 75 A in thickness. The triple-layered structure of the membrane usually was not apparent in these preparations but, when observed, the two dense layers are 25 A in thickness and separated by a clear space of 25 A (Fig. 10).[2] The leaflets appear of equal size and density. On no occasions in the apical junctions where the plasma membranes are closely apposed do the outer two leaflets of the adjacent membranes fuse to form an intermediate line as in *zonulae occludentes*. Instead, the outer

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[2] The plasma membranes of developing *Fundulus* are of the thin type with a diameter of ~75 A. It has been noted that the trilaminar nature of these thin membranes is poorly revealed by glutaraldehyde or osmium tetroxide fixation (10, 13, 15). The trilaminar structure of the membranes is more obvious after permanganate fixation, although this fixative was not utilized in the present study.

leaflets of the adjacent unit membranes remain separated by the intercellular gap of 60–75 A.

The apical junction in stage 12⅓ (mid-gastrula) differs somewhat from the basic pattern described above. Coarse cytoplasmic fibrils, about 75 A in diameter, are present in the cytoplasm adjacent to some junctions (Fig. 6). Most of the fibrils extend parallel to the junction, splaying out into the cytoplasm at its distal and proximal margins. Another modification consists of transverse dense bars extending across the intercellular space between adjacent membranes. These bars are ∼25 A thick and occur at intervals of 50 A (Fig. 7). These junctions are similar to the septate desmosomes of many invertebrates (23).

Beneath the apical junction in stages 3–10, the plasma membranes diverge to form a wide intercellular space. In the gastrula stages, however, the lateral membranes often are separated by a distance of 120 A beneath the apical junction (Figs. 5 and 6). This zone extends for a variable distance, usually about 0.5 μ, before the plasma membranes diverge to form a wider intercellular space or lake. The plasma membranes take a nearly parallel course that is straight or curved along the 120-A gap. No increased density or fibrils occur in the cytoplasm adjacent to the plasma membranes in this region. In many places, the plasma membranes follow a zigzag course so that cytoplasmic processes interdigitate with one another (Fig. 4).

A desmosome occurs between a few surface cells at stage 12⅓, and is situated below the apical junction or the region in which the intercellular gap is 120 A. The desmosome is characterized by the presence of a zone of dense material in the cytoplasm adjacent to the plasma membrane (Fig. 8). The density is greatest near the plasma membranes and tapers off into the cytoplasm. The intercellular space is ∼200 A in diameter in this region. The desmosome is about 0.1 μ in length. Coarse fibrils are not associated with these desmosomes.

Enveloping Layer—Periblast Junction

At the periphery of the blastoderm, the enveloping layer comes into close contact with the periblast. The pale, empty-appearing cytoplasm of the periblast can be distinguished from the somewhat richer cytoplasm of the enveloping layer cells (Fig. 9). The junction between the periblast and enveloping layer is identical to that uniting enveloping layer cells (Figs. 9 and 10). The adjacent plasma membranes are parallel and separated by a space of 60 A. They follow a curved course for 0.2–0.4 μ before diverging to form an intercellular space of 120 A or more.


Figures 1 to 3   Printed from time-lapse films of normally developing *Fundulus* blastoderms. Bright-field optics. × 100.

Figure 1   Deep blastomeres of a blastula (stage 9½). Most cells possess short lobopodia, two being apparent in profile (L). Translocation of the deep cells has not yet begun. The margin of the blastoderm, where it joins the periblast, stretches diagonally across the lower portion of the field (arrows). Three periblast nuclei (PN) are present.

Figure 2   Deep blastomeres of a mid-gastrula (stage 12½) near the ventral blastoderm margin. An elongate lobopodium (L) is adhering to another cell at its tip. Another blastomere possesses an adhering fanlike process (arrow), derived from a short lobopodium. These cells are actively motile. At the upper left, the outlines of the flattened cells of the enveloping layer (EL) may be discerned.

Figure 3 a   Cells at the lateral margin of a mid-gastrula (stage 12½). Marginal cells of the flattened enveloping layer (MEL) are visible where they adhere to the periblast. Note that the enveloping layer cells also adhere closely to each other. The margin of the periblast is located just beyond the margin of the enveloping layer, but is not visible in this micrograph.

Figure 3 b   The same region of the blastoderm illustrated in Fig. 3 a, 14½ min later, showing the progress of epiboly or downward migration of the enveloping layer and periblast over the yolk sphere (note position of marginal enveloping layer cells). A gap (arrow) has formed temporarily between three cells of the enveloping layer. Note that thin surface extensions appear to connect two of the cells. F, filopodium of a deep cell.
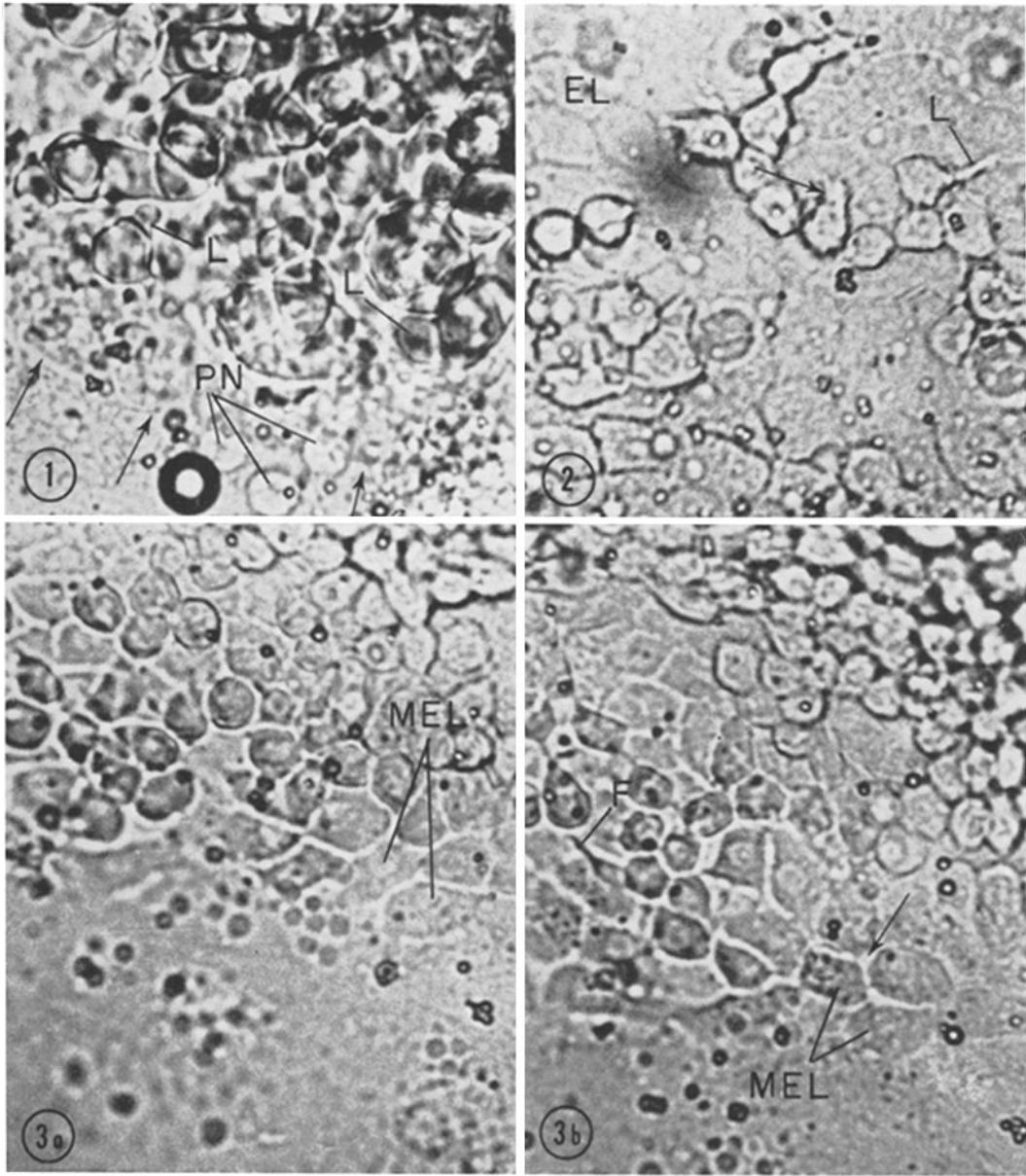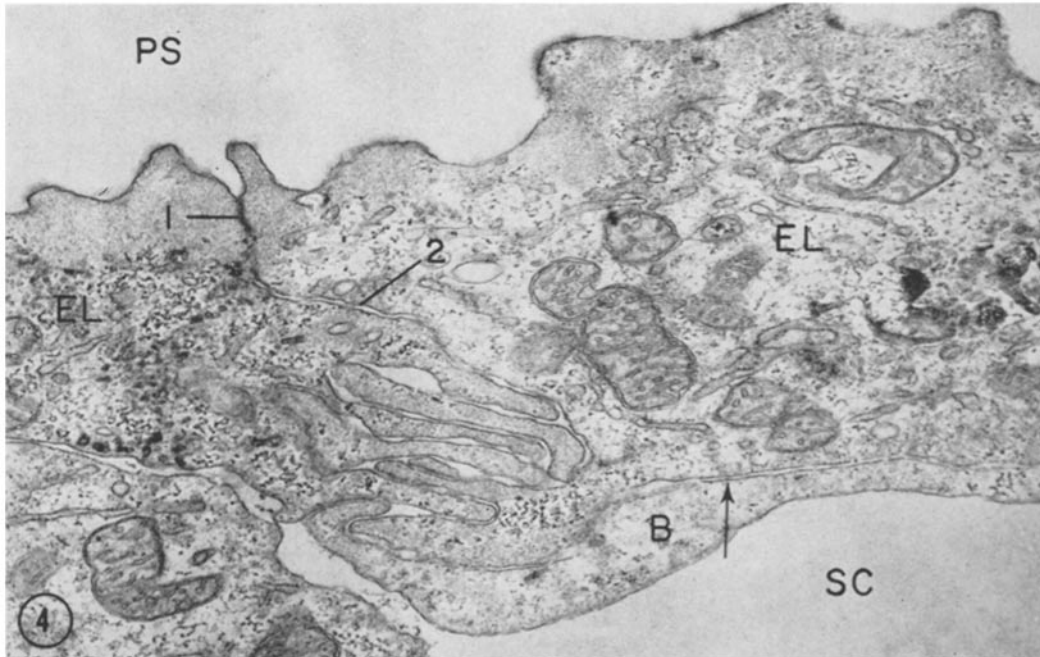
FIGURE 4 Enveloping layer cells (*EL*) of a mid-gastrula (stage 12⅓). The initial contact between adjacent cells is one in which the plasma membranes are closely apposed (*1*). Below this junction, the intercellular space widens (*2*). Several cytoplasmic arms interdigitate with one another. Note the undulating contours of the surface of the enveloping layer. The hyaloplasm beneath the plasma membrane bordering the perivitelline space (*PS*) is of medium density and contains few organelles. Other areas of the cytoplasm are less dense and contain mitochondria, membranous profiles, and glycogen. A portion of a deep blastomere (*B*) is separated from the under surface of the enveloping layer cells by a space of ∼200 A (arrow). *SC*, segmentation cavity. × 25,000.

Blastomere—Blastomere Contacts

The deep blastomeres are situated in the segmentation cavity between the periblast and enveloping layer. In some places, adjacent plasma membranes are closely approximated to form several types of junctions. The surfaces of many cells are undulated and come into contact at the tips of the projections or hillocks (Fig. 11). Some contacts occur at one point or only for very short distances. In some places, no intercellular space could be observed between the tips of the projections or undulations, but in other regions they are separated by a space of 120 A. In addition to these point contacts, the areas of contact between plasma membranes also extend over longer distances. In the more extensive junctions (∼0.5 μ), the plasma membranes are parallel and separated by spaces of either 120 or 60 A (Fig. 11). The latter are similar to the apical contacts between enveloping

layer cells, but only a few have been observed between deep blastomeres.

In contrast to the intermittent junctions, some blastomeres show an extensive region of contact with the undersurface of the enveloping layer (Fig. 4) or between themselves (Fig. 12). In these instances, the plasma membranes are roughly parallel over long distances. The intercellular space is about 200 A wide and slightly irregular. There are no cytoplasmic specializations adjacent to the contacts between blastomeres. Desmosomes were not observed between deep blastomeres.

Blastomeres of the blastula and gastrula stages send out pseudopodial projections or lobopodia. Only one type of lobopodium-blastomere contact was observed (Fig. 13), and this occurs only in late blastula and gastrula stages. In the region of contact the plasma membranes are parallel and separated by a space of 200 A. This junction usually extends for long distances although a few
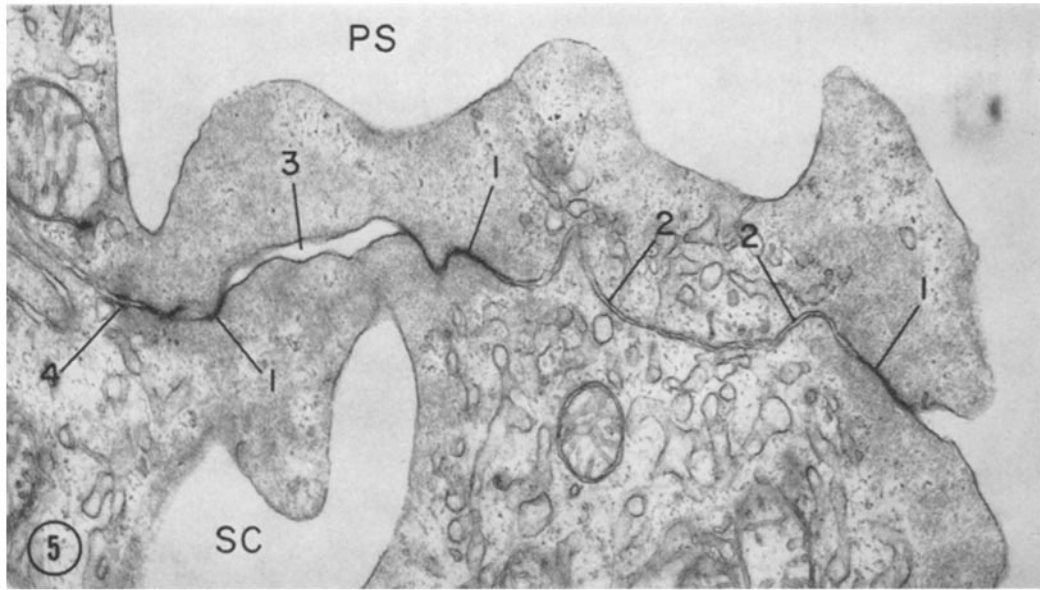
FIGURE 5 Contact specializations between enveloping layer cells of a mid-gastrula (stage 12⅓). In the initial junction and intermittently in other regions, the plasma membranes are closely apposed (1). Below the first contact, the plasma membranes are parallel and separated by a distance of 120 A (2). Another region of close apposition of plasma membranes occurs, after which the membranes diverge to form an intercellular lake (3) and then converge again. Below this last region of close contact a small desmosome occurs (4). Fibrils occur in the cytoplasm adjacent to the first two zones of close contact. The surface of the enveloping layer contains blunt undulations. The hyaloplasm beneath the plasma membranes is denser and contains fewer organelles than in deeper portions of the cells. PS, perivitelline space; SC, segmentation cavity. × 26,000.

short regions of contact were observed. Filopodia were observed in the films but not in the electron micrographs. Their absence in the latter could be explained by contraction of blastomeres upon immersion in fixative.

A large number of low power random survey micrographs of deep blastomeres were taken to estimate the approximate relative frequencies of occurrence of the different types of contacts. In the early blastulae (stages 8-9), about half the cell surfaces border the segmentation cavity and do not contact other cells. In the remaining cell surfaces, junctions at one point and contacts where the plasma membranes are separated by a 120-200-A space over short and long distances occur in approximately equal numbers. Only a few close junctions where the membranes are separated by 60 A were observed. The cell contacts increase in number significantly at the end of the blastula stage (stage 10) and become most numerous at the

mid-gastrula (stage 12⅓). Fewer areas of free cell borders occur in the late blastula and early gastrula and are extremely rare by the mid-gastrula (stage 12⅓). In the latter stage almost all cells are in contact. The most numerous junctions are the contacts at a single point or the short junctions characterized by a 120-A intercellular space. These contacts usually occur in series separated by wide intercellular spaces. Regions where the plasma membranes are parallel for long distances are not more numerous. The close junctions (60 A) are most frequent at mid-gastrula (stage 12⅓), but are still not nearly so numerous as the other types. Contacts between lobopodia and adjacent cell surfaces occur only in late blastula and in gastrula stages (10-12⅓). Lobopodia prior to this stage protrude freely into the segmentation cavity (see Fig. 10 in the preceding paper). Association of deep blastomeres and enveloping layer cells becomes common in gastrulae.
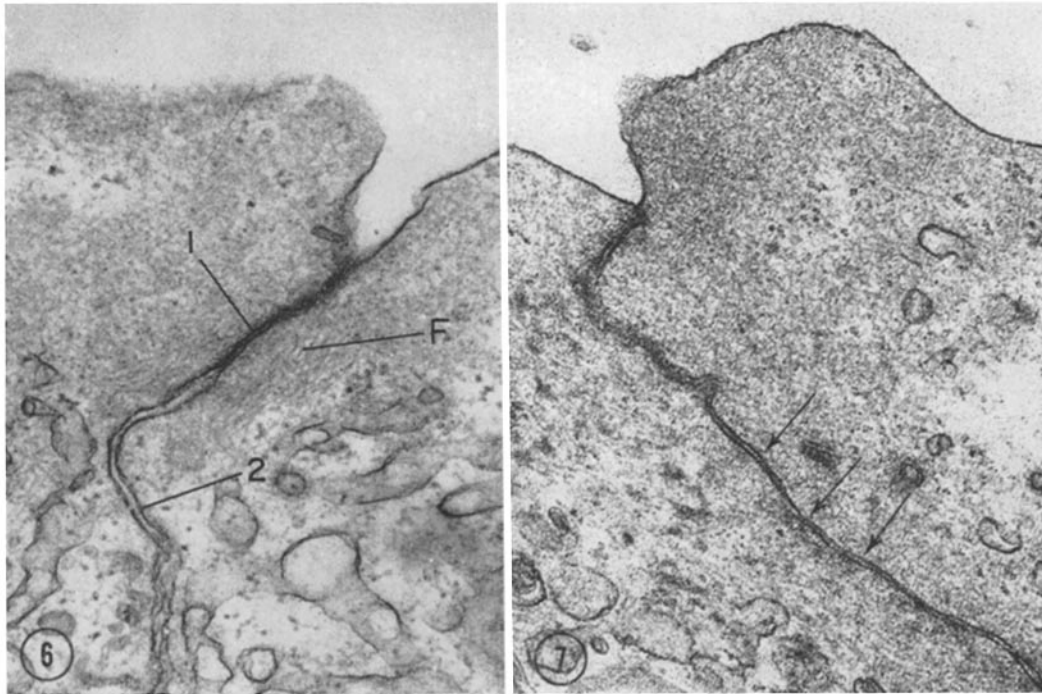
FIGURE 6   Enveloping layer junction (stage 12⅓, mid-gastrula). Where the section is normal to the junction (1), the plasma membranes are parallel and separated by an intercellular gap of 60 A. Below this region, the membranes diverge to produce a gap of 120 A (2). F, fibrils. × 56,000.

FIGURE 7   Contact between enveloping layer cells of a mid-gastrula (stage 12⅓). The zone of close contact between adjacent membranes extends for a considerable distance. Where the section is at right angles to the membranes, the intercellular gap is present and about 60 A across. In some regions (arrows), thin dense bars situated at regular intervals extend across the intercellular gap between the plasma membranes. × 65,000.

Periblast—Blastomere Contacts

Numerous short microvilli extend from the apical surface of the periblast which forms the floor of the segmentation cavity. The overlying blastomeres do not form any specialized contacts with the periblast. Some blastomeres, however, some into close proximity to the periblast and appear to brush the tips of the microvilli (Fig. 14). There are no extensive areas of contact or close junctions.

Cell Surfaces

Enveloping layer cells, deep blastomeres, periblast, and yolk cytoplasmic layer are bounded by a plasma membrane that contains no extraneous filamentous coat, even after lead hydroxide or uranyl acetate staining (Figs. 7, 9, 13, and 14).

Undulations occur on the surface of enveloping layer cells at all stages and are particularly evident during gastrula stages when the surface is irregular (Figs. 4 and 5). The basal surface of enveloping layer cells is relatively smooth (Fig. 4). The surface of deep blastomeres that borders the segmentation cavity is smooth in contour except for an occasional microvillus and the blunt lobopodia protruding from the cell (Figs. 13 and 14). The latter are numerous in blastula and gastrula stages. The surfaces bordering adjacent blastomeres contain numerous crests and indentations (Fig. 11) or are smooth (Fig. 12). Numerous microvilli extend into the segmentation cavity from the periblast (Fig. 14). Cytoplasmic arms and projections extend from the periblast into the yolk (21). The marginal portion of the periblast bordering the perivitelline space is irregular with blunt villous projections
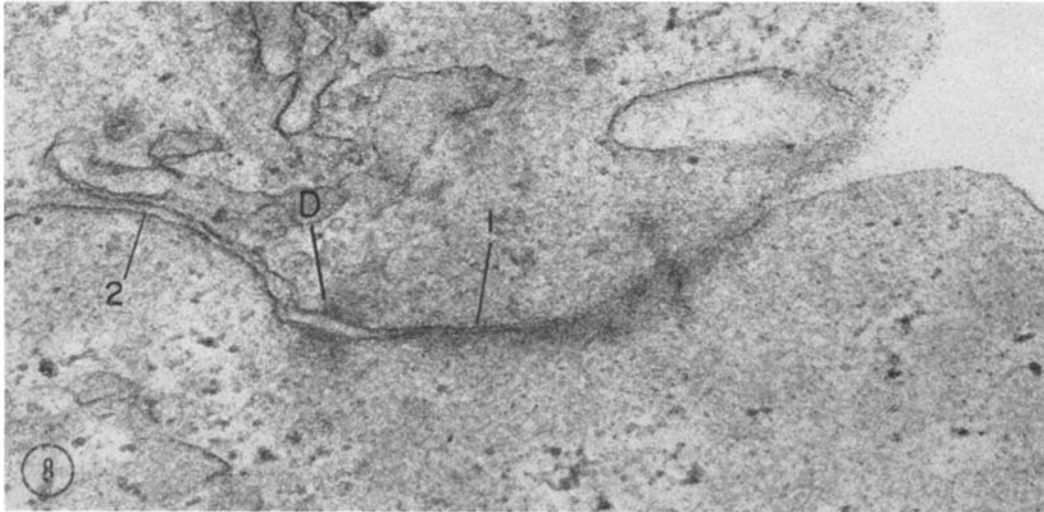
FIGURE 8   Enveloping layer cell contacts of the mid-grastrula (stage 12½). An initial region of close contact with an intercellular gap of 60 A (1) occurs between adjacent cells. A small desmosome (D) is present below the zone of close contact. In the desmosome, the membranes are separated by a space of 200 A. An accumulation of dense, amorphous substance occurs in the cytoplasm. Below the desmosome, the membranes are separated by a space of 120 A (2). The cortical hyaloplasm is of medium density and contains dense granules 200–300 A in diameter (glycogen). X 86,000.

(Fig. 9). Both surfaces of the yolk cytoplasmic layer are relatively smooth.

A dense cortical layer of cytoplasm occurs beneath all plasma membranes exposed to the perivitelline space: enveloping layer (Figs. 4–7) marginal periblast (Fig. 9), and yolk cytoplasmic layer. In the cortical layer, the hyaloplasm is denser and contains fewer organelles than in the deeper portion of the cell. Glycogen granules and a few vesicles sometimes occur in this layer (Fig. 8). There do not appear to be many fibrils in the cortical layer, except in the immediate vicinity of some intercellular junctions in the enveloping layer of gastrulae (Fig. 6). This layer is absent beneath cell surfaces bordering the segmentation cavity or yolk.

DISCUSSION

Cells of the enveloping layer of the blastoderm are bound together at their apical ends by junctions characterized by an intercellular gap of ∼60–75 A. These junctions differ from most other cell contacts, in which the intercellular gap is 150–200 A (16, 25), 240 A along a desmosome or macula adhaerens (13), or completely obliterated in a zonula occludens (13). Only a few other junctions other

than zonulae occludentes in which the intercellular space is less than 150 A have been observed (6, 7, 8, 17, 18, 22, 27), and the majority of these occur in either embryonic tissues or certain synapses. Beneath the initial zone of close contact is a region in which the plasma membranes are parallel and separated by 120 A. In stage 12½ gastrulae, a desmosome sometimes occurs below or between the other two contacts. This arrangement bears some resemblance to the tripartite junctional complexes of the zonula occludens, zonula adhaerens, and macula adhaerens described by Farquhar and Palade in different epithelia (13, 15). Some differences occur in Fundulus gastrulae: the plasma membranes are not fused in the initial zone of contact, dense material is not present in the cytoplasm adjacent to the 120-A contact, and the desmosome is small and lacks a central disk and cytoplasmic fibrils.

Dissections of Fundulus blastoderms show the enveloping layer to be a cohesive epithelial layer. Time-lapse cinemicrography of normally developing eggs confirms this impression and reveals that cells of the enveloping layer maintain their position relative to one another. The apical junctions are probably important in binding the enveloping layer cells together. As the enveloping layer spreads
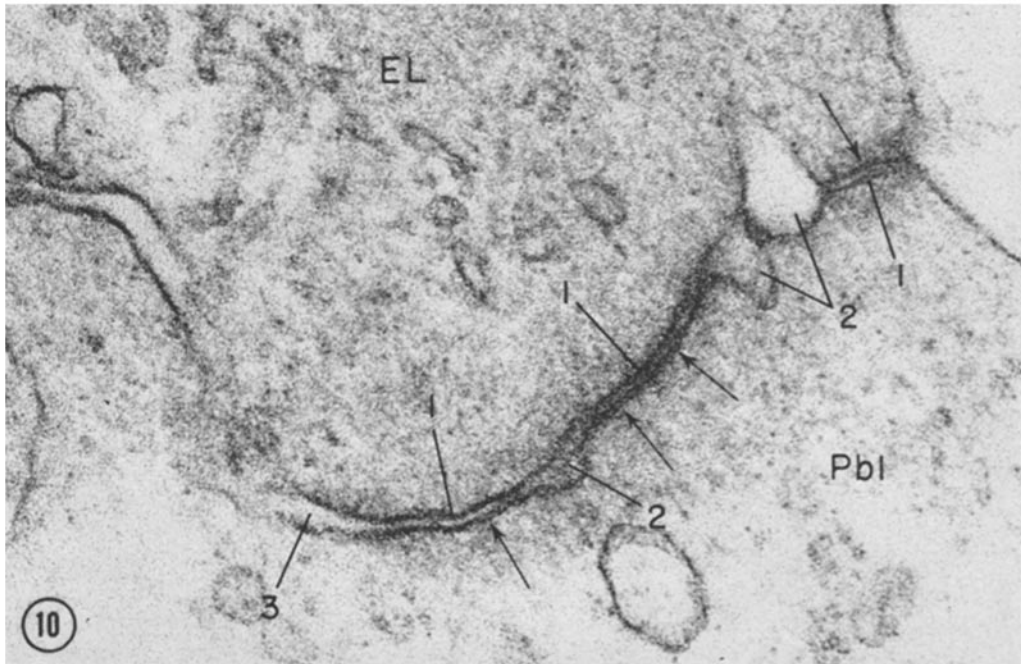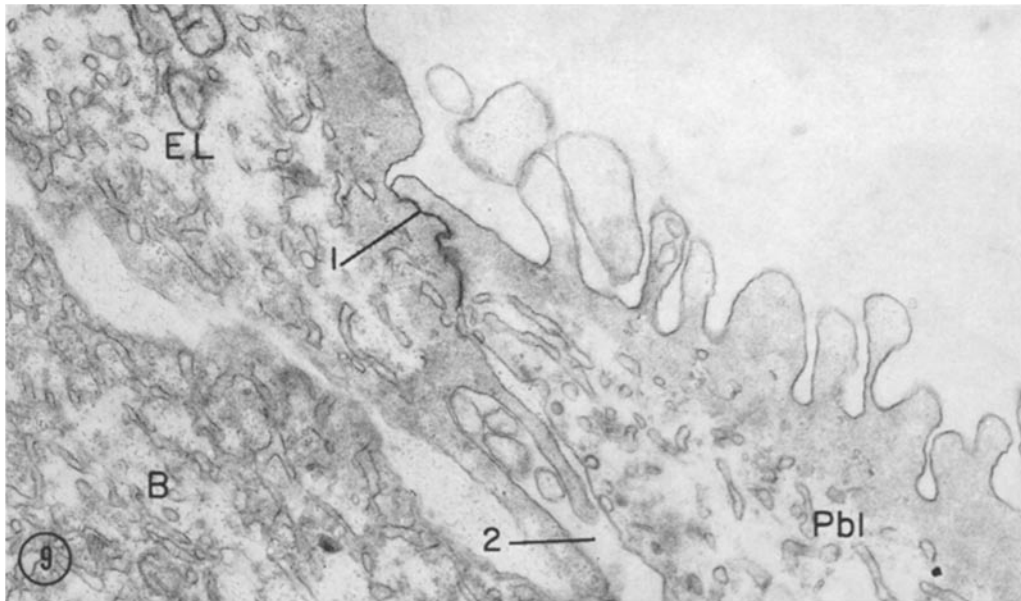
FIGURE 9   Enveloping layer (*EL*)–periblast (*Pbl*) junction. Stage 10, late blastula. The hyaloplasm of the periblast is of lower density than that of the enveloping layer. Blunt, villous projections extend from the periblast surface. In both regions (except in some of the blunt projections), the cortical cytoplasm beneath the surface membranes is of greater density and contains fewer organelles than elsewhere. The junction between these two layers consists of a close apposition of adjacent plasma membranes (*1*). Below this junction, the membranes diverge to produce a wide intercellular space (*2*) into which a few microvilli extend. *B*, deep blastomere, × 22,000.

FIGURE 10   Enveloping layer (*EL*)–periblast (*Pbl*) junction of an early gastrula (stage 11). The adjacent plasma membranes are parallel (*1*) and in close contact except in areas of focal dilatation (*2*). In the areas of close apposition (*1*), the intercellular gap is 60–75 A. In some regions (arrows), the trilaminar structure of the plasma membranes is evident. The intercellular space increases in width proximally (*3*). × 136,000.
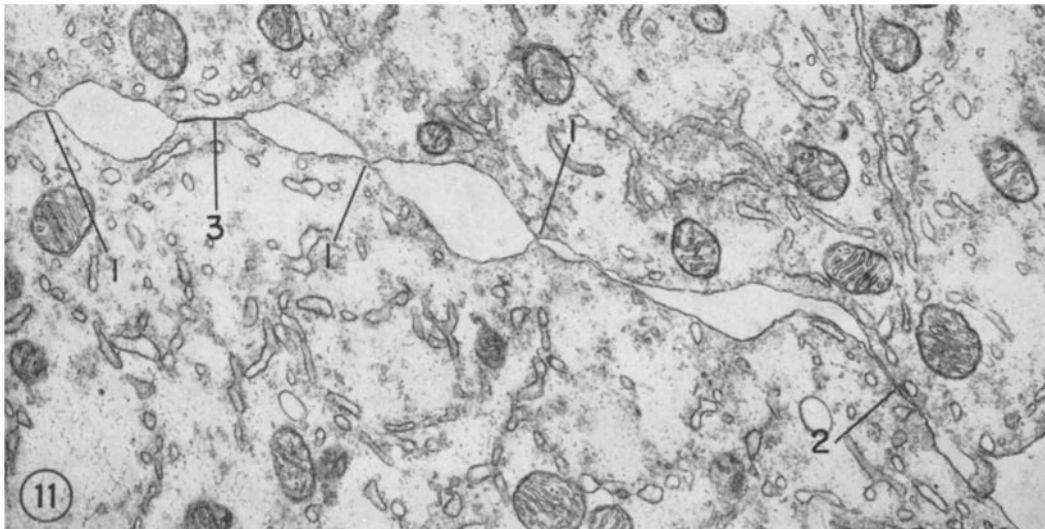
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FIGURE 11 Adjacent deep blastomeres of a stage 8 blastula. The cells make contact at single points at the crests of undulations of the plasma membranes (1). More extensive areas of contact are present in which the intercellular space is 120 A (2) or the plasma membranes are more closely apposed (3). × 19,000.

in epiboly, it becomes flattened and considerably stretched; and if its marginal adhesion to the periblast is severed during epiboly, it contracts vigorously (29). For these reasons, it appears that greater stress is placed on its intercellular junctions during epiboly. It is significant, therefore, that during this period of development the apical junction is supplemented by the appearance of desmosomes, since it suggests an increase in cell adhesiveness. Similarly, Bellairs has noted an increased incidence of desmosomes in the spreading chick epiblast (8). At the same time, cytoplasmic filaments become associated with some apical junctions, and transverse bars appear between some cells. The cytoplasmic filaments may transform a group of independent cells into a mechanically continuous structure (15).

Even though they are bound together in a sheet, individual cells of the enveloping layer are in constant activity during epiboly. Cells contract and expand to a small degree and show surface undulations. These undulations of the enveloping layer surface are also indicated in electron micrographs. In spite of this surface activity, enveloping layer cells remain in close contact most of the time, presumably because of their contact specializations. Occasionally, in the films, however, the surfaces of two adjacent cells appear to separate and the

exposed portions of their membranes show ruffling activity followed by closure of the gap. This activity is similar to that of a contact-inhibiting system, where formation of a ruffled membrane is inhibited at all contacted surfaces. When cells pull apart, however, the free surfaces form ruffled membranes and the cell spreads or moves in the direction of the ruffling (1, 3, 32). The separation of cells requires disruption of the apical junctions and desmosomes that unite enveloping layer cells, although disruption of these contacts was not noted in electron micrographs.

The contact relations of the marginal cells of the enveloping layer with the periblast are of special interest. The only strong connection of the blastoderm to the periblast is at this region (29). Moreover, the marginal cells of the enveloping layer show intense ruffled membrane activity at their outer edges as they spread over the expanding periblast in epiboly. Since adhesion to a substratum is necessary for cell movement (2, 31), the association of these two phenomena in the *Fundulus* blastoderm is not unexpected. It may be somewhat surprising to find a junction with an intercellular gap of 60–75 A in this region, however, because the adhesions of the marginal cells of the enveloping layer to the periblast appear to be intermittent in time-lapse films, the contacts apparently
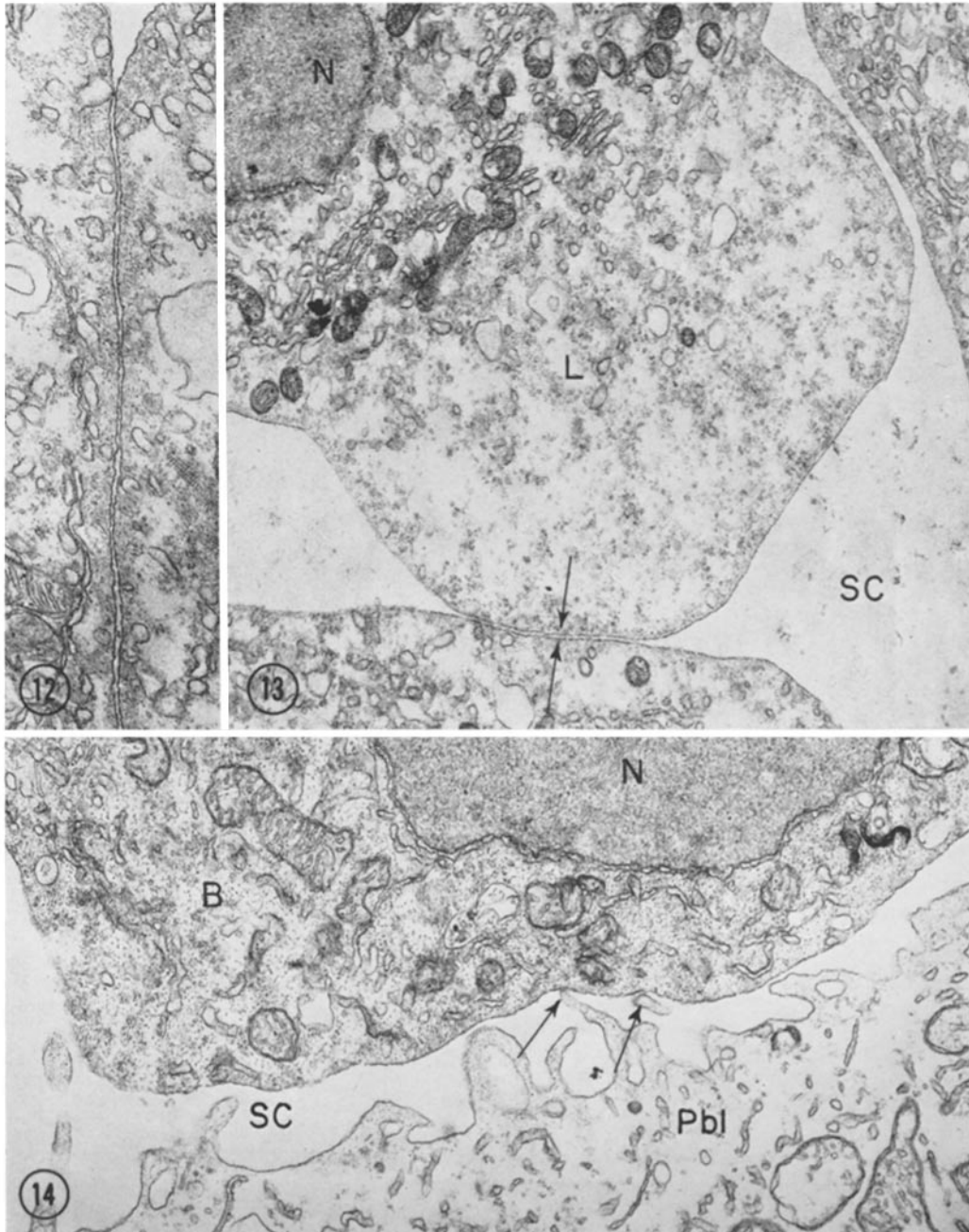
FIGURE 12 Adjacent deep blastomeres of a stage 8 blastula. The cells are separated by a slightly irregular intercellular space of about 200 A. The area of contact extends over a considerable distance. × 25,000.

FIGURE 13 Lobopodium (L)–blastomere contact at stage 11 (early gastrula). In the zone of contact (between arrows), the adjacent plasma membranes are parallel and separated by a gap of 200 A. SC, segmentation cavity; N, nucleus. × 21,500.

FIGURE 14 Blastomere (B)–periblast (Pbl) contact (stage 12⅓, mid-gastrula). The blastomere lying in the segmentation cavity forms no specialized junctions with the periblast but appears to brush the tips of some of the periblast microvilli (arrows). SC, segmentation cavity; N, nucleus. × 21,000.

being broken and remade continuously as the cells move out over the periblast (cf. reference 4). If this is the case, the cells must form junctions, separate again and form junctions anew, in a matter of minutes. If this process occurs in a step-wise fashion, with a distal junction forming before the proximal contact is disrupted, at least one region of contact would always be present between enveloping layer and periblast, as is the case in the electron micrographs.

In contrast to cells of the enveloping layer, the deep blastomeres are relatively loosely arranged. The fine structural observations correlate closely with the low power time-lapse observations of living blastomere activity. Lobopodia are evident in blastula and gastrula stages. During early and middle blastulae, there are no close contacts of lobopodia with other cells, an observation which is consistent with their evident lack of adhesiveness at this time. During late blastula and gastrula stages, however, close contacts of lobopodia with other cells appear regularly. The appearance of 200-A contacts at the time when lobopodia attach to other blastomeres constitutes evidence that these contacts are adhesive. No desmosomes and only a few junctions with an intercellular gap of 60–75 A occur between deep cells, in contrast to the enveloping layer where cells remain bound together in a unified sheet. The 200-A contacts between blastomeres represent strong enough adhesions for traction, but not so strong that they immobilize the cells. This difference in contacts between cells of the enveloping layer and deep cells is of interest since the former are contact-inhibiting whereas the latter are not.

It has not been possible to determine the relation of the deep cells to the underlying periblast in the films of living eggs. In blastoderms fixed for the light microscope, the lowermost deep cells (hypoblast) appear to flatten on the periblast, suggesting that they adhere to the upper surface of the periblast and use it as a substratum in some of their locomotory movements (29, 30). The electron micrographs of deep cell-periblast relations reveal no extensive junctions, although some blastomeres appear to lie on top of the periblast microvilli. If, therefore, the lowermost blastomeres use the periblast as a substratum for movement, they must "crawl" over the tips of the periblast microvilli.

What was formerly termed the yolk gel layer is in fact a cytoplasmic layer which is an extension of the periblast (21). This discovery forces a new view of the spreading of the periblast in epiboly. The yolk layer was thought to solate at its juncture with the periblast, as it is gradually replaced in epiboly (29). Now it appears that periblast cytoplasm flows into the yolk cytoplasmic layer, causing it to thicken and adding nuclei and some organelles. In consequence, periblast epiboly is not the spreading of a layer with abrupt margins over the noncytoplasmic surface of the yolk but must be a controlled flow of cytoplasm from the thicker animal part of an intact cytoplasmic layer (the periblast) into the thinner vegetal part (the yolk cytoplasmic layer). This process is, therefore, a reversal of blastodisc formation, whereby evenly distributed cortical cytoplasm flows toward the animal pole to form the thickened blastodisc.

In *Fundulus*, we observed no extraneous material applied to the outer surfaces of enveloping layer cells, even though the material was fixed in a manner that would probably reveal the presence of a mucopolysaccharide layer. A dense cortical layer of hyaloplasm occurs and has been noted in other embryonic tissues (5, 7, 19, 24). Balinsky suggested that the cortical layer may be contractile during neurulation of the frog (7). A dense layer in *Hyla* contains fibrils, and Baker suggested that contraction and expansion of the dense layer results in gastrulation (5). Although a similar process could be responsible for epiboly in *Fundulus* gastrulae because the enveloping layer contains a dense cortical layer, several observations are not consistent with this hypothesis. In *Fundulus* a system of fibrils, which could play a role in the mechanism of contraction, is not prominent. Some fibrils appear in gastrulae but these are largely confined to regions of adjacent cell contacts. Furthermore, the dense cortical layer in *Fundulus* is found not only in the migrating enveloping layer cells but also beneath all cell surfaces exposed to the perivitelline space (nonmotile surface blastomeres in cleavage stages, enveloping layer in blastulae and gastrulae, marginal periblast, and yolk cytoplasmic layer). Deep blastomeres, actively motile during epiboly, do not contain a cortical zone. For these reasons, the dense layer may not be so important in motility as in the permeability properties of the egg surface. This layer could be protective because the enveloping layer is highly impermeable to most substances (9, 20, 26). The apical junctions, in addition, may prevent passage of substances through the intercellular

spaces. As in other epithelia (13, 14), the enveloping layer probably maintains a chemical and an electrical potential gradient between the perivitelline space (corresponding to lumen) and segmentation cavity (subepithelial space.)

The new information presented in this paper compels a reexamination of our view of the mechanism of epiboly in *Fundulus*. The enveloping layer clearly spreads as a sheet. Cells do not change position relative to one another and are joined together by contact specializations. The importance of both the blastoderm margin (31) and the enveloping layer (12) in epiboly is confirmed. Like other epithelial layers, the enveloping layer appears to spread over its substratum (the spreading periblast) because of the activity of its marginal cells. These form junctional adhesive contacts with the periblast and spread over it as a result of ruffled membrane activity at their free margins. Once enveloping layer cells reach the periblast margin, they may be pulled by the spreading periblast. The adhesive contacts of the enveloping layer cells with each other communicate the pull of the outward (and downward) moving marginal cells to the entire enveloping layer. Nonmarginal cells are not completely passive, however. They show constant surface activity and, when they occasionally separate partially, presumably as a result of tension in the stretched membrane, they quickly form ruffled membranes and spread together again. Thus the marginal cells appear to be the prime movers, but other cells are potentially active at all times and aid sporadically in the spreading of the sheet by tiny spreading movements of their own. The tendency of enveloping layer cells to form a ruffled membrane only when an edge is free of contact with other cells and then to spread in the direction of the ruffling suggests that the enveloping layer is a contact-inhibiting system. When the enveloping layer has spread entirely over the yolk, its marginal cells contact each other in closing the blastopore. As expected of a contact-inhibiting system, their spreading now ceases. The mechanism of periblast epiboly appears to involve a flow of cytoplasm from the thicker periblast into the thinner yolk cytoplasmic layer with which it is continuous.

The deep cells of the blastoderm translocate in a different manner. They are not bound together to form cohesive sheets, but move about individually. At first, they appear to move largely at random, filling in space in the expanding segmentation cavity. Later, the movements of those near the blastoderm margin (germ ring) take on a directional character as they converge dorsally to form the embryonic shield. The deep blastomeres appear to acquire their capacity to move in two stages: (*a*) the formation of nonadhesive lobopodia during early blastula stages; and (*b*) an increase in surface adhesiveness during late blastula and early gastrula stages. There are three lines of evidence for the increased adhesiveness toward the beginning of gastrulation: observation of adhesion of lobopodia to other cells in time-lapse films of normal development with the consequent formation of taut filopodia and fans; the appearance of 200-A junctions where lobopodia contact other cell surfaces; and the tendency of dissociated gastrula cells to adhere to and flatten on glass in cell cultures (30). The lobopodium is the organ of locomotion, and the increased adhesiveness gives it traction. When an adhering lobopodium contracts, the cell is pulled along and translocation of deep cells begins. The uppermost deep cells are closely applied to the under surface of the enveloping layer, while some of the lowermost cells are in close relation to the microvilli of the periblast. Thus, deep cells apparently move by adhering to each other, to the inner surface of the enveloping layer, and possibly to the periblast.

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