

Hydra regeneration from recombined ectodermal and endodermal tissue

II. Differential stability in the ectodermal and endodermal epithelial organization

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SUMMARY

Hydra tissue consists of the ectodermal and the endodermal layers. When the two layers were separated by procaine treatment and then recombined, the ectodermal epithelial cells spread as a single cell layer over the endoderm as in epiboly in vertebrate embryogenesis, and the resultant spherical structure subsequently regenerated into a complete hydra. In this study, light and electron microscopy were used to examine the structural changes which took place in the cells and tissue during this epibolic ectodermal spreading process. Within a few hours after tissue recombination, the endoderm underwent dramatic changes; it lost its epithelial sheet organization, and turned into a mass of irregularly shaped cells without the apical-basal cell polarity initially present. In contrast, the ectoderm maintained its basic epithelial sheet organization as it spread over the endoderm. Later, the endodermal epithelial cells reorganized themselves into a single-layered

epithelial sheet underneath the spreading ectodermal layer. The resultant spherical structure consisted of a single layer of ectodermal epithelial cells outside, a single layer of endodermal epithelial cells inside, and an empty cavity in the center as in normal hydra tissue. This structure regenerated into hydra in the following days. These and other observations demonstrate that the two-layered epithelial sheet organization is highly dynamic, and that its stability is maintained by strong interactions between the two layers in normal hydra. It is suggested that this dynamic nature of the hydra tissue, particularly the high plasticity of the endodermal epithelial sheet organization, may be an important element for the high regenerative capacity of this organism.

Key words: Hydra, Epiboly, Regeneration, Electron microscopy, Epithelial organization

INTRODUCTION

The body plan of hydra is very simple (Campbell and Bode, 1983). Its main body column is a hollow cylinder consisting of an outer layer of ectodermal epithelial cells and an inner layer of endodermal epithelial cells. The two layers are separated by a thick layer of extracellular matrix, the mesoglea. A head consisting of a hypostome and tentacles is located at one end of the body column, while a foot with a basal disk and peduncle exists at the other end.

Epp et al. (1979, 1986) showed that ectodermal and endodermal layers can be separated from each other by treatment with an anesthetizing drug, procaine, and that the separated tissue layers can be reassembled to regenerate into a new hydra. This reassembly system was used to show that both the ectoderm and endoderm are involved in determining the polarity of regeneration from the reassembled tissue (Smid and Tardent, 1982), and that gland cells present in the endoderm

can arise by differentiation from the interstitial cells initially located in the ectoderm (Bode et al., 1987).

In these studies, the ectodermal and endodermal tissues were separated from each other without altering the original cylindrical organization of each tissue. The separated tissues were then reassembled by slipping the endodermal cylinder into the central hollow space of the ectodermal cylinder in a parallel or anti-parallel direction.

In a previous study (Kishimoto et al., 1996), we modified the procedure by deliberately destroying the cylindrical configuration of one or both of the tissues separated by procaine. The two tissue pieces were then recombined to allow them to regenerate. We then examined the interaction which took place between the recombined ectodermal and endodermal tissue at the light microscope level. Soon after recombination, the ectodermal tissue spread as a thin layer to cover the entire endoderm in 1-2 days. This process of ectodermal spreading over the endoderm was termed 'epiboly' after the name of

similar processes in early embryogenesis in various vertebrates, including chick (New, 1959), amphibian (Keller, 1980), and fish (Trinkaus, 1984). During the epibolic process, extensive cell rearrangement took place in the tissue, particularly in the interface between the recombined ectoderm and endoderm.

In the present study, we used light and electron microscopy to examine structural changes which took place in the ectodermal and endodermal epithelial cells during the epibolic process. We found that both the ectoderm and endoderm underwent major structural changes, but in significantly different ways. The ectoderm maintained its basic epithelial sheet organization, whereas the endoderm lost the epithelial sheet organization within a few hours after separation from the ectoderm, and turned into a mass of irregularly shaped cells. These cells later reestablished an epithelium underneath the spreading ectoderm.

The roles of epithelial and interstitial cell lineages in hydra regeneration are discussed based on observations made in this and related previous studies.

MATERIALS AND METHODS

Strain and culture

Hydra strain and culture conditions used in this study were described in detail by Kishimoto et al. (1996). Briefly, strain 105 of *Hydra magnipapillata* (Sugiyama and Fujisawa, 1977) was cultured at a low density in large flat plastic trays at 18°C. They were fed daily to satiation with freshly hatched brine shrimp nauplii, and a few hours later transferred to new trays containing fresh culture solution. Relatively young polyps cultured for 7-8 days after detachment from parental polyps were harvested 24-30 hours after the last feeding, and used for preparing the ectodermal and endodermal tissue described below.

Ectodermal and endodermal tissues

The procedures originally described by Epp et al. (1979) and modified by Kishimoto et al. (1996) were used to separate the ectodermal and endodermal tissues and then to recombine the separated tissues.

Equal volumes of a 1% solution of procaine-HCl (Wako Pure Chemicals, Tokyo) in distilled water, hyper-osmotic (70 mOsmol) salt solution for tissue dissociation and cell reaggregation (DM solution) (Gierer et al., 1972), and modified M solution for hydra culture (mM solution) (Sugiyama and Fujisawa, 1977) were mixed and adjusted to pH 4.5 (A solution) or pH 2.5 (B solution) immediately before use. Heads and feet were removed from 7-8 day old hydra by amputations made below the tentacle ring and above the budding zone (a in Fig. 1).

To prepare the ectodermal tissue, the body column tissue obtained was treated in A solution for 5 minutes, followed by treatment in B solution for 1½ minutes, both at 4°C. The treated tissue was gently transferred to DM solution kept at 18°C. Within several minutes, the ectodermal tissue contracted into a ring-shaped tissue at one end of the rod-shaped endoderm (c in Fig. 1). Using a pair of forceps with fine tips, the two tissue pieces were teased apart to obtain the ectodermal tissue (d in Fig. 1). To prepare the endodermal tissue, the body column tissue was treated in the same way, except for treatment in B solution for 6 minutes instead of 1½ minutes. This treatment produced partial disintegration of the ectodermal tissue whereas the endodermal tissue remained in the rod-shape in DM solution. The former was flushed away from the latter by a stream of liquid directed against the former using a Pasteur pipette. The rod-shaped endodermal tissue (e in Fig. 1) obtained in this way was used.

Recombination of the ectodermal and endodermal tissues

The ring-shaped ectodermal tissue obtained by procaine treatment was radially cut into 6-8 equal-sized pieces (d in Fig. 1), and the freshly cut-surface of one of the pieces (f in Fig. 1) was gently placed in direct contact with an endodermal tissue (e in Fig. 1). A firm adhesion was established between them in less than half an hour (g in Fig. 1). The recombined tissue produced in this way was allowed to regenerate. The entire process was carried out in DM solution.

Scanning electron microscopy

Specimens of recombined tissue were harvested at various times after recombination, and fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.5, at 4°C for overnight or longer. The fixed specimens were rinsed in distilled water, conductively stained by the tannin-osmium method described by Murakami (1974), dehydrated through a graded series of ethanol, transferred to isoamyl acetate, dried at critical point using CO₂, coated with gold-palladium vapor, and examined in a Hitachi S-450 LB SEM at an acceleration voltage of 10 kV.

Transmission electron microscopy

Specimens were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.5, at 4°C for overnight or longer. The fixed samples were post fixed in 1% OsO₄ for 1½ hours, dehydrated through a graded series of ethanol and propylene oxide, and embedded in Araldite resin. Semithin sections were cut at a thickness of 0.5 µm and stained with Toluidine blue for examination under a light microscope. Ultrathin sections were cut at a thickness of 95 nm using an ultramicrotome with a diamond knife, double-stained with uranyl acetate and lead citrate, and examined under a Hitachi H-7000 TEM at an acceleration voltage of 75 kV.

Layouts of photographs were made electronically using a Nikon Cool Scan and a computer program Adobe Photoshop 3.0J (Adobe Systems Inc., Mountain View, California), and printouts were made using a Pictography 3000 (Fuji Photo Film, Tokyo).

RESULTS

An outline of the experiment carried out in this study is illustrated in Fig. 1. Hydra body column tissue (b in Fig. 1) was treated with procaine to separate the ectodermal and endodermal tissue layers from each other (c in Fig. 1) (see Materials and Methods). The ring-shaped ectodermal tissue (d in Fig. 1) was cut into smaller pieces (f in Fig. 1), and one of them was placed in the middle of a rod-shaped piece of endodermal tissue (e in Fig. 1). The two tissue types established a firm adhesion quickly (g in Fig. 1). This was followed by gradual spreading of the ectodermal tissue as a thin layer over the endoderm (h and i in Fig. 1). During this process, the endoderm gradually rounded up. The resultant structure formed in 1-2 days was a small sphere completely enveloped by ectodermal tissue (j in Fig. 1). This structure regenerated into a new hydra in the following 3-4 days (Kishimoto et al., 1996).

At various times in the first 1-2 day period, the recombined tissues undergoing the epibolic process (g-j in Fig. 1) were harvested, fixed, and examined by light, scanning and transmission electron microscopy. Based on the results obtained, we divided the morphogenetic processes in this period into 4 stages. Stage I represents initial adhesion soon after tissue contact. Stages II, III and IV represent the early, middle and late phases, respectively, of the ectodermal spreading over the endoderm. Photographs of typical specimens from each of these stages are presented below.

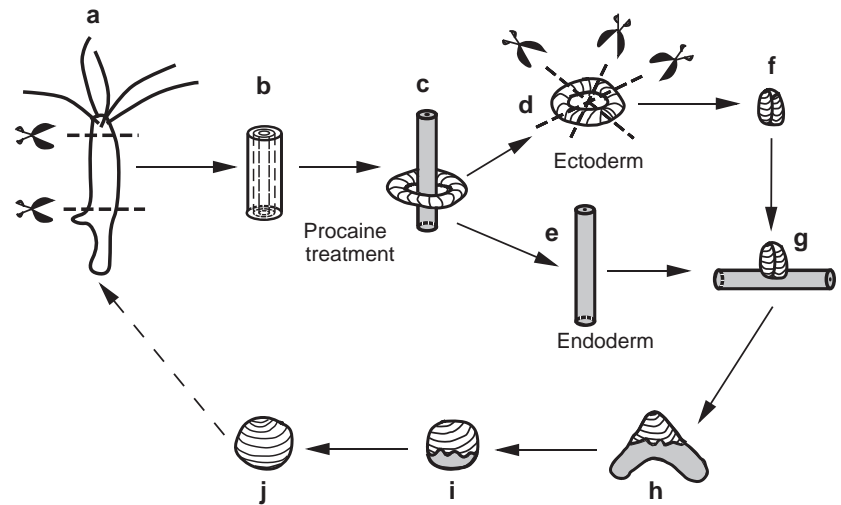


Fig. 1. An outline of the experiment carried out in this study. See main text for explanation (also Materials and Methods).

Scanning electron microscopy Stage I

Fig. 2A shows a typical recombined tissue soon after recombination (about 20 minutes). A small round piece of ectoderm was firmly attached to the middle of a rod-shaped piece of endoderm. A higher magnification of the ectodermal piece (Fig. 2B) showed the original external (apical) surface of the ectodermal tissue. The surface displayed distinct polygonal boundaries of the ectodermal epithelial cells measuring 12-18 μm in length and 5-9 μm in width. The surface within the

boundaries was smooth and flat, without any obvious fine structure.

Fig. 2C shows the external surface of the endodermal piece which corresponds to the basal surface of the endoderm before separation. The surface was covered in part by short fibrous materials running in random directions. Judging from TEM observation of similar specimens (see Fig. 6B), these fibrous materials were bundles of mesogleal fibers.

Fig. 2D shows the area of contact between the ectodermal and endodermal pieces. The ectodermal cells extended processes,

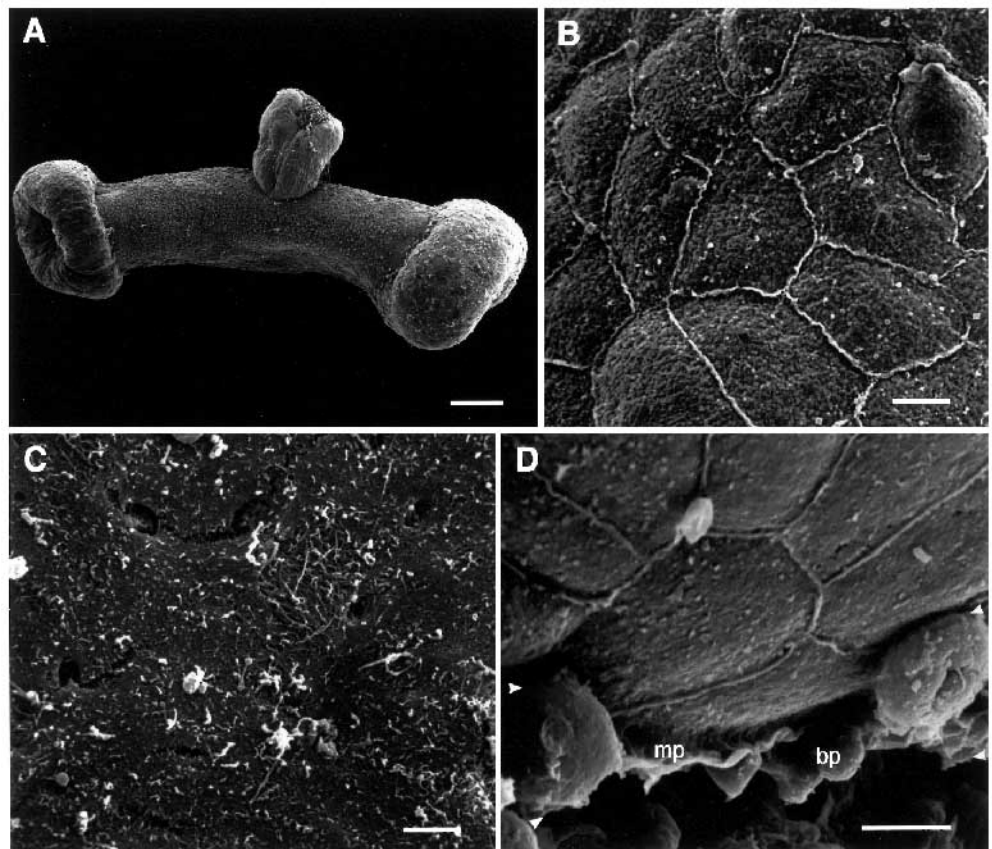


Fig. 2. SEM pictures of recombined ectodermal-endodermal tissues immediately after recombination. (A) An overall view showing the smaller ectoderm (upper) and larger endoderm (lower) firmly attached to each other. (B) Ectodermal part of the same tissue at a higher magnification showing polygonal boundary lines of the apical surface of the ectodermal epithelial cells. (C) Surface of the endoderm showing bundles of mesogleal fibers. (D) The area of contact between the ectoderm (upper) and endoderm (lower). Arrowheads indicate the zone of membranous or bulbous processes at the border between the two tissues. mp and bp: membranous and bulbous process, respectively, extended from the basal part of the ectodermal cells. Bars: 100 μm (A); 5 μm (B,D); 2 μm (C).

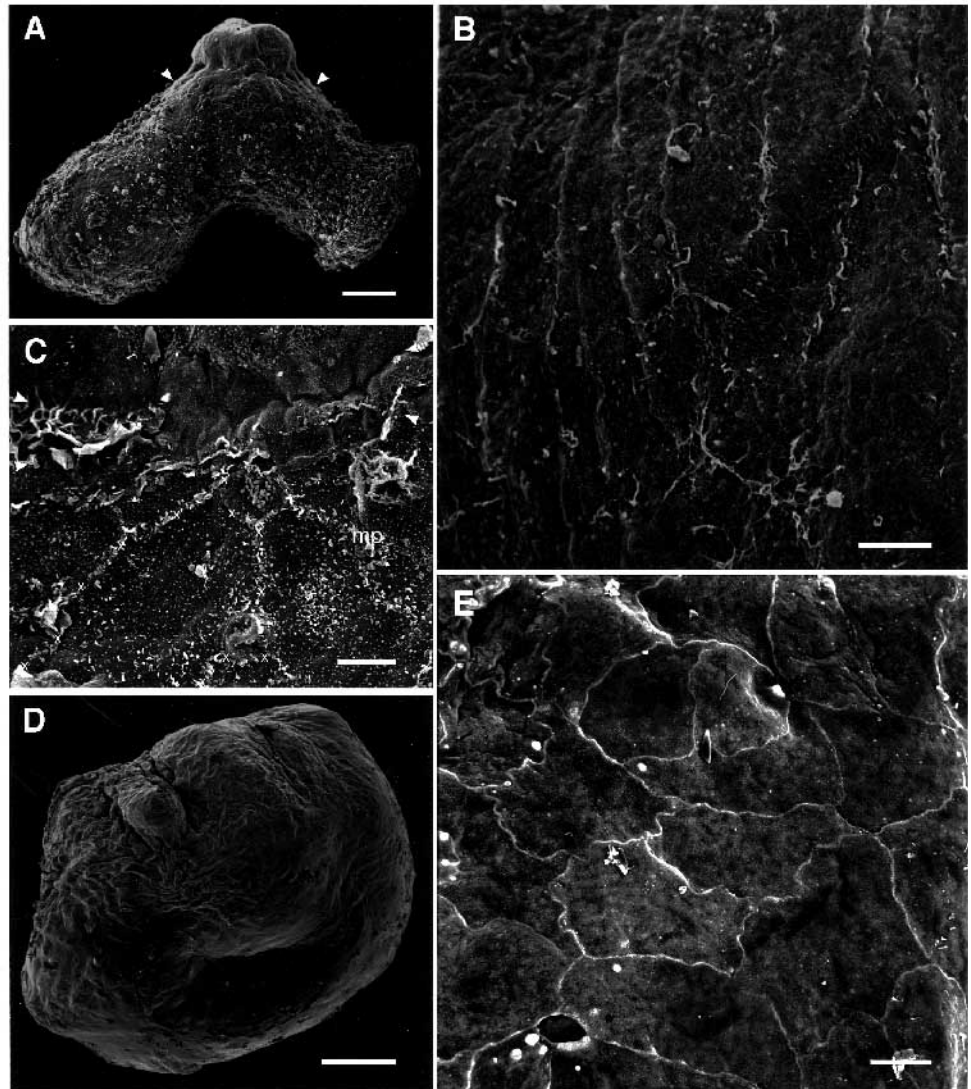


Fig. 3. SEM pictures of recombinants. (A) Overall view at 4 hours. Arrowheads indicate the border between the two tissues. (B) Ectodermal surface of the same tissue at a higher magnification showing altered boundary lines of the ectodermal epithelial cells. (C) Area of contact between the ectoderm (upper) and endoderm (lower) of the same tissue. Arrowheads indicate the zone of membranous processes extending from the ectoderm. Very small irregularly shaped microprojections are present along the basal boundary lines of the epithelial cells in the endoderm. The outline for one of the cells is indicated by small crosses. (D) Overall view of another recombinant at 40 hours. (E) The same tissue at a higher magnification showing polygonally shaped boundary lines of the ectodermal epithelial cells. mp: microprojections present on the boundaries of the endodermal epithelial cells. Bars: 100 μm (A,D); 5 μm (B,C); 10 μm (E).

which were membranous or bulbous in shape, onto the endoderm. They were present all along the edge of the ectoderm.

Stage II

A typical recombined tissue 4 hours after recombination is shown in Fig. 3A. The ectodermal part located above was present as a round main body having a smooth surface. Its basal part spread over the endodermal piece which had a less smooth surface. The endodermal piece had become shorter and more rounded than that in stage I shown in Fig. 2A. It was also bent into an L-shape from the point where the ectoderm was attached. This type of tissue bending was observed only at the site of contact with the ectoderm in many other similar specimens (see Fig. 3B in Kishimoto et al., 1996), suggesting that the bending is caused by the ectoderm (see Discussion).

Fig. 3B shows the main body of the ectodermal piece at a higher magnification. The apical boundary lines of the epithelial cells, uniformly polygonal in stage I (Fig. 2B), had become narrow (3–5 μm) and long (30–50 μm). They were oriented parallel to each other as if they were flowing from the top of the ectoderm toward the endoderm below. A few cells were observed which were extremely long (>120 μm , not shown).

Fig. 3C shows the area of contact between the two pieces. In the endodermal piece, fibrous residues of mesoglea, previously present in stage I (Fig. 2C), were absent. Judging from the TEM observation of similar specimens (Fig. 9C), the surface corresponded to the basal surface of the endodermal epithelial cells without the mesogleal layer on it. The border lines between these cells had irregularly shaped small projections (indicated by a series of small crosses).

The ectodermal cells extend onto the endoderm membranous processes (indicated by arrowheads) having multiple layers of wave-like structures with fine ruffles on the surface (see also TEM sections in Fig. 9A).

During the period from about 4 to 24 hours (stages II and III), the ectodermal tissue continued to spread onto the endoderm. The main ectodermal body became gradually smaller and flatter, and the area covered by the spreading ectoderm became gradually larger. The endoderm became gradually shorter and nearly spherical in shape. Throughout the course of this period, the features shown in Fig. 3B and C continued to be present on the surface of the ectoderm, endoderm and the border between them.

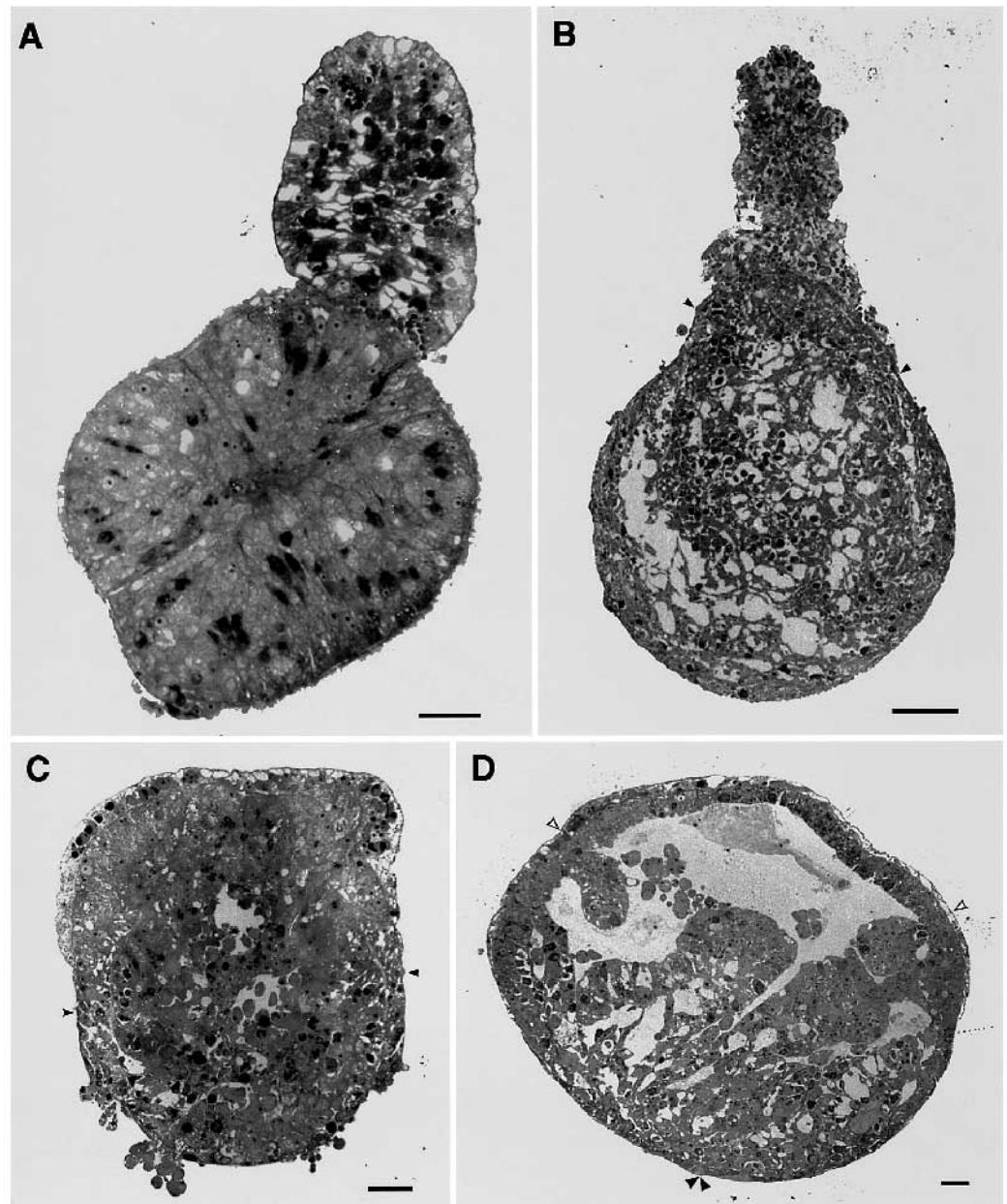


Fig. 4. Light microscope pictures of semi-thin sections of recombinants soon after recombination (A), at 4 (B), 19 (C) and 40 hours (D). Arrowheads indicate the front end of the ectoderm spreading downward onto the endoderm. White triangles in D indicate the area where the two-layered epithelial organization has been established. Bars: 100 μm .

Stage IV

Fig. 3D shows recombined tissue at 40 hours. The tissue had become nearly spherical in shape, and was apparently completely enveloped by the ectoderm. Fig. 3E shows a typical area on the ectodermal surface at a higher magnification. The boundaries of the ectodermal epithelial cells, narrow and long in stage II (Fig. 3B), had returned to a polygonal shape once again as in stage I (Fig. 2B). However, the surface area in stage IV (about 450 μm^2) was significantly larger than in stage I (about 100 μm^2).

Light and transmission electron microscopy

Stage I

Fig. 4A shows a semithin section of recombined tissue soon after recombination. The smaller ectodermal piece on the top and larger endodermal piece below are firmly attached to each

other. In the ectodermal piece, the external surface corresponds to the original apical surface of the ectoderm exposed to the outside environment in intact hydra before tissue separation (see Fig. 2B). In the endodermal piece, in contrast, the apical surface originally surrounding the gastric cavity in intact hydra is tightly contracted in the center (see Fig. 6A), and the external surface corresponds to the original basal surface covered by mesoglea layer (Fig. 2C).

Fig. 5A shows a layer of ectodermal epithelial cells in stage I. The outline of one of these cells is marked by a series of small crosses. Parts of these and similar cells are shown at higher magnifications in Fig. 5B and C. All the structures present in these cells resembled those in normal hydra. For example, the apical surface was covered by a mucous layer about 0.1 μm in thickness (marked ml in Fig. 5B and C). Small granules (presumably mucous granules) were numerous just

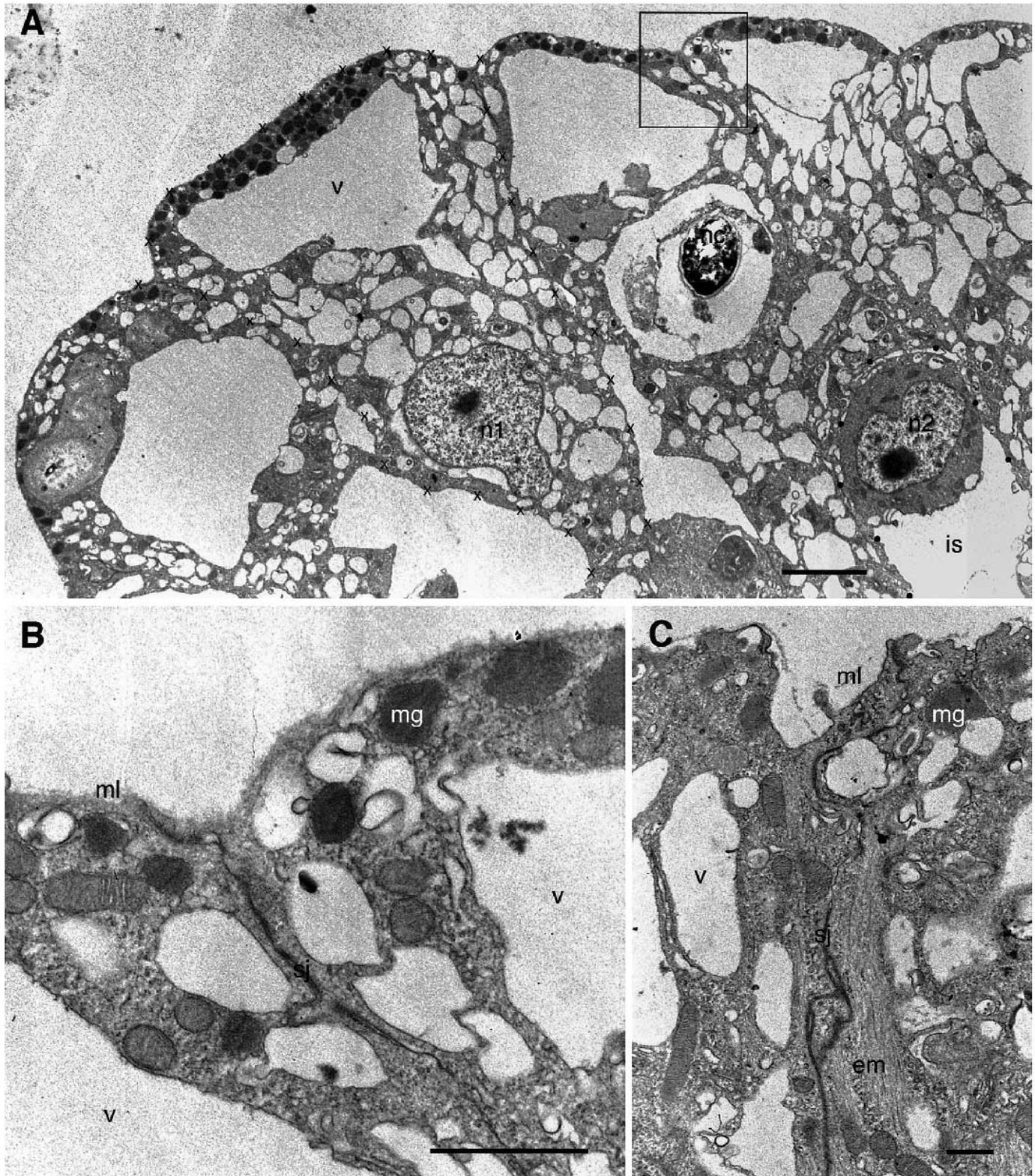


Fig. 5. TEM pictures of the ectodermal epithelial cells in a recombinant soon after recombination. (A) An overall view of several ectodermal epithelial cells lined up in a layer as in normal tissue. The outline of one of these cells is indicated by a series of crosses. The black dots indicate an interstitial space containing an interstitial cell. (B) A higher magnification of the boxed area in A showing a septate junction located between the lateral sides of two neighboring cells. (C) Another area of two neighboring endodermal epithelial cells, one of which contains myofilament-like structure in the lateral cytoplasm. Note that the filaments, normally located only in the basal cytoplasm, is ectopically located and run in parallel with the septate junction. em: ectopic myofilament; is: interstitial space; mg: mucous granule; ml: mucous layer; n1 and n2: nucleus of ectodermal epithelial cell and interstitial cell, respectively; nc: nematocyst capsule; sj: septate junction; v: vacuole. Bars: 5 μ m (A); 1 μ m (B,C).

below the apical surface (Fig. 5A and B). Numerous vacuoles of various sizes were present in the cytoplasm. A nucleus containing a distinct nucleolus was present in the central cytoplasm in each cell. On the lateral surface, neighboring cells were tightly connected to each other by septate junctions located immediately below the apical surface (Fig. 5B and C). An interstitial cell was present in the interstitial space between the epithelial cells (Fig. 5A).

One unusual feature, however, was noted in the ectodermal epithelial cells in stage I. In normal hydra, these cells extend from their basal areas long muscle processes filled with characteristic myofilaments (Wood, 1979; Campbell, 1980). In the ectodermal epithelial cells of stage I not in contact with the endoderm, myofilaments, or structures closely resembling them, were found in abnormal locations in the cytoplasm. Fig. 5C shows an example of the ectopic localization of myofilaments in the lateral cytoplasm close to the septate junctions (see Discussion).

Fig. 6A shows a section of the endodermal piece in stage I. Long columnar endodermal epithelial cells were present which stretch from the basal surface (top of figure) to the apical surface (bottom). The outline of one of these cells is indicated by a series of crosses. The apical surface of this cell was exposed to a small empty space (presumably original gastric cavity) located near the center in the bottom (indicated by black dots).

Fig. 6B and C show the basal and apical surfaces of these cells at a higher magnification. A mesoglea layer 0.6-2.2 μm in thickness was present on the basal surface (Fig. 6B). This was found in every specimen examined, indicating that mesoglea remained on the endoderm when the ectoderm and endoderm were separated by procaine treatment. The cytoplasm immediately below the mesoglea was filled with myofilaments running parallel with the mesoglea (Fig. 6B) as in normal hydra tissue.

Deeper in the cytoplasm, lipid droplets of various sizes and high in electron density were abundant (Fig. 6A). A large clear nucleus containing a distinct nucleolus is located in the central cytoplasm in each cell. The rest of the space in the cytoplasm was occupied by many large and small vacuoles.

The apical surface had many short microvilli and long cilia (Fig. 6C) both of which are normally extended into the gastric cavity. On the lateral side, cells were tightly connected to each other by septate junctions located very close to the apical surface (Fig. 6C).

All these features found in the endodermal epithelial cells in stage I are very similar to those present in normal intact hydra.

Fig. 7 shows the contact area between the ectodermal and endodermal pieces. Processes extending from the ectodermal cells, previously observed in a SEM picture (Fig. 2D), were present in the triangular space formed by the mesogleal layer below and the large ectodermal mass above. The processes were filled with filamentous materials similar to myofilaments present in the muscle processes of normal hydra. At least four layers of the processes are recognizable. One of them was connected to an ectodermal epithelial cell located at the right as indicated by a series of small crosses. The other processes appear to be connected to different cells some of which are not visible in the section.

Stage II

Fig. 4B shows a semithin section of a specimen at 4 hours. Approximately one sixth of the endoderm was covered by the ectoderm. At the electron microscope level, the ectodermal tissue in stage II (Fig. 8A) showed basically the same features as in stage I, with a few minor differences. For example, the large vacuoles present in the apical cytoplasm in stage I (Fig. 5A) were absent and only small vacuoles were present throughout the cytoplasm.

In contrast, the endoderm in stage II (Fig. 8B) showed a greatly different organization from stage I. The mesoglea, which was present in stage I, had disappeared from the basal surface of endodermal cells not covered by the ectoderm (but not from the area covered by the ectoderm). The epithelial cells, previously long and columnar (Fig. 6A), were contracted and irregular in shape. They were loosely and randomly connected to each other, leaving large empty spaces between them.

One of the irregularly shaped cells is outlined in Fig. 8B. The cell had a large nucleus (and a large phagosome) in the center surrounded by cytoplasm which contained only small vacuoles. Numerous large vacuoles which occupied a large part of the cytoplasm in stage I (Fig. 6A) were totally absent. Structures normally present in the apical surface (microvilli and cilia) or basal cytoplasm (myofilaments) were also absent. Well-developed septate junctions similar to those observed between the endodermal cells in stage I (Fig. 6C) cannot be found in this or any other similar irregularly shaped cells when examined at higher magnification (not shown).

These features indicate that the endodermal tissue loses its original organization as a single-layered epithelium in stage II, and that it turns into a mass of loosely connected cells having no apical-basal cell polarity (see Discussion). A similar loss of epithelial organization also occurs in the endodermal tissue kept alone without recombining with the ectoderm (not shown).

Previous investigators described similar abnormally shaped cells which appeared in the regenerating endodermal tissue soon after removal of the original head (for example, see Mookerjee and Bhattacharjee, 1966; Rose and Burnett, 1968; Znidarić, 1970) (see Discussion).

Stage III

Fig. 4C shows a light microscope section of recombined tissue at 19 hours. The spreading ectodermal layer had enveloped more than half of the endoderm. A few small cavities were present in the central part of the endoderm.

At the electron microscope level, the ectoderm which had moved onto the endoderm began to show a nearly normal epithelial organization (Fig. 9A). The layer is about 10 μm in thickness (cf. 20-30 μm in normal hydra tissue; Greber et al., 1992). The outlines of two neighboring epithelial cells are indicated by small crosses and black dots. Both cells were flat in shape, and partially overlapping each other. Each of them had many mucous granules just below the apical surface, a large nucleus near the basal surface, and many small vacuoles around the nucleus. Although not visible at the magnification shown, these cells had myofilaments in the basal cytoplasm. The cells in the most advanced edge had layers of relatively short processes. These processes, like those in stage I shown

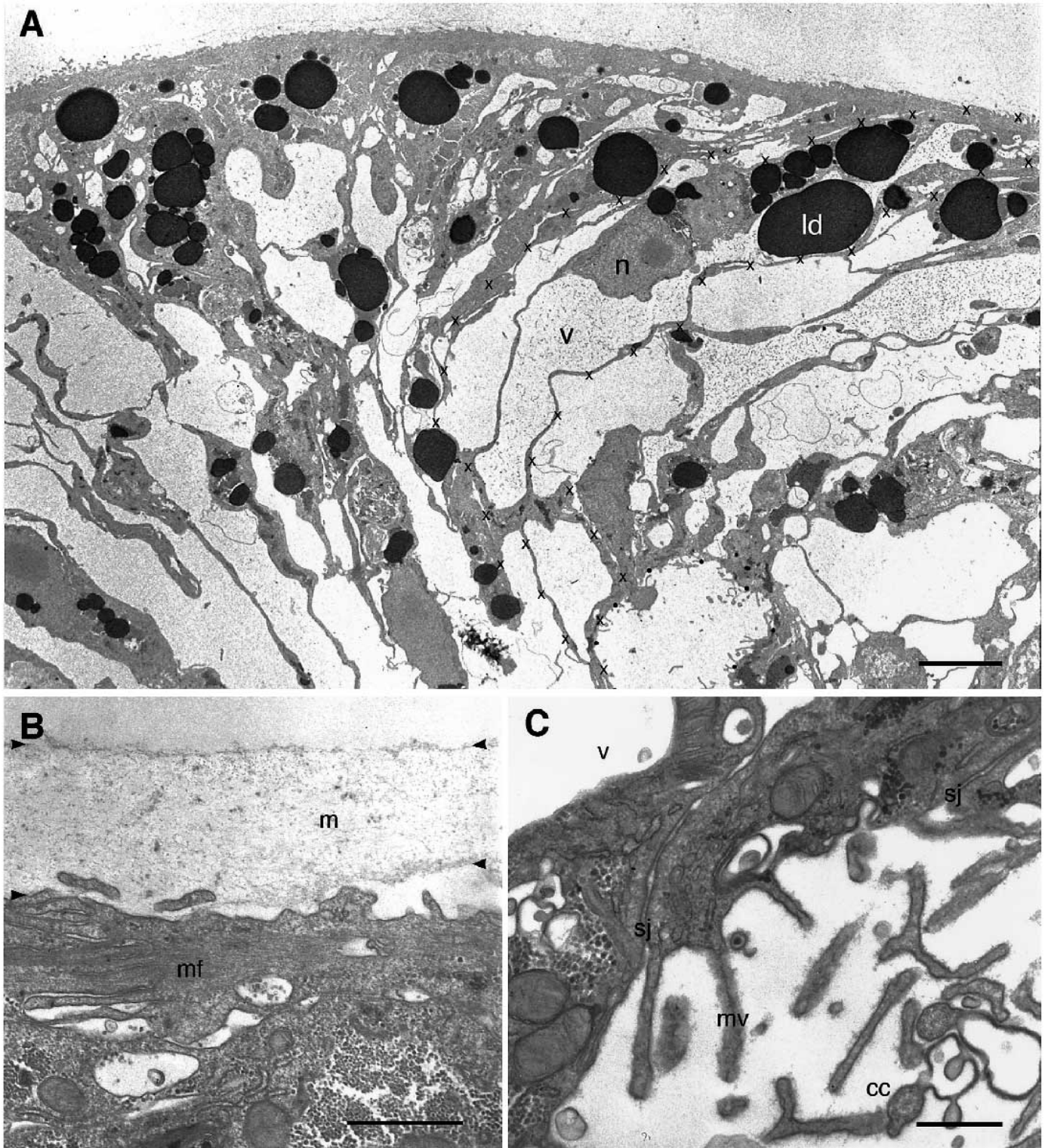


Fig. 6. TEM pictures of the endodermal epithelial cells in a recombinant soon after recombination. (A) A single layer of long columnar endodermal epithelial cells. Crosses indicate the outline of one of these cells. Note that the columnar cell extending from the basal surface above to the gastric cavity below is bent sharply into an L-shape, presumably by uneven tissue contraction. Black dots indicate the outline of a small part of the gastric cavity remaining in the tissue. (B) Basal surface area covered by a thick mesogleal layer (indicated by arrowheads). (C) Apical surface area projecting numerous microvilli and long cilia. cc: cross section of cilium; ld: lipid droplet; m: mesogleal layer; mf: myofilament; mv: microvillus; n: nucleus; sj: septate junction; v: vacuole. Bars: 5 μ m (A); 1 μ m (B); 0.5 μ m (C).

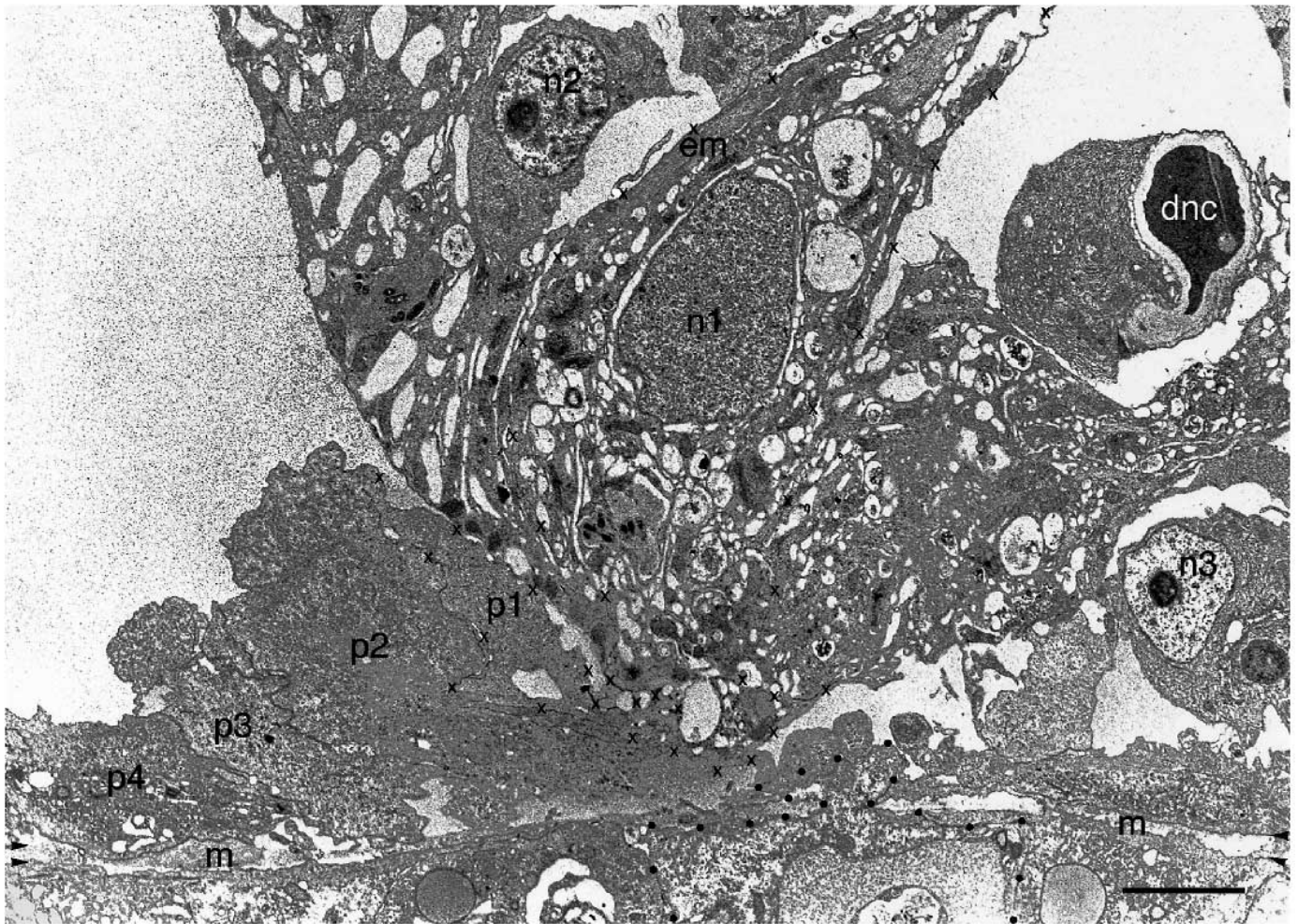


Fig. 7. The area of contact between the ectoderm and endoderm soon after recombination. The mesogleal layer (marked by two arrowheads, bottom left and right) indicates the boundary between the two tissues. Crosses show the outline of an ectodermal epithelial cell and the process extending from the cell. Note that the process is filled with myofilaments. Black dots indicate the outline of an endodermal epithelial cell. This and another cell on the right extend a cytoplasmic process through the mesogleal layer into the space between the mesoglea and the ectodermal tissue above. dnc: developing nematocyst capsule in nematoblast, m: mesogleal layer; n1, n2 and n3: nucleus of ectodermal epithelial cell, interstitial cell and nematoblast, respectively; p1, p2, p3, p4: processes extended from different ectodermal epithelial cells. Bar, 5 μ m.

in Fig. 7 at a higher magnification, were filled with myofilaments, and had small secondary processes extending upward from the surface.

These ectodermal epithelial cells were in direct contact with the endoderm; there is no mesoglea between them. In some spots, however, a layer of flocculent deposit, possibly newly formed mesoglea, was present (not shown).

The endoderm in stage III shows two distinctly different organizations depending on its location in the recombined tissue. Fig. 9B shows the area where the endoderm had been covered by the spreading ectoderm for some length of time. In the area directly below the ectoderm, endodermal epithelial cells began to exhibit some (but not all) of the characteristics of normal endodermal tissue in intact hydra. The cells, irregularly shaped in stage II (Fig. 8B), were now cuboidal or columnar, and neighboring cells were located side by side in a single layer. In each of these cells, the nucleus was located in a position just below the surface exposed to the ectoderm. Vacuoles of various sizes had reappeared throughout the cytoplasm.

These cells, however, differed from normal endodermal cells in one important way. They lacked an apical surface with characteristic microvilli or cilia exposed to the central cavity. Instead, these cells faced a random cell mass.

A gland cell is present in the periphery of the partially developed epithelial organization in Fig. 9B. It is ovoid in shape, and contains characteristic secretory granules (marked sg). The number of gland cells was examined and compared in an area of the partially developed epithelial organization and the adjacent area of the random cell organization of nearly equal size. The number in the former was about 20% as many as in the latter, suggesting a significant reduction in the number of gland cells during the transition from random organization to the partially developed epithelial organization (see Discussion).

Fig. 9C shows an area of the endoderm not yet covered by ectoderm. The tissue consisted of a random mass of irregularly shaped cells as in stage II (Fig. 8B) and had a smooth surface exposed to the external environment.

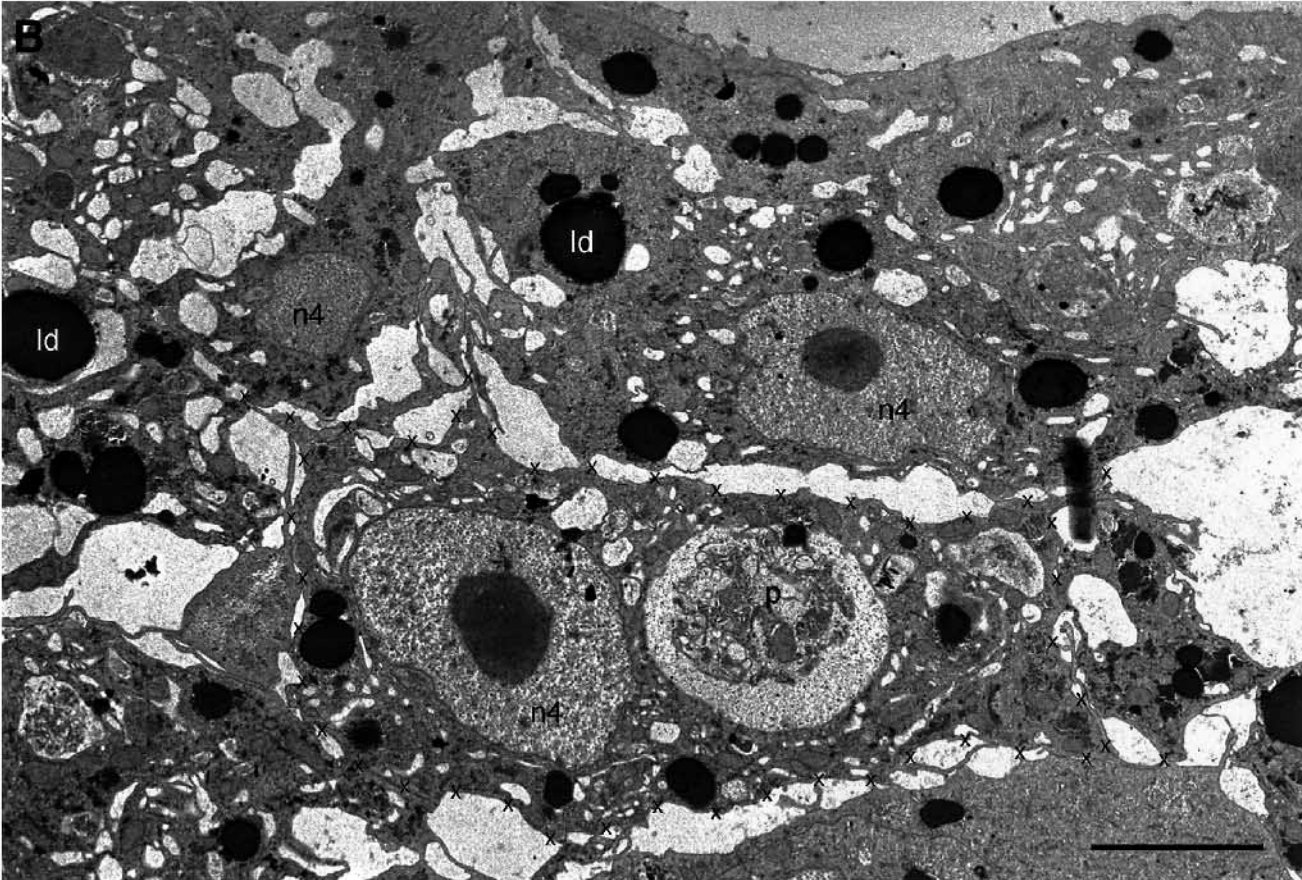
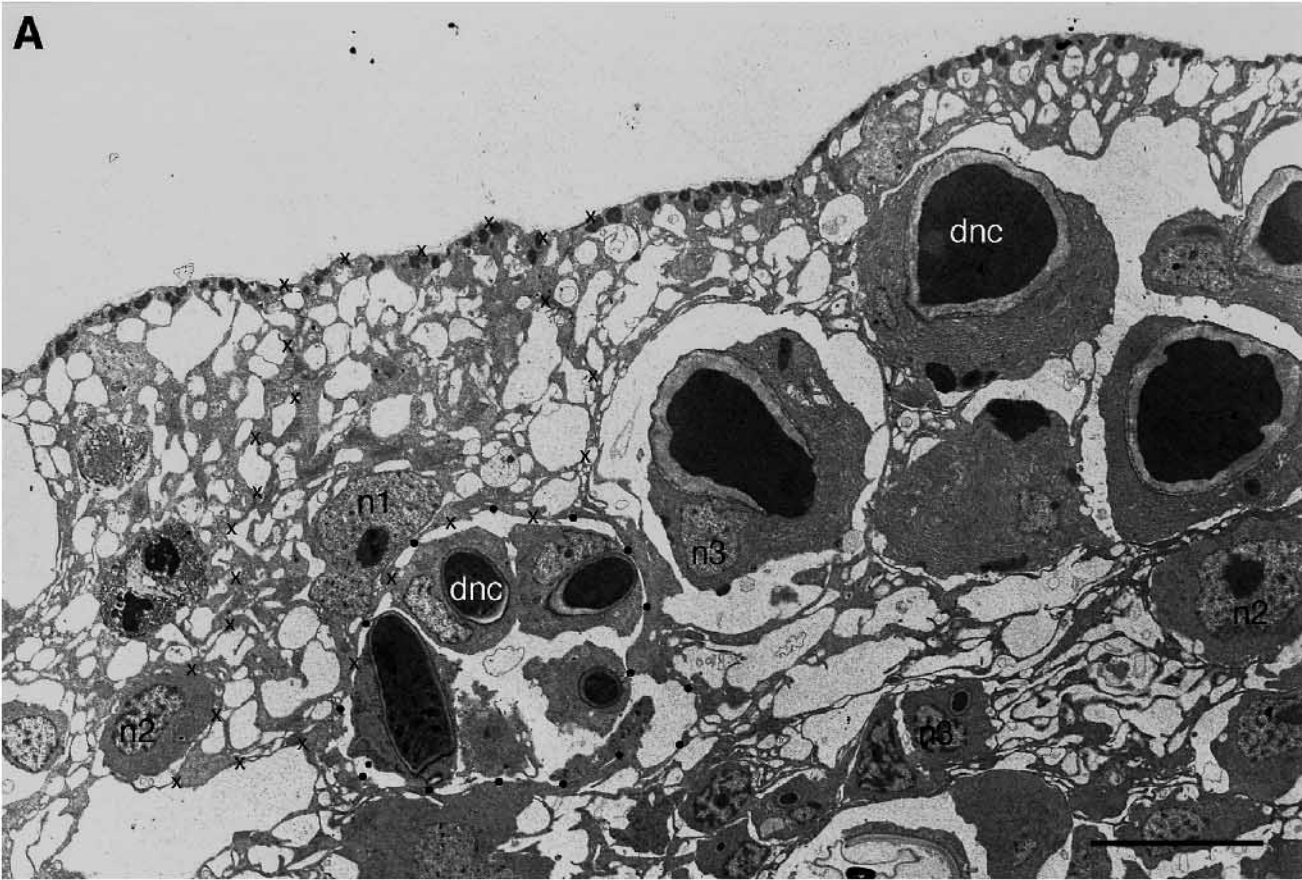


Fig. 8. The recombinants in stage II. (A) The ectodermal tissue at 2 hours. Crosses and black dots indicate the outlines of an ectodermal epithelial cell and a large interstitial space containing 4 developing nematoblasts, respectively. (B) The endodermal tissue at 4 hours consisting of a random mass of irregularly shaped cells. Crosses indicate the outline of one of these cells. dnc: developing nematocyst capsule in nematoblast; ld: lipid droplet; n1, n2, n3, and n4: nucleus of ectodermal epithelial cell, interstitial cell, nematoblast, and irregularly shaped endodermal cell, respectively; p: phagosome contained in the irregularly shaped cell. Bars: 10 μm (A); 5 μm (B).

Stage IV

Fig. 4D shows a light microscope section of a typical recombinant at 42 hours. A thin layer of the ectoderm had completely enveloped the endoderm. About two fifths of the recombinant in the upper side showed an organization of two cell layers with an empty cavity below it as in normal hydra. The remaining part showed a more complex organization consisting of partially organized tissue and many empty spaces.

Fig. 10A shows the arrangement of individual cells in the well-organized area. The ectodermal and endodermal cells both showed an organization similar to that in intact hydra. The ectoderm consisted of a single layer of epithelial cells which partially overlap each other. Each cell had a large nucleus with a distinct nucleolus, and many vacuoles in the cytoplasm. Small mucous granules were present in the apical cytoplasm, and muscle processes were located just above the basal membrane. Cells were tightly joined together by septate junctions located just below the apical surface.

The endodermal part consisted of a single layer of columnar epithelial cells and a large empty cavity below. These cells had cilia and numerous microvilli on the apical surface, many small empty vacuoles in the apical cytoplasm, and a thin layer of myofibers close to the basal surface. Cells were joined tightly by septate junctions located near the apical surface. A gland cell also occurred in the cell layer, projecting its apical tip into the central cavity (Fig. 10A).

Newly formed mesoglea 0.7-1.3 μm in thickness was present between the ectoderm and endoderm throughout the area of the organized layered structure (compared to 0.5-2 μm in normal hydra tissue; Haynes et al., 1968).

Fig. 10B shows a representative area in the complex organization in the main endodermal mass. Many cells were tightly but randomly packed together, and surrounded by large empty spaces and connecting narrow empty spaces. Cell surfaces exposed to the empty space had numerous microvilli similar to the apical surfaces of normal endodermal cells. However, myofibers characteristic of the basal cytoplasm could not be found in any of these cells.

DISCUSSION

Four stages of structural changes

The present study has shown a dramatic series of structural changes in the ectodermal-endodermal tissue recombinant of hydra. The changes observed are divided into 4 stages as schematically shown in Fig. 11.

Stage I occupied a short period immediately after tissue recombination. A firm adhesion was established between the

ectoderm and endoderm (Figs 2A and 4A). The ectodermal epithelial cells started to extend membranous or bulbous processes onto the endoderm (Figs 2D and 7).

Stage II began immediately following stage I, and lasted for about 24 hours. About a quarter of the endoderm was covered by the ectoderm (Fig. 4B). The endodermal epithelial cells lost epithelial organization and turned into a random mass of irregularly shaped cells (Fig. 8B).

Stage III started at about 18 hours, partially overlapping with stage II. Half to three-quarters of the endoderm was covered by the ectoderm (Fig. 4C). Epithelial-like organization began to be restored in the part of the endoderm directly covered by the ectoderm (Fig. 9B).

Stage IV started at about 20-30 hours. The endoderm was completely enveloped by the ectoderm (Figs 3D and 4D). The two-layered tissue organization similar to that present in normal hydra was re-established in a part of the recombinants (Fig. 10A).

Migratory ability of the ectodermal epithelial cells

In stages I to III, the ectodermal epithelial cells located at the advanced edge had processes filled with myofilaments (Figs 2D, 3C, 7 and 9A). These processes are probably a modified form of the muscle processes of the ectodermal epithelial cells. In intact hydra, the ectodermal muscle processes are long and narrow in shape, and extend longitudinally onto the mesoglea along the hydra axis (Otto, 1977). In the recombinant, they were short and irregular in shape, and extended onto the mesoglea or directly onto the endoderm in apparently random directions (Fig. 2D).

The processes in the recombinant exhibited a fine ruffled structure on the surface (Figs 3C and 9A), and contained a dense network of myofilaments (Fig. 7). In these respects, they appear similar to lamellipodia of migrating cells in culture (Abercrombie et al., 1971; Rajaraman et al., 1974), suggesting a possibility that these processes may provide locomotive ability for the epibolic ectodermal spreading over the endoderm.

Evidence exists, however, which suggests that the epibolic ectodermal spreading may be driven by a different mechanism. Kishimoto et al. (1996) examined movement of individual cells during the epiboly by spot vital staining of cells on the recombined ectodermal and endodermal cell aggregates. The results had shown that extensive cell rearrangement took place in the interface between the recombined two aggregates. Cells initially located in the inside of the endodermal aggregate migrated to the contact surface, and placed themselves among the cells located in the contact surface. This cell intercalation produced a gradual growth of the contact surface area, suggesting that this process is responsible, at least in part, for producing the spreading of the ectoderm.

Thus, two different mechanisms, not mutually exclusive, can be considered as the driving force for the epiboly. One is active locomotion of the ectodermal epithelial cells over the endoderm. The other is passive spreading of the ectodermal cells as the result of growth of the contact surface area generated by the endodermal epithelial cell migration and intercalation. Whether or not either of them really plays a crucial role in the epibolic ectodermal spreading remains to be examined.

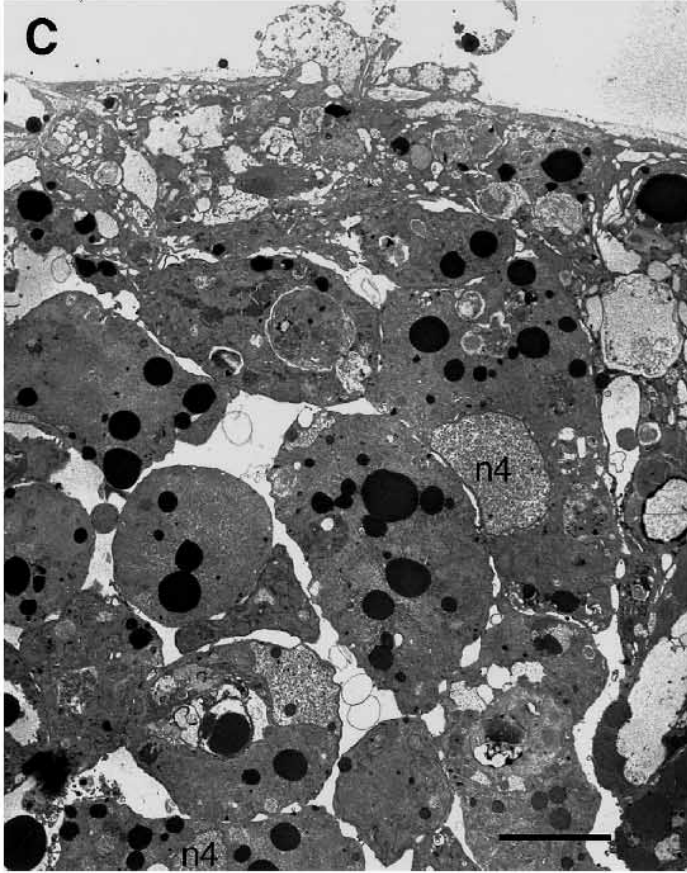
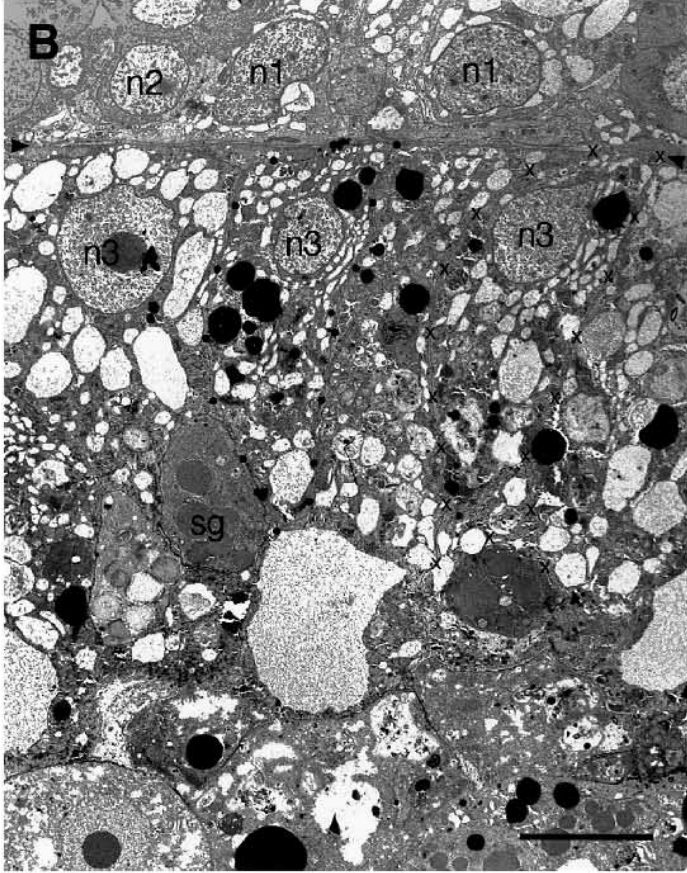
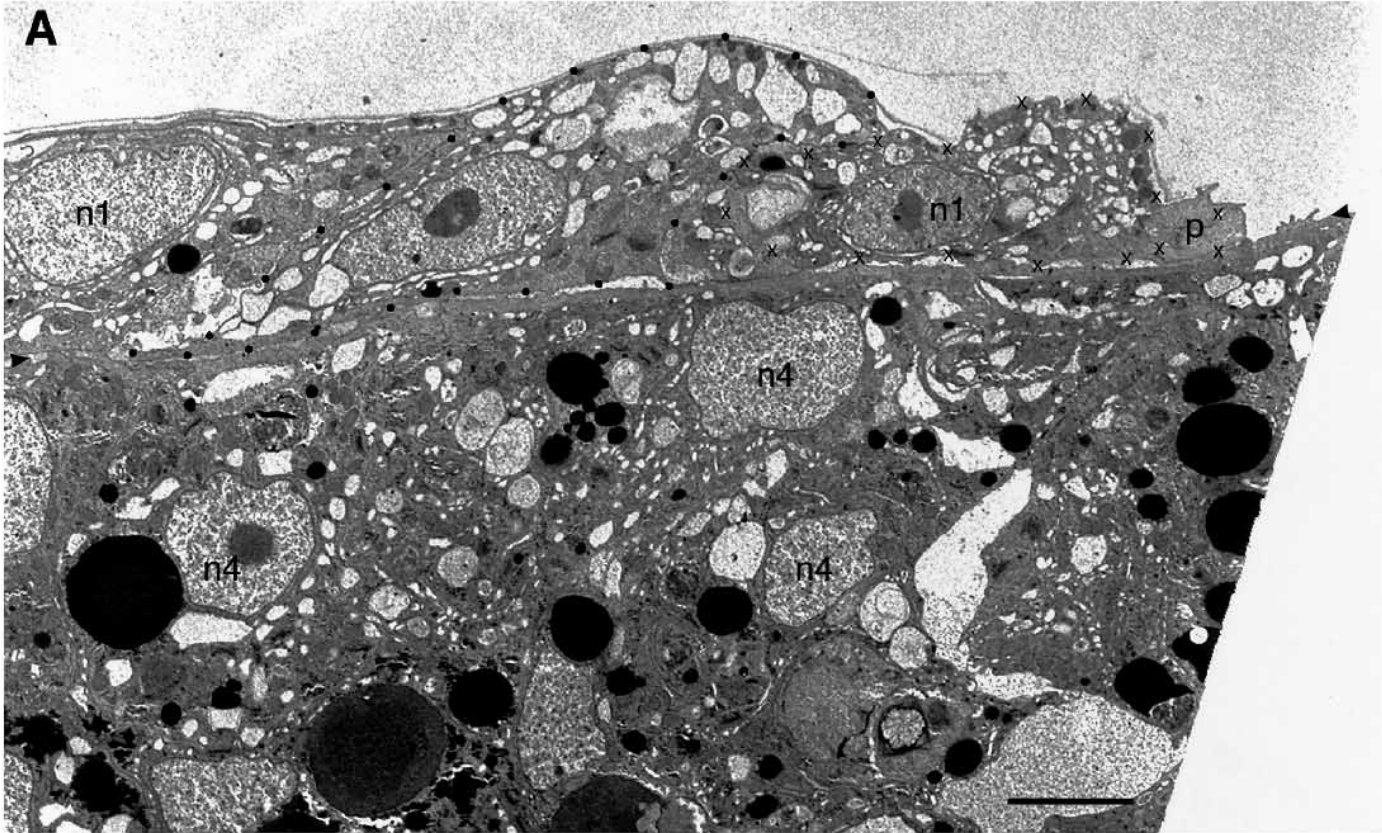


Fig. 9. The recombinants in stage III. (A) The area of contact between the advanced edge of the ectoderm and the endoderm at 19 hours. The border between the two tissues is indicated by arrowheads. Crosses and black dots indicate the outlines of two neighboring ectodermal epithelial cells. The cell on the right extends a lamellipodium-like process in the advancing direction. (B) The endodermal part covered by the ectoderm. Arrowheads indicate the border between the ectoderm and endoderm. Crosses and black dots indicate the outlines of two short columnar endodermal epithelial cells. Note that these 2 cells and another cell between them are

arranged parallel to each other in a single layer. (C) The endodermal part not covered by the ectoderm. Note that the tissue consists of a random mass of irregularly shaped cells, but that these cells form a distinct boundary at the periphery. n1, n2, n3 and n4: nucleus of ectodermal epithelial cell, interstitial cell, endodermal epithelial cell in partially organized area and irregularly shaped endodermal cell, respectively; p: process extended by an ectodermal epithelial cell onto the endoderm; sg: secretory granules in gland cells. Bars: 5 μm (A); 1 μm (B); 0.5 μm (C).

Epithelial organization

During the 4 stages of structural changes, an important difference was found between the ectodermal and endodermal tissue organization. The ectodermal epithelial cells exhibited significant changes in the cell shape and other properties. For example, the apical surface of these cells, which is initially polygonal (Fig. 2B), became narrow and stretched in stage II (Fig. 3B), or grew 4-5 times larger in the surface area in stage IV (Fig. 3E). When not in contact with the endoderm, some ectodermal epithelial cells formed myofilaments ectopically in unusual locations in the cytoplasm (Fig. 5C).

In spite of these changes, however, the ectoderm maintained the basic epithelial organization of a single-layered cell sheet throughout the 4 stages. All the epithelial cells continued to be tightly connected to each other by septate junctions (Figs 5A, 8A, 9A and 10A), and contained numerous mucous granules below the apical surface (Figs 5A,B, 8A, and 9A). They also had myofilaments above the basal surface whenever they were in contact with the endoderm (Figs 7, 9A and 10A). These observations indicate that the apical and lateral sides of individual ectodermal epithelial cells never lose their characteristic features present in normal tissue, suggesting that the basic

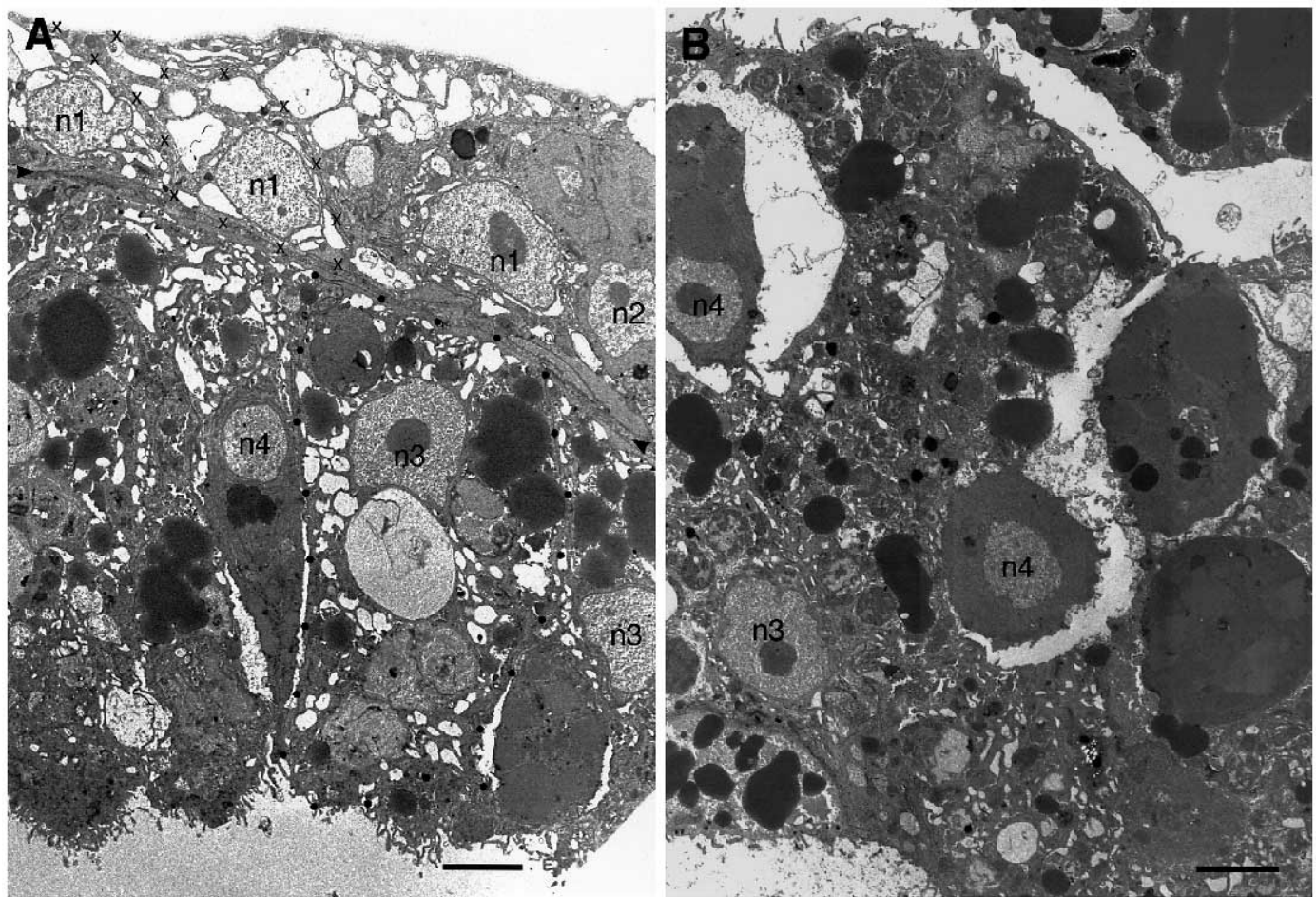
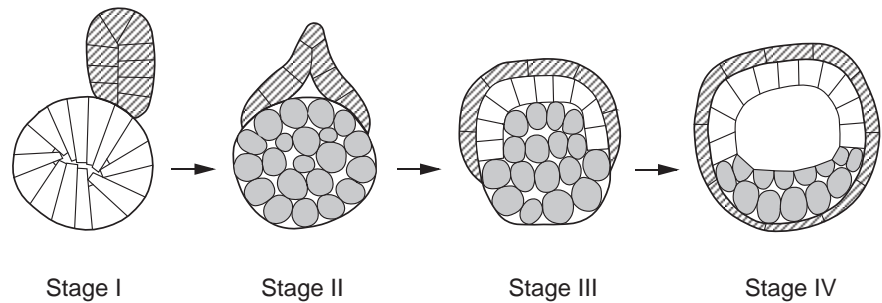


Fig. 10. Two types of tissue organization in a recombinant in stage IV (42 hours). (A) The area displaying a newly established epithelial organization. Arrowheads indicate the border between the ectoderm and endoderm. Crosses and black dots indicate the outlines of an ectodermal and an endodermal epithelial cell, respectively. Note that both the ectoderm and endoderm consist of single epithelial cell layers, and that numerous microvilli are present on the apical surface of the endodermal epithelial cells. (B) An internal part of the recombinant consisting of randomly packed endodermal cells. n1, n2, n3 and n4: nucleus of the ectodermal epithelial cell, interstitial cell, endodermal epithelial cell and gland cell, respectively. Bars: 5 μm .

Fig. 11. Schematic presentation of 4 stages of structural changes occurring in the recombined ectodermal-endodermal tissue. Ectodermal epithelial cells (cross-hatched) maintain epithelial organization throughout the 4 stages. Endodermal epithelial cells have epithelial organization in stage I (unshaded), lose it in stage II (lightly shaded), and re-establish it in stages III and IV (unshaded).



epithelial sheet organization is very stable and unaltered in the ectoderm during the entire 4 stages of structural changes.

The situation, however, was totally different in the endoderm. The typical epithelial sheet organization was completely lost, and the entire endoderm turned into a random mass of irregularly shaped cells in stage II (Fig. 8B). The apical-basal cell polarity of the normal endodermal epithelial cells, represented by cilia and microvilli on the apical surface and myofibers in the basal cytoplasm, could not be found in any of the irregularly shaped cells. Neighboring cells were joined together only at limited sites and without forming well-developed septate junctions, leaving major areas of the cell surface unattached to neighboring cells.

This lack of rigid tissue organization in the endoderm in stage II may provide motility to the endodermal cells, allowing them to participate in the active cell rearrangement processes in stages III and IV discussed below.

Re-establishment of the endodermal epithelial organization

The epithelial organization was re-established in the endoderm in stages III and IV. It is important to note that this process only occurred directly below the ectodermal layer (Fig. 9B) and never in other places, suggesting that the ectoderm is essential in some way for the formation of the endodermal epithelial sheet organization.

As already mentioned, the spot-labeling experiment in the recombined aggregates had shown that active cell migration and intercalation occurred at the contact surface between the ectoderm and endoderm (Kishimoto et al., 1996). It is conceivable that this cell rearrangement process plays a crucial role in re-establishing the endodermal organization directly below the ectoderm. The irregularly shaped endodermal cells may be attracted by a signal from the ectoderm to move to the contact surface, where they line up in a single cell layer, and subsequently establish the normal epithelial organization (Fig. 10A).

The same process of cell migration and intercalation may be also responsible for the bending of the endoderm into an L-shape (Fig. 3A). As more endodermal cells migrate from the interior toward the ectoderm, the number of cells increases at the contact surface but decreases in the interior. As this process occurs only in the part of the endoderm in contact with the ectoderm, it may cause the gradual bending of the endoderm at the contact site.

Another piece of evidence exists which supports active migration of the endodermal cells toward the ectoderm. The number of gland cells in the partially organized ectodermal tissue below the spreading ectoderm was only about 20% as

many as in the adjacent region of unorganized tissue. This observation is consistent with the view that only the endodermal epithelial cells selectively migrate to the contact surface to participate in forming the partial epithelial organization below the ectoderm, whereas the gland cells are left behind to remain in the unorganized cell mass.

Mutual dependency between the ectodermal and endodermal tissue

Observations made in this and previous studies by Kishimoto et al. (1996) have revealed interesting features of the interactions between the ectodermal and endodermal tissue layers in hydra. In normal hydra, these two tissue layers are separated by a thick layer of mesoglea. However, the cells located on both sides of the mesoglea maintain direct contact by means of long tissue projections which penetrate through the mesoglea to reach the cells on the other side (Wood, 1979).

When the two tissue layers are separated from each other by procaine, and each tissue is kept alone without recombining with the other, both tissue types invariably disintegrate within a few days. However, both types survive if they are recombined. This is also true when each tissue type is dissociated and then reaggregated before recombining with the other. An ectodermal or endodermal aggregate disintegrates if kept alone, but survives if the two are rejoined (Kishimoto et al., 1996).

Another type of relationship also exists between the two tissue layers. As already discussed, the endoderm rapidly loses its epithelial organization once it is separated from the ectoderm. This is true even in endoderm to which the ectodermal tissue was attached soon after tissue separation. Later, the endoderm regained its epithelial organization in the area directly below the spreading ectoderm. In contrast, the ectoderm did not lose its epithelial organization as long as it is attached to the endoderm and alive.

Taken together, these observations suggest that there is strong mutual interaction between the ectoderm and endoderm. The endoderm, either as a cell mass or as an organized epithelium, can support the survival of ectoderm and maintenance of its epithelial organization. The reverse, however, is only partially true. Ectodermal cell mass can support endodermal survival, but not maintenance of its epithelial organization. Only intact ectodermal epithelium can induce the endoderm to form and maintain its epithelial organization.

Temporary loss of the epithelial organization and 'dedifferentiation'

The observations made in this study clarify an important controversial issue on the role of the interstitial cell lineage in hydra regeneration. Previous investigators observed abnor-

mally shaped cells similar to the irregularly shaped cells observed in this study. They appeared in the endoderm of the regenerating tissue soon after the removal of the original head (Mookerjee and Bhattacharjee, 1966; Znidaric, 1970), or in the endoderm separated from the ectoderm by enzyme treatment (Davis et al., 1966). These cells were referred to by various names such as 'embryonic cells' (Rose and Burnett, 1968), 'neoblasts' (Lui and Znidaric, 1968) or 'amoeboid interstitial cells' (Mookerjee and Bhattacharjee, 1966). As implied by their names, these cells were thought to arise by 'dedifferentiation' from endodermal cells, and have the ability to differentiate into other cell types (Davis et al., 1966; Mookerjee and Bhattacharjee, 1966; Znidaric, 1970). In short, they were considered to be the equivalent of the blastema cells or neoblasts which appear in regenerating tissue of urodele amphibians or planarians, respectively.

We now suggest that these cells are identical to the irregularly shaped endodermal epithelial cells observed in this study. Although the abnormally shaped cells observed by previous workers may appear similar to normal interstitial cells at light microscope level, distinct morphological differences are present in the two cell types at the electron microscope level. For example, the abnormally shaped cells always contain numerous small vacuoles and sometimes phagosomes (for example see the marked cell in Fig. 8B), whereas such structures are never found in any of the interstitial cells (for example see the n2 cell in Fig. 5A). In addition, there is no evidence which directly shows that the abnormally shaped cells have a strong ability to proliferate or to later turn into different cell types other than the endodermal epithelial (or gland) cells.

Furthermore, it is well established that the interstitial cell lineage is not essential for hydra regeneration. Epithelial hydra completely free of interstitial stem cells, nerve cells or nematocytes can regenerate nearly as well as normal hydra (Marcum and Campbell, 1978; Sugiyama and Fujisawa, 1978). Thus, the cellular mechanisms of regeneration in hydra differ fundamentally from those in urodele amphibians or planarians. In the latter organisms, blastema cells or neoblasts accumulate in the regenerating tissue, and are thought to play a crucial role in the strong regenerative abilities of these animals (for review see Chernoff and Stocum, 1995). In contrast, cell dedifferentiation and redifferentiation plays little or no role in hydra regeneration.

At present, the roles of the irregularly shaped endodermal epithelial cells in hydra regeneration is uncertain. One may speculate that the remarkably high plasticity of the endodermal tissue organization in hydra is correlated in some way to the strong regenerative capacity of this organism.

Control of epithelial organization

Morphogenetic processes of epithelial sheet formation are widely observed in vivo in various embryonic systems and in vitro in many types of cultured cells. In the case of chick skin tissue, loss of epithelial organization occurs by removal of the basement membrane from its tissue, suggesting the importance of the basement membrane for maintenance of the epithelial organization in this system (Dodson, 1967).

In the present system, however, loss of epithelial organization takes place in the endoderm to which the basement membrane (mesoglea) is attached, but not in the ectoderm

which is free from the basement membrane after procaine separation. Therefore, the basement membrane may not be too important, but direct interaction with the ectoderm may be crucially important for the maintenance and formation of the endodermal epithelial organization in hydra.

In the case of *Drosophila*, two genes have been identified which play crucial roles in controlling epithelial organization (Knust, 1994). One is *crumbs* which encodes a large transmembrane protein localized in the apical surface of ectodermal epithelial tissue. Mutation in this gene results in the loss of apical-basal polarity (Tepass et al., 1990; Tepass and Knust, 1990), whereas its over-expression leads to expansion of the apical surface and reduction of the baso-lateral surface in the ectodermal epithelial tissue (Wodarz et al., 1995). Mutation in the other gene, *stardust*, also produces loss of cell polarity. Furthermore, zonula adherens are not properly formed in both mutants (Grawe et al., 1996).

Hydra may have genes which play similar roles to these two *Drosophila* genes. When the endoderm is separated from the ectoderm, functioning of such genes in the endoderm may be disrupted to convert the endodermal tissue into a mass of irregularly shaped cells without apical-basal polarity. The identity of such genes in hydra, however, is unknown at present.

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