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Cell Fusions in the Developing Epithelia of *C. elegans*

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In this paper we characterize the order of hypodermal cell fusions in the *Caenorhabditis elegans* hermaphrodite. Somatic cell fusions are part of the developmental program of many tissues in a variety of organisms. The formation and remodeling of tissues and organs can be studied at the cellular level in *C. elegans*. Here we establish a system for studying cell fusion by characterizing somatic cell fusions during morphogenesis in *C. elegans*. Fusion is a common cell fate in this nematode; numerous epithelial fusions occur in the hypodermis, vulva, uterus, and excretory gland cells (Sulston *et al.*, 1983. *Dev. Biol.* 100, 64–119). Some but not all pharyngeal muscles also fuse (Albertson and Thomson, 1976. *Philos. Trans. R. Soc. London Ser. B* 275, 299–325). We have studied the behavior of epithelial adherens junctions before and during cell-to-cell fusions in embryonic and postembryonic development. Our results define the timing and sequence of short-range migrations followed by fusions that generate syncytia. We have made use of an antibody that stains adherens junctions to study the behavior of hypodermal cells during development. Fusion of specific cells in the hypodermis causes rearrangements of the adherens junctions between cells. Fusion events usually start in the anterior part of embryos or larvae. There is some variation in the specific order in which cells fuse, but the final positions, boundaries, and sizes of syncytia are the same. In some cases fusion causes isolation of a mononucleate cell or group of cells by a surrounding, growing syncytium. Our characterization of the order of cell fusions will provide a basis for the identification of molecular events required for regulated membrane fusion during development.

INTRODUCTION

Cell-to-cell fusion is a universal process involved in fertilization (Yanagimachi, 1988), conjugation in yeast and other fungi (McCaffrey *et al.*, 1987; Trueheart *et al.*, 1987), entry of enveloped viruses (Stegmann *et al.*, 1989; White, 1990), and syncytia formation in placenta (Pierce and Midgley, 1963), muscle (Wakelam, 1988), and osteoclasts (Baron *et al.*, 1986; Jee and Nolan, 1963). Little is known about the mechanisms involved in cell fusion. Plasma membrane fusions are spatially and temporally regulated at the subcellular, cellular, and organismal levels. It is believed that specific polypeptides mediate different fusion events (White, 1992). To date the best characterized fusion proteins are those responsible for viral penetration (Stegmann *et al.*, 1989; White, 1990). These spike glycoproteins contain hydrophobic peptides that are inserted into membrane bilayers overcoming hydration forces between them and triggering fusion (Harter *et al.*, 1989; Stegmann *et al.*, 1991). However, the mechanisms for induction of membrane fusion remain controversial (Gallaher *et al.*, 1992; van der Goot *et al.*, 1992).

The nematode *Caenorhabditis elegans* is a good model system for analyzing cell fusions during development *in vivo*. The lineage of all the 959 somatic nuclei in the adult hermaphrodite is known, and it is essentially invariant (Sulston and Horvitz, 1977; Sulston *et al.*, 1983). Around one-third of all the nuclei in the adult reside in various multinucleate cells (syncytia) formed by cell-to-cell fusion. The largest syncytium in the adult has 133 nuclei; other large syncytia contain 15, 6, and 4 nuclei, and there are numerous trinucleate and binucleate cells in different tissues. Syncytia are generated by cell fusions that occur during embryonic and postembryonic development. They are formed in pharyngeal muscles, during organogenesis of the vulva and uterus, and in the morphogenesis of the hypodermis (epidermis) (Sulston and Horvitz, 1977; Sulston *et al.*, 1983; White, 1988).

Since *C. elegans* is transparent throughout the life cycle, it is possible to follow the nuclei of all cells during development using differential interference contrast (Nomarski) microscopy. However, when cells are closely apposed to each other, boundaries are not generally resolved by this method. Since it is necessary to have close contact between membranes in order for cell membranes to fuse, it is in general not possible to follow

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membrane fusion using Nomarski optics. The generation of all mononucleate cells in embryos and larvae of *C. elegans* was originally studied by light microscopy (Sulston and Horvitz, 1977; Sulston *et al.*, 1983; White, 1988), and the existence of syncytia was deduced from ultrastructural reconstructions of larvae and adults by serial section electron microscopy (Albertson and Thomson, 1976; White, 1988; White *et al.*, 1976).

The spatial and temporal sequence of events in the formation of syncytia has not been studied in any detail. This paper describes a developmental analysis of epithelial cell fusions in *C. elegans*. We followed changes in cell boundaries during embryonic and postembryonic development using immunofluorescent staining of an antigen associated with adherens junctions (Baird *et al.*, 1991; Priess and Hirsh, 1986; Waterston, 1988). We followed the order of fusions using 3D[3] reconstructions of confocal images of embryos, larvae, and adults at different stages in development. The first embryonic fusions start after a stereotyped pattern of migrations in the dorsal hypodermal cells has occurred; anterior cells fuse first. Although the order in which cells fuse is not completely invariant, the final boundaries between syncytia connected by adherens junctions are the same in all individuals analyzed. Our observations define the order of cell fusions between epithelial cells of known invariant lineage. We discuss the implications of cell fusion in a developmental program.

MATERIALS AND METHODS

Nematode Strains and Maintenance

Wild-type (N2) *C. elegans* variety Bristol was cultured at 20°C on agar plates carrying *Escherichia coli* strain OP50 as food source (Brenner, 1974).

Preparation and Fixation of Embryos

A population of embryos from different stages was obtained by washing 5-cm plates containing gravid adults and eggs with 0.8 ml of M9 buffer (Brenner, 1974). The suspension was mixed with 0.4 ml of alkaline hypochlorite (4 vol of 2 *M* NaOH:3 vol of 14% NaOCl) in an Eppendorf tube. After 5–10 min with occasional mixing the tube was spun in a microfuge (1 min 6000 rpm) (Sulston and Hodgkin, 1988). The pellet was taken up in 0.4 ml of M9 buffer and mixed with the same volume of ice-cold 2 *M* sucrose/100 m*M* NaCl. Distilled water (0.2 ml) was placed on the sucrose cushion and the embryos were floated to the interface after 5 min centrifugation at 6000 rpm (Goh and Bogaert, 1991). The embryos were recovered from the sucrose–water interface with a Pasteur pipette and washed once with PBS. Embryos were collected by centrifugation and resuspended in distilled water. Ten-microliter aliquots of the embryo suspension were placed per well on 8-well multitest slides (Flow Laboratories, UK) coated with 0.1% polylysine. A 22 × 40 mm coverslip was placed on top of the embryos. The embryos were frozen by placing the slides on dry ice. After freezing, the coverslips were flipped off and the slides were immersed in methanol followed by acetone for 5 min each at −20°C. The slides were washed in PBS for 10 min followed by PBS containing 0.5% Tween 20 (Albertson, 1984; Hyman and White, 1987).

Immunofluorescence

Fixed embryos were incubated in PBS-Tween supplemented with 2% dried milk for 15 min to reduce background, and all the antibodies were diluted in PBS-Tween-milk. The incubations with antibodies were done with 5 μl per well in a humidified chamber at room temperature. The monoclonal antibody MH27 (initially identified by Ross Francis and generously provided by R. Waterston) was used to visualize adherens junctions (Baird *et al.*, 1991; Priess and Hirsh, 1986; Waterston, 1988). It was used at an optimal 1:300 dilution for 45 min. After three washes for 5 min each in PBS-Tween, the embryos were incubated in FITC-labeled goat anti-mouse IgG (Sigma) at a 1:50 dilution for 45 min. Where indicated, nuclei were stained with 0.2 μg/ml propidium iodide for 5 min, after the secondary antibody incubation that was supplemented with 200 μg/ml RNase at 37°C. The slides were washed three times in PBS-Tween and once in PBS for 5 min each. They were briefly immersed in distilled water and permanent mounts were prepared using polyvinyl alcohol (Elvanol 51-05 or Mowiol 4-88; Hoechst) prepared as described (Heimer and Taylor, 1974) containing 20 mg/ml DABCO (Sigma). The specimens were observed after 20 min with a Zeiss Axioplan microscope equipped for epifluorescence. In experiments with larvae and adults, 10–20 animals of the indicated stage were placed in 10 μl of distilled water per well on 8-well slides coated with polylysine. The animals were then frozen, fixed, processed for immunofluorescence, and mounted as described above.

Confocal Microscopy

The specimens were analyzed using a MRC-600 Series laser scanning confocal microscope (Bio-Rad). Extended focus projections and stereo pair images were obtained

[3] Abbreviations used: 3D, three-dimensional; ZA, zonula adherens; VPC, vulva precursor cell; L1, L2, L3, L4, 1st, 2nd, 3rd, and 4th larval stages, respectively; PBS, Dulbecco's phosphate-buffered saline; FITC, fluorescein isothiocyanate; DABCO, triethylenediamine.

from 10 to 30 serial optical sections collected every 0.5 to 1 μm in the *Z*-axis. Images were recorded on IBM 3363 optical disks and photographed from the screen on Ilford FP4 film. Color images were printed on a Mavigraph color video printer (Sony). Unless otherwise stated, all figures are oriented with anterior toward the top of the page. All developmental times were estimated from the shape and length of the animals (Sulston and Horvitz, 1977; Sulston *et al.*, 1983). At least 50 animals of each developmental stage were analyzed.

RESULTS

Cell-to-Cell Fusion in the Hypodermis of an Organism with Invariant Cell Lineage

The lineage of all the 959 somatic nuclei in the *C. elegans* hermaphrodite is known to be essentially invariant (Sulston and Horvitz, 1977; Sulston *et al.*, 1983). Extensive cell fusions occur during different stages of development. The hypodermis in *C. elegans* embryos is the outer monolayer of 78 epithelial cells that secrete the cuticle. Nuclei forming the hypodermis are generated 210–230 min after first cleavage (Sulston *et al.*, 1983). One of the functions of the hypodermis is likely to be to establish the basic body architecture of the nematode (Priess and Hirsh, 1986; White, 1988).

Figure 1 shows cylindrical projections of the 65 larger cells in the hypodermis of an embryo cut open along the ventral midline and viewed from outside the animal. Smaller cells from the head and the tail are not drawn. At about 250 min after the first cleavage, the larger hypodermal cells are arranged in six parallel longitudinal rows (Fig. 1A). Between 250 and 390 min the central two rows of cells migrate contralaterally and interdigitate to form a single row of dorsal hypodermal cells (Figs. 1B and 1C) (Sulston *et al.*, 1983).

The large hypodermal cell, hyp7, is formed in the embryo by the fusion of 23 cells (Sulston *et al.*, 1983) including most of the middorsal row of cells, which are labeled arbitrarily 1 to 23 in Fig. 1C. During postembryonic development, an additional 110 cells fuse with hyp7, forming the largest syncytium in the adult body (Sulston and Horvitz, 1977). Hypodermal cells other than hyp7 also fuse forming cylindrical syncytia linked by circular adherens junctions or zonula adherens (ZA) (White, 1988).

Migration of Dorsal and Ventral Cells Prior to First Fusions in the Embryo

To study cell boundaries during migrations and fusions we used the MH27 monoclonal antibody (Waterston, 1988), which stains the ZA of all epithelial cells in embryos, larvae, and adults. Extensive serial section electron microscopy of *C. elegans* has shown that patterns of MH27 staining correlate with the presence of adherens junctions (White, 1988); J. G. White, unpublished results). Disappearance of a particular cell junction (ZA) between two cells represents the fusion of two apposed membranes and correlates with observations of syncytia by serial section electron microscopy (J. G. White, unpublished results). Thus, we were able to visualize changes in cell boundaries taking place during embryogenesis.

The first staining observed by indirect immunofluorescence with the anti-ZA antibody MH27 was detected after hypodermal cells were generated. ZAs were first seen as a discontinuous punctuate staining around the hypodermal nuclei stained in red with propidium iodide, revealing the cell boundaries of epithelial cells (Fig. 2A). The antigen was first detected dorsally in the posterior region (Fig. 2B). Six rows of hypodermal cells can be seen: two central that are dorsal hypodermis; two lateral that will form the seam cells (specialized epithelial cells that are situated along the lateral lines (Sulston and Horvitz, 1977), left and right); and two external rows that will become the ventral hypodermis (Figs. 2A and 1A). As shown in Fig. 3A, the two rows of dorsal hypodermal cells migrate and interdigitate to form a single row of dorsal cells (Figs. 3A and 3B). Simultaneously the two outer rows migrate toward the ventral side (Figs. 3A and 3C). While dorsal cells interleave (Fig. 3A), ventral cells (Fig. 2B) migrate to encounter the cells coming from the contralateral side and attach to them by means of adherens junctions. Thus, symmetric and simultaneous short-range migrations in the dorsal and ventral hypodermis enclose a spheroidal embryo before it elongates to become a cylindrical embryo. Small hypodermal cells in the head and tail are attached to many sensory receptors via interfacial hypodermal cells forming ZAs (White, 1988); these were not resolved in the light microscope.

Cell Fusions during Embryonic Development

To study fusion events in the developing epithelium, we obtained serial optical sections using confocal microscopy as described under Materials and Methods. Figure 4A shows a stereo pair of projections generated from 1-μm-thick serial optical sections of an embryo where all the hypodermal cells have migrated to their prefusion positions. Once migration is completed in the dorsal and ventral sides, the embryo starts to elongate. The first cell-to-cell fusion was detected in the ventral side as the embryo is being enclosed by hypodermal cells (340 min after first cleavage).

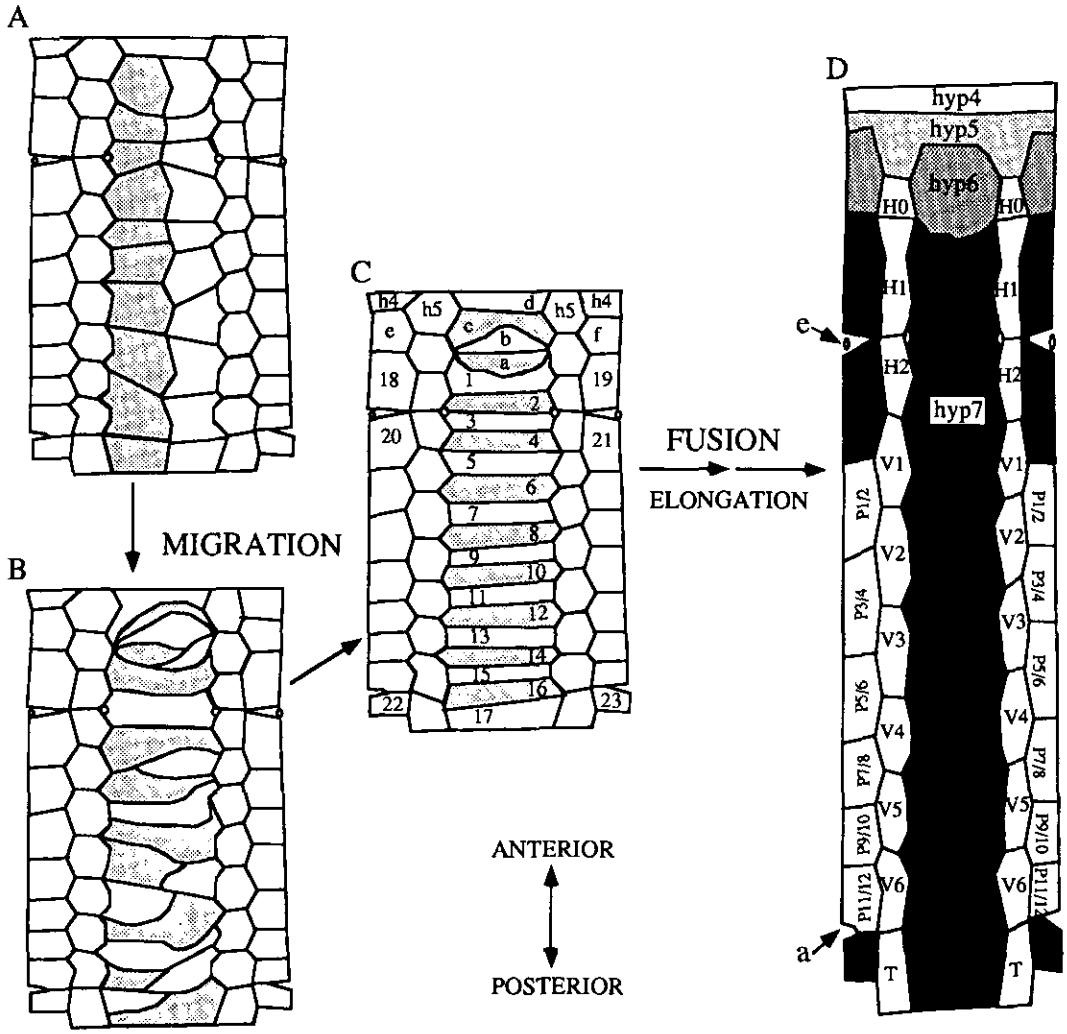

FIG. 1. Migration, interdigitation, and fusion of epithelial cells in the embryo. Cylindrical projection of the 65 cells making up the central body region of the hypodermis (epidermis) of an embryo cut open along the ventral midline and viewed from outside. (A) Epithelial cells are arranged in six parallel rows. (B) The central two rows of cells migrate and interdigitate to form a single row of hypodermal dorsal cells. (C) After migration of hypodermal cells, 17 cells from the dorsal hypodermis fuse with cells 18–21 in the ventral hypodermis (around the excretory pore) and with cells 22–23 (around the anus) to form hyp7, the largest syncytium. Hyp6 syncytium is formed by the fusion of cells a–f. The left and right lateral h5 cells migrate and fuse to form hyp5. Left and right ventral h4 cells fuse to form hyp4. The fusions in the embryo occur before and during elongation. (D) Left and right cells in D are labeled according to (Sulston *et al.*, 1983) e, excretory pore; a, anus.

Figure 4B shows the first embryonic fusions after egg and sperm fusion. The embryo viewed from the ventral side (Fig. 4B right, arrowhead) shows the disappearance of the ZA between cells 18 and 19 (depicted in Fig. 1C). The MH27 staining between these two anterior cells of the ventral epithelium (arrowhead) becomes fainter and disappears before the comma stage, indicating that these two mononucleate cells have fused. An earlier embryo in Fig. 3C also shows cells 18 and 19 fused, although other posterior ventral hypodermal cells have not completed their migration toward the ventral midline (arrowheads). Thus, cells 18 and 19 meet in the ventral midline, form a new ZA (Fig. 4A, left arrow), and become fusion-competent during enclosure (Fig. 3C). Another early fusion occurs in the dorsal hypodermis between two cells located anterior to the deirids (sensory sensilla; (White *et al.*, 1986)). This is the second intermediate in the formation of the hyp7 syncytium and corresponds to the fusion of cells 1 and 2 in Fig. 1C. Thus, the earliest fusion intermediates that we have detected in the developing *C. elegans* embryo occur just before the onset of elongation, between two cells of the anterior ventral hypodermis and another pair in the dorsal hypodermis that fuses independently.

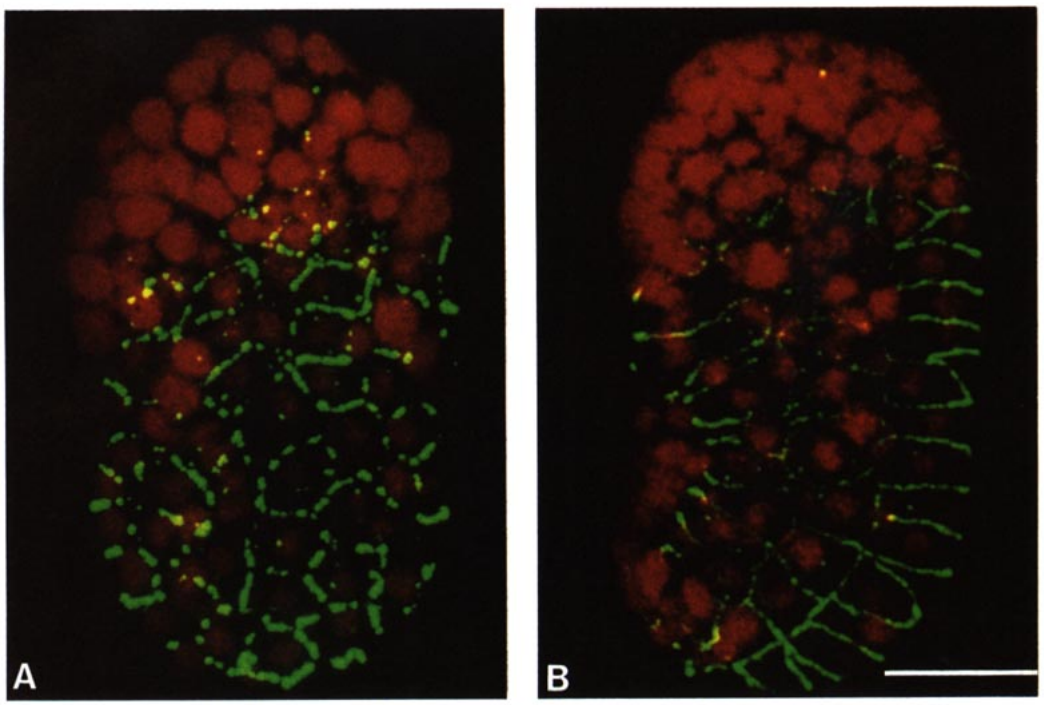

FIG. 2. Indirect immunofluorescence of adherens junctions (ZA) of fixed embryos visualize dynamic changes in cell boundaries. (A) Double staining of early embryo (~250 min after first cleavage). ZA is formed as a discontinuous punctate staining around hypodermal nuclei stained in red with propidium iodide. Six parallel rows of nuclei surrounded by punctate MH27 stain (see Materials and Methods) form a flat monolayer on the dorsal surface. (B) Lateral left view of a later embryo where the flat monolayer seen in A is now surrounding the embryo (250–300 min). The cells on the left are migrating toward the ventral side (left) to enclose the embryo. Epithelial nuclei of the head (top) do not express the MH27 antigen at this stage. The antigen recognized by MH27 appears first in dorsal posterior cells where it remains brighter than in lateral and ventral cells during migration. Times of development were estimated from Sulston *et al.* (1983). About 100 embryos of each developmental stage were observed. Bar, 10 μm.

Order in Which 23 Cells Fuse to Form the Large Hypodermal Syncytium (hyp7)

The hypodermis contracts circumferentially, thus elongating the body (Priess and Hirsh, 1986; Sulston *et al.*, 1983). Most of the hypodermal fusions occur as the embryos elongate. To analyze the order of fusions of 23 mononucleate cells that will form hyp7 (Figs. 1C and 1D), we studied the patterns of fusions in hundreds of elongating embryos as revealed by the MH27 staining. We found that fusions start around 3 hr after the cells are generated. Fusions start in the anterior part of hyp7 and in general progress toward the posterior part of the elongating embryo. Most dorsal and ventral fusions are completed between 1.5- and 2-fold elongation. In the section above we showed the earliest fusions at the onset of elongation and here we will describe four typical embryos with fusion intermediates. In an embryo that had elongated 1.5-fold (3-fold is completely elongated), cells 1 + 2 and 18 + 19 fused early as shown in less elongated embryos (e.g., Fig. 4B), five cells of the dorsal epithelium had fused (Fig. 5A; large arrowheads correspond to cells 1 + 2, 18 + 19, and 4 to 8 in Fig. 1C). This pentanucleate cell is separated from a binucleate cell, also present in Fig. 4B, by one cell that has not yet fused (cell 3 in Fig. 1C).

Figure 5B shows an unusual embryo in which the two anterior dorsal cells that normally fuse first (e.g., Figs. 4B and 5A) had not fused (cells 1 and 2 in Fig. 1C). This embryo had elongated 1.5-fold (~430 min after first cleavage) and was an absolutely typical embryo apart from the lack of fusion between cells 1 and 2. Very few embryos out of hundreds observed to date have shown cells 1 and 2 unfused when most other cells in the dorsal hypodermis are already fused (arrows in Fig. 5B; cells 3–16 from Fig. 1C). Thus the order in which hypodermal cells fuse is not completely invariant. Twenty cells that will form the hyp7 have fused in four independent groups in the embryo shown in Fig. 5B. These include a group of 14 cells in the dorsal hypodermis (cells 3 to 16 in Fig. 1C), and three binucleate cells in the ventral hypodermis (18 + 19; 20 + 21; 22 + 23 in Fig. 1C). The 1.5-fold stage embryo shown in Fig. 6A contained all the fused groups observed in Fig. 5B plus the binucleate cell formed by the fusion of cells 1 + 2, and cell 17 fused to 3–16.

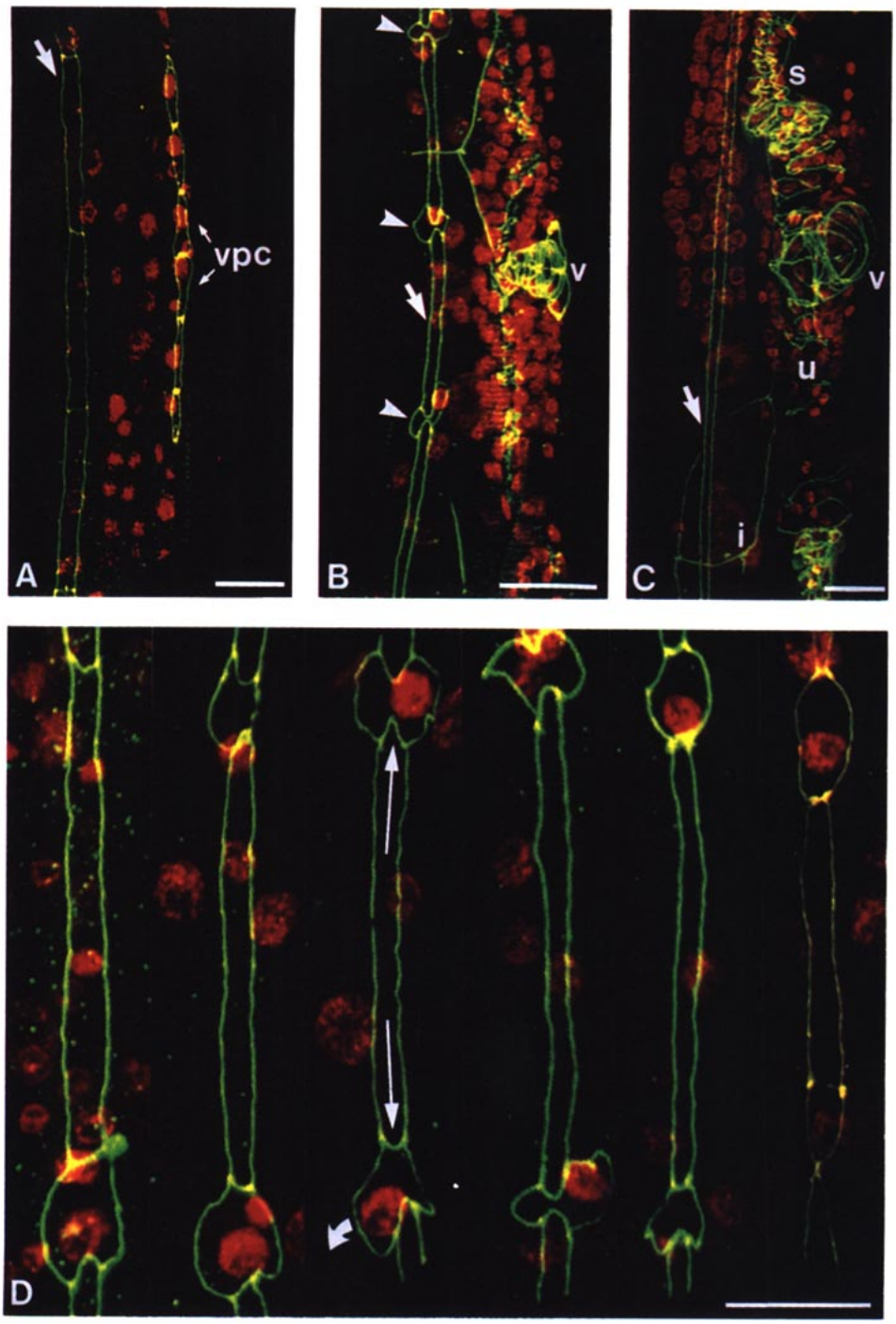

FIG. 9. Postembryonic migrations and fusions in the L4. Double staining of adherens junctions ZA (green) and nuclei (red). (A) Projection of L3 larva; arrow points to right seam cells at around 30 hr after hatching. Vulva precursor cells (vpc) on the ventral side are the daughters of P5.p, P6.p, and P7.p. They have become isolated by the surrounding syncytium of hyp7. (B) Anterior daughters (arrowheads) of seam stem cells (arrow) at around 35 hr in L4. The vulva (v) was formed by the 22 descendants of vpc after migrations and fusions (manuscript in preparation). (C) After fusion of their anterior daughters, seam cells stop dividing and fuse to each other forming a continuous syncytium (arrow) containing 15 nuclei (around 41 hr after hatching). Left-ventral view of v, vulva; s, spermatheca; u, uterus; i, intestine. The uterus contains around 50 nuclei organized in syncytia. (D) Higher magnification of the same animal as in B. Migration of 10 of the 13 anterior daughters of seam cells toward the surrounding hyp7 syncytium where they will fuse (small arrow). On the left side of D are the most anterior seam cells; toward the right are more posterior seam cells. As the anterior smaller daughters are sinking into the syncytium, seam cells elongate on top of the apical surfaces of their daughters until they touch each other (long arrows). The nuclei of the fusing cells are brighter because they have undergone endoreduplication of their DNA. Bars, 10 μm.

Thus, fusions during embryogenesis are fairly ordered but do not follow a completely invariant sequence. Two lateral rows of hypodermal cells (known as seam cells) are not fusogenic during embryogenesis while dorsal cells fuse starting in the anterior area close to the deirids and six ventral cells fuse by pairs. Cells 1 and 2 usually fuse first (Fig. 5B is an exception) around the same time as cells 18 + 19 in the ventral hypodermis, but their pairwise fusion is independent of the fusion of cells 3 to 17. The ZA between cells 2 and 3 disappears only after all the other dorsal and ventral fusions have occurred. Ventral fusions are by pairs between left and right mononucleate cells that fuse along the ventral midline (18 + 19; 20 + 21; 22 + 23; Fig. 1C). The 23-nucleate hyp7 syncytium covers most of the dorsal area of the three-fold embryo and L1. This syncytium also surrounds the animal in the anterior ring around the excretory canal and in the posterior ring around the anus. Using MH27 staining we were not able to resolve the fusions between dorsal (1–17) and ventral (18 + 19, 20 + 21, and 22 + 23) cells.

Some Hypodermal Fusions Cannot Be Easily Resolved by MH27 Immunofluorescence

It is difficult to imagine how ventral syncytia fuse with dorsal syncytia to form hyp7 and hyp6 despite the presence of two lateral rows of seam cells that separate them (e.g., Figs. 1 and 4–6). There are no ZAs between fused binucleate ventral cells (e + f, 18 + 19, 20 + 21, and 22 + 23) and the dorsal syncytia (a–d and 1–17), thus it is not possible to use the disappearance of MH27 staining as an indication of fusion. Serial section electron microscopy of embryos at about 1.5-fold elongation has shown continuous syncytial cells located in the dorsal hypodermis, under the seam cells, and in the ventral hypodermis (R. Durbin personal communication and J. G. White unpublished results). Thus, dorsal cell 1–17 fuses with ventral 18 + 19, 20 + 21, and 22 + 23 under the seam cells without the involvement of ZAs. The six cells of hyp6 are fused in two groups, the dorsal formed by cells a–d and the ventral binucleate cell e + f (Figs. 5, 6, and 1), and these two syncytia also fuse during elongation.

Once the embryo has elongated 3-fold, toward the end of embryogenesis (Fig. 6B), hyp7 contains 23 nuclei. Six of these nuclei are derived from the ventral hypodermis and the rest are derived from the dorsal hypodermis. The hypodermis in the head is formed by six concentric rings, hyp1 through hyp6, containing 3, 2, 2, 3, 2, and 6 nuclei, respectively (White, 1988). Figure 6B shows the hyp6 annular syncytium anterior of hyp7, as well as hyp5 and hyp4. Hyp1–3 cannot be resolved by MH27 staining in this region, which is very rich in sensory endings that form multiple small adherens junctions with the hypodermis. Posterior of hyp7 there are four cells hyp8 through hyp11, containing 1, 1, 2, and 1 nuclei, respectively (White, 1988). Hyp10 fusion occurs during elongation between 1.5- and 3-fold stages (Figs. 5B and 6A; see tip of the tail, or most posterior concentric ring).

Epithelial Cell Fusions during Postembryonic Development

During larval development the lateral seam cells H1–H2, V1–V6, and T (Fig. 1D) on each side act as hypodermal stem cells, dividing at each larval stage. These two laterally placed, longitudinally oriented rows of cells on each side of the animal are embedded in the hypodermal syncytium hyp7 and are also in close contact with the P cells (ventral epithelial cells that also act as ventral cord blast cells; Fig. 1D). In general the posterior daughter of each seam division is a seam cell while the anterior daughter fuses to hyp7, except H1 for which this pattern is inverted during the first two divisions. Three of the seam cells (H2, V5, and T) also give rise to neuroblasts (Sulston and Horvitz, 1977).

To study intermediates in postembryonic fusions we followed the same strategy as with embryonic fusions. L1s were fixed and stained with MH27 antibody to visualize the adherens junctions (ZA) that define boundaries between cells. Using this approach we were able to reconstruct the order of generation, migration, and fusion of cells to the hyp7 syncytium. Figure 7 summarizes these observations from a newly hatched L1 to the end of the first cycle of cell divisions, migrations, and finally fusions about 7.5–9.5 hr later. After hatching, the L1 is surrounded by a set of cylindrical syncytia generated during embryogenesis and joined by ZAs (Sulston *et al.*, 1983). The hyp7 syncytium is cylindrical in its most anterior part around the excretory pore and in its posterior section around the anus (these cylindrical regions of the syncytium are generated by fusion under the seams as described in the previous section). However, the hyp7 syncytium is not cylindrical in midbody at this time (Fig. 7A). Two rows of 10 lateral seam cells that run on each side of the L1, and two rows of 6 P cells located on either side of the ventral midline, are all surrounded by syncytial hypodermis (Fig. 7A). The seam cells round up (Figs. 7B and 8A) and divide (Fig. 7C) about 5 hr after hatching (except H0 which does not divide). H1 and H2 divide 2.5 and 2 hr, respectively, after the other seam cells (Sulston and Horvitz, 1977). About the middle of the L1 stage the anterior daughters of V2–V6 send cytoplasmic processes toward the ventral midline (Fig. 7D). These seam cell migrations start between the most anterior P cells (Fig.

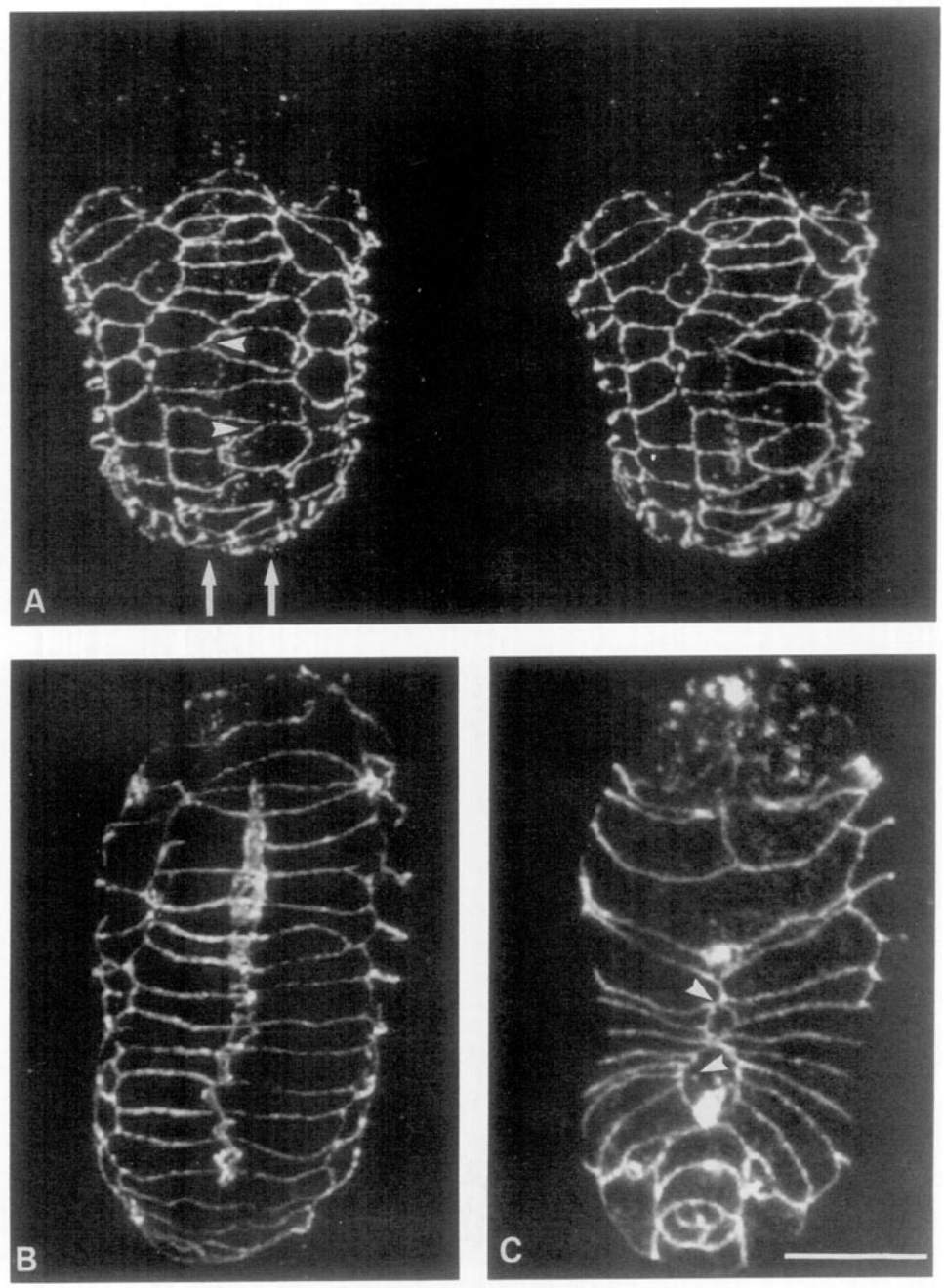

FIG. 3. Migration of epithelial cells before fusion during embryogenesis. (A) Stereo pair image obtained by confocal microscopy (dorsal view). Six rows of cells: Two central rows of dorsal hypodermal cells (arrows) migrate and interdigitate to form a single row. Two lateral rows of seam cells (left and right). Two external rows migrating toward the ventral side (away from the viewer). Arrowheads point to the leading tip of two migrating dorsal cells. Simultaneously two outer rows migrate toward the ventral side (Fig. 1B shows a cylindrical projection of the hypodermis corresponding to this stage). (B) Dorsal view of an embryo after migration of dorsal cells. Developing pharynx and intestine are under dorsal cells in the middle. (C) Ventral view of the same embryo shown in B. Ventral cells have nearly enclosed the embryo. Arrowheads point to two regions that have not yet come together (Fig. 1C is a schematic representation of an embryo around the same developmental stage). Bar, 10 μm.

8D). The cytoplasmic processes from these seam cells open up the adherens junctions between anterior and posterior pairs of P cells causing the formation of six pairs of left and right P cells instead of two longitudinal rows (Figs. 7D and 8E). The anterior daughters of V and T cells fuse to hyp7 (Figs. 7E and 8F), as well as the posterior daughter of H1 (H1.p) and H2.ap (asterisk in Fig. 8C). Fusion of seam cell descendants occurs about 3

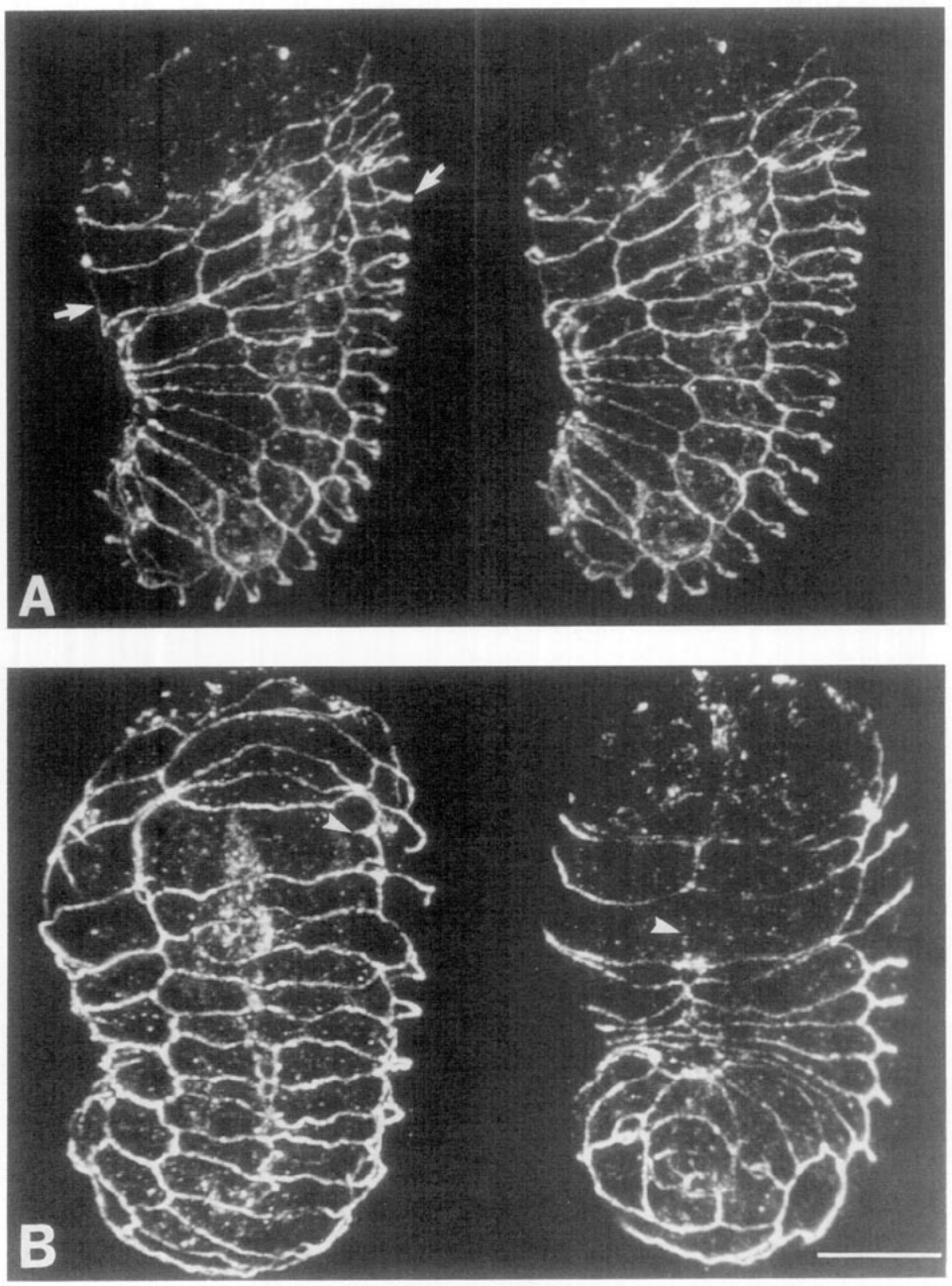

FIG. 4. Projections of embryos before and during first epithelial fusions. (A) Lateral stereo view of an embryo enclosed by an epithelial monolayer before fusions (~350 min). This is the total projection of 21 serial optical sections (1-μm-thick) through the embryo obtained as described under Materials and Methods. Arrows in A point to adherens junctions in the dorsal (right arrow) and ventral (left arrow) hypodermis that disappear after fusion in B (arrowheads) forming binucleate cells. (B) Dorsal and ventral views (left and right sides, respectively) of an embryo containing the first two dorsal cells fused after egg–sperm fusion (arrowhead on the left projection) and the first two ventral cells fused (arrowhead on the ventral projection on the right) (~400 min). MH27 staining is very weak in the head but there is a faint staining of cellular junctions in the epithelium of the developing pharynx and gut in the middle of the embryo. Bar, 10 μm.

hr after they are generated (times estimated from morphology and (Sulston and Horvitz, 1977)).

Once the ventrolateral P cells are isolated from their hypodermal neighbors, each P cell retracts its lateral margin and its nucleus enters the ventral cord (Sulston and Horvitz, 1977; White *et al.*, 1976). As this occurs, pairs of P cells rotate to form a single row of epithelial blast cells (Fig. 8F). The six cells derived from the poste-

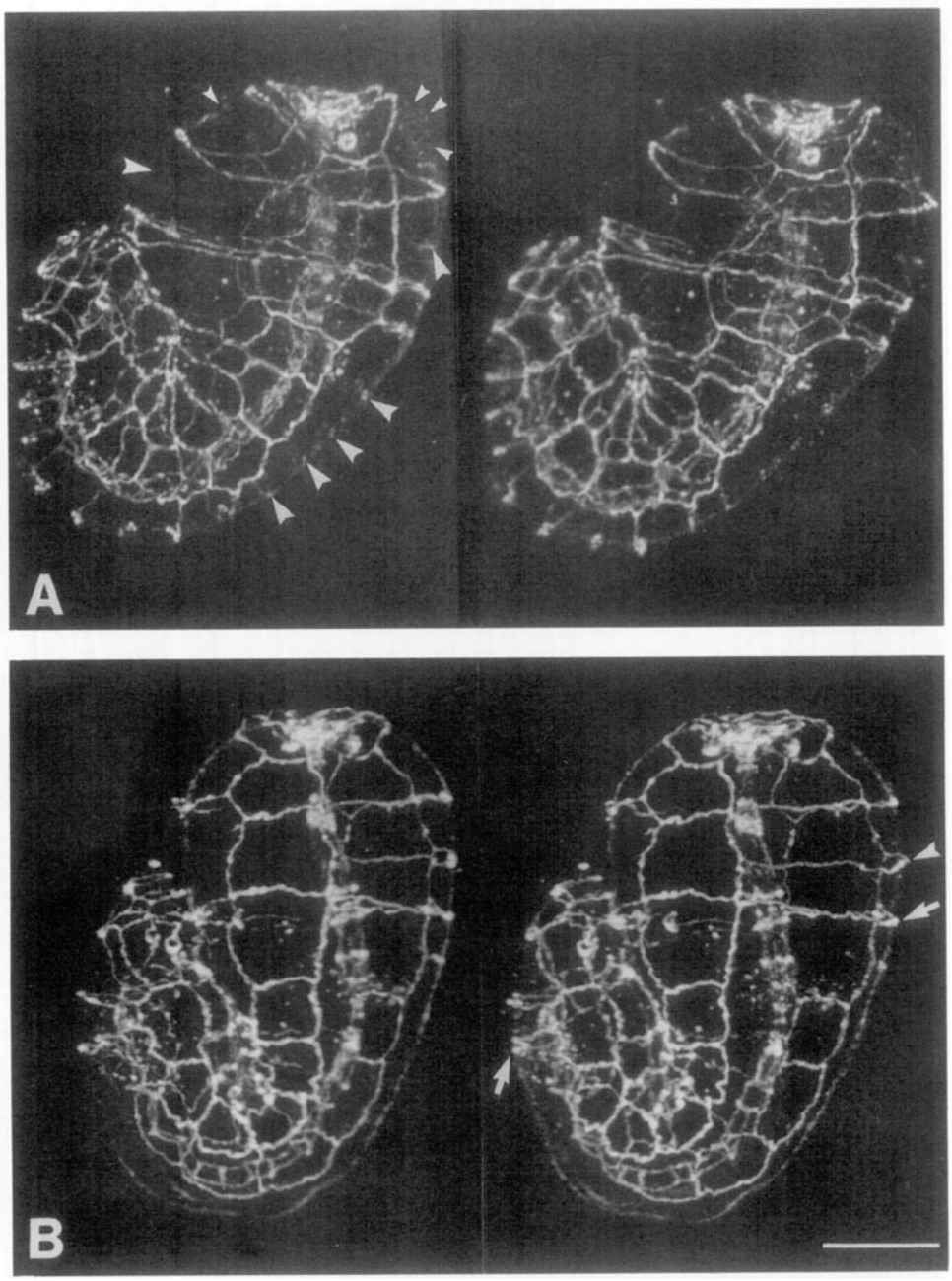

FIG. 5. Stereo pairs of fusion intermediates in elongating embryos. Dorsal and ventral epithelial cells are fused in groups; the elongating embryos are viewed from the left side; pharynx and intestine are in the middle (~430 min). (A) Small arrowheads point to the position where the boundaries between four dorsal and two ventral cells were before fusion; this syncytium will form hyp6 in the adult. Large arrowheads point to the location of fused cells that will form hyp7 syncytium at the end of elongation. The staining in the head, pharynx, and gut is brighter than in earlier stages and smaller syncytia in the head can be observed (hyp6, small arrowheads corresponding to cells a–d and e + f in Fig. 1C). (B) Arrowhead points to an adherens junction that had already disappeared in A. The embryo in B is unusual because cells 1 and 2 (arrowhead) are usually fused at this stage. Only a few embryos elongated 1.5-fold have shown this pattern of fusion out of hundreds observed to date. The two arrows point to the ZAs that limit an intermediate syncytium containing 15 nuclei. Bar, 10 μm.

rior daughters of P1, P2, and P9–P12 fuse with hyp7 at the end of the L1 stage.

In summary, about 5 hr after hatching, the seam cells round up and divide, and then the anterior daughters of V2–V6 (left and right) send cytoplasmic processes that open up adherens junctions between unfused ventral hypodermal cells (P1/2–P11/12, left and right). It is the fusion of these cytoplasmic processes with hyp7 that causes detachment of anterior pair P1–P2 cells from posterior P3–P4 and eventually produces six pairs of iso-

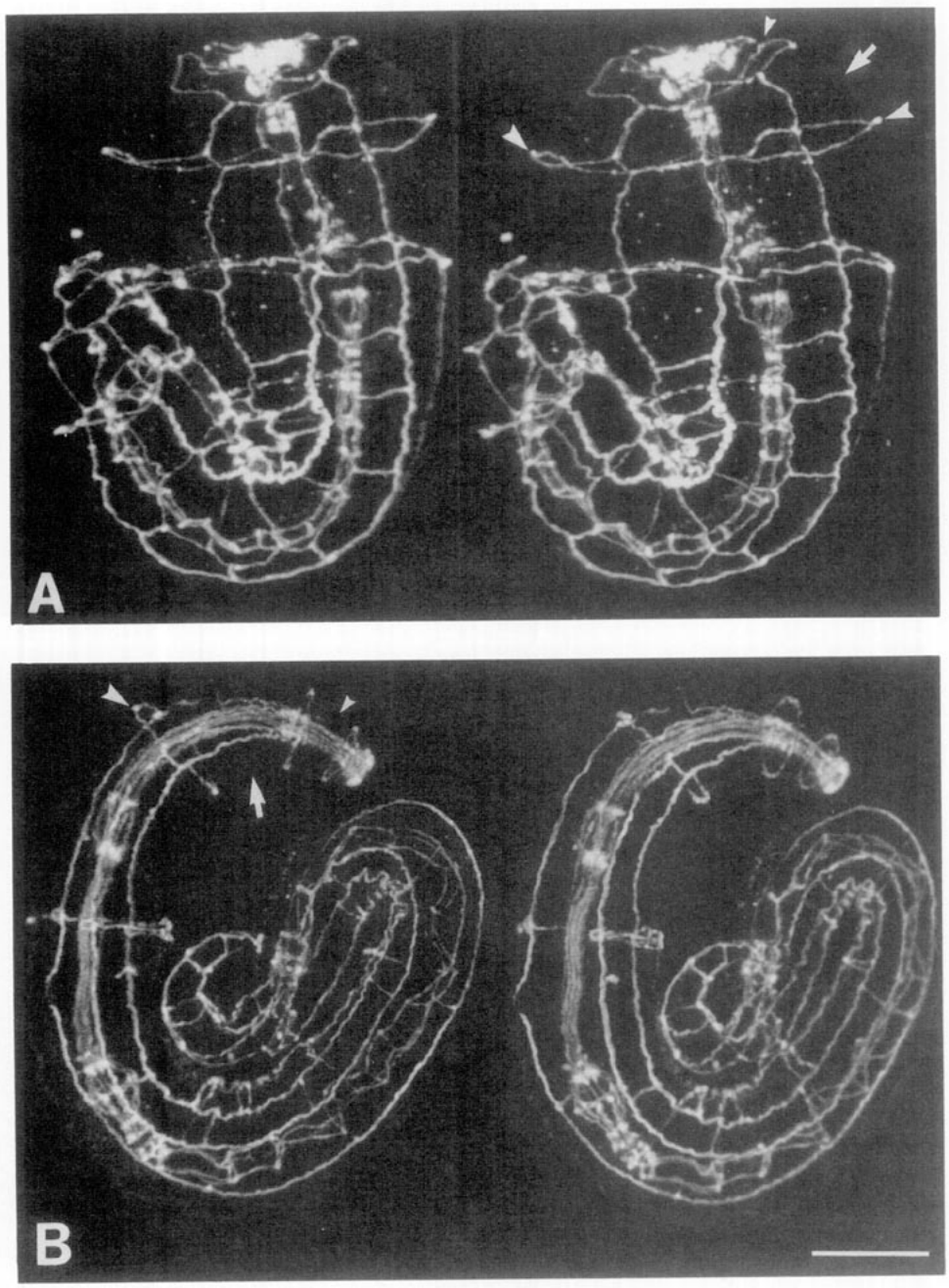

FIG. 6. Stereo pairs of fusion intermediates in 1.5- and 3-fold embryos. Embryos elongated 1.5-fold (A) and 3-fold (B) show an anterior binucleate syncytium. Small arrowhead, hyp5; arrow, hyp6 syncytium; and large arrowheads point to the boundaries between hyp6 and the largest syncytium hyp7. In B it is clear that the syncytia are joined by circular ZA that completely surround the embryo. Bar, 10 μm.

lated P cells. P cells do not send cytoplasmic extensions toward the ventral cord as was originally reported based on Nomarski observations (Sulston and Horvitz, 1977). Once the pairs of P cells are surrounded by hyp7 the nuclei migrate into the ventral cord, and the cells rotate by 90° to form a single row of ventral P cells. Concomitantly the lateral margins of the P cells retreat into the ventral cord. In some cases, postembryonic fusions discussed here are specific for the hermaphrodite. Recent studies have described cellular events in the development of the male tail involving at least six genes and a reproducible pattern of cell rearrangements and fusions in the posterior lateral hypodermis (Baird *et al.*, 1991).

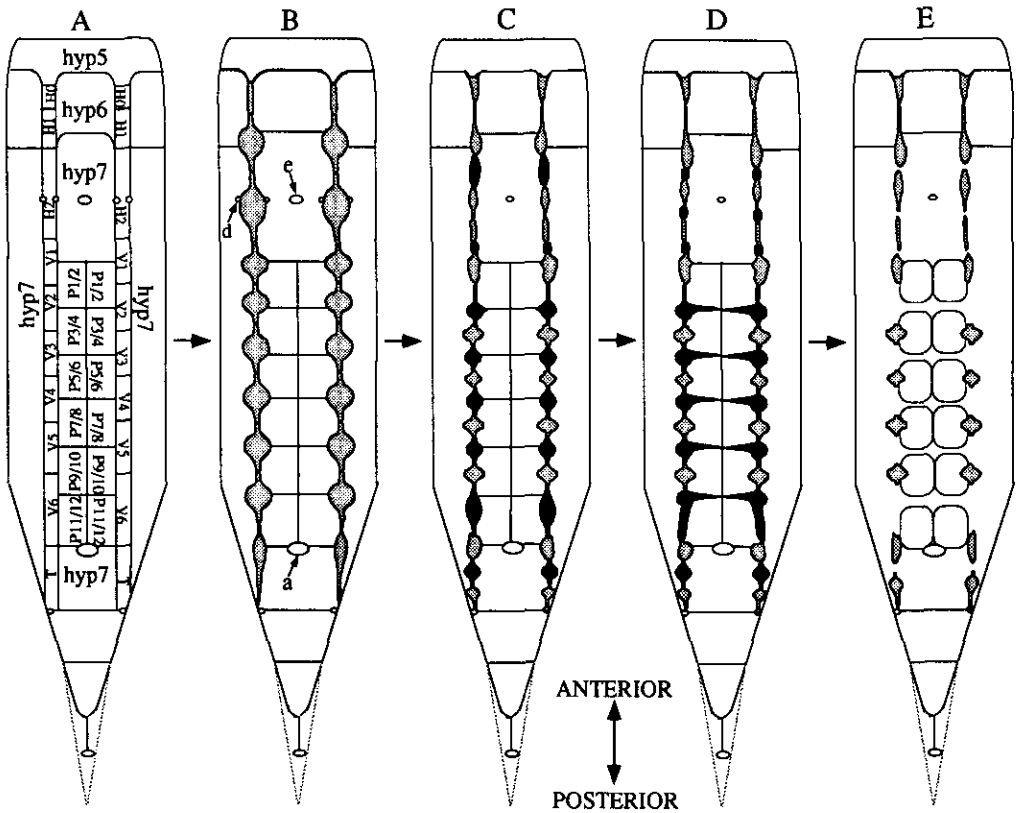

FIG. 7. Generation, migration, and fusion of postembryonic epithelial cells. Cylindrical projections of the body hypodermis of first larval stage (L1) nematodes, cut open along the dorsal midline and viewed from inside. (A) Newly hatched L1 cells are labeled according to Sulston and Horvitz (1977) and Sulston *et al.* (1983) (Fig. 1C). There are two longitudinal rows of cells, called seam cells (H0–H2, V1–V6, T), that run on the left and right sides of the L1, and two rows of ventral P cells (P1/2–P11/12) all embedded in cylindrical syncytia generated during embryogenesis (hyp5, hyp6, hyp7). (B) The seam cells round up and divide (C) except H0 left and right that do not divide. (D) The anterior daughters of V2–V6 (left and right) send cytoplasmic processes toward the ventral midline along the junctions between P cells. (E) Black daughter cells fuse to the surrounding hyp7 syncytium isolating pairs of P cells. e, excretory pore; d, deirid socket or sensory sensilla accessory cell (not all d are shown); a, anus.

Late during the L3 stage the posterior daughters of P3–P8 divide, generating 12 cells, 6 of which fuse with hyp7 (the daughters of P3.p, P4.p, and P8.p; not shown), the remaining 6 become vulva precursor cells (VPC, Fig. 9A). The 22 descendants of these VPCs will form six toroids connected to form a tube that communicates the uterus to the exterior (Figs. 9B and C). Vulva morphogenesis also involves migrations and ordered fusions of epithelial cells (manuscript in preparation).

The seam cells undergo a stem cell division at the beginning of each larval stage (L2, L3, L4). The nonstem daughters endoreduplicate their DNA without chromosome condensation (Hedgecock and White, 1985). These tetraploid daughters constrict their apical surfaces as shown by the ZA stain (Fig. 9D). As their apical surfaces become smaller these cells fuse with the surrounding hyp7 syncytium. Simultaneously, the seam stem cells elongate longitudinally on top of their sisters to reach both anterior and posterior seam cells and to regenerate a continuous row of seam cells on each side of the animal. Around 3 hr after being born, the tetraploid nonstem daughters join the hyp7 syncytium as revealed by the disappearance of ZA stain.

The above migrations coupled to fusions are shown in Figs. 8B and 8C for L1 division, and in Figs. 9B and 9D for L4 division. About the middle of the L4 stage and after the fusion of 13 anterior daughter cells (Figs. 9B and 9D), 15 seam cells on each side fuse longitudinally forming two continuous lateral syncytia embedded in hyp7 but distinct from it (Fig. 9C). These are the last postembryonic somatic cell fusions in *C. elegans.*

DISCUSSION

Cell Fusion Is a Common Cell Fate during Development in C. elegans

The young adult contains 186 hypodermal nuclei, 183 of which are part of different syncytia, and overall,

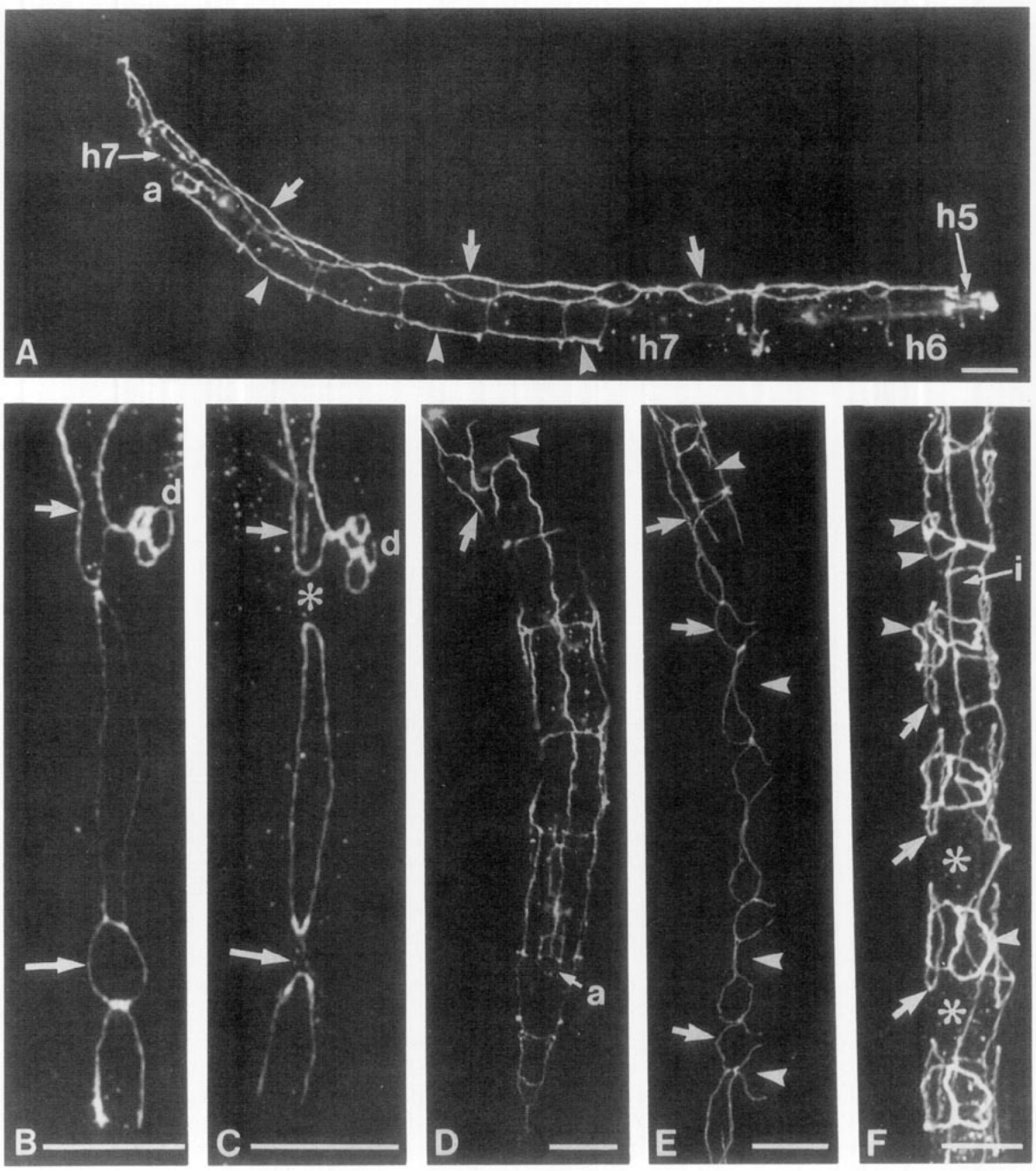

FIG. 8. Epithelial cell migrations and fusions in L1 hermaphrodites. Adherens junctions of L1 larvae stained with antibody MH27. Partial confocal microscope projections of sections of hypodermis. (A) Ventral-left view, seam cells are beginning to round up (arrows) and boundaries of 6 P cells (arrowheads pointing to ventral midline) are surrounded by hyp7 syncytium (h7). a, anus; hyp5 and hyp6 are labeled h5 and h6, respectively. Anterior is to the right. (B-F) Anterior is to the top. (B) Left and (C) right seam hypodermal cells of the same L1; short arrows point to H2 descendants (H2.aa, left and right, respectively) that function as deirid socket (d, sensory sensilla accessory cell); long arrows point to seam daughters (V1.al) before (B) and (V1.ar) after or during fusion (C) since the ZA staining is very weak; asterisk, fused H2.ap cell. (D) Ventral view of an L1 slightly later in development than the animal in A; arrowhead points to P1/2 and arrow to a left seam cell V2.a that is sending a cytoplasmic process along an adherens junction toward the ventral midline; a, anus. (E) Right view of an L1 later in development than the L1 in D. Arrows point to seam cells that have sent cytoplasmic processes that opened up the ZA between P cells and reached the ventral midline; arrowheads show the positions of some P cells situated in a lower focal plane. (F) Ventral view of an L1. Seam cells that sent processes in D and E have fused to hyp7 (asterisks; not all possible asterisks are shown). Arrows point to some of the blast seam cells that did not fuse and appear to be associated to pairs of P cells (arrowheads; not all shown). The top pair of P cells (top two arrowheads) have rotated 90°; P1 is anterior and P2 is posterior. Bottom arrowheads points to a pair of P cells (P7/8 left and right) that have not rotated; i, intestine. Bars, 10 μm.

around one-third of all somatic nuclei are contained within multinucleate cells formed by cell-to-cell fusion. We have described epithelial cell fusions that occur during development in embryos and larvae of the nematode *C. elegans*. We have followed the order and sequence of fusions and associated short-range migrations.

The time of fusion of a cell appears to be linked to its time of birth. We found that embryonic cells fuse around 3 hr after their generation. In developing *anc-1* larvae mutants the nuclei of certain epithelial cells during postembryonic development are not elastically anchored, but float freely within the syncytial cytoplasm (Hedgecock and Thomson, 1982). Making use of the *anc-1* phenotype it was possible to record the earliest times at which new syncytial nuclei enter the syncytial cytoplasm. It was found that fusion occurs 3 hr after the generation of the daughters of the seam stem cells (Hedgecock and Thomson, 1982). Taken together these results suggest that fusion competence is acquired after a defined and invariant period of time during which cells that will fuse participate in migrations and change contacts within epithelia.

There is some natural variation among embryos in the precise order and timing in which dorsal cells fuse to form hyp7. Generation of cells that will fuse as well as the order in which cells fuse generally starts in the anterior part of the embryo or larva and continues posteriorly. Though we found individuals with variation in the fusion intermediates, the final boundaries of the cylindrical syncytia are well defined, for example hyp6 does not fuse with hyp7.

Like most other cell fates in *C. elegans*, cell fusions are specific and generate an invariant pattern of syncytia from one animal to the next.

Cell Fusion Is a Significant Morphogenetic Process

Cell fusion between somatic cells is part of the developmental program that participates in the formation of specific tissues and organs in *C. elegans*. It is possible to study dynamic changes in cell shapes, positions, and fusions in undisturbed tissues and organs in *C. elegans*.

Figure 10 summarizes three examples in which specific cells at specific times and locations migrate and fuse in the course of morphogenesis. Fusion between mononucleate cells after short-range migration and reshaping of adherens junctions occurs in the dorsal hypodermis during early embryogenesis (Fig. 10A). Similar cell rearrangements occur in embryonic epithelial monolayers of *Xenopus, Drosophila, Fundulus* (Trinkaus, 1984), and sea urchins (Hardin, 1989); to our knowledge fusions have not been identified in these epithelia. These cell rearrangements are intriguing since they occur in spite of the continuous presence of tight junctions, adherens junctions, and desmosomes (Trinkaus, 1984). Here also we have shown how adherens junctions are dynamic structures that reshape the apical boundaries of cells in *C. elegans*. These cell rearrangements are associated with elongation of epithelia. It is conceivable that interdigitation and fusion of dorsal cells are necessary for the asymmetric dorsal elongation that is a prelude to the actin-mediated elongation of the embryo from a spheroid to a cylindrical worm (Priess and Hirsh, 1986).

Why Do Epithelial Cells Fuse?

We do not have an answer for this question but our observations suggest at least four possible explanations.

(i) To eliminate potential routes for migration. Since epithelial cells tend to migrate along cell junctions, by interdigitation (Fig. 10A) or by sending cytoplasmic processes that open up adherens junctions (Fig. 10B), fusion would restrict potential avenues between cells that could be used by migrating epithelial cells.

(ii) To isolate cells. Fusions in the ventral hypodermis of L1 in *C. elegans* (Fig. 10B) cause the isolation of groups of cells and dramatic rearrangements of cell boundaries. These changes in cell contacts could be important for the induction or repression of different fates. For example, it has been proposed, based on mosaic analysis, that expression of *lin15*(+) in hypodermis directly represses a vulval fate in uninduced vulva precursor cells or promotes fusion of these mononucleate cells to the surrounding syncytium (Herman and Hedgecock, 1990). Thus, fusion may act to isolate an interactive group of cells such as the vulva precursors to prevent spurious interactions with surrounding cells.

(iii) To exit from a division cycle. Fusions in the lateral hypodermis are coupled to the ectoblastic activity of the seam lateral cells. A total of 98 daughters of seam stem cells and 12 descendants of P cells fuse to hyp7 during postembryonic development (Sulston and Horvitz, 1977). Seam cell daughters are generated in a cyclic way and fuse to the surrounding syncytium after their apical domains become constricted. Seam stem cells migrate on top of their fusing sisters until they reach their neighbors and start the cycle again. In the L4 larva 15 seam cells fuse on each side of the animal (Fig. 10C). These final fusions may ensure that the seam cells will stop dividing. Therefore fusion may be a strategy to pull cells out of the cell cycle. A precedent for this regulatory activity exists in yeast, where *FUS3* encodes a cdc2$^+$/CDC28-related kinase required for the transition from mitosis to fusion between haploid cells during mating (Elion *et al.*, 1990).

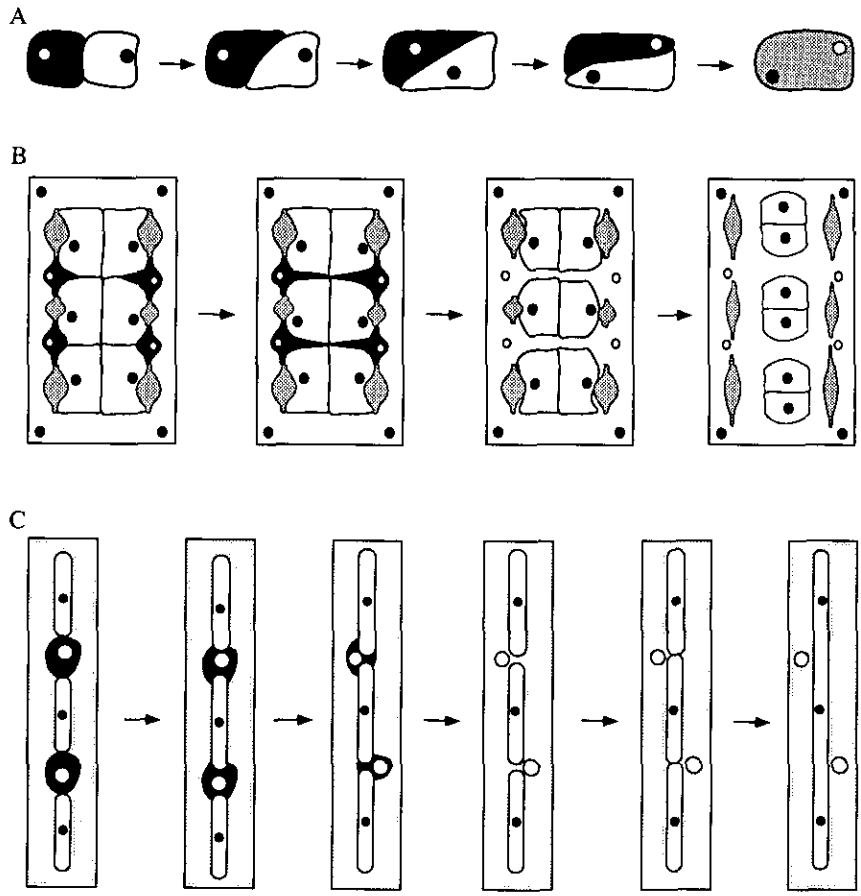

FIG. 10. Cell fusion as a morphogenetic force. (A) Cell rearrangements within epithelia involve changes in cell contacts. Fusion occurs after short-range migrations, such as is seen in embryonic dorsal hypodermis. In this case fusion starts before elongation of the spheroidal embryo. (B) During postembryonic development fusion causes the remodeling and isolation of ventral cells as well as growth of preexisting hyp7 syncytium. Black lateral cells send cytoplasmic processes that change boundaries and adherens junctions before they fuse releasing white nuclei into the hyp7 syncytium. (C) Anterior daughters of seam cells (black cells, with white nuclei) embedded in hyp7 syncytium (grey) fuse as their sisters (white cells with black nuclei) elongate to maintain a continuous row of lateral cells. In the L4 lateral seam cells fuse with their sisters but not with the surrounding hyp7.

(iv) To extinguish the regulatory state in one of the fusing cells. If a cell fuses to the hyp7 syncytium it is likely to be reprogrammed to become part of a multinucleate epithelia to which it contributes membrane, cytoplasm, and nucleus. Fusion may act to reprogram a partially committed or uncommitted cell. Cells generated as sisters of epithelial precursors would escape from the hypodermal committment by not fusing with a preexisting epithelial multinucleate cell. Putative neurons, muscle, and uncommitted cells would have become epithelial cells if they had fused to hypodermal syncytia. An example for this fusion-mediated regulation of cell fate has been shown during myogenesis in postnatal rodent development (Hughes and Blau, 1992), as well as during the formation of the placenta (Coutifaris *et al.*, 1991). The placenta is an epithelium that reshapes itself, grows, and differentiates into an endocrine organ upon fusion of mononucleate cytotrophoblasts. After fusion the invasive cytotrophoblast cells become noninvasive syncytiotrophoblasts (Strickland and Richards, 1992). The use of *in vitro* systems will help to continue the analysis of cell fusions in muscle (Cross and Sang, 1978), osteoclasts (Baron *et al.*, 1986), and placenta (Coutifaris *et al.*, 1991). However the role of somatic cell fusion during development may be difficult to study in these complex systems.

To Fuse or Not to Fuse: A Developmental Question

There are six founder cells in *C. elegans* (AB, MS, E, C, D, and P_4). The cell types and tissues derived from each founder cell are known (Sulston *et al.*, 1983). Most epithelial cells that fuse are descendants of AB. There are cases in which fusion occurs between cells of different

lineages: during embryogenesis 12 descendants of C fuse with 11 descendants of AB to form hyp7. In pharyngeal muscle, pairwise fusions occur between descendants of founder cells AB, MS, and both. Also two gland cells that fuse embryonically are both descendants of MS. Patterns of fusion therefore appear to be independent of cell lineage.

Toward the end of the L4, seam cells fuse using a very small domain of their plasma membrane. Fusion is restricted to the area of close contact between sister seam cells in the anterior and posterior tips of the cells (Fig. 10C). They do not fuse with the surrounding hyp7 on the left and right sides. Cell fusion therefore appears to involve specific cell–cell recognition as seam cells only fuse to seam cells and not to the adjacent hyp7 syncytium. Likewise, hyp7 is distinct from hyp6, and so on with all the other smaller syncytia.

At least three sequential steps exist in the developmental program responsible for pattern formation through fusion in the hypodermis of *C. elegans*. First, cells are generated and in some cases they become tetraploid. Second, epithelial cells undergo short-range migrations, changing their relative positions and contacts with neighbors. Third, specific fusions between mononucleate cells or with preexisting syncytia occur. Mutations that affect all but the last step in this process have been isolated in *C. elegans*. For example, there are mutations that affect postembryonic lineages and cell determination, timing of cell divisions, and long-range migrations in neurons, muscles, and gonad epithelial cells (Hedgecock *et al.*, 1987). Other mutations identify genes that may mediate cell interactions (Hedgecock *et al.*, 1987).

To date no fusion-defective mutant has been characterised in *C. elegans*. The identification and characterization of mutants affecting cell fusions during embryonic and postembryonic development in the hypodermis as well as in the morphogenesis of organs like vulva, uterus, and pharynx will help us to understand the roles and mechanisms of cell fusion during development.

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