Role of bilateral zones of ingressing superficial cells during gastrulation of *Ambystoma mexicanum*

CATHY LUNDMARK*

Department of Zoology, University of California, Berkeley, CA 94720, USA

SUMMARY

  Vital dye staining and cell lineage tracers were used to mark superficial cells of early *Ambystoma mexicanum* gastrulae. Superficial marks placed between the equator and the blastopore, on the dorsal midline, stained notochord, whereas marks or injections made at similar animal–vegetal levels but 90° to either side of the dorsal midline were later found in somitic mesoderm. Notochord marks remained on the dorsal surface of the archenteron throughout gastrulation, though they became elongate and narrow by the morphogenetic movements of extension and convergence. Marked somitic mesoderm disappeared from the superficial epithelial layer soon after passing over the blastoporal lip and could not be found on the archenteron surface. A possible mechanism for this de-epithelialization is proposed on the basis of correlated SEM. The significance of a method of gastrulation so distinctly different from that of certain other amphibians is discussed in terms of amphibian phylogeny.

INTRODUCTION

  Amphibian gastrulation can be seen as the integration of several morphogenetic movements: epiboly of the animal cap, bottle cell formation, migration of cells on the gastrula wall, and convergent extension (Keller, 1985). The dissection of amphibian gastrulation into these component processes is complicated by the fact that not all amphibians gastrulate the same way, and that the degree to which the various morphogenetic movements participate may vary among different species (see del Pino & Elinson, 1983; Keller, 1985). The idea that amphibian gastrulation is the same for all species stems in part from Vogt's original fate maps for amphibians (1925, 1929), corroborated by Nakamura (1938) and Pasteels (1942), which show essentially the same map for all the anuran and urodele species studied (Fig. 1). This notion was reinforced by one of the earliest concepts in embryology (Haeckel, 1879), that early development is highly conserved in vertebrates, particularly those thought to be closely related. Since very few species of amphibian were studied and since none of the amphibians with unusually large eggs were studied (for example, see del Pino & Elinson, 1983), this notion went unchallenged. By comparing details of the gastrulation of an anuran and a urodele we are just beginning to understand which processes are and which are not conserved throughout amphibian evolution.

  * Present address: Bodega Marine Laboratory, Bodega Bay, CA 94923 USA.

Until relatively recently (Keller, 1976; Løvtrup, 1975) the amphibian fate maps (Vogt, 1929; Pasteels, 1942) represented all germ layers in the superficial cell layer and gave few details about deep layer contributions. The differences between species was primarily in the placement of the boundaries of each region (Fig. 1). It was then necessary to explain how mesoderm got from the surface to its place between ectoderm and endoderm during gastrulation. Vogt postulated that the blastopore formed on the mesoderm–endoderm boundary and, by separating them, allowed their independent morphogenetic movements: elongation and convergence of mesoderm; and dorsalward migration of free endodermal edges (lateral endodermal crests) over the mesoderm temporarily lining the roof of the archenteron (Fig. 2). Vogt did not explain how mesoderm is separated from
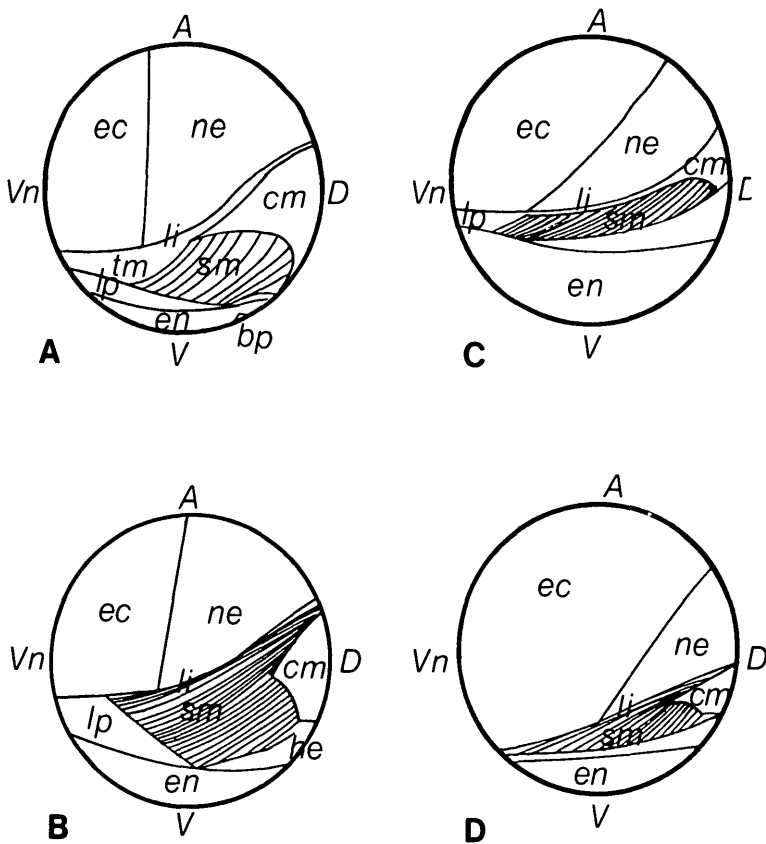

Fig. 1. Maps depicting the presumptive areas of different amphibians at the onset of gastrulation. The regions shown represent those structures which will ultimately contain marks placed on the surface of the early gastrula. Maps of urodeles are shown in A and B: (A) early gastrula of *Triturus* (formerly *Triton*), redrawn from Vogt (1929); (B) early gastrula of the axolotl, redrawn from Pasteells (1942). Maps of anurans are shown in C and D: (C) blastula of *Bombina* (formerly *Bombinator*), redrawn from Vogt (1929); (D) early gastrula of *Discoglosse*, redrawn from Pasteels (1942). *A*, animal pole; *bp*, blastopore; *cm*, chordamesoderm; *D*, dorsal; *ec*, ectoderm; *en*, endoderm; *he*, head endoderm; *li*, limit of involution; *lp*, lateral plate mesoderm; *ne*, neural ectoderm; *sm*, somitic mesoderm; *tm*, tail mesoderm; *V*, vegetal pole; *Vn*, ventral.
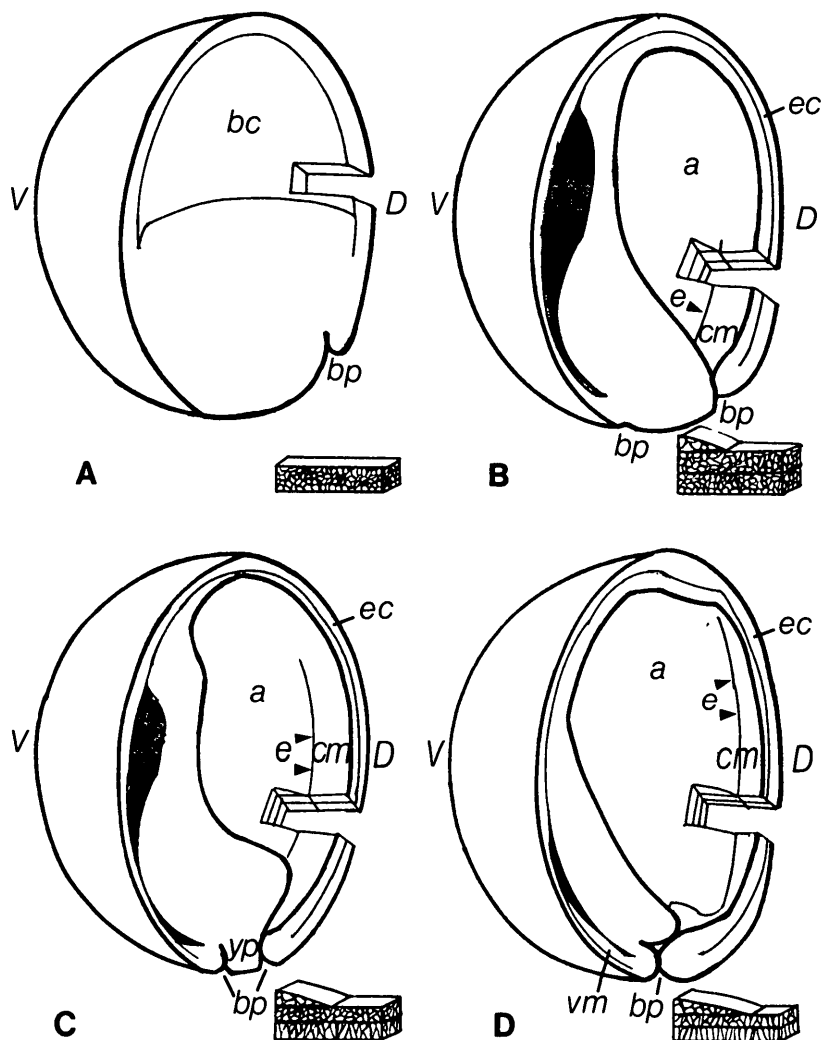
Fig. 2. Diagrams redrawn from Hamburger's (1960, figs 6–9) reconstruction of urodele gastrulation (Vogt, 1929). All figures are fractured midsagitally and viewed at an angle for more perspective. Cut-out fragments depict the approximate number of cells in each cell layer at the different stages. (A) Onset of gastrulation (stage 10½), (B) midgastrula stage (stage 10¾), (C) late gastrula stage (stage 12), (D) early neurula stage (stage 13). *a*, archenteron; *bc*, blastocoel; *bp*, blastopore; *cm*, chordamesoderm; *D*, dorsal; *e*, edge of endoderm; *ec*, ectoderm; *V*, ventral; *vm*, ventral mesoderm; *yp*, yolk plug.

ectoderm, and may have thought them continuous, divided by the limit of involution. In a reanalysis of Vogt's experiments on urodeles, Løvtrup (1966, 1975) argued for a multilayered map, as the embryo is made up of 'membrane-bound' (superficial) cells and 'free inner' (deep) cells. Løvtrup found the relative positions and distortions of Vogt's marks irreconcilable with his fate maps and claimed Vogt's mistake was that he stained deep cells (mesoderm) through the superficial layer. Thus Løvtrup resolved the issue by relegating all the mesodermal

anlagen except the notochord to the deep layer. By independently mapping the deep and superficial layers of *Xenopus laevis* gastrulae, Keller (1975, 1976) clearly demonstrated that mesoderm resides solely in the deep layer, and that deep and superficial layers of the marginal zone differ not only in their fates but also in their mechanical roles in gastrulation (Keller & Schoenwolf, 1977; Keller, 1981, 1984, 1985; Lundmark, Shih, Tibbetts & Keller, 1984).

Does this explanation hold for other species of amphibians? Several recent comparative studies (Nieuwkoop & Sutasurya, 1976; Nakasutji & Johnson, 1983; Smith & Malacinski, 1983; Brun & Garson, 1984; Boucaut, Darribere, Boulekbache & Thiery, 1984) have shown there are fundamental differences between *Xenopus laevis* and *Ambystoma mexicanum* organization and development. Nakasutji & Johnson, interested in the role of cell migration in gastrulation, have used SEM to compare the surface of the blastocoel roof, which is the putative substratum for mesodermal cell migration, in various species of amphibians. They found dense extracellular fibrils appearing just prior to the onset of gastrulation in the three species of urodele studied, but far fewer in the two anuran species looked at, possibly implying that migration plays different roles in the two orders of amphibians. Keller, Danilchik, Gimlich & Shih (1985) have shown that when the blastocoel roof is removed from *Xenopus* embryos prior to gastrulation, involution occurs as fast as or faster than in unoperated controls, suggesting migration is not necessary for the major movements of gastrulation in *Xenopus*. By surface labelling of superficial cells with the Boulton–Hunter reagent, Smith & Malacinski (1983) demonstrated the contribution of superficial cells to the deep mesodermal structures in *A. mexicanum* neurulae and the lack of any such contribution of superficial cells to mesodermal structures in *X. laevis*. Brun & Garson (1984) argue on the basis of ultrastructural studies that, during gastrula and neurula stages of *A. mexicanum* development, the cells that will form the notochord are epithelial, lining the dorsal roof of the archenteron, and form a continuous cell sheet with the ectoderm. They claim, therefore, the notochord should be considered ectodermal in origin.

To ascertain the extent to which superficial mesoderm exists in the axolotl, superficial cell fates were followed using vital dye marks and injected cell lineage tracers. Correlated SEM was used to examine the rearrangements and shape changes occurring at the internal blastoporal lip to pinpoint where and how these cells disappear from the surface layer.

MATERIALS AND METHODS

Embryos

Ambystoma mexicanum embryos were supplied by Indiana University Axolotl Colony and the Center for Reptilian and Amphibian Propagation in Reno, NV.

Cell marking

Agar blocks containing Nile blue sulphate were used to stain manually dejellied embryos by the method of Keller (1975) with two modifications. First, marks were made through the

vitelline envelope which was shrunk to the surface of the embryo by adding 5 % Ficoll (Sigma) to the Steinberg's solution. Second, embryos were fixed overnight in 2·5 % glutaraldehyde, 0·1 M-sodium cacodylate, pH 7·4, dissected and sketched, unless they were being prepared for LM or SEM (see below).

Lysinated fluorescein dextran (200 mg ml^{-1}), supplied by Dr R. Gimlich (see Gimlich & Braun, 1985), was used as a cell lineage tracer. It was diluted 3:1 in 4 % fast green so that filling the cells could be visually monitored, since the volume delivered could not be regulated. Injections were carried out using Dr D. Weisblat's pressure injection apparatus, in which the electrical potential of the micropipette was signalled by a d.c.-modulated audiomonitor. During injections embryos were kept in Steinberg's solution, left to develop in one-third strength Steinberg's and fixed overnight in 4 % paraformaldehyde, 0·1 M-sodium cacodylate, washed for 2–3 days in sodium cacodylate, dehydrated, cleared, paraffin embedded, serial sectioned and counterstained in 0·2 % Evan's blue, which appears red through the fluorescein filter (Zeiss 48 77 16).

Scanning electron microscopy (SEM)

Embryos were fractured and prepared for SEM by the method of Keller & Schoenwolf (1977).

RESULTS

Vital dye marking experiments: notochord

Marks placed on the dorsal midline of early gastrulae midway between the blastopore and the equator come to lie, after involution, on the dorsal roof of the archenteron (Fig. 3). A circular mark becomes greatly elongate and narrow, and undyed cells become interspersed among the dyed cells. This is probably due to (1) the cell rearrangements (mediolateral intercalation) of extension and convergence, and possibly also to (2) deep cells moving into the superficial layer lining the archenteron as the notochordal anlage decreases in the number of cell layers (radial intercalation, see Fig. 5).

SEM of notochord

When the surface of the archenteron is viewed, the notochordal anlagen can be distinguished readily from endodermal cells in LM by virtue of their darker pigment (not pictured) and in SEM (Fig. 4A,B) by virtue of the smaller size of the cells. As notochord formation occurs, this area narrows, elongates, and brings the two lateral endodermal crests, to which it is firmly attached, closer together. Marks placed on the notochord, near its junction with the lateral endodermal crest, are not covered by migrating edges of endoderm (Vogt, 1929), but remain visible on the surface of the archenteron roof until the notochord is completed (not pictured). As this process continues, the notochord is removed from the superficial layer, and the endodermal crests fuse (Fig. 4C).

In cross sections of early and late gastrulae, the number of cell layers in the notochordal mesoderm decreases as gastrulation proceeds (Fig. 5). At the early gastrula stage the chordamesoderm is about four or five cell layers thick, and by the late gastrula stage it is two or three cell layers thick. The cell rearrangement responsible for this thinning is probably radial intercalation, whereby deep cells interdigitate into the superficial layers, reducing the number of deep cell layers.

Vital dye marking experiments: somites

When marks are placed on early gastrulae below the equator and 30–90° to either side of the dorsal midline, they usually cannot be found on the surface of the archenteron after involution, but are seen in the mesenchymal layer lateral to the notochord, where somites will form, and below unmarked endoderm (Fig. 6). The marked population of cells remains fairly coherent, with few cells ranging out of contact with their original neighbours. In three experiments where embryos were fixed and dissected as or soon after marks involuted over the lip, a single mark could be seen both on the archenteron surface and partially beneath unstained cells, as if they had been caught in the act of disappearing from the surface (Fig. 6).
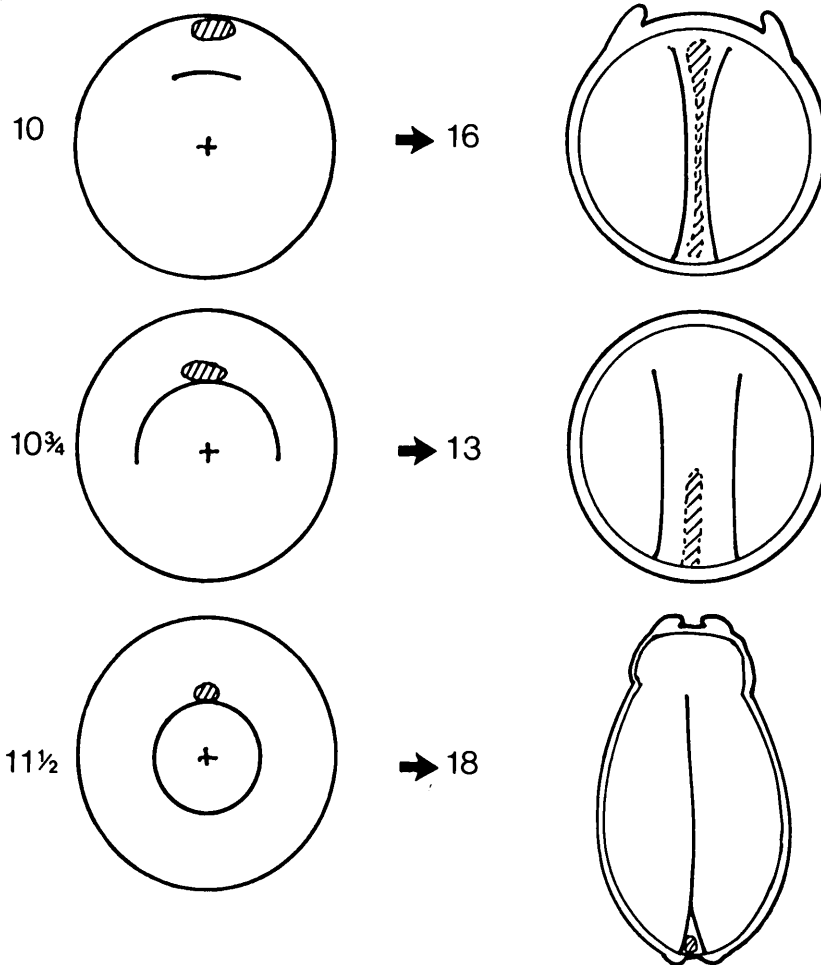

Fig. 3. Results of vital dye experiments marking the chordamesoderm. Superficial marks placed on gastrulae at various times, shown as shaded regions on left, are found on the roof of the archenteron (shaded areas) at the stages shown on right. Marks placed on early gastrulae become hugely distorted by the cell rearrangements of convergent extension (see text), such that single marks may span almost the entire length of the notochord.
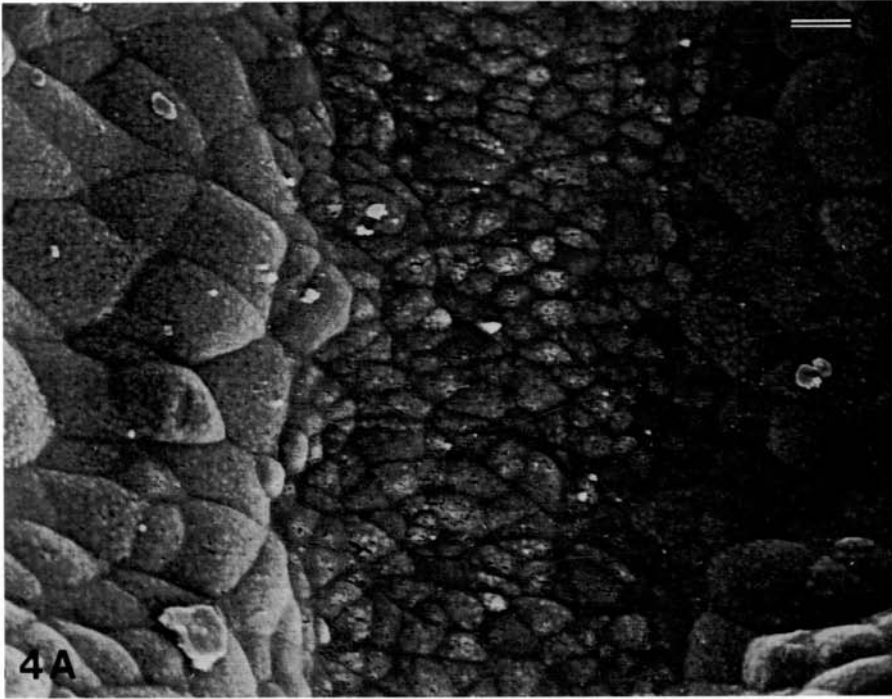
Fig. 4. SEM of dorsal surface of archenteron showing distinct appearance of notochordal cells. (A) Late gastrula–early neurula, smaller cells of the notochord flanked by larger endodermal cells. (B,C) Higher magnification of dorsal roof of archenteron at late gastrula (B) and late neurula (C) showing fusion of lateral endodermal crests as notochord drops out of the superficial layer. Bars (A,B), 25 μm; (C), 10 μm.
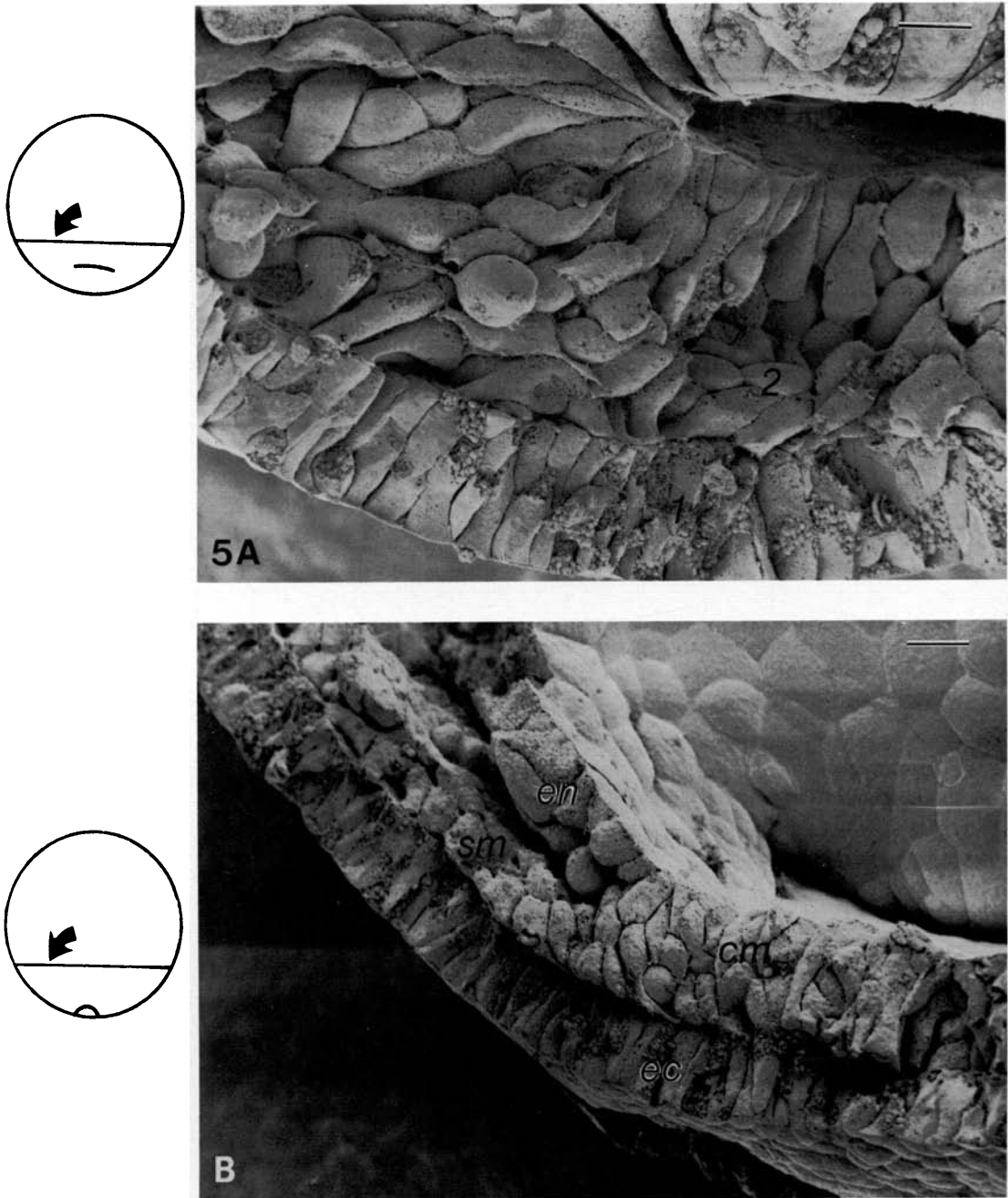
Fig. 5. SEM of early (A) and mid- (B) gastrulae fractured as shown on left, and rotated forward so the dorsal aspect is down. When notochord is viewed in cross section, the reduction of number of cell layers can be seen. At the early gastrula stage, postinvolution material (2) can be distinguished from preinvolution material (1), but germ layers are not easily delineated. By midgastrula stage, chordamesoderm (cm) and overlying ectoderm (ec) are seen in the dorsal region, and, laterally, the somitic mesoderm (sm) is now distinct from the overlying ectoderm (below in micrograph) and underlying endoderm (en) (above in micrograph). Bars, 50 µm.

Injection experiments

To show conclusively that superficial cells leave the surface, early gastrula cells in the area shown in Fig. 7 were injected with the lysinated fluorescein dextran cell lineage tracer. Approximately a dozen cells were injected on either side of each embryo, and 12–15 embryos were marked per experiment (n = 3). Late neurulae were histologically prepared and screened for filled cells. Only a fraction (roughly, a fifth) of the cells injected could later be found, since the volume delivered by pressure injection varied considerably, from too little to be detected to too much, lysing the cells. Labelled cells are found adjacent to the notochord and neural tube in midbody somites (Fig. 8), but not in the epithelium lining the archenteron. This suggests that superficial cells reach the deep, mesenchymal layer by leaving the surface (ingression), not by division of superficial cells into deep and superficial
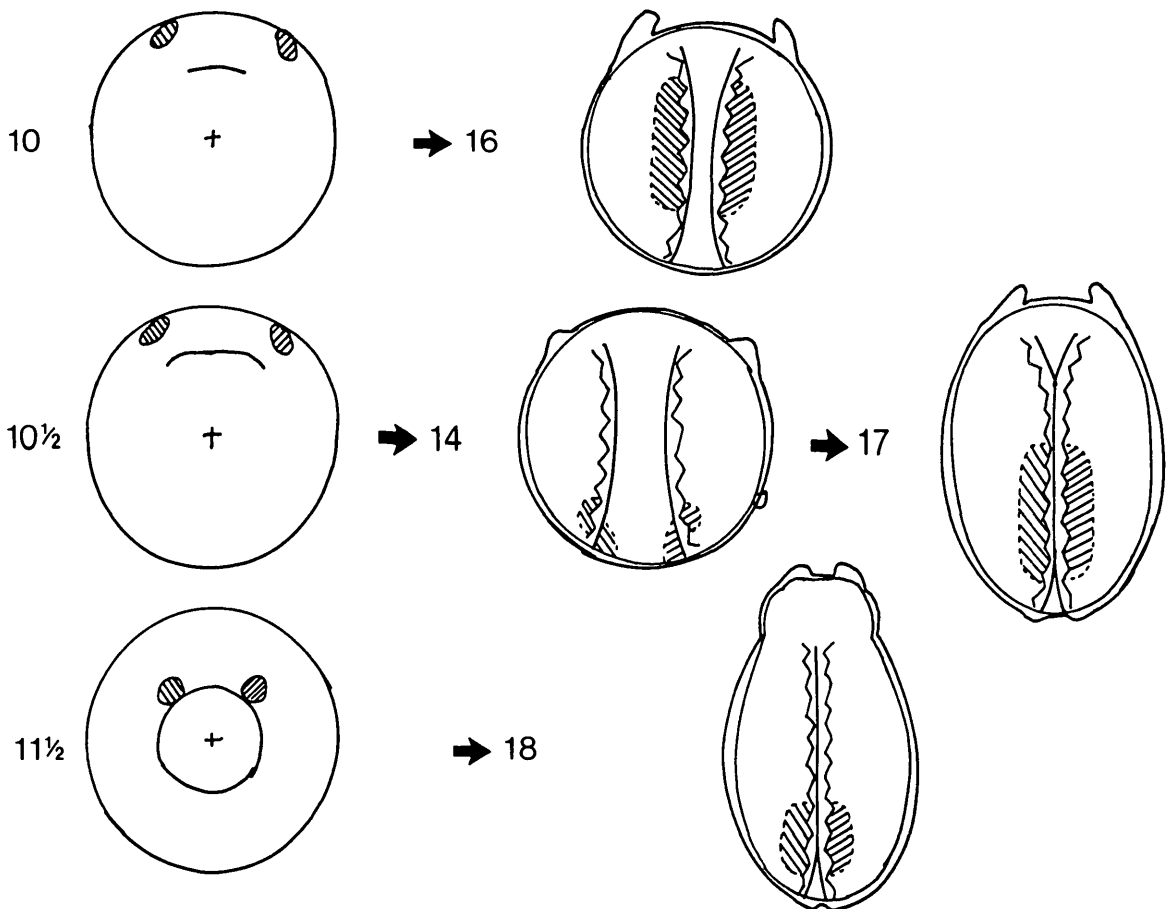

Fig. 6. Results of vital dye experiments marking somitic mesoderm. Superficial marks placed on gastrulae at various times, shown as shaded regions on left, are found later beneath unmarked endoderm (jagged lines depict edge where endoderm has been cut away). Marks placed on early gastrulae are found in more anterior somites, while marks placed on late gastrulae are found in more posterior somites. The middle figure represents embryos in which marks were caught in the act of disappearing.
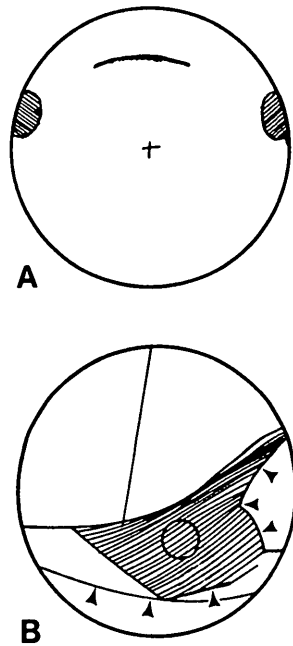
Fig. 7. (A) Early gastrula viewed from vegetal pole showing sites of cell lineage tracer injections (shaded regions). (B) Map of early gastrula (Pasteels, 1942) showing presumptive region where sites of injections fall. Arrows indicate edges of regions that are brought together due to the removal of superficial mesoderm.

daughter cells, since no labelled superficial cells can be found to account for such divisions.

A single experiment (Fig. 8), in which more ventrally located cells were injected with tracer, showed that lateral plate material also comes in part from the superficial layer of the blastula. The sites chosen for all the marking experiments lie well within the boundaries outlined by Vogt (1929) and others (Fig. 1). The extent to which these areas are continuous, or composed of similarly fated cells, has not been thoroughly investigated.

SEM of region of ingression

No obvious, large discontinuity has been seen in SEM to account for the disappearance of large numbers of cells at a time, but small pockets or dimples can be found on the archenteron surface near the blastopore, which may be signs of de-epithelialization of individual cells or small groups of cells (Fig. 9). Embryos fractured obliquely through the dorsal and lateral lips show many bottle cells which appear to be forming near the edge of the notochord soon after involution (Fig. 9). These may be superficial cells in the process of leaving the surface. More laterally, cells with many protrusions appear to be contacting, and possibly migrating over, each other and the blastocoel wall.

The extent to which the deep layer contributes to mesodermal structures cannot be quantified on the basis of these experiments, but it is significant. At the early

gastrula stage the embryo is several cell layers thick. As gastrulation proceeds, the superficial and deep layers move over the blastoporal lip in concert, so that as cells leave the surface, they join an already significant population of mesenchymal cells.
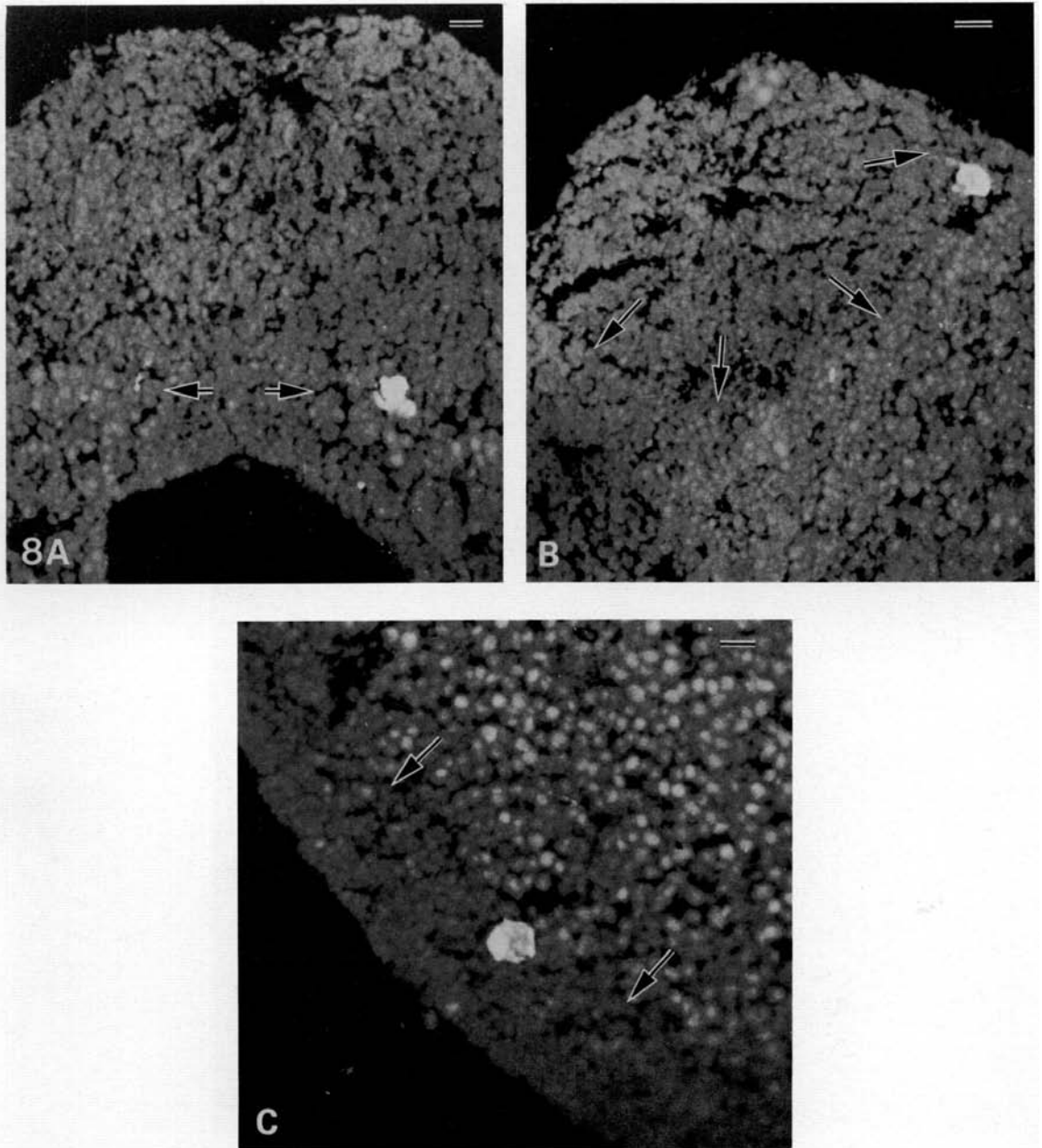

Fig. 8. Light micrographs of serial sections of tailbud stage embryos containing labelled cells. A and B show sections in which labelled cells are found in somitic mesoderm. Arrows delineate notochord in A and neural tube in B. (C) Micrograph shows a labelled cell found in lateral plate mesoderm when more ventrally located cells were injected. Bars, 100 μm.
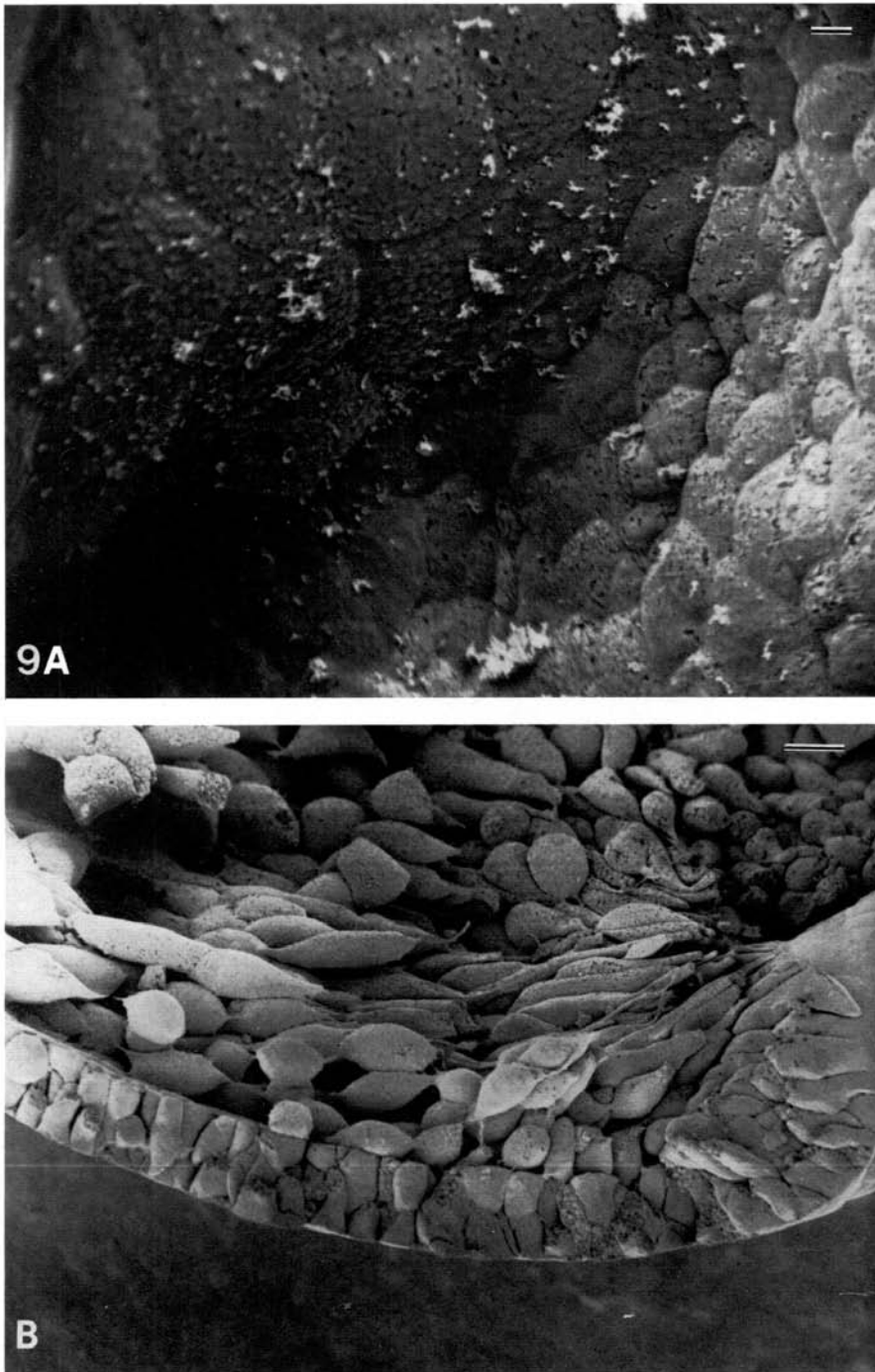
Fig. 9. (A) SEM of archenteron surface of late gastrula near blastopore (lower left) showing putative region of ingression. Larger endodermal cells can be seen in the upper left, separated from smaller cells on lower right by 'pockets' where cells might be leaving the superficial layer. (B) SEM of oblique fracture through the lateral lip of the blastopore. A small patch of the dorsal surface of the archenteron lining is visible on the right, the rest of the archenteron lining has been removed. Deep cells lateral to the notochord are visible and appear bottle-shaped near the notochord. More laterally, deep cells appear to be migrating anteriorly and laterally, away from the blastopore. Bars (A), 10 μm; (B), 50 μm.

DISCUSSION

Vogt's map (Fig. 1) for urodeles is confirmed by these results, in that large areas in the marginal zone of the blastula surface layer are fated to become mesodermal structures. Yet only the notochordal anlage can be found on the surface of the archenteron soon after involution. Large numbers of cells must therefore be removed from the superficial layer on both sides of the notochordal anlage soon after involution, bringing endodermal tissue immediately adjacent to the noto-chordal material (Figs 7B, 10). Thus Vogt's (1929) notion that the endoderm is brought into apposition with the lateral edges of the notochord is correct (Fig. 2). His results and their interpretation were summarized accurately by Hamburger (1960, figs 6–10), but some accounts still insist on an older, erroneous view in which the lateral endodermal crests are free edges which migrate dorsally across the chordamesoderm lining the archenteron roof, to meet in the midline.

Mechanism of removal: bilateral zones of ingression

It was never clear from Vogt's description how the divergence of endodermal and mesodermal paths occurred at the cell level. On the basis of correlated SEM (Fig. 9) it appears that a sizeable population of cells is removed by ingression. They appear to form bottle cells, detach from the superficial layer, and then migrate anteriorly and laterally along the blastocoel wall. The results of Nakasutji & Johnson (1983) are consistent with the notion that cell migration occurs along the blastocoel wall. They found denser networks of fibrils appearing just prior to the onset of gastrulation in urodeles than in anurans. Treating urodele gastrulae and neurulae with anti-fibronectin antibodies, Boucaut *et al.* (1983, 1984) have shown gastrulation movements require fibronectin, whereas neurulation does not. Taken together, these experiments imply a larger role for migration in urodele gastrulation than in anuran gastrulation (see Nakatsuji, 1975; Nakatsuji, Gould & Johnson, 1982; Keller, 1985). When the animal cap is removed from *Xenopus laevis* early gastrulae, leaving the marginal zone intact, they undergo convergent ex-tension, culminating in the closure of the blastopore, without the blastocoel roof as a substratum for any cell migration that might contribute to this process (Keller, 1985). This experiment should be carried out with *Ambystoma mexicanum* to determine whether mesodermal cell migration is required for gastrulation to occur (Keller, 1985).

Notochord formation

Based on the work of King (1902, 1903) and others cited therein, it appears there may not be a neat distinction between anuran and urodele orders based on the mechanics of gastrulation. In particular, *Bufo lentiginosus* and possibly other species of frogs have superficial mesoderm which persists for a time in the posterior dorsal roof of the archenteron before being incorporated into the notochord. In the anterior region of the gastrula, however, the notochord is found in deep mesoderm, beneath the endodermal lining of the anterior archenteron. For these reasons the notochord has been considered endodermal (King, 1903)
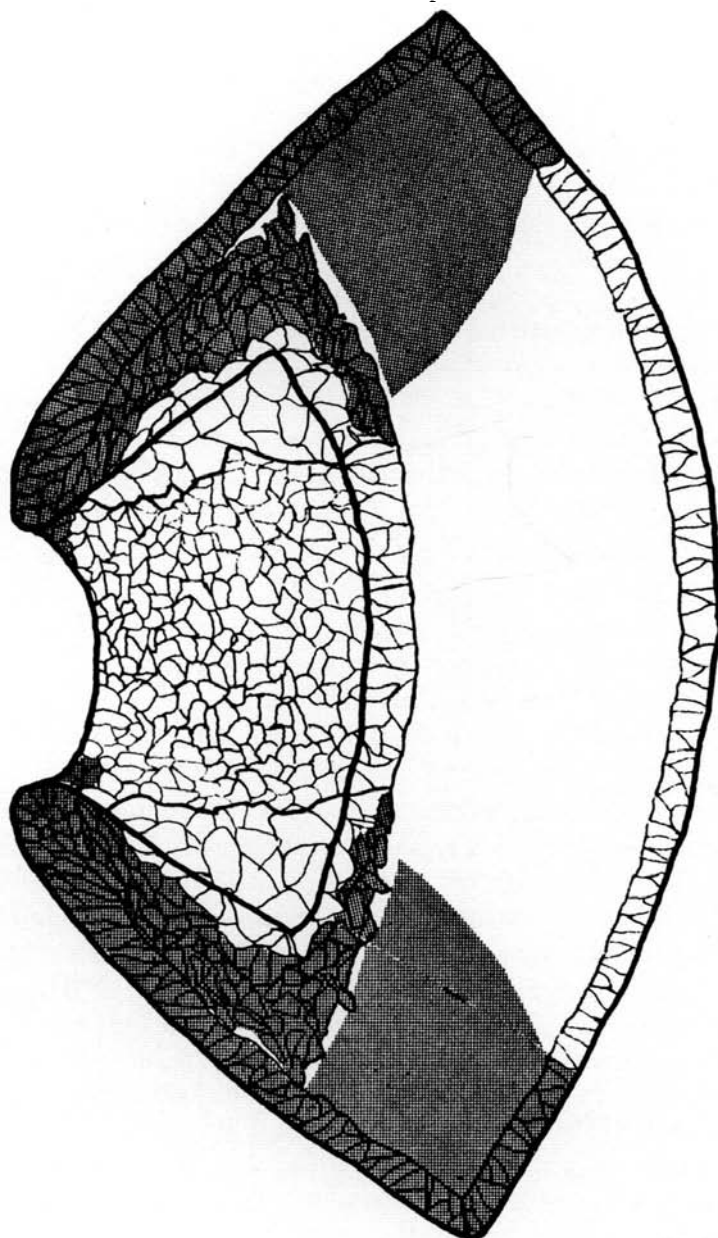
Fig. 10. Drawing of a dorsal quarter of a gastrula depicting involution of notochordal and somitic mesoderm. Soon after involution, somitic mesoderm (shaded) is shown undergoing ingression, bringing endodermal crests (large cells) into juxtaposition with notochordal mesoderm (small cells, heavy line demarcates boundary). The endoderm comes from subblastoporal endoderm (not pictured), which is immediately below the blastopore prior to involution, and moves laterally and dorsally after involuting. The somitic mesoderm then migrates on the inner surface of the gastrular wall. Together these movements generate a dorsalward convergence of large areas previously occupying the outer shell of the gastrula.

and even ectodermal (Perenyi, cited in King, 1903; Brun & Garson, 1984) as well as mesodermal in origin. In all cases, however, the notochord is located between endoderm and ectoderm by the time it is completely formed, hence its definition as mesoderm.

Some very basic concepts about amphibian development are at issue. How significant are the striking differences between *Xenopus laevis* and *Ambystoma mexicanum* gastrulae? Are these differences representative of all the members of each order? What if there are amphibians in which seemingly disparate developmental mechanisms are found, as described in *Bufo* (King, 1903)? Nieuwkoop & Sutasurya (1976) and Brun & Garson (1984) argue that the differences found between urodele and anuran embryonic organization imply at least a diphyletic origin for amphibians. Where do caecilians (the often forgotten third order of amphibians) fit into the picture? How distinct is amphibian development from that of out-groups (slightly more distant relatives) such as sturgeon or lungfish, for example? We need a more comprehensive picture before we can say whether the differences seen between *Xenopus* and *Ambystoma* represent a phylogenetic distinction between urodele and anuran orders. What we observe could be the result of different mechanical demands placed on the gastrula that have less to do with evolutionary history than with adaptable cell behaviours. Without any superficial mesoderm *Xenopus* may not use migration (just one of many cell behaviours in its putatively vast repertoire) as a convergence mechanism, while *Ambystoma* may use mesodermal cell migration because, for example, its convergent extension machinery cannot accommodate all the material located in a single cell layer. Does *Bufo*, displaying an intermediate form of notochord formation, also have an intermediate type of mesoderm organization prior to gastrulation? Is its anterior somitic mesoderm deep, while its posterior somitic mesoderm is superficial? If so, then there would not appear to be a phylogenetic distinction between urodeles and anurans on the basis of gastrulation mechanics. Instead there would be a variety of adaptable mechanical means to accomplish gastrulation.

Until we can answer some of these questions it is difficult to see the implications for amphibian origins. The studies reported here are not inconsistent with the notion that the urodele/anuran distinction is a very old one, or an embryologically significant one. More comparative studies are needed before it will be clear whether observed embryological differences are phylogenetically significant.

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