

Origin and organization of the zebrafish fate map

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Summary

We have analyzed lineages of cells labeled by intracellular injection of tracer dye during early zebrafish development to learn when cells become allocated to particular fates during development, and how the fate map is organized. The earliest lineage restriction was described previously, and segregates the yolk cell from the blastoderm in the midblastula. After one or two more cell divisions, the lineages of epithelial enveloping layer (EVL) cells become restricted to generate exclusively periderm. Following an additional division in the late blastula, deep layer (DEL) cells generate clones that are restricted to single deep embryonic tissues. The appearance of both the EVL and DEL restrictions could be causally linked to blastoderm morphogenesis during epiboly. A fate map emerges as the DEL cell lineages become restricted in the late blastula. It is similar in

organization to that of an amphibian embryo. DEL cells located near the animal pole of the early gastrula give rise to ectodermal fates (including the definitive epidermis). Cells located near the blastoderm margin give rise to mesodermal and endodermal fates. Dorsal cells in the gastrula form dorsal and anterior structures in the embryo, and ventral cells in the gastrula form dorsal, ventral and posterior structures. The exact locations of progenitors of single cell types and of local regions of the embryo cannot be mapped at the stages we examined, because of variable cell rearrangements during gastrulation.

Key words: blastula, gastrula, morphogenetic movements, epiboly, involution, clonal analysis, cell lineage.

Introduction

During the course of embryonic development in vertebrates, cells become allocated into separate lineages that each express a limited and stereotyped set of cell fates. Eventually, dividing cells can autonomously maintain commitments to their lineages, as shown by studies of their development in clonal cultures (e.g. Rutz and Hauschka, 1982) or by transplanting them singly to new positions in the embryo (Wylie *et al.* 1987). Commitment could depend upon the activity of relatively few specific regulatory 'lineage switching' genes, for example the MyoD gene (Davis *et al.* 1987; Hopwood *et al.* 1989). The commitment events themselves are not well understood.

A detailed description of the origin of lineage allocations is now available for the ascidian, a simple vertebrate relative with a determinate mode (Stent, 1985) of early development. Early blastomeres each produce descendants of several cell types, but by the 64-cell stage individual cells begin to be 'tissue-restricted' (Nishida, 1987), generating clones confined to a single type of tissue of the tadpole. Isolated cells can develop the same fates as they would have *in situ* (Whittaker *et al.* 1977; Deno *et al.* 1984), suggesting that the lineage restrictions are due to cell commitments.

In contrast, a similar analysis in vertebrates is hampered because many more cells are generated before

gastrulation, and because, among species that have been examined, the early cell lineages are indeterminate (Rossant, 1985; Kimmel and Warga, 1987a; Moody, 1989). Lineage indeterminacy, as well as the changes in cell fate resulting from transplantation (e.g. Hamburger, 1988; Wylie *et al.* 1987), suggest that in vertebrates the early assignments of cell fates require position-dependent cell interactions that begin, at least in *Xenopus*, during cleavage (Gimlich and Gerhart, 1984). Therefore, it is important to know how early cell positions relate to later cell fates. This information is contained in a fate map.

No fate map is available for the zebrafish, a vertebrate with a number of advantages for developmental study (Kimmel, 1989). The descendants of single early blastomeres scatter to diverse locations and differentiate into diverse cell types (Kimmel and Warga, 1987a). The scattering occurs in the late blastula (Warga and Kimmel, 1990), and precludes making a fate map for earlier stages as can be done, for example, for *Xenopus* (Dale and Slack, 1987a, Moody, 1987). However, by the gastrula stage zebrafish cells generate tissue-restricted lineages (Kimmel and Warga, 1986) as they do in ascidians. Here we describe when the clonal restrictions appear, and how the resulting fate map is organized. Our purpose is to provide a descriptive basis for learning about cellular commitment events, and eventually their underlying mechanisms.

We show that clonal restrictions are established by the onset of gastrulation. The restrictions of two kinds of blastoderm cells, in the enveloping layer (EVL) and deep layer (DEL), arise with different time courses, possibly as consequences of morphogenetic changes associated with epiboly. EVL cells generate periderm (Ballard, 1966), a specialized superficial epithelium (Bouvet, 1976). DEL cells generate all the deeper tissues (Warga and Kimmel, 1990). We show how the primary tissue rudiments are arranged in the DEL at the onset of gastrulation, and consider the degree of detail that is present in the fate map at this stage. Our fate map has some features that are distinctive from those made previously for other teleosts. Notably, it is closer in organization to those for other types of chordates than any teleost fate map made previously.

Materials and methods

Embryos and blastomere injections

We injected single cells, located either in the EVL or the DEL, with lineage tracer dye in individual embryos beginning with the midblastula (Fig. 1A), through the beginning of gastrulation (Fig. 1B). The letter h means standardized hours after fertilization at 28.5°C, as determined by morphological criteria. Midblastula period begins at 3.5 h, late blastula period at 4.0 h, and the gastrula period at 5.2 h. Description of the stages, and the detailed procedures used for labeling cells (usually with rhodamine-dextran) and following their development in live embryos are given in the accompanying paper (Warga and Kimmel, 1990). Whether we injected an EVL or a DEL cell we found it important to examine the labeled cell immediately after the injection by its fluorescence to confirm

that the labeling was confined to a single cell, and with bright-field or Nomarski illumination, to critically ascertain its depth in the blastoderm. In a significant number of embryos, some dye-leakage or spurious dye-filling of a cell or cells adjacent to the injected one occurred, which would confound a clonal analysis; we did not use these embryos for the analysis of clonal restrictions. We did include them in the fate map data set, for which only the position (not the lineage) of the labeled cell was important.

Making the fate map

At the beginning of gastrulation the blastoderm appears uniform in structure and has the shape of a cup inverted over the giant yolk cell (Fig. 1B). At this time its margin is exactly 50% of the distance between the animal and the vegetal pole (50%-epiboly stage; Warga and Kimmel, 1990).

Using a SIT video camera, with which the same clone could be viewed repeatedly during development, we examined the labeling in each embryo at least three times (see below, Fig. 3, for examples of clones mapped this way). At the time of injection we determined, along with the depth of the labeled cell in the blastoderm, its position as a percentage relative to the animal pole (0%) and the blastoderm margin (100%). This provides one of the coordinates necessary to locate the cell on the map, the cell's *latitude*. We have determined by time-lapse video recording (Warga and Kimmel, 1990) that this measure of cell position (as it is defined relative to the margin) does not change greatly as the blastoderm expands and thins by epiboly in the late blastula to acquire the form shown in Fig. 1B.

At 50%-epiboly, and when the injections were made earlier, there is no marker that we know of for the dorsal-ventral position, or *longitude*, of the injected cell. The first such marker, the embryonic shield at the dorsal side of the gastrula, appears at 6 h, and at this time we observed the clone again. We viewed it both from the side (the view shown in Fig. 1; see also Fig. 3B and G) to check its latitude again, and also from an animal polar view (e.g. Fig. 3C and H), to determine its longitude, defined as the angular distance (from this view) between the clone and the embryonic shield. We used this measure to estimate the dorsal-ventral position of the clone at gastrula onset, which is about 40 min earlier. This method introduces an error of a few degrees, which we ignored, because cells are undergoing dorsalwards convergence movements during this 40 min (Warga and Kimmel, 1990).

We then examined the clones in the embryo at about 24 h, to map the positions and types of labeled cells. At this stage, the embryo is motile and many types of cells are differentiating and distinguishable by their morphologies when they are labeled with lineage tracer (Kimmel and Warga, 1987a). Endodermal and some anterior mesodermal derivatives differentiate relatively late, and embryos bearing clones within organs derived from these sources were reexamined a day or two later.

The fate maps are presented for the left side of the gastrula. In fact about half the clones were on the right side, and in our maps these have been translated to the left; assuming bilateral symmetry.

Results

Midblastula cells contribute to lineages of several tissues

Cleavage and early blastula stages in zebrafish are

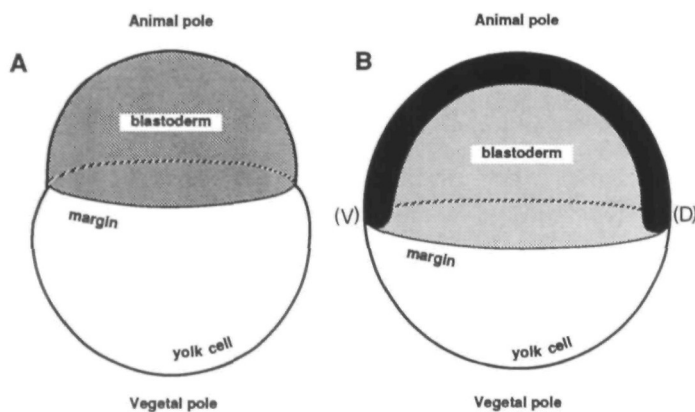


Fig. 1. Appearance of the zebrafish (A) midblastula at 3.5 h, and (B) at the onset of gastrulation, at 5.2 h, the stage for which the fate map was made. See Fig. 3, and Fig. 1 in Warga and Kimmel (1990) for photographs. (A) The blastoderm is a thick cap of about 2×10^3 cells positioned at the animal pole of the uncleaved yolk cell. (B) The blastoderm consists of about 6×10^3 cells and has the form of a cup inverted over the yolk cell. The maps presented in this paper (in Fig. 5 and subsequently), show only the hemispherical blastoderm; the yolk cell being omitted. The dorsal (D) and ventral (V) sides are given in parentheses as a reminder that these positions cannot be distinguished until 40 min later in development when the embryonic shield appears (see Materials and methods).

characterized by rapid and synchronous mitotic divisions of the blastoderm cells. At first, the cleavages are incomplete. The furrows do not pass through the yolk-rich region of the egg, and this region eventually forms a single giant (uncleaved) cell, the yolk cell. Those blastomeres located on the rim or margin of the blastoderm, where it borders the yolk cell, are connected to it, and to one another, by cytoplasmic bridges (Kimmel and Law, 1985a). At the beginning of the midblastula period of development, at the 10th cycle, the marginal blastomeres undergo a change in form, essentially leaving the blastoderm and contributing their nuclei and cytoplasm to the yolk cell, which thereafter remains a multinucleate syncytium. After this cycle the blastoderm itself consists only of single mononucleate cells, none of which are bridged to the yolk cell (Kimmel and Law, 1985b).

Injections of lineage tracer dye into single blastomeres in midblastula embryos (Fig. 1A) yields clones that contribute cells to several tissues. In the example shown in Fig. 2, a cell of the surface enveloping layer (EVL) of the blastoderm was injected at the 1k-cell stage (3 h); and eventually gave rise to three classes of descendants at three separate locations, as determined by inspection of the labeled cells in the live embryo at 24 and 48 h (Fig. 2B). A compact group of periderm cells

contributed to the outer epithelial covering of the embryo. A group of mesodermal cells was dispersed among two adjacent somites and included a differentiated muscle fiber. The third class included two clusters of neural cells in the spinal cord. Present in each of the neural clusters were motoneurons, as well as interneurons that possessed either ascending or descending axons confined to the spinal cord.

The dispersed nature of the cells in this clone is typical of injections made into midblastula cells, and demonstrates the extensive cell rearrangements that accompany morphogenesis after the time of the injection.

Using enhanced time-lapse video recording, we followed these cell rearrangements and the cell lineage generated by the injected cell for about 8 h after the injection, well after the end of epiboly at 10 h. The lineage diagram in Fig. 2A shows the origin of the three separate tissue-restricted lineages founded by this cell. At the end of the recording interval (11 h), there were nine labeled cells represented by the nine vertical lines at the end of the diagram. These cells were already arranged into well-separated groups along the anterior-posterior axis of the embryo that we could identify later when the cells had differentiated. The three groups of cells were also separated into the three

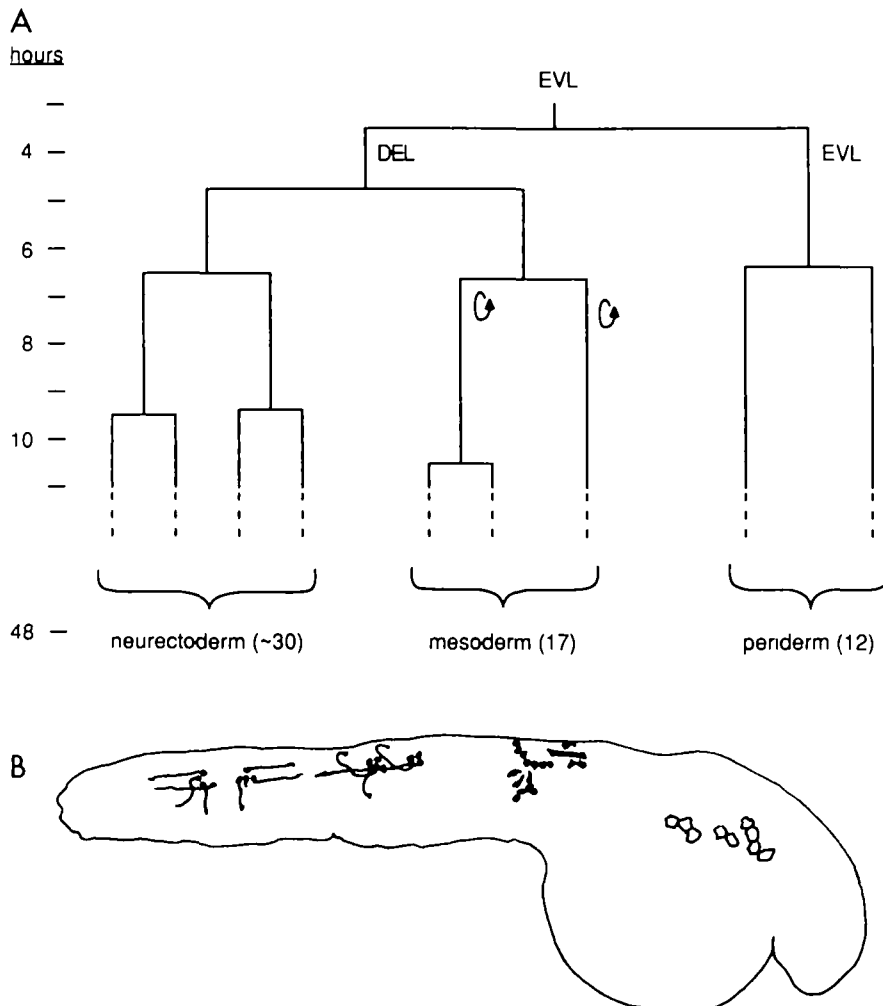


Fig. 2. Segregation of tissue-restricted lineages in the blastula. (A) Lineage diagram beginning with an injected enveloping layer (EVL) cell, and showing numbers, in parentheses, and fates of its EVL and deep layer (DEL) descendants. Vertical lines are cells and horizontal lines are divisions. Time after fertilization is shown on the left. The curved arrows indicate the time when two cells (that went on to generate mesoderm) underwent involution at the blastoderm margin (see Warga and Kimmel, 1990). The lineage was obtained from a video time-lapse recording of the clone developing in the intact embryo. (B) A view of the same clone in the live embryo (right side with dorsal to the top) at 24 h, drawn from the face of the video monitor. The three clusters of labeled cells are of three different tissue types, corresponding to the descriptions shown just above the cells in (A).

primitive germinal layers of the blastoderm that are present at the end of epiboly; the EVL, the epiblast, and the hypoblast (Wilson, 1891; described for zebrafish in Warga and Kimmel, 1990). Except for the developing tail tissue and special cases like the neural crest, large-scale cell movements are nearly completed by the end of epiboly at 10 h. Thus when the same embryo was reexamined at 24 and 48 h there was no difficulty in unambiguously determining the fates of the cell groups that had been present at 11 h. We could not, however, determine the closer lineage relationships of the cells present within each of the groups, because cell divisions continued during the time the embryo was left unobserved.

The first division after the injection of the EVL cell, amounting to its eleventh zygotic division, was oriented parallel to the surface of the embryo. Thus one of the daughter cells (labeled EVL in Fig. 2A) remained in the surface layer and its sibling arose beneath the surface layer; this cell had entered the deep layer (DEL) of the blastoderm. EVL cell divisions of this nature, termed delamination divisions, occur frequently before the midblastula period of development (Kimmel and Warga, 1987a). Afterwards, however, delamination divisions are very infrequent, if they occur at all, as we have learned by direct inspection in a few cases. Thus, in the example in Fig. 2, when the EVL-daughter of the injected cell divided again at 6.5 h both of its daughters remained in the EVL. These cells did not divide again by 11 h, but by 48 h had generated a clone of 12 periderm cells. Hence the lineage restriction of the EVL cell to periderm occurred in this case at the eleventh zygotic division, at 3.5 h.

The DEL daughter of the injected cell was not tissue-restricted; its descendants contributed to both neurectoderm and somitic mesoderm. However, these two fates were segregated after the following division, the twelfth, occurring in the late blastula at 4.8 h. Thus lineage restrictions to different fates were completed just before gastrulation, which begins at 5.2 h.

EVL cells generate periderm

In the example just described, the EVL restriction to periderm arose at 3.5 h. To learn whether this finding could be generalized, we examined the differentiated fates in clones derived from single injected EVL cells in a series of embryos. In most cases, continuous development of the labeled clones was not recorded as described above, since only one embryo could be analyzed in a single experiment of that kind. Rather, the labeled cells were viewed once at the time of the injection (Fig. 3A), usually again in the early gastrula (shield stage; Fig. 3B and C), and then finally after the cell migrations had ceased and differentiation of the labeled cells had occurred (Fig. 3D and E). The example in Fig. 3A–E is a case in which a single EVL cell injected at 3.7 h yielded a periderm-restricted clone.

The solid curve in Fig. 4 summarizes the principle finding from these experiments. We usually observed multiple fates arising after EVL injections in midblastulae, whereas near the end of the blastula period the

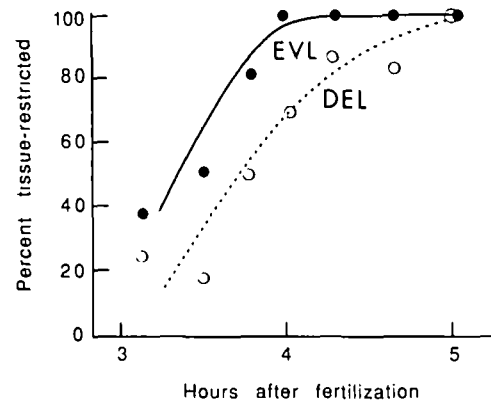


Fig. 4. Clonal restrictions to single tissue fates develop during blastula stages. Percent tissue-restricted means the fraction of clones observed that generated only a single type of tissue. Cells were injected with lineage tracer at times shown on the horizontal axis, and the cell types formed by their descendants were ascertained in embryos 24- to 72 h old. Each point represents the average of at least 5 clones. The closed circles and solid line show the fraction of clones restricted to a single fate, the periderm, resulting from injections of EVL cells ($n=52$). The open circles and dashed line show clonal restrictions to single embryonic tissues (e.g. blood, nerve, muscle, notochord) resulting from injections of DEL cells ($n=54$).

clones were restricted to the periderm. The transition from essentially no restrictions to complete restriction occurred during about one hour, which at this stage amounts to one or two cell cycles, and was complete at the beginning of the late blastula period of development, at 4 h.

Single EVL cells were marked in 52 cases for this study, and as shown in the first row of Table 1, every one of them included periderm among the derivatives. To look for evidence that restrictions occur in a hierarchical fashion, we asked whether only a subset of other embryonic cell types were present in clones that were not periderm-restricted. In such cases, injected at the earlier times, the other fates included cell types classically assigned to any one (or in some cases two) of the three standard embryonic germ layers; ectoderm, mesoderm and endoderm (Table 1). Presumably these diverse cell types arose from DEL cells originating by delamination divisions after the tenth zygotic division, as shown directly in the case in Fig. 2. Thus even though the EVL daughter derived from such a division will usually be tissue-restricted to the periderm, these experiments provide no evidence that the sibling DEL cell bears any particular lineage-dependent restriction of a similar nature.

Fate map of cell position within the periderm

Cells located anywhere in the EVL do not involute (Ballard, 1966; Warga and Kimmel, 1990), but remain on the embryo's surface and differentiate as periderm (Fig. 5). EVL cells also do not undergo dorsalwards convergence movements (Kimmel and Warga, 1987a). Thus the only morphogenetic movement exhibited by the EVL is epiboly, and we can learn how epiboly,

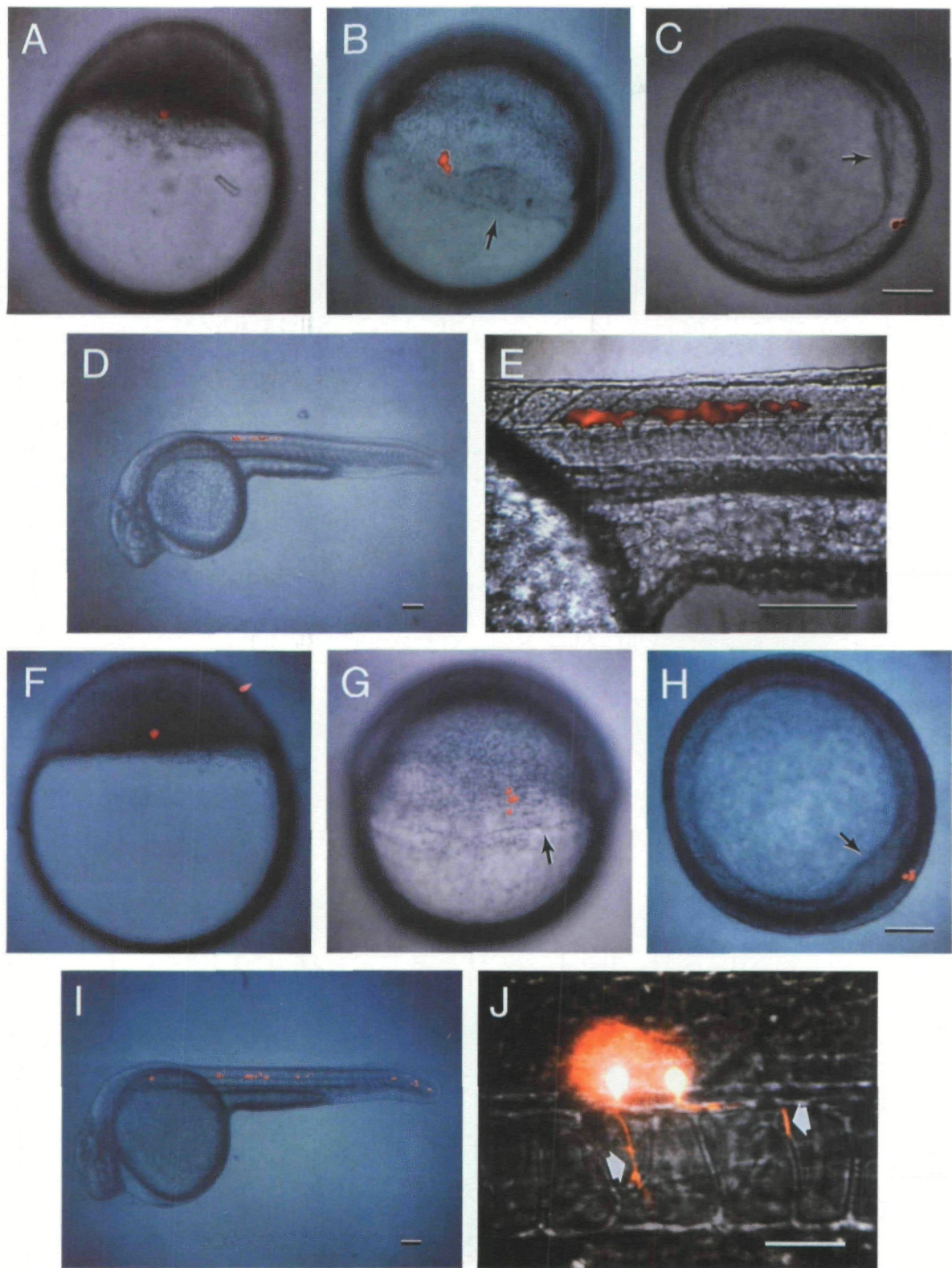


Fig. 3. Clonal restrictions to the periderm (upper panels) and neuroectoderm (lower panels). Data from two different embryos are presented in the same fashion in this figure; all of the photographs are computer-enhanced images that combine fluorescence of the labeled cells in color, and the unlabeled background to show morphological features of the embryos. (A–E) A single cell in the EVL was injected with lineage-tracer dye at 3.7 h (oblong stage), and examined immediately afterwards (A) to confirm that only a single cell was labeled and that it was indeed within the EVL. At 6 h (shield stage), the clone (now containing 2 cells) was examined from both an animal polar view (B) to determine its longitude and a side-view (C) to determine its latitude. Longitude is the position relative to the embryonic shield (arrows in B and C) at the dorsal side. Latitude is position relative to the animal pole and the blastoderm margin. In the embryo at 24 h, at low (D), and at higher magnification (E), the clone consisted of a labeled stripe of cells in the dorsolateral periderm, a squamous epithelium covering the embryo. It consisted of a single coherent group of 9 epithelial cells, a configuration frequently observed of clones within the periderm. (F–J) In the second embryo a single DEL cell was injected (F), which generated a clone of 4 cells by shield stage (arrows in G and H indicate the embryonic shield). The clone was entirely confined to the hindbrain and spinal cord of the embryo at 28 h (I). Two of these cells are shown at higher magnification in J. They are secondary motoneurons (Myers *et al.*, 1986), lying near the floor of the spinal cord. Their axons (arrowheads) are growing within the ventral roots of two adjacent segments. Scale bar: 100 μm , for all panels except J, which is 25 μm .

Table 1. Fates, according to germ layer, of cells located at different depths in the blastoderm

Location of injected cell	No.	Fate ^a			
		Periderm	Ectoderm	Mesoderm	Endoderm
EVL	52	52	7	3	3
First layer DEL ^b	40	2 ^c	25	15	4
Layer 2-4 DEL	14	0	10	4	0 ^d

^aData were pooled from embryos injected between 3.2 and 5.2 h (midblastula through gastrula onset). Many of the clones generated more than single germ layer fates; thus the sum of the several fates will be greater than the total number of clones studied.

^bThe DEL is several cells thick but not obviously layered before gastrulation (Warga and Kimmel, 1990). None-the-less we use 'first layer' to describe those cells that lie immediately beneath the EVL, and 'layer 2-4 DEL' to mean cells lying farther beneath the EVL, estimated in terms of cell diameters by optical sectioning.

^cWe assume we misidentified the location of the injected cell in these two cases, and actually EVL cells were injected. During mitosis EVL cells become rounded and could be mistaken for DEL cells.

^dEndoderm fate maps at or very near the blastoderm margin, where it borders the yolk cell (Fig. 7). Near the margin specifically, because of flattening of the blastoderm on the yolk cell before germ-ring stage, the DEL is only a single cell thick. This explains why endoderm was not observed after injections into deeper DEL blastomeres.

specifically, changes cell position during early development by examining this layer. Accordingly, we have cross-correlated the latitudes (Fig. 6A-E) and longitudes (Fig. 6F-I) of injected EVL cells. The summary map obtained from these correlations (Fig. 6J) shows that latitude in the gastrula approximately coincides with anterior-posterior position in the embryo, and longitude in the gastrula approximately coincides with dorsal-ventral position in the embryo. However, there is substantial distortion: the ventral side of the gastrula forms ventral structures, but also more posterior structures in the embryo. We show below that this same distortion also applies for blastomeres present deep to the EVL.

Origin of tissue-restricted lineages of DEL cells

In contrast to the EVL, DEL cells appear to confine their lineages to the deeper tissues of the embryo. To show this, and to learn when DEL lineages become restricted to single tissues, we directly injected lineage tracer into cells lying superficially in the DEL. As for the EVL injections, it was particularly crucial in these experiments to confirm the specificity of labeling and the location of the cell by combining fluorescence and Nomarski microscopy immediately following the injection (Fig. 3F). As described above for the EVL clones, the DEL clones were also mapped in the gastrula (Fig. 3G and H), and in the one- or two-day embryo to learn their fates. In this example, the labeled cells were remarkably distributed along the embryonic axis (Fig. 3I), but entirely within the confines of the neural tube. Derivatives included recognizable types of neurons, including secondary motoneurons (Fig. 3J).

Except for two cases that are likely to have resulted

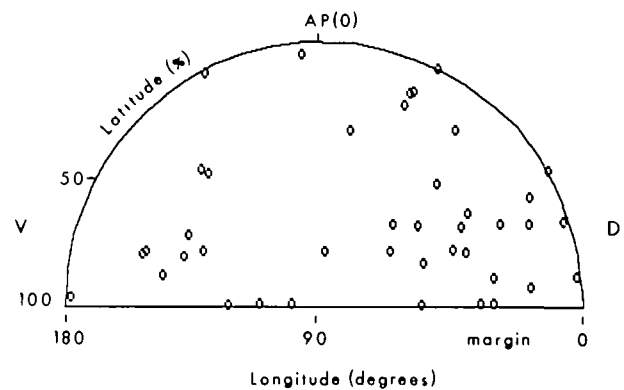


Fig. 5. EVL cells generate periderm, irrespective of their blastoderm positions. The outline represents a surface view of the left side of the blastoderm at 50% epiboly (see Fig. 1B), the time gastrulation begins. Each point represents the blastoderm position of a single EVL cell that was injected with lineage tracer in a separate embryo, and projected to this stage as explained in Materials and methods. Periderm was either the exclusive fate, or included among the fates observed in all of these cases. Latitude is defined as percent of the distance between the animal pole (AP, 0%) and blastoderm margin (100%). Longitude is defined in degrees of arc, with the embryonic shield (dorsal midline) set to 0°. All of the injected cells are shown as if they were on the left side of the blastoderm, in fact about half (24/52) were on the right side.

from experimental error, cells derived from injected DEL cells did not contribute to the periderm, but formed other cell types (Table 1, rows 2 and 3). As for the EVL restriction to periderm, DEL restrictions to single tissues appeared progressively during mid- and late blastula stages (Fig. 4). Evidently however, the tissue-restricted DEL lineages appear more gradually than the EVL restriction to periderm. Many DEL cells, in contrast to the EVL, are not tissue-restricted at 4 h, the beginning of the late blastula period. As in the example in Fig. 2, most DEL cells divide only a single time in the late blastula (unpublished data of R. M. Warga and D. A. Kane), and this division yields the cells whose lineages are tissue-restricted.

The type of tissue derivative a DEL cell will form, or even the germ layer it will enter, is not predicted by its depth within the DEL during blastula stages. As pointed out above, DEL cells arising by delamination divisions of injected EVL cells in the midblastula could contribute to any of the three embryonic germ layers. The DEL cells so produced by delamination divisions at this stage are located very superficially in the DEL. As expected from those results, the most superficial DEL cells that were injected directly with lineage tracer yielded clones contributing to any of the germ layers; ectoderm, mesoderm and endoderm (Table 1, row 2). Several of these included two germ layers. Advancing the injection pipette still deeper into the DEL, to inject cells removed by 1-3 cell diameters from the EVL, made little difference in the results (Table 1, row 3). Although not shown in Table 1 (where data from both mid- and late blastula injections are pooled) these

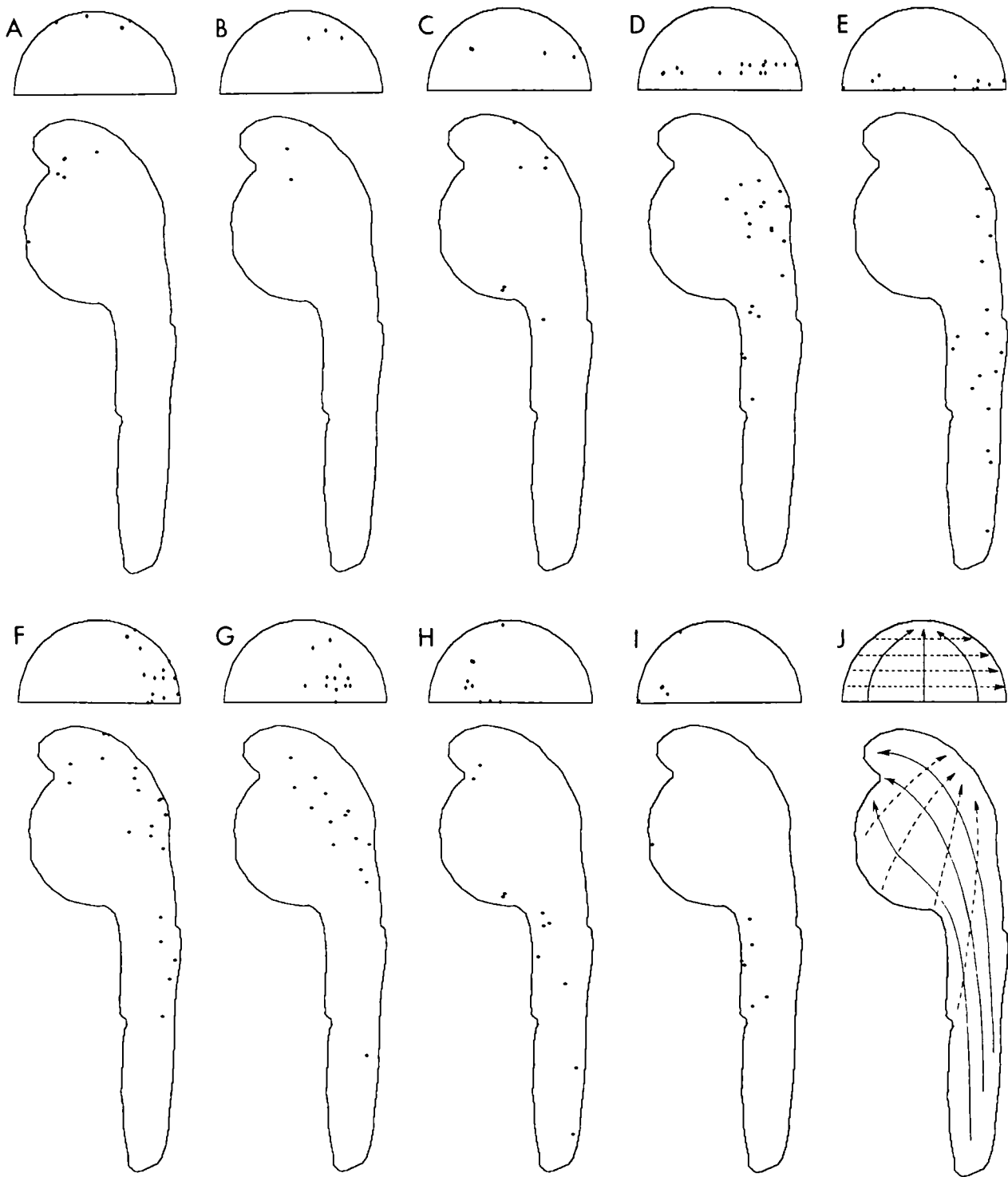


Fig. 6. Relationship of the positions of EVL cells at the onset of gastrulation and the positions of their descendent clones in the periderm of the embryo. A point in the upper drawing of each pair shows the position of each injected cell, as in Fig. 5. A point in each lower drawing represents the approximate center of the group of descendent periderm cells. For A–E, the data are collected into bins of 20% latitude. For F–I, the same data are collected into bins of 45° longitude. J shows these distributions in summary form. At first, the dashed lines point dorsally and solid lines point to the animal pole. Morphogenetic movements subsequently skew these vectors.

findings do not depend on the stage when the cell was injected, until gastrulation begins and the DEL subdivides into two layers (Warga and Kimmel, 1990).

Germ layers are mapped by latitude

At the beginning of gastrulation, cells that will generate tissues beneath the periderm are present exclusively

within the deep layer (DEL) of the blastoderm. At this stage the DEL is several cells thick, but prospective cell fates are apparently not segregated by depth within it (above, and Kimmel and Warga, 1990). Rather, time-lapse studies showed that DEL cells initially lying near the blastoderm margin involute during gastrulation and generate mesodermal and/or endodermal derivatives, whereas those lying farther from the margin generate ectoderm (Warga and Kimmel, 1990). Consistent with this observation, we found a strong correlation between the latitude of a DEL cell in the early gastrula and the germ layer to which it would later contribute (Fig. 7). Ectodermal derivatives arise from cells located over most of the blastoderm, from the animal pole to near (but not at) the margin. Endodermal fates come from

cells at or very near the margin, and mesodermal fates from cells in between.

Comparisons among the panels of Fig. 7 reveal that boundaries of these germ-layer fields overlap to a considerable extent. In part this is due to the fact that the cells were labeled in blastulae (see Discussion), not at the onset of gastrulation. Overlaps are more extensive for clones initiated at earlier stages.

Positions of tissue and organ rudiments

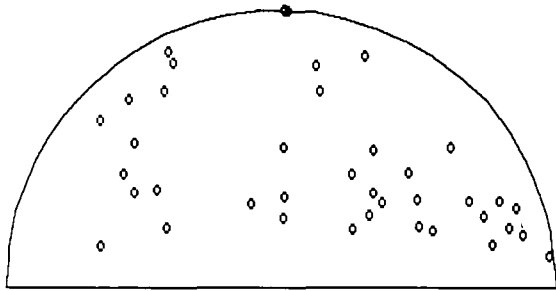
We can subdivide the germ layer fields into areas fated to form different tissues and organs. Thus prospective ectodermal cells located near the animal pole of the gastrula generate brain if they come from the dorsal side and epidermis (an epithelial layer just beneath the periderm) if they come from the ventral side (Fig. 8). There is an anterior-posterior mapping of brain regions according to latitude in the gastrula. Forebrain structures (including nasal and eye tissues; see Kimmel and Warga, 1987a) come from the region around the animal pole, and midbrain and hindbrain structures come from latitudes nearer the margin (Fig. 8). Cells that generate brain are located in the dorsal half of the gastrula, but some cells generating spinal cord stem from the ventral side. A few of the clones formed neural crest derivatives; the crest appears to arise in the ventral half of the map, nearer the margin than epidermis. As for the germ layer maps (Fig. 7), tissue-fate map areas overlap substantially. For example, prospective neural cells located near the dorsal margin might form midbrain, hindbrain, or spinal cord.

There is an interesting mapping of spinal motor and sensory neurons. The cell types were determined by identifying the positions of their axons, which were often brightly labeled with lineage tracer. Spinal motoneurons (Fig. 9A), located ventrally in the spinal cord, come from the dorsal side of the gastrula (e.g. Fig. 3). Rohon-Beard sensory neurons (Fig. 9B), located dorsally in the spinal cord, map with neural crest cells on the ventral side of the gastrula (data not shown).

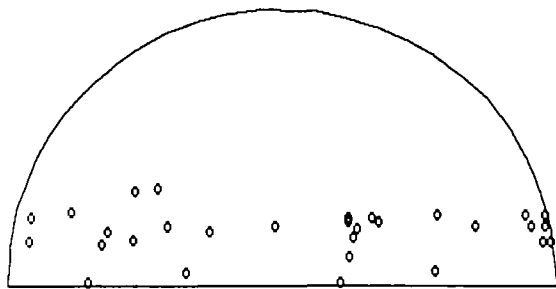
Our data also provide some key features of the mapping of mesoderm (Fig. 10). Muscle progenitors are found in a circumferential ring: head muscles (extrinsic eye, jaw, and gill arch muscles) map to dorsal positions in this ring, and myotomal (somite-derived) muscles of the trunk and tail map to intermediate and ventral locations. The notochord comes from the dorsal side, blood from the ventral side, and pronephros in between.

We include the hatching gland in Fig. 10 because its fate map position is extensively overlapped with the notochord (compare Fig. 10C and D). However, the hatching gland is a teleost speciality, and in fact we do not know whether it should be assigned to the mesoderm or endoderm. We have some observations about this curious tissue, but do not show the data: notochord and hatching gland cells frequently share the same clones when injections are made at early blastula stages. Prospective hatching gland cells involute in the very early gastrula, to enter the hypoblast, and they lead the notochord cells in the anteriorwards movement that

A. ectoderm



B. mesoderm



C. endoderm

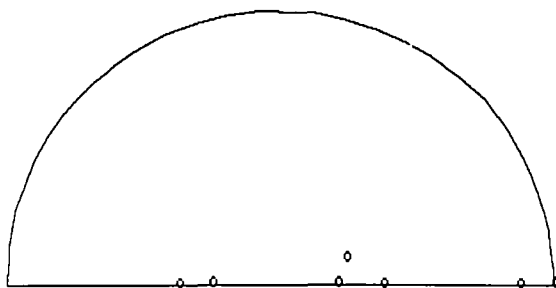


Fig. 7. Ectoderm, mesoderm and endoderm map to different latitudes of the fate map. The presentations are as in Fig. 5. The data set includes both EVL blastomeres that produced deep-lying descendants as well as DEL cells injected directly.

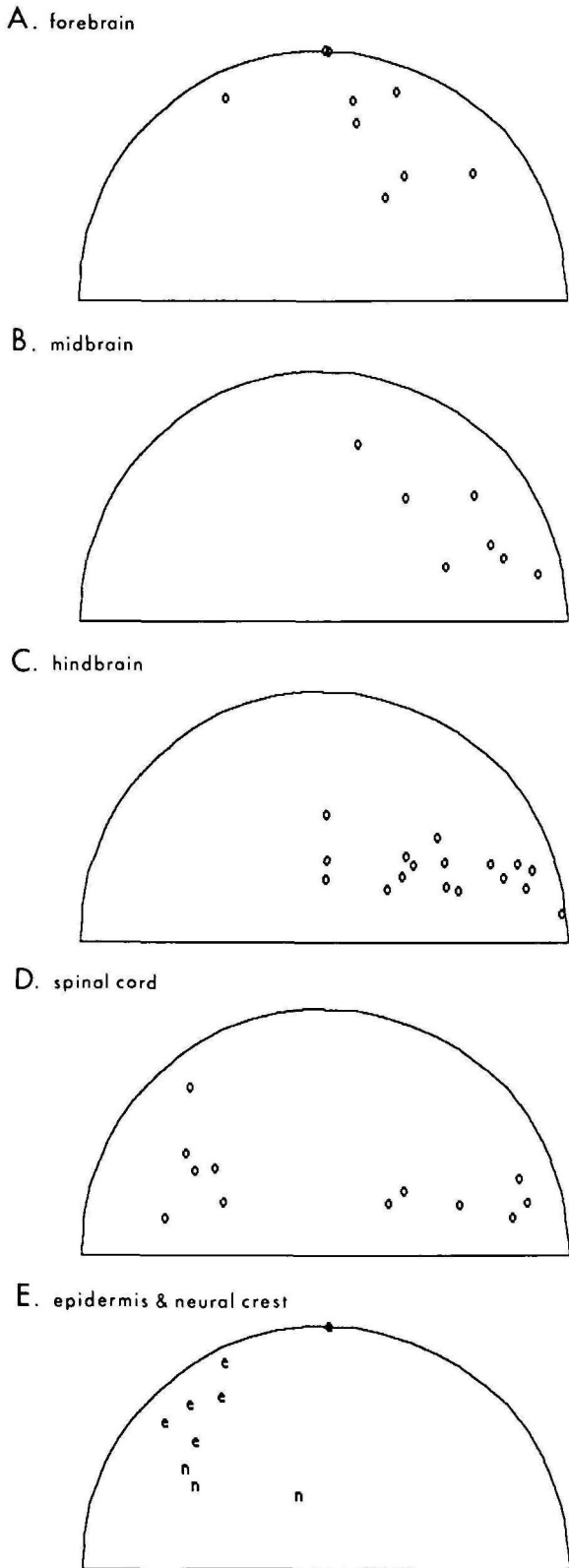


Fig. 8. Fate map positions of derivatives of the ectoderm. The presentations are as in Fig. 5. The entire data set for ectoderm, including all of these derivatives is shown in Fig. 7A. Here the forebrain map (A) includes neural retina. (E) A single map combining the positions of cells generating epidermis (e) and neural crest (n).

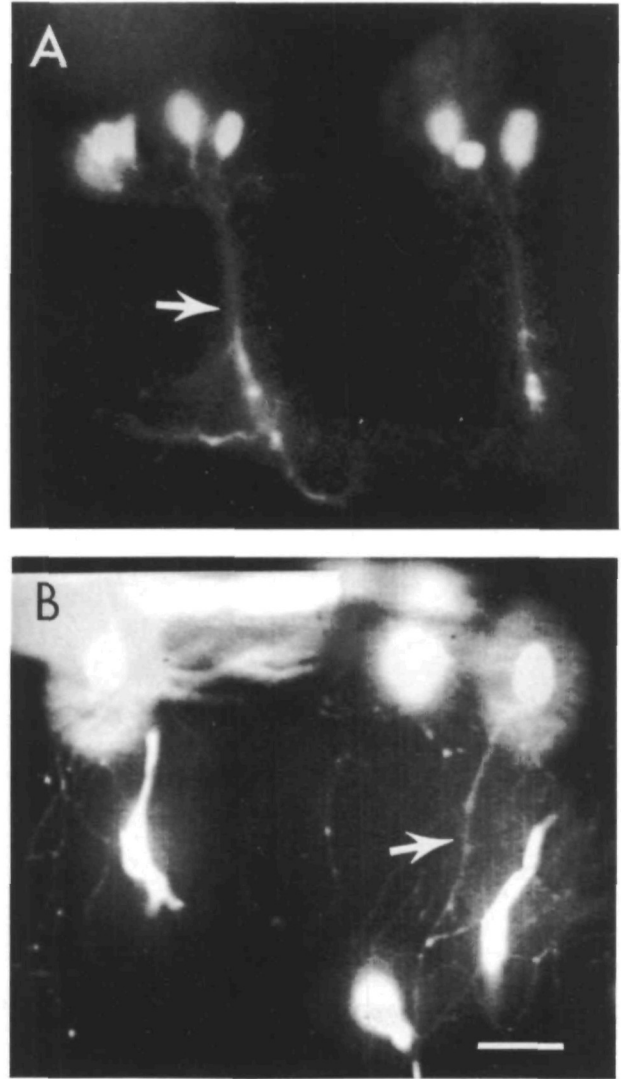


Fig. 9. Identifiable types of neurons labeled with lineage-tracer dye. The live images were recorded onto an optical disk with a high-sensitivity video camera, and using a computer several focal planes are combined for each image, background noise subtracted, and the contrast of the final image enhanced. Dorsal is up, and anterior is to the left. Arrows show labeled axons. (A) Clusters of primary motoneurons (Myers *et al.* 1986) in two adjacent spinal segments each contribute to a bundle of axons (a motor nerve) innervating somite-derived muscle. (B) Rohon-Beard neurons form peripheral axons that branch repeatedly within the skin (Myers *et al.* in preparation). Scale bars: 25 μ m.

occurs in the hypoblast along the dorsal side of the gastrula. Later, after gastrulation, the hatching gland precursors underlie the rudiment of the brain in a head mesodermal/endodermal region of distinctive structure that has been termed the polster (i.e. 'pillow', see Ballard, 1982), and that may be equivalent to the prechordal plate region of amphibians. Shortly afterwards, the hatching gland cells take up their definitive positions over the pericardium (see Fig. 3B in Kimmel and Warga, 1987a), i.e. at an anterior and ventral location. The hatching gland is rather singular in that it

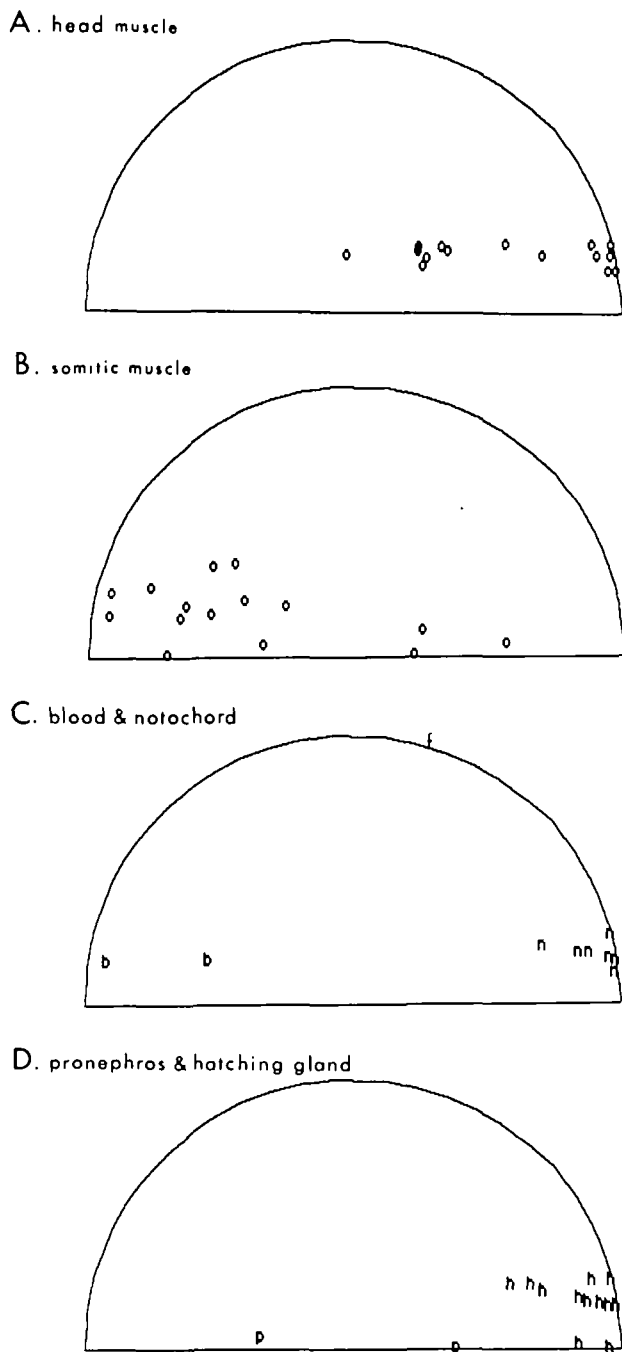


Fig. 10. Fate map positions of derivatives of the mesoderm. The presentations are as in Fig. 5. The entire data set is shown in Fig. 7B. (A) The map includes gill arch, as well as head muscle. Endothelium was also observed after injections into cells at the same latitudes, but our data are incomplete with respect to longitude and this fate is not illustrated. (B) Somite-derived muscle; (C) blood (b) and notochord (n); (D) pronephros (p) and hatching gland (h).

is a ventral tissue formed by progenitors located dorsally in the gastrula.

On the other hand, many DEL cells on the ventral side of the gastrula converge dorsally and have dorsal fates (Fig. 11), primarily in the tail. We showed above

(Fig. 6J) that much of the periderm of the tail was derived from ventral EVL progenitors. Similarly, ventral DEL cells contribute nerve (Fig. 11A and B) and muscle (Fig. 11C and D) exclusively to tail segments. Although our data set includes only a few endodermal clones, we also found their anterior–posterior location to be roughly according to dorsoventral position of the gastrula progenitor.

Lineage restrictions and the origin of the fate map boundaries

We have shown that at late blastula stages when tissue-restricted lineages appear, DEL cells that have similar fates are collected together into local regions of the blastoderm. The restrictions appear progressively (Fig. 4), suggesting the possibility that they arise in particular regions at particular times. If this were so, then at the time that the restrictions appear, cells remaining unrestricted should be localized to one or another region of the blastoderm. However, cells contributing descendants to two or more different tissues were widely distributed (Fig. 12). More were observed in the ventral than in the dorsal half of the blastoderm, but this difference was not significant by Chi-squared analysis (data not shown). Thus our data provide little evidence for a model in which the time DEL cells become tissue-restricted depends on their locations in the blastula.

Restrictions of DEL cells could also occur hierarchically. For example, a cell might acquire a lineage restriction to a single germ layer, and afterwards to a single tissue within this germ layer. Most of the clones in Fig. 12 generated two fates, and we can address this issue by seeing whether both of these fates are from the same germ layer. Among these 14 clones there were 8 cases where both derivatives were ectodermal (e.g. epidermis and nerve) or mesodermal (e.g. blood and muscle), and 6 cases where the derivatives were from separate germ layers (e.g. nerve and muscle). The data do not support the idea that lineages are germ layer-restricted before they are tissue-restricted.

Discussion

Lineage restrictions arise before gastrulation

We have described when tissue-restricted cell lineages arise in the zebrafish and where cells founding these lineages are located in the blastoderm. There are two distinctive cell types in the blastula, EVL and DEL cells, and their restrictions arise with separate time courses. All EVL cells present at the beginning of the late blastula period (4 h) generate lineages restricted to periderm, whereas some DEL cells continue to generate unrestricted lineages throughout the late blastula period. The different time courses suggest that whatever changes bring the restrictions about occur separately in the EVL and the DEL.

Moreover, the tissue-restrictions arise separately from a lineage segregation described previously, between the blastoderm and yolk cell (Kimmel and Law,

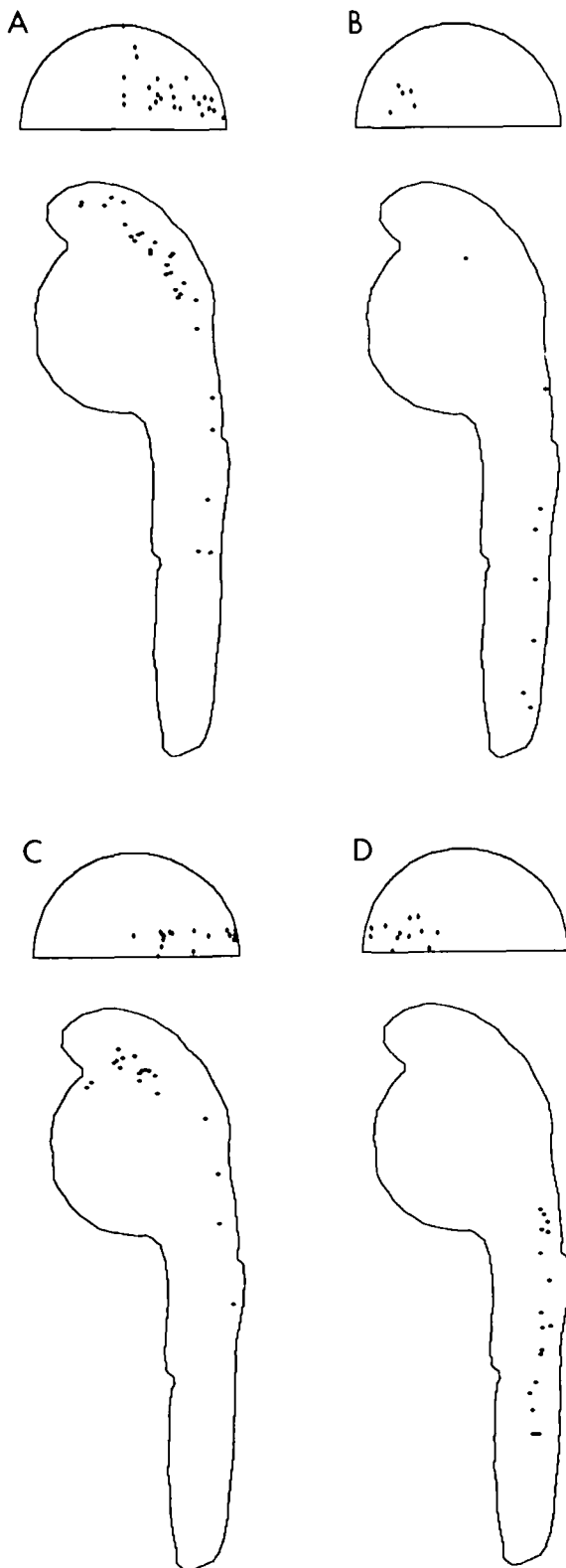


Fig. 11. DEL cells located at different dorsoventral levels in the gastrula generate axial tissues at different anterior-posterior levels of the embryo. (A,B) Ectodermal fates; (C,D) mesodermal fates. Dorsal neural progenitors form brain and trunk spinal cord (A), while ventral progenitors form spinal cord of the lower trunk and tail (B). The isolated anterior clone present in the view of the embryo is within the cranial sensory ganglion of the trigeminal nerve, not within the CNS. Dorsal mesodermal progenitors generate extrinsic eye muscle cells, gill muscle cells and muscle cells in anterior myotomes (C), whereas more posterior myotomal muscle comes from ventral progenitors (D). In both comparisons, there are positional overlaps in the region of the lower trunk.

between the blastoderm and yolk cell. It is likely that the yolk cell is an extraembryonic structure; it appears to make no cell contribution to the tissues of the embryo proper. This restriction occurs an hour before the periderm restriction described here, and is the first lineage restriction that we have observed in the zebrafish.

Like the yolk cell restriction, the EVL restriction to periderm represents a lineage compartmentation in the embryo. All EVL cells present after 4 h generate clones of periderm cells, and DEL cells were never observed to enter the EVL to generate periderm, either before (Fig. 3 in Warga and Kimmel, 1990) or after 4 h (Table 1). Thus the 4 h EVL represents the unique precursor pool of cells for the peridermal lineage. Also like the yolk cell, the periderm might be extraembryonic; Bouvet (1976) suggested that in the trout the periderm does not contribute to the definitive skin of the fish (or to any other tissue), but is sloughed late in embryogenesis. The definitive fate of the periderm has yet to be examined in zebrafish.

Similarly, at the beginning of gastrulation, when nearly all DEL cells are tissue-restricted, we have shown that the progenitors of one tissue are located

1985b). At cleavages before the tenth one, marginal blastomeres contribute progeny variably both to the yolk cell (a multinucleate syncytial cell) and to the blastoderm. The yolk syncytial layer forms rather abruptly at the tenth cleavage and, afterwards, in the midblastula (3 h) there are no further lineage exchanges

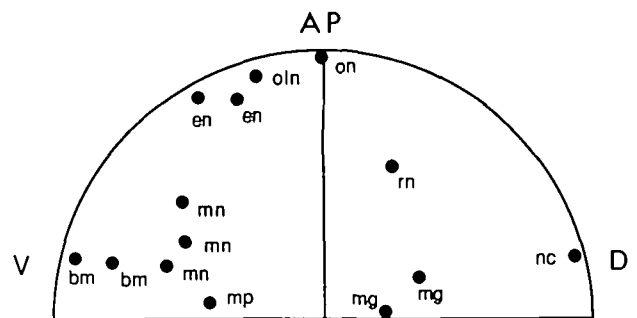


Fig. 12. DEL cells that generate unrestricted lineages are distributed widely in the blastoderm. The presentations are as in Fig. 5. The points show the positions of injected DEL cells that generated lineages including more than single fates. Tissue abbreviations: b, blood; c, notochord; e, epidermis; g, gut epithelium; l, lens; m, skeletal muscle; n, nervous tissue; o, olfactory epithelium; p, pronephric duct; r, pigmented retinal epithelium.

together in the blastoderm. It may be however, that the fate map boundaries are not completely determinate (see below), as contrasted with the YSL and EVL segregations and, for example, with compartmental boundaries in *Drosophila*. All of the lineage restrictions in early zebrafish also seem different from *Drosophila* compartments in that they segregate functionally distinct, not just geographically distinct, classes of cells.

Morphogenesis may underlie the appearance of tissue-restricted lineages

In the case of the ascidian embryo, it is likely that at least some tissue-restricted blastomeres are committed, or determined, to express their normal lineages (Whittaker *et al.* 1977; Deno *et al.* 1984). We cannot, however, equate lineage restrictions with restriction in developmental potential in the zebrafish (see Kimmel and Warga, 1988). We observed neither systematic spatial ordering nor hierarchy in appearance of DEL cell restrictions. Furthermore, transplantation experiments carried out many years ago with teleost embryos of other species, *Salmo* (Luther, 1936) and *Fundulus* (Oppenheimer, 1936; 1938), suggest that development is regulative during gastrulation in fish. Similar results have recently been obtained in cell transplantations made in the early zebrafish gastrula (R. K. Ho, unpublished observations). Thus, cells generating at least some tissue-restricted lineages in the early zebrafish may not be committed to them.

Changes in the morphogenetic behavior of cells during development of the blastula, rather than cell commitment, can provide explanations for the origin of all of the lineage restrictions we have observed. The yolk cell restriction is coincident with formation of the yolk syncytial layer, after which only nuclear divisions occur in the yolk cell; the restriction may be a consequence of the cessation of yolk cell cytokinesis.

The EVL restriction to the periderm may be a consequence of EVL blastomeres orienting their cell division planes such that delamination divisions (divisions in which one daughter remains in the EVL and the other daughter enters the DEL) no longer occur. Delamination divisions are frequent at earlier stages (Kimmel and Warga, 1987a), but we have not observed them in the late blastula. Delamination divisions cease at the time the blastula is developing a spherical shape (Compare Figs. 1A and B, and see Warga and Kimmel, 1990). At this stage tension at the blastoderm surface could be increasing, and influence the orientation of divisions occurring in cells at the surface.

The appearance of DEL restrictions to single tissues is correlated with the cessation (or a marked decline) in radial cell intercalations, rearrangements that appear to mediate the thinning of the blastoderm at the end of the blastula period, and that scatter DEL cells (Warga and Kimmel, 1990). Once this mixing slows, then cells that are neighbors might generally have similar fates whether or not they are related by lineage, and whether or not their potentials are restricted at the beginning of gastrulation.

The fate map boundaries are overlapped

The fate map appears as cell lineages become restricted. Clearly, it is the pronounced intermixing of cells during epiboly that precludes making a map earlier, as can be done, for example, even during cleavage stages in *Xenopus* (Dale and Slack, 1987a; Moody, 1987). Radial intercalations mediate blastoderm thinning in the *Xenopus* late blastula (Keller, 1980), but the cell intermixing is much more limited (Wetts and Fraser, 1989) than in zebrafish.

Our fate map is for the beginning of gastrulation, but because it is derived from injections during blastula stages, we show the positions of small clones of cells, rather than single cells present at gastrulation. Thus we can only approximate the positions of the boundaries separating regions of different fates. Nevertheless, the rather extensive overlaps that we observed, including overlaps between different germ layers (Fig. 7) suggest that the boundaries are not completely determined at the time gastrulation begins. More likely they are statistical ones, as in *Xenopus* (Keller, 1975; 1976).

During gastrulation, DEL cells in both hypoblast and epiblast undergo mediolateral intercalations (Warga and Kimmel, 1990). Unlike the earlier radial intercalations, these rearrangements do not usually scatter clonally related cells from one tissue primordium to another, but cells in one clone do scatter widely within a single tissue primordium (e.g. Fig. 3I). Hence one can map a region in the early gastrula that will form, for example, somitic muscle, but not be able to predict to which somite a given clone will contribute (see also Kimmel and Warga, 1987b). Similarly, the two sides of the CNS (Kimmel and Warga, 1986), and, as we have shown here (Fig. 8), the regional subdivisions of CNS cannot be mapped to unique, non-overlapping regions in the early gastrula.

Fig. 13 summarizes the positional shifts that occur after gastrulation begins for three representative populations of cells, generating periderm from the EVL (A), neurectoderm from the epiblast (B), and muscle from the hypoblast (C). Within the EVL, cell movements are less extensive, but even so, the general flow of cells during development within these three separate layers of the gastrula is quite similar. These summary diagrams are inadequate to show the very extensive longitudinal dispersion of clones of DEL cells.

Comparative aspects of the fate map

A summary fate map for the DEL at gastrula onset is shown in Fig. 14A. There are some differences between this fate map and previous maps made for other teleosts. Thus, fate maps for *Fundulus* (Oppenheimer, 1936) and *Salmo* (Pasteels, 1936) show the ventral side of the blastoderm as largely 'extraembryonic'; contributing to the yolk sac membrane, whereas our map reveals a variety of embryonic structures (e.g. epidermis, neural crest, blood, and tail axial tissues) stemming from ventral locations in the gastrula. The differences are likely due to the fact that the zebrafish egg is considerably smaller than these others (hence less cells might contribute to the yolk sac), and that we use a

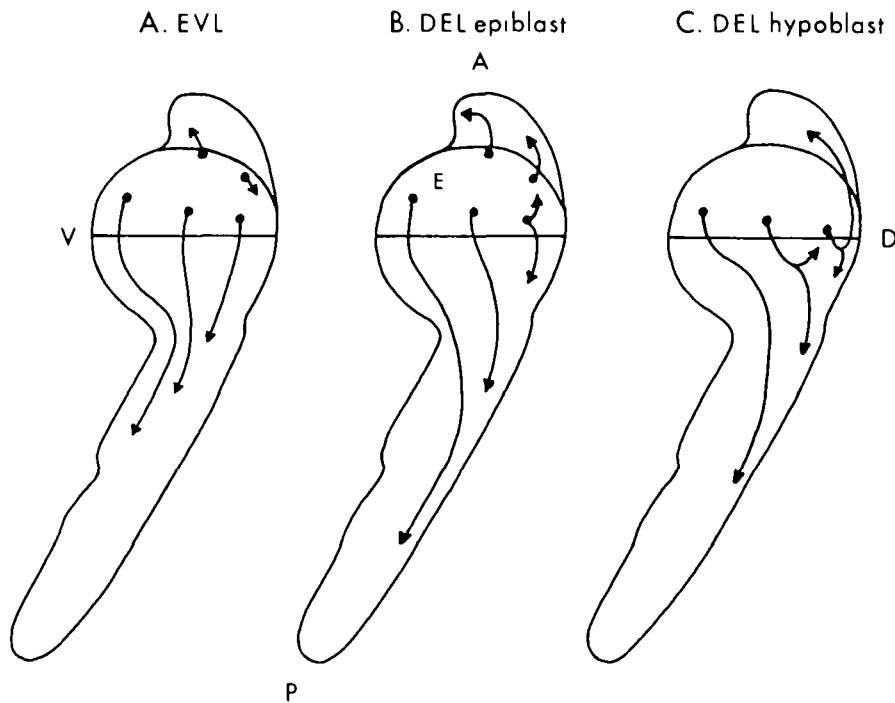


Fig. 13. Morphogenesis during gastrulation and subsequently, of (A) the EVL, (B) a subset of DEL cells that form epiblast and later contribute to neuroectoderm, and (C) a subset of DEL cells that form hypoblast and later contribute to paraxial mesoderm. Side views of the early gastrula and 24 h embryo are superimposed and arrows show the general nature of the cell movements between the two stages. The major component of the movement of EVL cells is epiboly, whereas the DEL cells also show dorsalwards convergence and extension along the axis, such that their movements are generally more extensive than those of EVL cells present at the same location in the early gastrula. In the case of C the DEL cells also involute. Except for involution (see Warga and Kimmel, 1990), the movements are generally very similar to those described by Ballard in other teleosts (Ballard, 1981; 1982; 1986). For example, the region labeled 'E' in (B) was named the 'evacuation zone' by Ballard, because (as we confirm) during gastrulation DEL cells move out of this zone, without others moving in (as they do in other regions of the blastoderm). We observed, as did Ballard, that many of the DEL cells of the evacuation zone generate epidermis.

considerably more refined cell marking technique. Pasteels' map showed the boundaries of individual somites, and considering the overlaps we have described, we suspect imagination rather than data produced these boundaries.

In many respects the zebrafish fate map is similar to maps prepared for several teleost species by Ballard (1973, 1981, 1982, 1986), who, for example, also described posterior somites originating from ventral locations in the gastrula. Ballard found even greater overlaps of the boundaries than we did. He interpreted his data to mean that the fate map must be organized three-dimensionally; cells generating different fates would be located at different depths. The overlaps could then be explained away as artifacts because the three-dimensional structure is represented in two-dimensions in the fate map. However, this explanation is not supported by our findings in zebrafish. Except for the EVL, which does indeed generate the periderm as shown by Ballard, indeterminacy rather than three-dimensional organization accounts for the overlaps: First, we observed (e.g. Fig. 2) that clonal descendants of single cells present just before epiboly begins, form a number of different cell types representing different germ layers. There are no differences in fates of cells derived from blastomeres located at different depths

beneath the EVL (Table 1). Second, the thinning of the blastoderm during epiboly occurs by deep cells intercalating radially among more shallow ones (Warga and Kimmel, 1990). This would thoroughly stir up any three-dimensional organization that had been present before epiboly. Third, cells developing as mesoderm and endoderm acquire their deep positions as they undergo involution during gastrulation (Warga and Kimmel, 1990), as in many other creatures. We suspect that Ballard observed more extensive overlaps than we did because he marked cells at an earlier stage, before cells are mixed during epiboly.

There is a key difference between our map and Ballard's. We find head mesoderm to originate near the dorsal blastoderm margin (Fig. 10A). Based upon chalk particles thrust into the blastoderm, Ballard (1973) thought that the head mesoderm mapped deep to the surface at the animal pole. Yet we specifically examined the fates of these deep-lying cells (Warga and Kimmel, 1990) and observed that they made ectoderm. We think it unlikely that a major difference in fate map organization really exists between zebrafish and *Salmo*. Ballard's implanted chalk particles may possibly have adhered to the yolk cell at the animal pole, the region to which prospective head mesodermal cells migrate.

More than any other previously published teleost fate

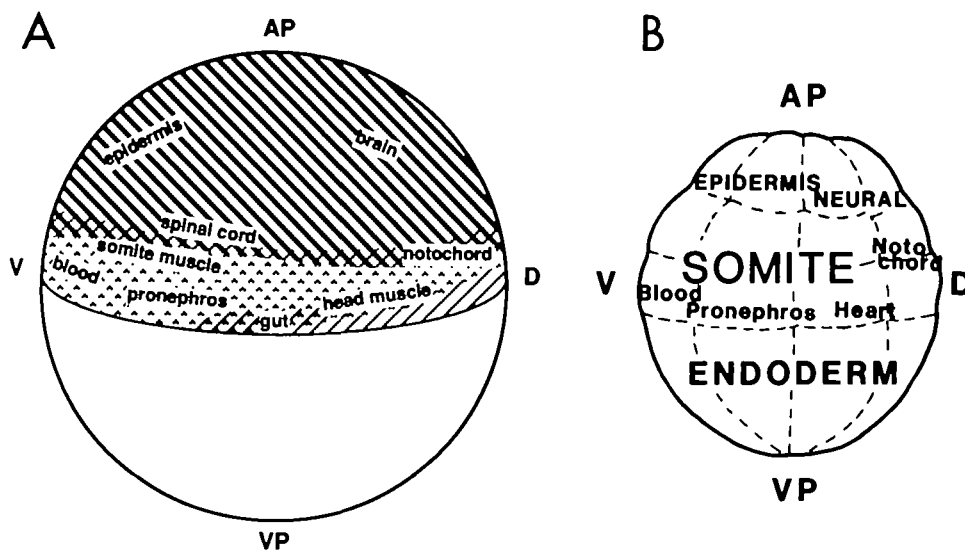


Fig. 14. Summary fate maps for (A) the zebrafish at the beginning of gastrulation and (B) *Xenopus* at the 32-cell stage (adapted from Dale and Slack, 1987b). In A, shadings of different textures show regions where DEL cells give rise to ectoderm, mesoderm and endoderm (compare with Fig. 7). As can be seen, the regions are considerably overlapped. Probably a great deal of the overlap is really present in the embryo at this stage (see the text). Areas that form different tissues are labeled. This presentation gives a ready overview of the way the map is organized, but we caution the reader that we do not show the boundaries of these areas: Figs 8 and 10 need to be examined for the approximate boundaries and the extent of their overlaps. For example, the notochord position is well shown in this summary map, but the representation of the spinal cord is incomplete; its progenitors are present in the dorsal half as well as the ventral half of the gastrula; indeed, some of them lying within the 'notochord' area.

map, the zebrafish map looks similar to those of other chordates, e.g. ascidians (Nishida, 1987) and *Xenopus* (Fig. 14B). The zebrafish embryo is organized like a frog or ascidian embryo, but with its vegetal pole opened up and the yolk cell inserted at this location. Remarkably, even particular kinds of neurons, spinal sensory and motoneurons, map similarly in the two vertebrate species (Fig. 9 and see Moody, 1989), in spite of the fact that neural tube formation differs. The basic similarity in fate map organization among different vertebrate embryos (including fish) was already remarked upon by Waddington more than thirty years ago (Waddington, 1952). Our study reinforces the notion that the machinery that patterns early development has been highly conserved during vertebrate evolution.

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