INTRODUCTION

To understand zebrafish embryo development, we need a knowledge of how descendants from early blastomeres contribute to adult tissues. Provided these patterns of contribution are predictable we can construct fate maps from them. We previously mapped the fates of individual blastomeres of the zebrafish embryo at the 8-cell stage (Strehlow and Gilbert, 1993). These studies supported the notion that each of the eight blastomeres have distinct fates. Moreover, analysis of these fates suggested that all three body axes arise at this stage and observed embryos agreed with the postulated axes in 82 of 92 cases. To confirm and extend these studies, we present an analyses of the 16-cell-stage embryo.

We also asked whether we could distinguish 16-cell-stage sister blastomeres by their fates. This question is interesting because different fates would imply the presence and expression of mechanisms that lead to some distinction between these blastomeres. This could be achieved in two fundamental ways. The fourth cell division could differentially apportion cellular components to each of the two daughter cells. Or the differences could come about because each of the daughter cells, by necessity, will occupy a different space — with different neighbors — in the cleavage-stage embryo. A combination of these mechanisms may also be at play. To ascertain whether blastomeres at the 16-cell stage may have distinct fates, we analyzed two offspring from two injected cleavage-stage blastomeres in each of 56 embryos. We labelled blastomeres such that we could follow the progeny derived from both the 8-cell stage and its daughters at the 16-cell stage.

Our results confirm our previous analyses and furthermore indicate that indeed distinct fates can be discerned among the 16 blastomeres.

Another issue is whether our fate maps from the 8-cell stage are consistent with maps constructed from zebrafish embryos at the gastrula stage (Warga and Kimmel, 1990). The present analyses of the 16-cell-stage embryos suggest that indeed the early and later fate maps are consistent with each other.

MATERIALS AND METHODS

All zebrafish used here were undefined strains obtained from commercial sources and maintained at 26°C. After injection, we raised the embryos at 28°C until observation at 26 hours.

Commercial sources supplied one of the fluorescent dextran (2 million-dalton (MDa; 2 × 10^6 M_r ) fluorescein-conjugated dextran from Sigma). We synthesized 2 MDa Texas Red dextran (Debelder and Dranath, 1973) from the isothiocyanate derivative of the fluor (Texas Red isothiocyanate from Molecular Probes) and commercial sources of the unconjugated 2 MDa dextran (Sigma). We dissolved the dyes at 25 mg/ml in 50 mM phosphate buffer at pH 7.0 and added phenol red (final concentration 3 mg/ml) to aid in visualization during injection. We then filtered the solutions using 0.22 mm Millipore Millex-GV4 filters before injection. We obtained borosilicate capillary tubing (catalog number BF100-78-15) from Sutter Instrument Co., pulled them using a Sutter Brown-Flaming puller and filled them with dye by capillary action.

We placed the embryos in a 5 cm plastic Petri dish containing a grooved plexiglass bottom and injected them through the intact chorion under a stereo dissecting microscope. We observed all embryos within 15 minutes after injection using epifluorescence to...
ensure that dye was in only the expected cells. We rejected embryos that contained dye in inappropriate cells as incorrectly injected. We inspected the embryos again at 4 hours and 24 hours after fertilization. Those that showed non-uniform fluorescence among the fluorescent cells or those that exhibited fluorescence not confined to cells (perhaps indicating cell lysis) were also rejected. Observations of fluorescence patterns took place at 26 hours.

For observation, we anaesthetized living embryos using 125 mM 3-aminobenzoic acid (Sigma) and mounted them in one of two ways depending on the desired axis of view. For lateral viewing, we dechorionated and mounted animals in a small drop of water between two coverslips. For dorsal viewing, we immobilized the embryo in a 1% agar cylinder and placed it in a grooved plastic plate.

We observed a total of 112 fluorescent patterns, two in each of 56 embryos. We scored 31 tissues in the 26-hour embryos for their fluorescence. We assigned scores for each pattern following the precedent of Moody (Moody, 1987), who separated the labelled tissues into four categories: no labelling; 10 or fewer labelled cells in that region; more than 10 but not all of the cells in that region were fluorescent; all or nearly all the cells in the region were labelled. Representative examples of these observations are shown in Figs 2, 3. A summary of the probabilities of each blastomere’s contribution to each of the 31 tissues is in Fig. 4*.

We documented observations with both video tape using a SIT camera fitted to the microscope and 400 ASA Kodak Ektachrome 35 mm film. Color slides were digitized, composed and labelled using Adobe Photoshop. Files from Photoshop were recorded on 35 mm film before printing on Ilfachrome direct from the color slides. Photographs of fluorescent sections in Fig. 3 were digitally adjusted before printing to increase the fluorescence signal approximately twofold compared to background.

For sectioning, embryos were injected as described above, mounted live and photographed for the whole-mount views. They were then fixed using a modification of freeze-substitution (Feder and Sidman, 1958). This was necessary in order to fix the 2 million dalton dextrans, which contain no free amine groups to facilitate fixation protocols such as those based on paraformaldehyde. Anaesthetized embryos were placed directly in liquid nitrogen. Frozen embryos were placed in pure ethanol at −70°C for 3 days before embedding and sectioning in JB-4 medium (Polysciences, Inc.) as recommended by the manufacturer. Sections were cut at 4 μm intervals. Nearby sections were observed for fluorescence or stained with hematoxylin and eosin using standard methods.

RESULTS

Blastomere designations

Fig. 1 shows the labels of the 8- and 16-cell-stage blastomeres. We name the cells by three letters, whose order reflects the chronological appearance of the cleavages that result in the respective axes (Strehlow and Gilbert, 1993): the first cleavage results in two cells, one dorsal and one ventral (D and V). The second cleavage results in left and right dorsal and ventral cells (DL, DR, VL and VR). The third results in the appearance of anterior and posterior cells (DLP, DLA, DRP, DRA, VLP, VLA, VRP and VRA). The eight cells that result from the third cleavage are labelled in Fig. 1A. We designate the 16-cell-stage blastomeres so that we can also recognize sisters by their names (Fig. 1B). For example, the 8-cell-stage blastomere DLP gives rise to the sisters DLP2 and DLP1.

The 16-cell-stage fate map

The 16-cell-stage blastomeres are arranged as a 4×4 array atop a large yolk. This array usually appears square and symmetric through rotations of 90° making it difficult to identify uniquely a single injected blastomere. To simplify this problem, we injected each embryo twice. The first injection, using the green fluorescent dye fluorescein-dextran (2 MDa), was into an 8-cell-stage blastomere. Since the 8-cell-stage embryo is a 2×4 array, we can identify each blastomere as one of two possibilities upon injection. After observing the fluorescence pattern derived from the 8-cell-stage injection at 26 hours, we can identify the injected cell uniquely based on the 8-cell-stage fate map (Strehlow and Gilbert, 1993). A second injection into one of the two green daughters of the 8-cell-stage injection, using a red fluorescent dye Texas Red dextran (2 MDa), allowed us to map both of the daughters at the 16-cell stage. Progeny of one of the daughter cells were both red and green (and appear yellow in a double exposure); progeny of the other were green but not red. Since we can identify the blastomere injected at 8-cell stage uniquely, as described above, we can also identify the blastomere injected at 16-cell stage uniquely.

This strategy allowed us to inject a large number of embryos each with two different dyes. We then were able to compare the fates of blastomeres that are sisters at the 16-cell stage. If sister blastomeres have different fates, one would expect to see one tissue that contains only green cells and another tissue that contains yellow cells (in double exposures). Embryos injected in this manner and photographed with double exposures 26
hours later are shown in Fig. 2A-D. Here, we injected three different embryos in blastomere DRA at the 8-cell stage with fluorescein dextran (the same animal is seen in both A and C; B and D are different). We then injected one of the two daughters of DRA, namely DRA2, with Texas Red dextran. The double exposures show the progeny of DRA2 — the cells that inherited both the red and green dyes — in yellow. The progeny of DRA1 are in green. Note how in all four pictures the yellow cells primarily contribute to the notochord and the dorsal epithelium. The green fluorescent cells contribute to the ventral epithelium in the tail region but little to the notochord. Thus, since different tissues are regularly labelled by different sisters, we conclude that two sisters at the 16-cell stage have noticeably different fates.

The labelling patterns are reproducible in different animals.

An important issue is the reproducibility of the fate map in separately injected embryos. We constructed a fate map of the 16-cell-stage animal by making detailed observations of live, anaesthetized whole-mount embryos at 26 hours. This allowed us to take advantage of the transparency of the embryo and score the fluorescence of 31 separate tissues without sectioning. Fig. 2 illustrates the reproducibility of the labelling patterns among different embryos. Fig. 2E-H,U,V shows six different live, whole-mount embryos displaying the dorsal pattern. The four hallmarks of the dorsal pattern are the notochord, floorplate, dorsal epithelial strip and hatching gland (see figure legend for anatomical references).

Fig. 2I-P show eight embryos displaying the ventral pattern. The five hallmarks of ventral labelling include profuse muscle labelling, and labelling of the blood, heart, pronephric tubules and ventral epidermis. The first four ventral animals, in Fig. 2I-L, illustrate the ventral pattern with anterior contributions while the next four, Fig. 2M-P, illustrate the ventral pattern without anterior contributions (the posterior pattern). The posterior cells usually fail to label rostral to the hindbrain.

Fig. 2Q-T,W,X show dorsal views of 6 embryos injected in blastomeres on opposite sides of the left-right axis. (Of course these embryos were injected in blastomeres that were not sisters at the 16-cell stage — 16-cell-stage sisters are both on either the left or the right side of the left-right axis.) Fig. 2U-X are four whole mounts that correspond to the thin sections in Fig. 3 and are described below.

These results, as well as additional injected embryos that are not shown (see the footnote) reveal consistent patterns of labelling that clearly relate the position of the injected blastomere at the 8-cell and 16-cell stages to the labelled tissues at the 26-hour stage.

Plastic sections of labelled embryos verify the whole-mount observations

To confirm the whole-mount observations, we injected a number of embryos as described above and then fixed and mounted them (see Methods). The last set of four panels in Fig. 2 are representative whole-mount images of animals which were then fixed and sectioned and are shown in Fig. 3. The first column in Fig. 3 shows sections of five different animals with dorsal patterns — labelling mainly the notochord, floorplate and dorsal epithelium. The last column shows five views of four different animals with ventral patterns — labelling mainly the pronephric tubules, blood and muscle in the somites. (Fig. 3C and L show two sections of the same animal at different axial levels.) The middle column of panels shows representative haematoxylin and eosin-stained sections (see the figure legend for a description). Comparing Fig. 2U,V,W,X with the sections in Fig. 3A,D,C,F, respectively, shows that the structures in the labelled whole mounts correlate well with the structures in the sections. Thus, similar features are easily recognized in both the thin sections and the whole mounts, validating our use of whole mounts in the construction of the fate map. Figs 2W,X and 3C,F also illustrate labelling across the left-right axis in whole mounts and sections. This exemplifies the extent of contributions of both the left and right blastomeres to the spinal cord and the lack of contributions of the same blastomeres to muscle on the opposite sides of the left-right axis.

The fate map

Fig. 4 summarizes the extent to which descendants of each of the 16-cell-stage blastomeres contribute to each of the 31 scored tissues. The scores represent the percentage of the designated blastomeres which, after injection, gave rise to labelling at least 10 of the cells in the designated tissue. The number of times each blastomere was injected is indicated in the bottom-right of the figure. For example, from data available according to the footnote*, six embryos were injected in blastomere DRP2. Of these, four labelled at least 10 cells in the hatching gland. Since four of six, or ~70% of the injected embryos had cells labeled in the hatching gland, the DRP2 blastomere is shaded appropriately for the 75%-100% range in the ‘HATCH’ blastomere array. As a second example, five of six (~80%) embryos injected in DRP2 labelled at least 10 cells in the notochord. Thus, DRP2 is shaded appropriately for the 75%-100% range in the ‘NOTO’ blastomere array.) The original fluorescence scores categorized the degree of fluorescence into four groups. Fig. 4, however, divides the same data into two groups: fewer than 10 cells in a tissue labelled, and 10 or more cells in a tissue labelled. Hence, Fig. 4 is a less detailed but more abbreviated representation of the data. Two features of this figure should be noted. First, not all of the scored tissues are labelled in accordance with the axes predicted in Fig. 1. Second, all tissues are labelled by progeny of more than one blastomere.

Violations of the predicted axes (Fig. 1) can easily be seen in the lens and the retina. Both the left lens and retina are formed with contributions from the right blastomeres. Similarly, the right lens and retina are made with contributions from the left blastomeres. Posterior blastomeres sometimes make contributions to anterior tissues such as the olfactory epithelium and telencephalon even though the primary contributions come from anterior blastomeres. Ventral blastomeres often contribute to dorsal fin and trunk epithelium, and the spine, while dorsal blastomeres contribute to ventral epithelium in a similar fashion. Other violations can also be seen in the figure. These can be summarized as follows: with the exception of the notochord, hatching gland, nephros, blood and heart, there are occasional violations of the predicted axes for all of the observed tissues.

As in other vertebrate fate maps, each scored tissue was made from the descendants of more than one blastomere. Hence, each blastomere has a probability for contributing to each tissue. Fig. 4 represents this probability as the degree of
Fig. 2. (A-D) Double exposures of animals injected with green fluorescent 2MDa dextran dye into one blastomere at the 8-cell stage, then injected into one of the two green daughter cells at the 16-cell stage using red fluorescent 2MDa dye. The patterns that contains both red and green dyes (derived from the 16-cell stage) appears yellow. The descendants of the sister to the yellow progenitor are green. All four views are of embryos injected in blastomeres DRA with green and DRA2 with red dextran. A and C are two different views of the same animal.

(E-H) Four different animals exhibiting the dorsal pattern. The animals were injected as follows: (F,G) in blastomere DLP2; (E,H) in blastomere DLP (8-cell stage). (I-L) Four different animals exhibiting the ventral, anterior pattern. Compare these pictures to those in the next row (M-P) to notice the difference between the anterior and posterior labelling patterns. (I) yellow=VLA2; green=VLA1; (J) yellow=VRA2; green=VRA1; (K) yellow=VLA2; green=VLA1; (L) yellow=VRA2; green=VRA1. (M-P) Four different animals exhibiting the ventral, posterior pattern. (M) yellow=VRP2; green=VRP1; (N) yellow=VRP2; green=VRP1; (O) yellow=VRP2; green=VRP1; (P) yellow=VRP2; green=VRP1. (Q-T) Dorsal views of four different animals exhibiting the left-right axis, and injected at the 8-cell stage. (Q) DLP and DRP; (T) VLP and VRP; (R) DLP2 and DRP2; (S) VLP and VRP. (U-X) Whole mounts of animals whose sections are displayed in Fig. 3. (U) Animal in Fig. 3A; (V) animal in Fig. 3D; (W) animal in Fig. 3C; (X) animal in Fig. 3F. Scale: 1 cm approx. 40 μm in A,F,G,I-Q; 1 cm approx. 16 μm in others. Abbreviations: e, epidermis; f, floorplate; n, notochord; m, somitic muscle mesoderm; p, pronephric tubule; s, spine; y, yolk.
Fig. 3. Thin plastic sections of the dorsal and ventral fluorescent patterns with corresponding hematoxylin and eosin sections. Five separate animals with dorsal patterns are in A, D, G, J and M. Five views of four animals with ventral patterns are in C, F, I, L and O. (Two different axial views of the same animal are seen in C and L.) The two different animals in C and F also demonstrate labelling on opposite sides of the left-right axis with red and green. Representative h & e-stained sections correspond to other views as follows: B=A; E=D; H=G; K=C; and N=F. Representative whole mounts of animals A, D, C and F are in Fig. 2U, V, W and X, respectively. The animals were injected as follows: (A) DLP; (C) green in VRP2 and with red in VLP2; (D) DLP; (F) green in blastomere VLP2 and with red in VRP2; (G) red in DLP2; I: green in VRP; (J) red in DLP2; (L) green in VRP2 — same embryo as in C; (M) red in DLP2; (O) green in VRP. The dorsal pattern is reflected in fluorescent sections as notochord, floorplate and dorsal epithelium. The ventral pattern contributes to pronephric tubule, mesoderm and blood staining. Notice the muscle mesoderm at the arrow in L and the blood at the arrow in O. The left-right axis is reflected by green and red contributions to the opposite sides of the midline in fluorescent sections in C and F. Abbreviations as in Fig. 2.
shading of given blastomeres. For example, four blastomeres had a high probability (75-100%) of labelling most of the cells in the notochord. These blastomeres are DLP2, DLA2, DRP2 and DRA2. Twelve other blastomeres were unlikely to contribute to the notochord. Similarly, eight blastomeres contributed to the blood; eight others did not. Some tissues, such as the right gill (RGILL), are constructed from four particular blastomeres’ descendants with only a poor likelihood. In these cases, we could not label the tissue frequently enough from any injection. In other instances, such as the spine (SPI) a large number of blastomeres had a high probability of contributing to the fluorescence.
Differences between the fates of sisters at the 16-cell stage

There are several differences between sister blastomeres at this stage. (Note that the sisters closer to the dorsal-ventral axis are named with a ‘1’ appended. The sister which is further from the axis has a ‘2’ appended.) The two cells, DLPL1 and DRPL1, contribute more than their respective sisters to the motor neurons in the spine; to epithelial cells in the dorsal and lateral trunk and the dorsal fin. Their sisters, DLPL2 and DRPL2 (further from the bilateral plane of symmetry), contribute rarely or not at all to these tissues, but they contribute significantly to the mes- and rhombencephalons, to the head epithelium, and to the hatching gland and notochord. The anterior sister blastomeres behave similar to the posterior sister blastomeres: the cells that are closer to the bilateral plane, DLAL1 and DRAL1, label the spine and most of the epithelial tissue more than their sisters; while they label the hatching gland and notochord less. Contributions to the brain from DLA2 and DRA2 are considerable, but DLAL1 and DRAL1 contribute less to the brain except for the telencephalon.

We can point out several differences between the sister ventral-anterior blastomeres. The blastomeres that are close to the bilateral plane (VLA1 and VRA1) have a much greater tendency to label the epithelial tissues than their sisters. In addition, they have a slightly greater tendency to supply the anterior somitic muscle although contributions to the middle and posterior somites arise from all four blastomeres. All four ventral anterior blastomeres contribute to the telencephalon and other regions of the brain, although VLA1 and VRA1 may do so more consistently. As to the ventral posterior descendants, the two cells that are next to the bilateral plane (VLPL1 and VRPL1) contribute heavily to the epidermis and the anterior somite muscle cells. These cells also appear to contribute somewhat more than their sisters (VLP2 and VRP2) to the rhombencephalon and the spinal cord, and somewhat less than their sisters to the otocysts and the primordial gills.

**DISCUSSION**

We traced descendants of two injected cells in each of 56 embryos. Analysis of these tracings allowed us to describe a fate map for the 16-cell stage. Just as in *Xenopus laevis*, the fate map is probabilistic and imperfect. That is, the daughters of a designated blastomere can be said to have a quantifiable probability of contributing to a particular tissue and not all tissues arise according to the axes presented in Fig. 1.

The percentage scores in Fig. 4 represent the per cent of embryos injected in a given blastomere that gave rise to labelling of at least 10 of the cells in the designated tissue. The notochord is considered a dorsal tissue. The 4 blastomeres that labelled the notochord in all embryos — DLPL2, DLPL2, DRPL2 and DRA2 — can therefore be designated as dorsal. Since they are always in the same half of the 4×4 array, we suggest that they define a dorsal region of the fate map. The nephros, considered a ventral tissue, is labelled always and only by blastomeres from the other half of the array. This half can therefore be designated as a ventral region. Taken together, these two regions describe the dorsal-ventral axis of the embryo. Similar reasoning can be applied to describe the left-right and anterior-posterior axes.

There are imperfections in the fate map for all three axes: dorsal blastomeres sometimes contribute to ventral tissues; left blastomeres sometimes contribute to right tissues and posterior cells sometimes contribute to anterior tissues. These are illustrated in Fig. 4. There are two logical sources for these imperfections: technical difficulties such as injection or marker dye construction and biological events such cell mixing during epiboly. Some combination of these may be at play, but we suspect that, as in other vertebrates, cell mixing plays a large role in the imperfections of the fate map.

Warga and Kimmel analyzed labelling patterns of zebrafish embryos injected at the early cleavage stage (1990). They found no consistent relationship between injected blastomeres and labelled tissues. They explained this finding by postulating extensive cell movements in the late blastula and early gastrula stages. Similar cell movements are observed in the cleavage-stage *Xenopus* embryo (Slack and Tannahill, 1992), for which extensive fate map data exists. Our data are not inconsistent with some cell movement. For example, the right ear receives contributions from both sides of the left/right axis. Similarly, both retinas are derived from left and right blastomeres. Our data, however, suggest that these movements follow patterns and that some of them are reproducible. The reproducible patterns could be missed unless the progeny of all blastomeres at the 8- and 16-cell stages are repeatedly followed as we have done here. Wilson et al. (1993) speculate that the early positions of blastomeres correlate with lineage positions at gastrulation, when a ‘tissue-specific fate map’ arises. This suggests that cell mixing is insufficient to disrupt the relative positions of lineages completely. Whether position alone guides a cell’s fate from the cleavage stage remains to be elucidated. Nonetheless, this view also suggests that the positions
of the early blastomeres should have a predictive value. Our data in Fig. 4 attempts to quantitate this value.

Warga and Kimmel (1990) studied the fates of the gastrula-stage zebrafish blastomeres. Our 8- and 16-cell-stage fate maps are consistent with the maps derived from the gastrula. For example, the notochord and hatching gland progenitors are derived from dorsal progenitors in both maps. Our data indicate that both tissues map to the same blastomeres in the 16-cell-stage embryo. Both tissues are derived from the same region of the gastrula according to Warga and Kimmel (1990). Similarly, the progenitor for ventral tissues — the blood, pronephros and somite muscles — all arise from common ventral blastomeres at the 16-cell stage. The progenitors to these tissues also co-localize to one region of the gastrula. Thus, both early and late maps are consistent with each other.

Another conclusion from our analysis is that each organ has a reproducible and specific pattern of contributions from each of the first 16 blastomeres. Interestingly, every organ is populated by daughters of more than one blastomere; that is, there is no organ that is derived from only a single 16-cell-stage blastomere. The notochord, hatching gland and left gill are derived from the smallest number of blastomeres. The left and right otocysts are also populated by less than one half of the blastomere daughters. Most tissues receive contributions from more than one half of the 16 blastomeres. However, not a single tissue or organ that we scored is derived from all blastomeres. One tissue to which at least one half of the blastomeres contribute is the retina. Streisinger et al. (1989) concluded, on the basis of somatic mutation analysis, that many of the blastomeres of the 32-cell embryo contribute to the retina. Thus, two independent experimental approaches lead to a similar conclusion.

Finally, our extensive analyses reveal that the sister blastomeres of the 16-cell stage have different fates. This finding implies distinctions between these sisters — perhaps simply differences in location; perhaps in more complex properties. Our previous analysis suggests that such differences are already present at the 8-cell stage (Strehlow and Gilbert, 1993). By what mechanisms are they interpreted in the developing embryo? Could they be present in the egg? The analysis reported here provides a strong impetus for addressing these questions further.

Some of the data used in preparing the figures for this paper were too lengthy to include in the published manuscript. With the permission of the publisher, we offer the original fluorescence scores separately. They can be obtained either from the corresponding author or from an electronic bulletin board available via any Internet connection. See the footnote for more detailed information.

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