INTRODUCTION

As embryos develop, distinctive cell lineages arise from apparently homogeneous cell populations by a process termed specification (Davidson, 1990; Kimmel et al., 1991). When cells become specified, they begin to express tissue-specific molecular markers, display characteristic cell movements and undergo restrictions in developmental fate. Specifications may be conditional, since specified cells can alter their fates when transplanted to new locations (Ho, 1992) and are thus different than commitment events, which represent irreversible changes in cell fate (Kimmel et al., 1991). To learn when and where cells become specified, we have observed trunk neural crest cells in living embryos of the zebrafish, \textit{Brachydanio rerio}.

The neural crest is useful to study the time course of specification because it begins as a seemingly uniform cell population atop the neural tube that then migrates along distinct pathways and generates diverse cell types, including sensory and sympathetic neurons, Schwann cells and pigment cells (Horstadius, 1950; Weston, 1970; Le Douarin, 1982; Newgreen and Erickson, 1986; Erickson, 1988). By determining where and when neural crest cells become specified, we hope to begin to understand what cues influence crest cell fate.

As cells become specified, they undergo fate restrictions, so that they generate only a subset of all possible types of progeny. Fate restrictions are operationally defined by observing cell lineages and identifying cell divisions after which all progeny give rise to a single phenotype. Fate restrictions should not be confused with restrictions in potency, which are the result of commitment events. Cells that have undergone fate restrictions so that they produce only a single phenotype of progeny are defined as type-restricted precursors. For most species studied, the temporal sequence of neural crest fate restrictions has been difficult to determine. Studies performed in vivo are typically analyzed in fixed and sectioned material, which cannot adequately describe dynamic temporal processes. Since specification may be conditional, studies in vitro may not accurately reproduce the normal fates of crest cells or reflect when different fates first become apparent. These difficulties are overcome by studying neural crest cells in zebrafish embryos, because individual cell lineages can be followed in the living embryo (Kimmel and Warga, 1986). We have shown previously that neural crest cells in the trunk of the zebrafish are similar to those of other vertebrates in their initial location, pathways of migration and the types of derivatives that they produce (Raible et al., 1992). Zebrafish trunk neural crest cells initially migrate ventrally from the dorsolateral aspect of the neural tube along a medial pathway between somite and neural tube. Later, other crest cells migrate ventrally along a lateral pathway between somite and overlying ectoderm. After migration, zebrafish trunk neural crest cells give rise to neurons of the sensory and sympathetic ganglia, Schwann cells and at least two types of pigment cells.

We have undertaken a temporal analysis of neural crest cell development in zebrafish by labeling individual trunk crest cells with fluorescent vital dyes, following them in living embryos and constructing lineages showing temporal sequences of divisions and phenotypes of all progeny. These studies have allowed us to learn when neural crest cells acquire unique characteristics and to determine when differences in cell fate first arise. We provide evidence that zebrafish trunk neural crest cells become lineage-restricted, generating type-restricted precursors that produce a single kind of derivative. Additionally, we find that some type-
restricted precursors display unique characteristics while still migrating. Taken together, these observations suggest that some trunk neural crest cells are specified before reaching their final locations.

**MATERIALS AND METHODS**

**Animals**

Embryos were obtained from the University of Oregon zebrafish colony. Embryos were staged by hours postfertilization at 28.5 °C (h). Chorions were removed with watchmaker’s forceps and embryos were maintained in embryo medium (Kimmel and Warga, 1987). Living embryos were mounted in agar (Eisen et al., 1989) or placed between coverslips held apart by spacers (Raible et al., 1992) and observed under Nomarski (DIC) optics. When necessary, embryos were immobilized in a dilute solution of tricaine methylsulfonate (MS-222; Sigma, St Louis, MO).

**Cell labeling and imaging**

Individual neural crest cells were labeled by intracellular injection with lysinated rhodamine dextran (10,000 M₉, Molecular Probes, Eugene, OR) as described previously (Raible et al., 1992). Premigratory crest cells at the level of somite 7 were labeled in 15-18 h embryos; only embryos that contained single labeled cells were analyzed. Premigratory neural crest cells were defined as cells that had segregated from the neural tube but were still on the dorsal aspect of the neural tube and were dorsal to the somites. Cells were monitored using low light level, video-enhanced fluorescence microscopy, and images were captured on a Macintosh Ilci using AxoVideo (Myers and Bastiani, 1991; available from Axon Instruments, Foster City, CA). White-light and fluorescent images were combined using Adobe Photoshop (Mountain View, CA).

**Clonal and lineage analysis**

We performed both clonal analysis, where sibling relationships between cells were not determined, and lineage analysis, where cells were monitored frequently, sibling relationships recorded and lineage trees constructed. For both kinds of analysis, individual labeled cells were first monitored at ½ hour intervals to determine the onset of migration, defined as the time when the labeled cell extended a substantial process ventrally between somite and neural tube. For lineage analysis, cells were subsequently monitored at hourly intervals over the next 18 hours and at 4 hour intervals over the next 16 hours.

The phenotypes of progeny were determined at 72 h by morphology, location and, in some cases, by specific antibody staining (Raible et al., 1992). Sensory neurons were identified by their position lateral to the spinal cord, and by their central and peripheral processes. Sympathetic neurons were identified by their position ventrolateral to the notochord and by their round cell bodies; occasionally their fine processes could be detected. In some cases, putative sympathetic neurons were confirmed to be immunopositive for tyrosine-hydroxylase (Raible et al., 1992). We were not able to determine whether all labeled cells in the sensory and sympathetic ganglia were neurons. However, in all sensory ganglion lineages, at least 1 cell extended a process into the spinal cord; processes cannot reliably be detected on sympathetic cells because of the limited optical resolution in that area of the embryo. Schwann cells were identified by their position along the ventral root, and by their elongated morphology. In some clones that also contained sensory neurons, Schwann cells were in close apposition to their peripheral processes. Zebrafish have several types of pigment cells, including melanophores, xanthophores and iridophores. However, we simply classified pigment cells as those that contained melanin granules (melanophores) and those that did not (xanthophores or iridophores). Melanin-negative pigment cells fluoresced under fluorescein optics.

**Fig. 1.** Lineage analysis of neural crest progeny. Trunk neural crest cells generate different cell types by first producing type-restricted precursors. A and B represent alternate models for phenotypic segregation (see text). C-F are representative examples of general patterns in the 28 lineages analyzed. Type-restricted precursors, which divide and produce only a single type of progeny, are marked with stars. In a few cases, a neural crest cell divided to produce one or more type-restricted precursors and a sibling that did not divide before differentiating. It is unclear whether the sibling that did not divide (cell marked with § in F) represents a type-restricted precursor. Boxes indicate where sibling relationships were not determined; open circles, sympathetic cells; shaded circles, Schwann cells; checkered circles, sensory cells; black circles, melanized pigment cells; striped circles, unmelanized pigment cells; n, the number of lineages having 1 (C), 2 (D,F) or 3 (E) distinct phenotypes of progeny.
RESULTS

Crest cells that begin to migrate at different times produce different types of derivatives

Neural crest cells that migrate on different pathways in the zebrafish trunk make different subsets of derivatives (Table 1). Crest cells first migrate on the medial pathway between somite and neural tube, and produce progeny of all phenotypes, including cells of the sensory and sympathetic ganglia, Schwann cells and pigment cells. Later, other crest cells migrate on the lateral pathway between somite and epidermis, and only produce clones of pigment cells. These clones exclusively contained cells that were either all melanin positive or all melanin negative.

Neural crest cells begin to migrate on the lateral pathway 4-5 hours after migration has begun on the medial pathway (Raible et al., 1992). Furthermore, laterally migrating cells make only a subset of the progeny produced by cells that migrate medially. We therefore asked whether the times that individual crest cells began to migrate on the medial pathway predict the types of progeny that they will produce. We found that sympathetic and sensory ganglion cells only arose from crest cells that began to migrate on the medial pathway early, while all other phenotypes arose from cells that began to migrate on the medial pathway at any time (Table 2). These results suggest temporal differences in the fates of cells that begin to migrate at different times.

Zebrafish trunk crest cells act as type-restricted precursors

Most clones derived from trunk crest cells that migrated on the medial pathway contained derivatives of a single phenotype (Table 1). However, about 20% of medially migrating cells produced multiple-phenotype clones. Individual trunk crest cells could generate multiple derivative clones in at least two ways. Early divisions could produce type-restricted precursors, each of which divides to generate only a single type of derivative (Fig. 1A). These early divisions are segregating divisions, where restrictions in fate first arise. Alternatively, individual progeny could remain unrestricted (Fig. 1B). To distinguish between these possibilities, we labeled premigratory trunk neural crest cells and followed them at frequent intervals to determine the sibling relationships between cells. Most trunk crest cells generated progeny of a single phenotype in which all descendants of the labeled cell differentiated into the same type of neural crest derivative (Fig. 1C). We defined these cells as type-restricted precursors, because they generated progeny of a single fate. In contrast, some labeled cells generated lineages that contained progeny of multiple phenotypes (Fig.

Table 1. Composition of clones migrating on the lateral and medial pathways

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<tr>
<th>Pathway</th>
<th>Clone composition</th>
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<tr>
<td>lateral</td>
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<td>M</td>
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<td>dorsal stripe</td>
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<td></td>
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Premigratory crest cells were individually labeled, and the phenotypes of their progeny determined at 72 h. Cells migrating on the lateral pathway generated only pigment cells, while cells migrating on the medial pathway generated all cell types. Other cells contributed to the dorsal pigment stripe. Of 203 cells labeled, 115 migrated medially, 17 migrated laterally, 23 contributed to the dorsal pigment stripe, and 48 died. Clones containing single cells are included here. D, sensory ganglion cell; S, sympathetic ganglion cell; G, Schwann cell; P, unmelanized pigment cell; M, melanized pigment cell.

Fig. 2. Average number of cells for each derivative. Clones contain characteristic numbers of cells of each derivative type (A). The number of cells of each derivative type is the same for both multiple-derivative and single-derivative clones. (B). Multiple-derivative clones are significantly larger than single-derivative clones (C). Cell types are abbreviated as in Table 1. Averages were calculated for clones derived from labeled cells that began migrating during the same period, from 16 h to 18 h. The mean numbers of cells for each derivative (A) are significantly different by one-way ANOVA (P<0.001). Mean numbers of cells in both single-derivative and multiple-derivative clones (B) are not significantly different, P>0.1; means for total cells in single-derivative and multiple-derivative clones (C) are significantly different, P<0.001.
1D-F). Some cells produced two types of derivatives, which segregated with the first cell division, so that each daughter of the first division gave rise to progeny of a single phenotype (Fig. 1D). Other cells produced progeny displaying three phenotypes, which segregated with the first and second divisions (Fig. 1E). In these instances, the labeled crest cells produced type-restricted precursors for specific derivatives. In two lineages containing two phenotypes, segregation did not occur after the first division (Fig. 1F). However, one daughter of the first division produced two cells that acted as type-restricted

Fig. 3. Progeny of an individual pigment precursor migrate to different locations. Panels show lateral views of zebrafish embryos, with rostral to the left. (A), At 23 h, the labeled cell has divided, producing two daughters. (B) At 24.5 h, the daughter cells have separated. (C) At 28 h, the rostral daughter has begun to migrate dorsally, while the caudal daughter has begun to migrate ventrally. (D,E) 31 h. For each panel, the fluorescent image is left and the bright-field image revealing the pigment granules is right. (D) The rostral daughter formed a melanophore in the dorsal pigment stripe. (E) The caudal daughter migrated ventrally and also formed a melanophore. nc, notochord; nt, neural tube. Scale bar, 25 µm.
Fig. 4. Characteristic migratory behavior and division patterns of sensory precursors. Panels show lateral views of zebrafish embryos, with rostral to the left. (A) At 29 h, one daughter of a labeled neural crest cell has just divided to produce a sensory precursor (arrowhead) and a sympathetic precursor. The other daughter generated a single sympathetic cell. (B) At 42 h, the sensory precursor shown in A has moved dorsally, a characteristic movement seen for all 4 sensory precursors whose complete movements were followed. (C) At 54 h, the sensory precursor shown in A has migrated to the location of the DRG and divided to produce two sensory ganglion cells. (D) At 66 h, the sensory ganglion cells are extending processes to the spinal cord. Arrows indicate the boundary between notochord and neural tube. Scale bar, 50 µm.
The number of cells per clone seems to be a cell-type-specific attribute. Sensory and melanin-positive pigment clones were significantly smaller than sympathetic, Schwann and melanin-negative pigment clones. Moreover, the number of cells per clone for each derivative was the same in both single-phenotype and multiple-phenotype clones (Fig. 2B). These observations agree with the idea that type-restricted precursors are the source of the different phenotypes of progeny in multiple-derivative clones, as suggested by lineage data. Consistent with this idea, multiple-phenotype clones were about twice as large as single-phenotype clones (Fig. 2C).

Type-restricted precursors appear to be specified before reaching their final locations

Although we have presented evidence that trunk crest cells become cell-type restricted, they may still remain unspecified. If the progeny of a labeled cell all end up in the same location, the apparent lineage restriction of the labeled cell may arise not because the cell has become specified, but simply because its progeny are influenced by the same environmental signals. However, we observed that some pigment cell precursors divided while migrating and their progeny ended up in widely separate locations. The labeled neural crest cell shown in Fig. 3 began to migrate on the medial pathway, divided adjacent to the spinal cord and, over the next few hours, its daughters separated and migrated ventrally into two different trunk segments in the embryo. One daughter returned dorsally and contributed to the dorsal pigment stripe, while the other daughter migrated ventrally and contributed to ventral pigment over the yolk. Of 42 pigment cell precursors that generated clones of more than one cell, 10 gave rise to progeny that contributed to both dorsal and ventral pigment. That the progeny of a single crest cell migrated to different areas of the embryo yet differentiated into the same cell type suggests that some type-restricted precursors are specified before reaching their final position.

If type-restricted precursors are specified before they reach their final locations, they might be expected to exhibit characteristic division patterns or migratory behaviors, or to express tissue-specific markers (Davidson, 1990; Kimmel et al., 1991). We found that this was the case for at least some type-restricted precursors. For example, sensory-restricted precursors characteristically reversed their migration at a stereotyped location en route to the position where dorsal root ganglia (DRG) formed. The sensory-restricted precursor shown in Fig. 4 arose from a cell that migrated ventrally on the medial pathway, divided adjacent to the notochord, and then migrated dorsally to the position of the DRG, where it divided to generate two sensory cells. All four sensory-restricted precursors observed during lineage analysis displayed this characteristic behavior, suggesting that they were already specified before reaching their final locations. However, since sensory-restricted precursors typically did not divide until reaching the DRG position, they may have also responded to specific local cues.
Some zebrafish crest cells also start to exhibit specific markers while migrating. Fig. 5 shows a trunk crest cell that began to exhibit pigment granules while still adjacent to the spinal cord, soon after it entered the medial pathway. Melanin granules were first observed as a clump adjacent to the nucleus that then spread throughout the soma and processes. Over the next few hours, this cell migrated ventrally and contributed to the internal pigment layer over the gut. These observations strongly suggest that some pigment cell precursors are specified before reaching their final position.

DISCUSSION

We have analyzed when zebrafish trunk neural crest cells become specified, by determining when trunk crest cells undergo restrictions in developmental fate, begin to express tissue-specific markers and display characteristic cell movements. Our observations show that many premigratory trunk crest cells act as type-restricted precursors, which give rise to single types of progeny. This is consistent with observations of zebrafish cranial crest, in which all cells appear restricted as early as they can be identified (Schilling and Kimmel, 1994). In the trunk, however, some of the earliest migrating crest cells generate more than one type of progeny, but do so by first producing type-restricted precursors. We also provide evidence that some type-restricted precursors are specified before reaching their final locations, because they begin to express cell-type-specific characteristics while migrating. Our observation that some cells begin to exhibit pigment granules before reaching their final locations confirms and extends studies of others that demonstrate pigment cells in zebrafish embryos are dynamic, moving to new positions and changing morphology (Shephard, 1961; Milos and Dingle, 1978). We have also observed that sensory-restricted precursors undergo a characteristic migration, suggesting that they may be specified before reaching the position of the DRG. In avian embryos, neural crest cells transplanted ventrally are able to migrate dorsally (Weston, 1963; Erickson et al., 1980; Stern et al., 1991). Our results show that in zebrafish, dorsal migration is a normal behavior of some neural crest-derived cells.

Whether neural crest in other vertebrates produce type-restricted precursors that generate specific derivatives is unclear. Clonal analysis of premigratory (Bronner-Fraser and Fraser, 1988, 1989; Frank and Sanes, 1991) and migratory

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**Fig. 5.** Pigment cell precursors begin to express pigment granules while migrating. Panels show lateral views of zebrafish embryos, with rostral to the left. (A) The pigment precursor cell has pigment granules next to its nucleus while it is adjacent to the spinal cord. (B) The pigment granules have spread throughout the soma as the cell migrates ventrally. (C) The pigmented cell is now lateral to the notochord as it continues its ventral migration. (D) The pigmented cell has become part of the internal pigment layer below the notochord and over the gut. The pigmented cell did not divide over the time of our observation. nc, notochord; nt, neural tube. Scale bar, 50 µm.
(Fraser and Bronner-Fraser, 1991) crest cells in avian embryos, and analysis of single avian crest cells in vitro (Sieber-Blum and Cohen, 1980; Sieber-Blum, 1989; Baroffio et al., 1988, 1991; Dupin et al., 1990; Duff et al., 1991; Deville et al., 1992) has shown that some individual cells produce progeny of several phenotypes. These observations have shown clearly that there are multipotent neural crest cells and have been interpreted as evidence that neural crest cells are not specified until late, perhaps not even until reaching their final locations (Bronner-Fraser and Fraser, 1991). However, in these studies, it was not determined whether different derivative lineages segregated with subsequent divisions and thus generated type-restricted precursors.

Mammalian crest cells in culture undergo asymmetric mitoses, suggesting that they act as self-renewing stem cells (Stemple and Anderson, 1992). In our lineage analysis, we observed that four zebrafish trunk crest cells underwent at least one asymmetric division (Fig. 1E), but since they did not undergo unlimited self-renewal, they do not fit the strictest definition of stem cells (Potten and Loeffler, 1990). We do not yet know whether zebrafish trunk crest cells undergo asymmetric divisions at developmental stages prior to our observations.

The neural crest has been proposed to go through progressive fate restrictions in order to generate diverse derivatives (Weston, 1982; 1991; Le Douarin et al., 1991). In these models, as neural crest cells divide, daughter cells give rise to sequentially more limited subsets of crest derivatives. Neurogenic progenitors (Weston, 1991; Vogel et al., 1993) or pigment/Schwann cell progenitors (Nichols and Weston, 1977; Weston, 1991) have been proposed as intermediates during sequential fate restrictions. In our data, the combinations of phenotypes found in two-derivative clones should reveal whether zebrafish trunk neural crest cells generated different derivatives via progressively restricted intermediates. We observed almost all combinations of phenotypes in two-derivative clones (Table 1), not just specific combinations of two types. For example, we identified clones containing sensory-restricted precursors with all other type-restricted precursors, suggesting that sensory-restricted precursors are not derived from specific neurogenic progenitors. We thus have no evidence supporting the idea that individual crest cells undergo progressive restrictions.

Although our observations do not support the existence of individual neurogenic progenitors, they are consistent with the idea that different populations of trunk neural crest cells have different neurogenic potential (Weston, 1991; Vogel et al., 1993). In culture, populations of avian neural crest cells do not give rise to neurons if their dispersal is delayed (Girdlestone and Weston, 1985; Vogel and Weston, 1988). We have observed that, in the zebrafish trunk, sensory and sympathetic cells are derived only from neural crest cells that migrate early. Consistent with these observations, neurons are generated by the most lateral cranial crest cells in zebrafish, which are presumably the first to migrate (Schilling and Kimmel, 1994). However, we do not know whether later-migrating zebrafish trunk crest cells have lost the potential, or ability, to produce sensory and sympathetic cells if given the appropriate stimuli. The different fates of early- and late-migrating zebrafish trunk crest cells may be due to intrinsic differences, suggesting heterogeneity in the premigratory crest, or to extrinsic cues, such as length of interaction with the neural tube or changes in signals in the periphery.

Although we have provided evidence for sensory restricted precursors, it is important to point out that we cannot distinguish whether all cells derived from sensory precursors are neurons. All groups of labeled cells in DRGs had at least one neuronal process. However, it is possible that some of the cells do not differentiate as neurons and remain as precursor cells or form satellite glia. Zebrafish cranial neural crest cells generate some clones in the trigeminal ganglia that contain unidentified cells (Schilling and Kimmel, 1994), which may also be precursor cells or satellite glia. These observations suggest that crest cells become restricted to specific ganglia, but whether cells are restricted to produce different cell types within ganglia remains unknown.

In avian embryos, neural crest cells have been suggested to fill positions along the migration pathway in the order in which cells disperse, so that locations farthest from the dorsal neural tube are occupied first (Serbedzija et al., 1989). We have found that zebrafish trunk crest cells that begin to migrate at the same time end up in locations of different distances from the dorsal neural tube. Although these observations suggest that zebrafish trunk crest cells do not fill positions simply by distance from the neural tube, we cannot rule out the idea that positions are filled in a preferential order.

We propose that in zebrafish, premigratory trunk crest cells are not specified until after they undergo a restrictive cell division. For most cells, the restrictive division occurs before they begin to migrate; however for a subset of early-migrating crest cells, the restrictive division occurs along the medial pathway. We suggest that some trunk crest cells become specified before reaching their final locations, because they begin to exhibit identifiable characteristics while still migrating. Our observations are consistent with the neuroepithetic model of crest development (Anderson, 1989), which suggests that uncommitted crest cells stochastically produce committed progenitors for specific sublineages. Although our observations demonstrate differences in fate of individual neural crest cells, they do not address whether these cells are committed to specific developmental pathways, since commitment involves changes in developmental potential (Kimmel et al., 1991). Whether zebrafish crest cells have restricted potential will only be determined by challenging them individually with new environments.

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REFERENCES


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