

Segment and cell type lineage restrictions during pharyngeal arch development in the zebrafish embryo

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SUMMARY

In zebrafish, the segmental series of pharyngeal arches is formed predominantly by two migratory cell types, neural crest and paraxial mesoderm, which arise in the early embryo. Neural crest cells migrate ventrally out of the neuroepithelium and into the arches to form cartilage, neurons, glia and pigment cells. Surrounding mesoderm generates muscles and endothelia. We labeled individual pharyngeal precursor cells with fluorescent dyes and found that their clonal progeny were confined to single segments and generated single cell types. When a neural crest or mesodermal cell was marked before migration into the pharynx, its progeny dispersed but generally remained confined to a single arch primordium. Such segmental restrictions arose first in the most rostral arches, mandibular and hyoid, and progressed caudally. The phenotypes of progeny generated by single cells were examined in the mandibular arch. Clones derived from premigratory

neural crest cells generally did not contribute to more than one cell type. Further, the progenitors of some cell types were spatially separated in the premigratory crest. In particular, neurogenic crest cells were situated further laterally than cells that generate cartilage and connective tissues, while pigment and glial cell progenitors were more evenly distributed. Based on these results we suggest that arch precursors may be specified as to their eventual fates before the major morphogenetic movements that form the arch primordia. Further, cell movements are restricted during segmentation establishing a group of arch precursors as a unit of developmental patterning, as in the fashion of vertebrate rhombomeres or segmental lineage compartments in *Drosophila*.

Key words: zebrafish, neural crest, pharyngeal arch

INTRODUCTION

During vertebrate embryogenesis, cells diversify to contribute to different body segments and tissues. Cues that specify different cell fates are thought to initiate developmental pathways in precursor cells that lead to recognizable characteristics such as patterns of gene expression or the appearance of restricted cell lineages. In the vertebrate head, cells diversify to form a segmental series of pharyngeal arches. Cranial neural crest cells migrate into the arches to form elements of the peripheral nervous system, pigment cells, connective tissues and cartilage. Adjacent paraxial mesoderm forms segmented pharyngeal muscles and endothelia (reviewed by LeDouarin, 1983; Noden, 1988; Kimmel et al., 1991). The complex patterns of cell migration, segmentation, and differentiation in the arches require mechanisms to specify different cell behaviors within what appear to be homogeneous embryonic populations.

In avian and mammalian embryos, several lines of evidence suggest that cell diversity in the arches results from positional specifications of pharyngeal precursors just after gastrulation. Heterotopic transplantation studies have shown that cranial neural crest cells at premigratory stages have some intrinsic

patterning information since they form pharyngeal structures unique to their original axial positions when transplanted into ectopic locations (Noden, 1983). Migrating neural crest cells exhibit a segmental organization consistent with their particular hindbrain rhombomeric origins (Lumsden, 1991). In addition, subsets of both neural crest and paraxial mesoderm in the pharyngeal arches are characterized by the expression of particular genes, including Hox genes, which are thought to be involved in positional specification by regulating transcription of other genes (Hunt et al., 1991a; reviewed by Hunt et al., 1991b).

To understand mechanisms of cell specification in the pharyngeal arches requires that the normal range of fates generated by individual precursors be well characterized. Studies using cultured cells have suggested that a cell's lineage as well as its environment may influence its eventual fate (Barald, 1989; Baroffio et al., 1991; Cohen and Konigsberg, 1975). However, because it has been difficult to mark individual cells in embryos and precisely identify their progeny or segmental distributions, the studies that have been done have been limited to only a few species. Therefore, little is known about the range of phenotypes that clonally related cells actually acquire in vivo.

Using the zebrafish embryo, *Danio (Brachydanio) rerio*, we have examined the possibility that prior to pharyngeal morphogenesis, precursor cells are already specified as to their future fates. Specified cells should be lineage-restricted, whereas naive cells may or may not be so restricted. We looked for restricted cell lineages by microinjecting single precursors at times just before the onset of neural crest migration, which in zebrafish occurs before the formation of a definitive neural tube. We found not one but two types of restrictions; the clonal progeny of single premigratory neural crest cells frequently are all confined to only single arch segments (segment restriction) and all make only a single type of differentiated derivative (cell type restriction). By recording the positions of cells at the time of labeling we generate fate maps that show particular segments and cell types arise from discrete locations. Progenitors of each individual segment are located in register along the anterior-posterior axis and in register with particular hindbrain neuromeres that later innervate them. Segment-restrictions arise in a rostral to caudal sequence, but occur at a similar stage in cells of both neural crest and mesoderm that contribute to the same segment. Furthermore, progenitors of different neural crest-derived cell types within a segment are spatially segregated within the premigratory crest population. Taken together with the results of lineage analyses of trunk neural crest (Raible and Eisen, 1994; reviewed by Eisen and Weston, 1993) the observed lineage restrictions lead us to propose a surprisingly early specification of zebrafish neural crest cells.

MATERIALS AND METHODS

Embryos

Embryos were obtained through natural crosses in the zebrafish facility at the University of Oregon, raised at 28.5°C in 60 mg/l of Instant Ocean aquarium salt, and staged according to Kimmel (in Westerfield, 1993; Warga and Kimmel, 1990) and Schilling (1993) in hours postfertilization at 28.5°C (h). In some experiments, embryos homozygous for the *golden (gol^{bl})* mutation (Streisinger et al., 1981) were used because of their reduced pigmentation.

Microscopy and lineage tracer injections

Embryos were prepared for injection by first mechanically removing their chorions. Individual embryos were then mounted in a 1.2% agar solution and oriented appropriately (Westerfield, 1993). Agar was removed from a small region above the area to be injected, using a tungsten needle. Cells were injected intracellularly with a 3-5% solution of a fluorescent dye, tetramethylrhodamine-isothiocyanate dextran (RD; Molecular Probes, Eugene, OR; $10 \times 10^3 M_r$) dissolved in 0.2 M KCl, at early stages of somitogenesis (12-15 h). The second dye for double-label experiments was fluorescein-dextran (Sigma), dissolved the same way. Under these conditions, the dye remains confined to a cell and its progeny (Weisblat et al., 1980; Kimmel and Warga, 1986).

Fate maps and clonal analysis

The positions of cells were recorded at the time of labeling. In most cases, embryos with labeled cells were reexamined about 10 minutes after injection to confirm that only a single cell had been labeled. Embryos were mounted in depression slides in 3% methylcellulose in embryo medium, in which they could be oriented and visualized and then removed without injury. Cells were visualized using low light level, video-enhanced microscopy with a SIT camera.

The labeling in each embryo was examined at least three times: once at the time of labeling, again at a stage when pharyngeal arch primordia are prominent (~24 hours), and finally at 3 days after many cells have differentiated. Precise lineage relationships of individual cells within each of the clones were not determined.

At the beginning of somitogenesis, the premigratory neural crest in the head appears as a mass lateral to the developing neural keel, fairly uniform in structure and having a distinct lateral edge (see Figs 1 and 7). Cells in this mass appear segregated from the keel but do not have the filopodial extensions characteristic of migratory crest cells observed slightly later. Thus we call them 'pre-migratory'. Locations of cells within the mass were determined at the time of labeling by their tier, or distance in cell diameters, from this lateral edge. Each cell level was considered a tier; each tier was 10-20 µm wide. About 10 minutes after a cell was injected, it was reexamined to verify that only one cell had been labeled, and also to determine its position. All of the injections were made into the most superficial cells in the pre-migratory mass, lying close beneath the surface ectoderm and enveloping layers. The superficial cells appear to represent approximately 20% of the total neural crest population (see Discussion).

The proportion of cases in which neural crest or mesodermal clones were observed to cross pharyngeal arch segment boundaries was compared with the proportion expected if clones spread across boundaries without constraint. Expected frequencies of clones crossing (0.69 for neural crest; 0.73 for mesoderm) and respecting (0.31 for neural crest; 0.27 for mesoderm) the boundaries by chance were calculated given the average spatial extent of the clones (57% for neural crest; 64% for mesoderm of rostral-caudal length of an arch). At 24 h an arch has an average rostral-caudal length of 7 cell diameters. A chi-square analysis of the observed frequencies of crossing and respecting the boundaries compared with those expected rejects the null hypothesis ($P < 0.00001$, 1° of freedom in each category).

The expected frequencies of clones from any one tier containing each derivative by chance were calculated, as well as the expected frequency that derivatives are randomly spread among all tiers. For example, among 14 clones observed from tier 2, 9 (64%) formed neurons. If derivatives are scattered rather than positionally restricted we would expect to find a neuron in only 5-6 clones, since the overall frequency of neurons throughout the crest population was approx. 40%. That expectation was compared to the number observed and a chi-square analysis of the observed frequencies compared with those expected, was used to test the null hypothesis for each cell type.

Histochemical procedures

Embryos (24-30 h) were labeled with the zn-5 monoclonal antibody, which recognizes a cell surface antigen found on a subset of early developing endodermal and neural cell types, and visualized using the peroxidase anti-peroxidase method as described by Hanneman et al. (1988) and examined as whole mounts. Cartilage was stained with Alcian blue, using a modified version of the method of Kelly and Bryden (1983). Embryos (72 h) were fixed for 2-3 hours in 10% buffered formalin, rinsed in distilled water, and immediately transferred into a filtered, 0.1% Alcian blue solution (20% glacial acetic acid, 80% ethanol) and stained overnight. Both antibody and Alcian blue stained embryos were dehydrated in an ethanol series, cleared in methyl salicylate and mounted in Permount.

Identification of derivatives

One clear advantage of the fish embryo is that we can usually recognize cell phenotypes under Nomarski optics, without the necessity of markers. Neural crest and mesodermal derivatives were readily distinguished by their characteristic morphologies. Trigeminal neurons had stereotyped processes; their peripheral axons extended above and below the eye, while their central processes entered the hindbrain at the level of rhombomeres 2 and 3 (Trevarrow et al., 1990). Presumptive Schwann cell somata and nuclei were elongated and oriented longitudinally in the trigeminal nerve. Other ganglionic

cells that were neither neurons nor Schwann cells, appeared to be associated with the sheath of the ganglion or wrapping neuronal somata. Although these cells formed a heterogeneous group, they were all classified as satellite cells.

Neural crest cells also formed non-neural derivatives in the pharyngeal arches. Some of these cells became visibly pigmented (melanocytes) starting at about 26 h. Others that did not contain melanin were visible because they fluoresce at a wavelength near that of fluorescein. Skeletal cartilages formed characteristic stacks of vacuolated cells by about 72 h. Connective tissue cells in the muscle fascia and around the eye had characteristic shapes. Thus, when the same embryo was reexamined at 24 h and 72 h there was no difficulty in finding and determining the fates of the cells. Cells that were not recognizable as one of the types were classified as 'unidentified'.

RESULTS

Pharyngeal arch morphogenesis

We examined the fates of two embryonic cell types, neural crest and paraxial mesoderm, which contribute mesenchyme to the pharyngeal arches. Eventually, in the zebrafish larva, the first pharyngeal arch (mandibular) forms the jaw, the second (hyoid) forms jaw support structures and the last five form the branchial gill structures.

Cell rearrangements during zebrafish gastrulation produce two cell layers in the head at 12 h: a dorsal ectoderm in which dorsal and lateral regions form neural crest, and a ventral mesoderm/endoderm in which much of the lateral regions form paraxial mesoderm (Fig. 1A,B). Many cranial neural crest cells never appear to reach the dorsal midline, in contrast to tetrapods, but remain laterally segregated from the neural keel at premigratory stages as a coherent mass, with a distinct lateral border near the optic vesicle. This mass extends caudally to the otic placode, but no similar lateral extension of the ectoderm is visible at this stage in more caudal regions. A thin string (1-2 cells wide) of paraxial mesoderm lies ventrally, along the yolk cell, and is continuous caudally with the first somite.

Between 15-16 h, anterior neural crest cells just caudal to the developing eye change form, leaving the mass and becoming motile. Cells migrate from progressively more caudal regions thereafter (Schilling, 1993). Segmentation is evident a few hours later when mesenchyme as well as nearby endodermal epithelia form a reiterated series of six pharyngeal arch 'primordia' (Fig. 1C). At this time the mesenchymal cells line up in transverse rows with at least 4-5 rows in mandibular and hyoid arches, but two thin lines in more caudal pharyngeal arches (not shown). Two rows of endodermal epithelia separate each arch primordium from its neighbors starting around 19 h, as revealed by the monoclonal antibody ZN-5 (Fig. 1C). The stereotyped geometry of the arch and its small numbers of cells at this stage allow unambiguous regional assignments of individual cells.

By 72 h, many neural crest cells have differentiated, forming long rows of cartilage within each arch (Fig. 1D). Several muscles and an aortic arch are situated adjacent to each cartilage structure (not shown).

Segment fate maps

Some arch cell types (e.g. cartilage) consistently arise from neural crest and others (e.g. muscle) arise from mesoderm. Furthermore, cells that contribute to a single arch originate at the same axial level, as illustrated by double labeling with two

different colored lineage tracers (Fig. 2). The resulting clones colocalize within the same arch primordium and eventually differentiate within the same segment. For example, in one such experiment, labeled neural crest cells contributed to the ceratohyal cartilage within the hyoid arch while mesoderm from the same axial level at early stages contributed to the hyohyal muscle. This muscle is also within the hyoid arch.

There is a strong correlation between the early rostrocaudal position of a cell and the segment to which it later contributes. A summary fate map for all segments is shown in Fig. 3. Boundaries of these segmental fields overlap at their edges (black regions in Fig. 3), so much so that discrete regions cannot be defined for individual caudal pharyngeal arches. Rostrally, the progeny of a cell that contributes to the third pharyngeal (first gill) arch, for example, might in some extreme cases lie within the region that normally forms the hyoid. However, progeny of any one cell never contribute to more than one segment. The sharpness of the borders between adjacent fate map areas is unclear, due to potential errors in measurements of position as well as variability in distances among different animals. A conservative estimate of this introduced error would be around 2 cell diameters (15-20 μm).

Furthermore, progenitors of each individual segment are located in register along the AP axis and in register with particular hindbrain rhombomeres that later innervate them (Fig. 3). The *krx 20* gene product has been shown to localize to future rhombomeres 3 and 5 in the developing hindbrain, beginning around 9 h and persisting at least through a day or two of embryogenesis (Oxtoby and Jowett, 1993). Rhombomeres 2 and 3 eventually innervate the mandibular arch; rhombomeres 4 and 5 eventually innervate the hyoid arch. As shown in Fig. 3, expression of *krx 20* in 3 and 5 lines up with the caudal halves of the fate map regions that generate mandibular and hyoid arches.

Segment-restricted lineages

When premigratory neural crest cells were labeled at 13 h, most of the clones (84.7%) stayed within a single segment (Fig. 4A), significantly more than expected if clones spread without constraint (31% expected, see Methods). Clones derived from mesodermal cells labeled at 13 h were also predominantly segment-restricted (Fig. 4B,C). Of 44 mesodermal clones, most (77.6%) were restricted to single segments, also significantly more than expected if clones spread without constraint (27% expected).

Segment-restricted clones could arise in at least two ways. Cells could disperse freely but stop at boundaries, or cells could disperse little and seldom encounter a boundary. We found that many of the clones, both neural crest and mesoderm, spanned nearly the entire width of an arch between two adjacent boundaries, intercalating extensively with neighboring unlabeled cells within the arch (Fig. 4A,B). Other clones abutted endodermal boundaries and showed a tendency to expand in the transverse plane along them (Fig. 4C) corresponding in a rough way to the morphologically distinct rows described above. Outside the boundary region there was no evidence for subcompartments within segments (e.g. we never observed a similar line up of cells at any more central location). Segment-restricted clones, in both rostral and caudal regions of an arch primordium showed similar alignments along boundaries.

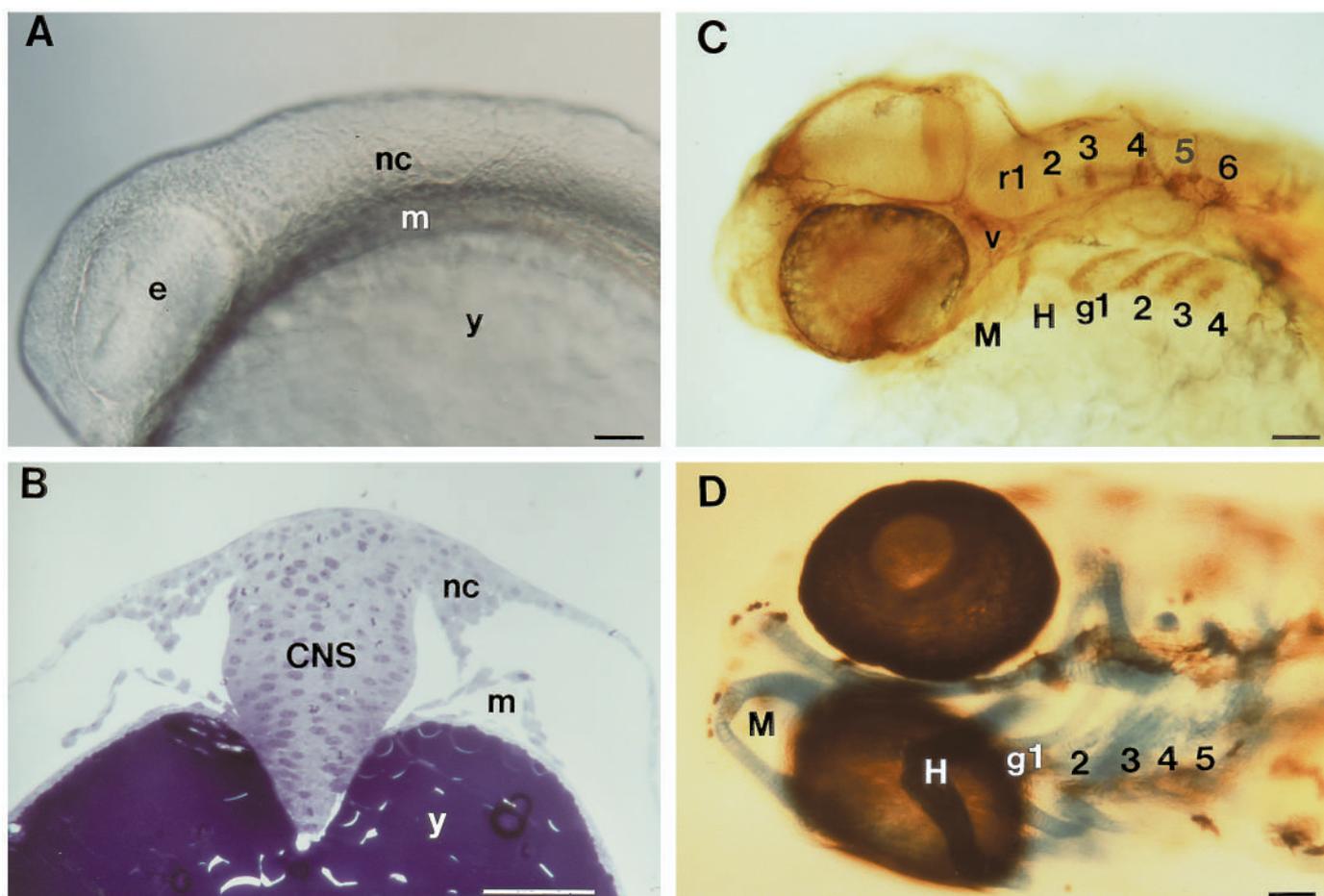


Fig. 1. Pharyngeal arch development in the zebrafish during the first 72 hours of embryogenesis. (A) Left side view of the head of a live zebrafish embryo at 12 h (the stage for which the fate maps were made) photographed using Nomarski contrast optics. The embryonic axis surrounds the large yolk cell (y), and a mass of cranial neural crest (nc) is visible lying dorsal to the paraxial mesoderm (m) and caudal to the eye (e). (B) Transverse section through the head in a region just caudal to the eye at 12 h, stained with toluidine blue to reveal general cellular architecture. (C) Pharyngeal arch primordia develop during the first 24 h. By 30 h, pharyngeal endoderm has formed a reiterated series of outpocketings at the boundaries between arch primordia. Endodermal cell surfaces label with a monoclonal antibody, zn-5, here shown in a lateral view of a whole-mounted embryo. Labeled pouches form dorsoventrally oriented, dark bands between unlabeled groups of mesenchyme that form mandibular (M) and hyoid (H) primordia and a series of 4 smaller gill bars (g1-4). Also labeled by the antibody are cells in the trigeminal ganglion (V) and rhombomeres (r1-6) in the developing hindbrain. (D) By 72 h, cartilage of the pharyngeal skeleton is labeled with Alcian blue and all 5 gill arches are visible. Cartilage of the otic capsule also stains. Scale bar, 25 μ m.

A third way that segment restrictions could arise is if cells that cross segment boundaries die. However, we saw the bright fluorescent spots that are evidence of cell debris and hence cell death in only about 5% of the clones. There seemed to be no correlation between cell death and spatial restriction.

Sequential appearance of segment restrictions

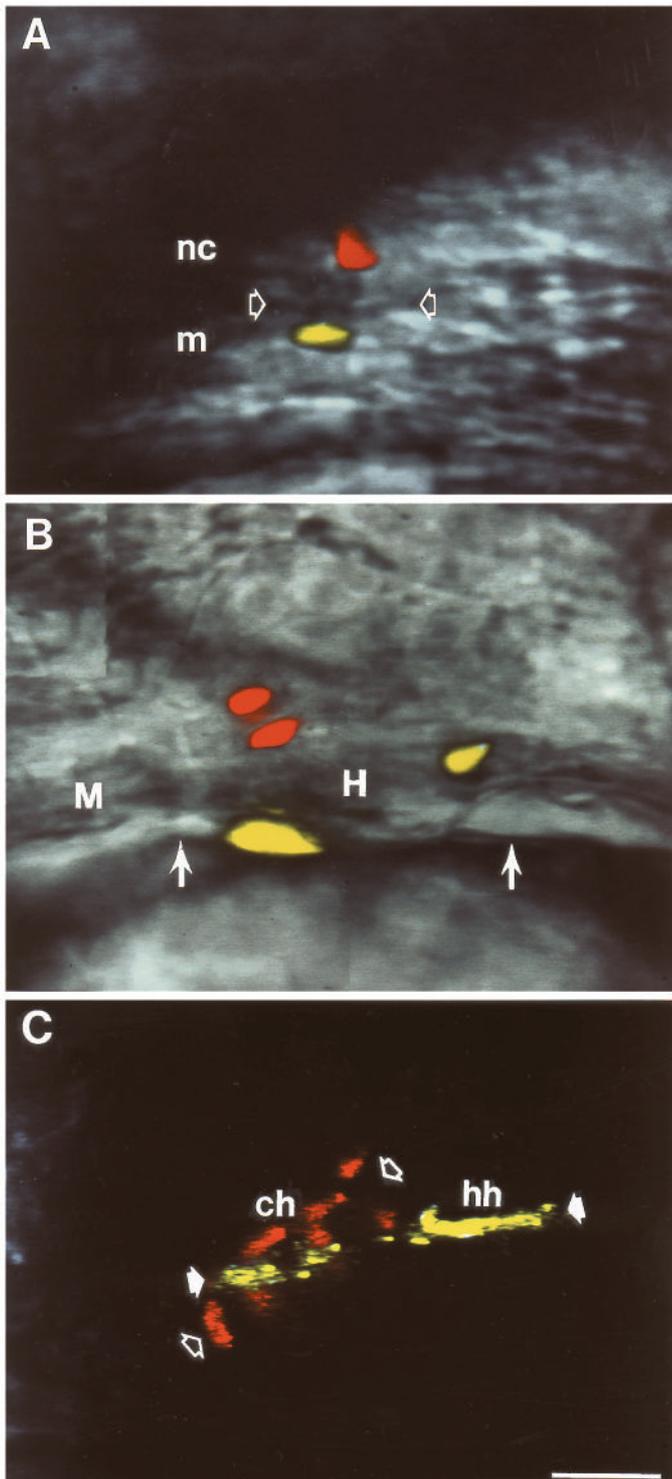
Cells at different axial levels behave differently with respect to segment restrictions (Fig. 5A). Even at early postgastrula stages (12 h), 100% of clones generated by cells in the rostral premigratory neural crest or paraxial mesoderm were restricted to mandibular or hyoid arches at 24 h. In contrast, progenitors of caudal gill arch segments from both neural crest and mesoderm, labeled at the same stage, contributed to up to 3 adjacent arches. Restrictions to these segments then appeared later, and did so in a rostrocaudal sequence. Single cells at both rostral and caudal levels were labeled at 13.5, 15, 16.5 and 18 h, during which time many, but not all, of the crest cells

migrated. The transition from essentially no restrictions to complete restriction occurred during this 5 hour period. Furthermore, the time course for the development of restrictions was almost identical for both the neural crest and mesodermal populations of arch progenitors.

Restrictions to some segments and not others could simply reflect clone size rather than segmental specification; larger clones would tend to be more widely distributed. We compared cell numbers in clones between different pharyngeal arches at 24h. Clone size was not correlated either with segmental restriction or, correspondingly, with rostrocaudal position (Fig. 5B). On average, clones consisted of 5 cells, with a range of 2-9, depending on the cell type.

Some cells may occupy only particular segments transiently and subsequently move into others. We therefore reexamined the spatial distribution of both neural crest and mesodermal clones at 72 h after most cell types had differentiated. Among 58 clones that were segment-restricted at 24 h, all but

6 (10%) remained restricted. Two of these clones produced neural crest-derived cartilage cells (out of 20 total segment-restricted clones at 24 h) and the other 4 produced mesoderm-derived endothelia (out of 6 total). Spreading of endothelium is perhaps expected, given its invasive nature. In general, however, clones observed to be segment-restricted at 24 hours remained so, at least throughout the period of embryogenesis.



Cell type fate maps

Whereas anterior-posterior position predicts the segment to which a neural crest cell will contribute, medial-lateral position in the premigratory crest predicts the type of derivative that a neural crest cell will form. Cells in the most lateral positions in the premigratory mass (tiers 1 and 2) gave rise predominantly to neurons of the trigeminal ganglion (Fig. 1, Table 1). The frequency of neurons was 100% in tier 1. While most cells in tier 2 also gave rise to neurons, a few gave rise to pigment cells and Schwann cells. Cells in tiers 3-5 gave rise predominantly to pigment cells, cartilage cells and some non-neuronal ganglionic cells. By tier 6 the clones consisted exclusively of cartilage and connective tissues. Thus the cranial neural crest, or at least the most superficial 20% that we labeled (see Discussion), can be subdivided into roughly two areas, a narrow marginal band that will form the ganglion and a large, medial area that will form all other cell types.

A significant fraction of clones (12%) contained cells that could not be classified as a particular type (Table 1). Such unidentified cells were found in the same clones as satellite and Schwann cells, cartilage, connective and pigment cells. Clones never consisted entirely of these unidentified cells. Furthermore, in most cases these cells continued to divide through the next several days. However, these unidentified cells did not differentiate. Most unidentified cells were probably precursor cells; it is unclear whether or not they were restricted to the same fate as their clonal relatives.

Cell-type restricted lineages

Most clones, neural crest as well as mesodermal, gave rise to only a single cell type. This cell-type restriction was examined in detail at 72 h for the neural crest in the mandibular arch (Fig. 6). Of a total of 84 clones, 75 gave rise to single cell types (88%). The other 9 did not contain multiple differentiated cell types, rather, they contained some subset of labeled cells that were 'unidentified' as to their type or types (see below). 10 of the 84 clones (12%) contained only a single differentiated cell.

Of the cells that could be identified, the majority were neural and located in the trigeminal ganglion. Typically, sensory neurons differentiated by 24 h (Fig. 6A). In contrast, clones that consisted of non-neuronal cells such as glia or satellite cells differentiated later at day 2 or 3 (Fig. 6B). Along with

Fig. 2. Two cells, one neural crest and the other paraxial mesoderm, labeled at the same axial level generate progeny in the same pharyngeal segment. One neural crest cell was labeled with rhodamine-dextran (red) and one mesodermal cell with fluorescein-dextran (yellow) in the same embryo and development was followed until cells completed differentiation. Fluorescent images were combined with bright-field background images showing surrounding morphology. (A) Initially the two cells were separated by a boundary between dorsal neural crest and ventral mesoderm at 12 h (open arrows). (B) After neural crest cells migrated their progeny were colocalized with mesoderm in the hyoid arch primordium (arrows) at 24 h. (C) They remained colocalized in the hyoid after differentiation at 72 h: crest in the ceratohyal (ch) cartilage and mesoderm in the hyohyal (hh) muscle. Crest cells formed disc shaped chondrocytes while paraxial mesoderm formed elongated, striated muscle fibers, as determined by Nomarski optics (data not shown). Open arrows indicate the long axis of the cartilage bar, while closed arrows indicate that of the muscle. Scale bar, 25 μ m.

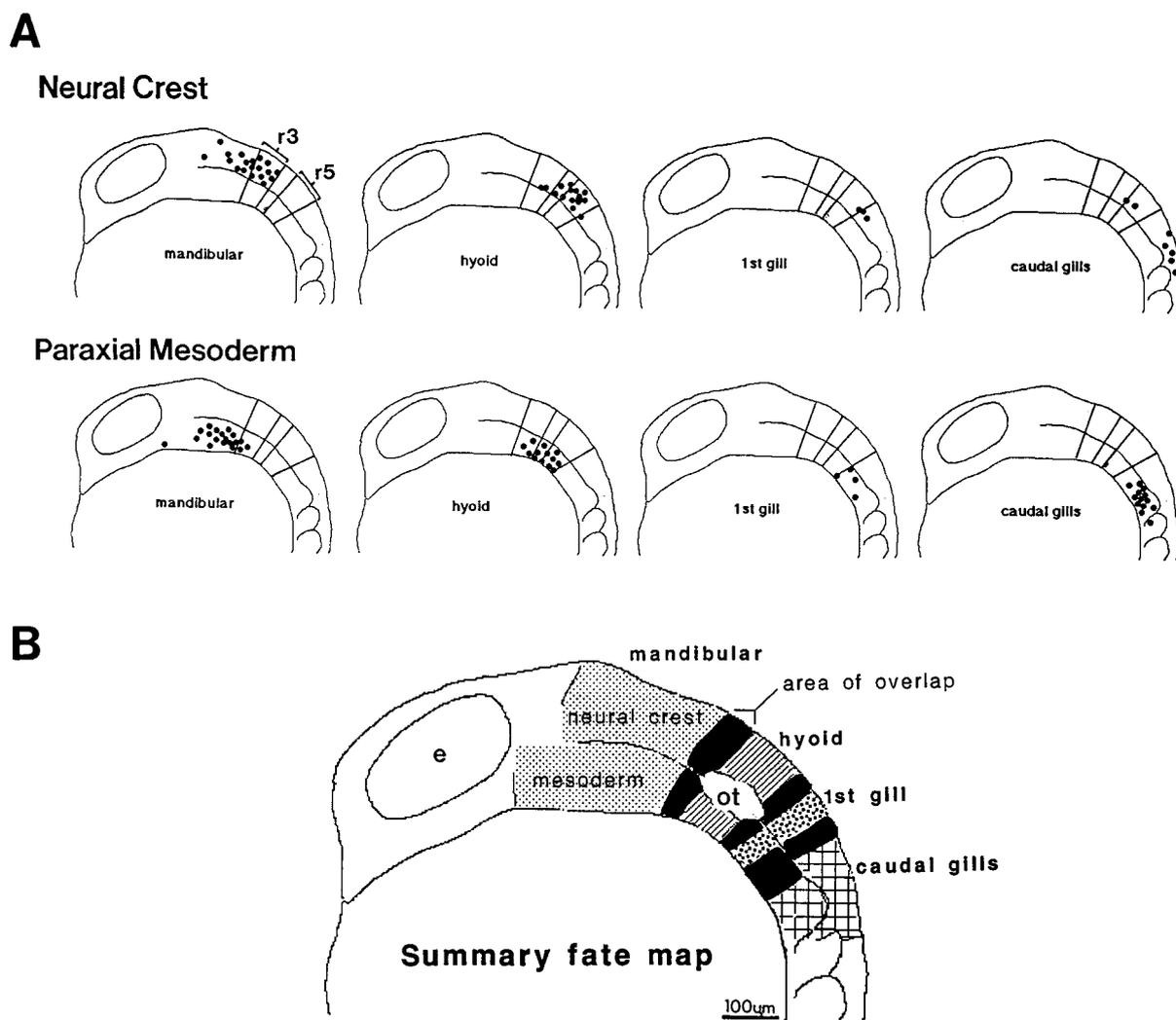


Fig. 3. A fate map of the positions of neural crest and paraxial mesodermal cells at 12 h and the positions of their descendent clones in pharyngeal segments. (A) A filled circle in one of the eight diagrams shows the position of an injected cell superimposed on an outline of a lateral view of the head. Position was measured in micrometers relative to the position of the eye, ear and somites at the time of injection. Labeled cells that gave rise to individual segments are grouped in different diagrams showing the approximate extent of each segment's origin. Each diagram also indicates the regions of the CNS that generate hindbrain rhombomeres 3 and 5 (r3 and r5), as determined by the expression of *krx 20* in whole mounted in situ at this stage. (B) The summary fate map was drawn by outlining the areas of neural crest and mesoderm that generate each arch. Black regions denote the overlap between fate map areas.

neural tissues, clones located outside of the nervous tissues in the pharyngeal arch mesenchyme also formed cell type-restricted clones. These included pigment cells, cartilage, and a collection of other connective tissues such as the scleral component of the eye (Fig. 6C-E). Pigment cell clones were primarily melanocytes. The clonal relationships between subtypes of pigment or connective tissue cells were not examined.

DISCUSSION

We have presented evidence that in the zebrafish embryo single neural crest cells and mesodermal cells that contribute to the pharyngeal arches, produce clones confined to single segments and limited to single cell types. Further, the fate of a cell can be predicted by its position within these precursor populations.

Based on these results we suggest that the precursor cells may already have been 'specified' (see Introduction) according to their positions: segment and cell type-restrictions are imposed in a spatially and temporally coordinated fashion in the neural crest and mesoderm.

Arches are polyclonal compartments

The clonal progeny of individual neural crest or mesodermal cells, labeled after the crest has formed a segregated mass adjacent to the neural tube, but before ventralwards migration, were subsequently restricted to single arch segments. Moreover, precursors of particular arches were colocalized along the anterior-posterior axis in the 'pre migratory' mass. Thus, clonal relatives migrate together and behave as though contained in compartments, because small cohorts of cells form at an early stage and generally do not mix among arches. Individual cohorts of arch precursors would be 'polyclones',

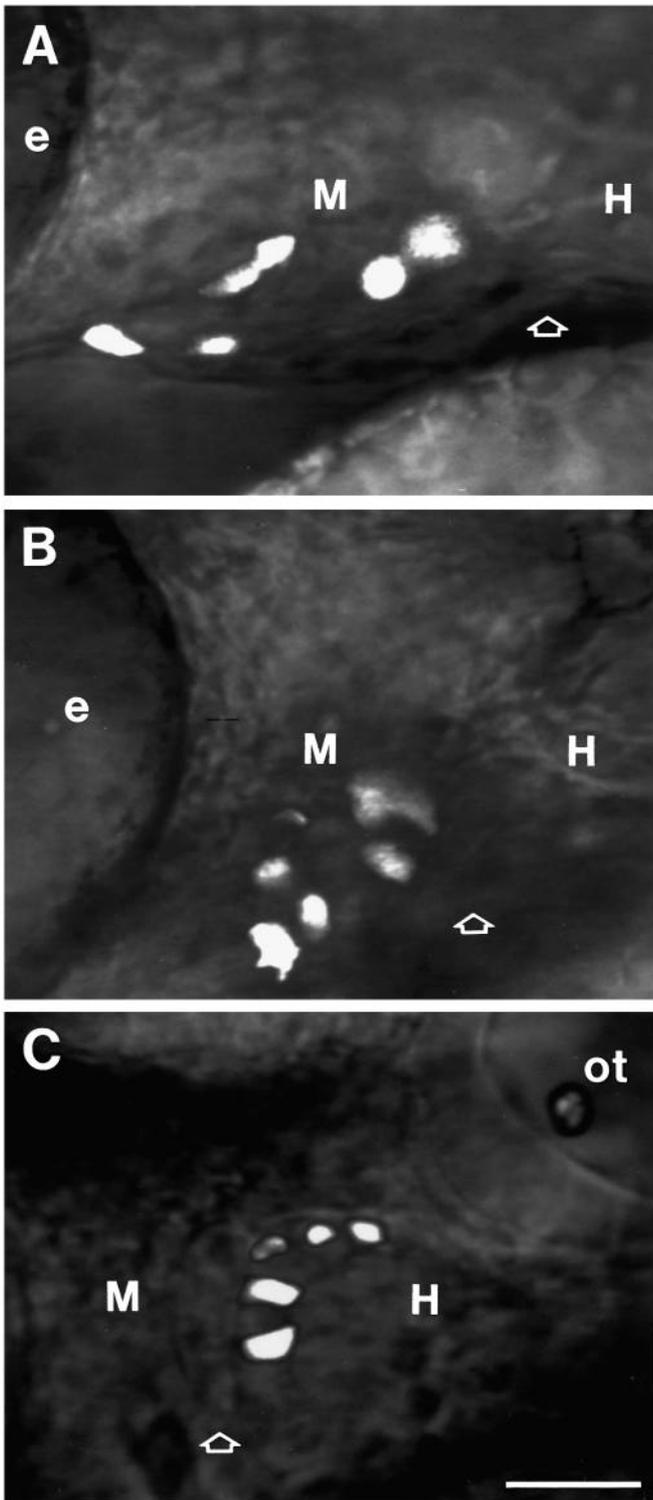
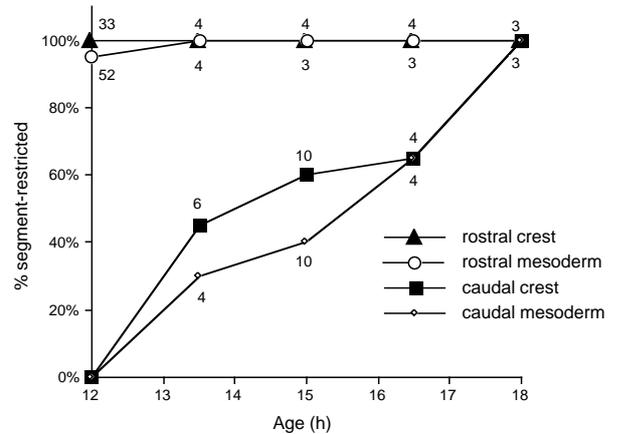


Fig. 4. Clones disperse freely within segments but line up along segment boundaries. Computer-combined fluorescence and bright-field images of live embryos show typical distributions of labeled clones. Open arrows indicate positions of segment boundaries. (A) Neural crest cells at 24 h were dispersed within but confined to the mandibular arch (M), ventral to the developing eye (e) and ear (ot). (B) Paraxial mesoderm cells at 24 h dispersed within the mandibular arch. (C) Paraxial mesoderm cells at 24 h aligned along the anterior boundary of the hyoid arch (H). Scale bar, 25 μ m.

A Segment-Restrictions Arise Progressively in Caudal Pharyngeal Arches



B Clone Sizes in Pharyngeal Segments at 24h

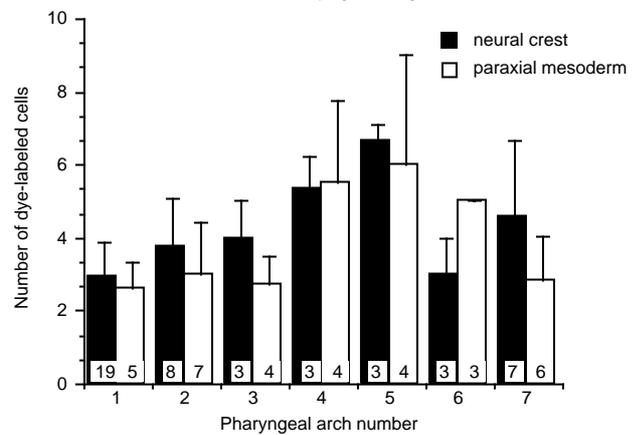


Fig. 5. Segment-restrictions develop in a rostral to caudal sequence and are not correlated with clone size. (A) Cells at different axial positions were injected with lineage tracer at times shown on the horizontal axis, and the segmental fates of their progeny were ascertained in 24 h embryos. Numbers associated with each line indicate the number of clones at each position at each time. The solid triangles and open circles show the fraction of *rostral* (labeled anterior to the otic placode) neural crest or mesodermal clones, respectively, that were segment restricted. The solid squares and diamonds show the fraction of *caudal* segment-restricted clones. (B) Neural crest clones (dark bars) averaged anywhere from 2-7 cells as did mesodermal clones (open bars), although there was significant variability. The size of clones of each type are compared for each of the 7 pharyngeal arches (horizontal axis). Standard deviations and the number of clones (at the base of each bar) are given for each case.

because they are not necessarily related by ancestry but rather by proximity, much like cells in the insect epidermis (Garcia-Bellido et al., 1973). Eventually, the endoderm forms boundaries between arches, but restrictions are observed long before these overt boundaries arise. The early appearance of segment restrictions at 12 h, after gastrulation but before crest migration and the formation of morphological segment boundaries, suggests that cell movements are constrained during arch formation.

Thus, in the zebrafish, there is evidently little mixing among segmental groups during migration, as also has been observed

Table 1. Fates, according to cell type, of cells located at different medial-lateral positions in the cranial neural crest

Location (tier*) of injected cell	No.	Fate†					
		N	S	P	C	CT	U
1 (lateral)	24	24	-	-	-	-	-
2	14	8	2	2	-	-	2
3	9	-	5	2	-	-	2
4	10	-	1	3	2	2	2
5	16	-	1	5	5	4	1
6 (medial)	11	-	-	-	6	3	2
Total	84	32	9	12	13	9	9

Column 1 gives the distance, in cell diameters, from the lateral margin of the neural crest which is designated as a cell's 'tier'. Column 2 gives the total number of clones in each tier. Columns 3-8 show the number of clones that contained each cell type. Most of the clones generated single tissue fates, other than those containing unidentified cells.

*Tiers were determined by counting the number of cell diameters between a labeled cell and the lateral margin of the premigratory neural crest population. For statistics see Materials and Methods.

†Fate was determined by cell morphology and position (as described in Materials and Methods) as neuron (N), Schwann or satellite cell (S), pigment (P), cartilage (C), connective tissue (CT) or unidentified (U).

to some extent in the chick (Lumsden et al., 1991; Serbedzija et al., 1992). Several factors may contribute to this restriction in mixing. In zebrafish, the rate of neural crest migration is fast compared with the length of the cell cycle and cells may undergo little anterior-posterior movement simply because of a clone's small size. In addition, cells may preferentially adhere to some dorsoventrally oriented guide, such as epithelia derived from pharyngeal endoderm and surface ectoderm, or extracellular matrix structures, such as fibronectin and laminin (Boucaut et al., 1984). The cells may be committed to their respective segmental fates, a possibility that can now be tested by heterotopic transplantations of cells between different regions of the fate map.

Segmental compartmentation is also suggested by the observation that clones line up after migration along arch primordium boundaries. Cells in regions away from boundaries disperse and mix with neighboring unlabeled cells, suggesting that boundaries specifically form barriers to movement. A likely candidate to restrict mixing is the endoderm, which forms overt pockets in between pharyngeal arch primordia. Similar restricted cell movements have been observed at segment boundaries in the hindbrain rhombomeres of the chick embryo (Fraser et al., 1990). Alternatively, cell surface properties might differ between the mesenchymal cells themselves in different segments as has been proposed for hindbrain rhombomeres (Guthrie and Lumsden, 1991). Neural crest or mesoderm destined for a particular segment might thus be essentially immiscible with cells of a neighboring segment.

Lineage restrictions could account for coordinated segmentation of head tissues

As revealed directly with double-labeling, segment progenitors in the neural crest and the mesoderm lie in register along the anterior-posterior axis prior to overt pharyngeal segmentation. Progenitors of individual pharyngeal arches also lie in register with the neuromeres that innervate them. The expression of *krx20* in neuromeres 3 and 5 at 12-13 h lines up precisely with

the positions of progenitors of mandibular and hyoid arches, respectively. In this case the head segment could be extended to include the CNS, which later shows a clear segmental relationship with the pharyngeal arches, in its innervation pattern as well as patterns of gene expression, at least in some vertebrate species (Couly and LeDouarin, 1990; Lumsden and Keynes, 1989; reviewed by Hunt, 1991b).

Surprisingly, while single cells are segment-restricted, fate map regions overlap (Fig. 4), suggesting that the boundaries are undetermined at the 12 h stage. Some or all of this overlap may be due to errors in measurements of the positions of cells at the time of labeling. It is possible that while every cell in the premigratory neural crest or mesoderm will contribute to only one segment, one cannot predict the segmental fate of a cell near the fate map boundary by its position. Thus, it remains unclear exactly when the boundaries form, and how precise they are until 17 h, the stage when the earliest marker we have used for pharyngeal endoderm first labels cells.

On the basis of previous observations, we were surprised to find such an early, strict pattern of segmental restrictions. That is, in accord with the developmental compartments described in the chick hindbrain (Fraser et al., 1990), or even in zebrafish somites (Kimmel and Warga, 1987), it seemed likely that pharyngeal lineages would be restricted only after arch boundaries were well established. Our results show that this is not the case in zebrafish pharyngeal arches. Restrictions are present before the migrations that form the arch primordia.

Cranial neural crest cells are cell type-restricted

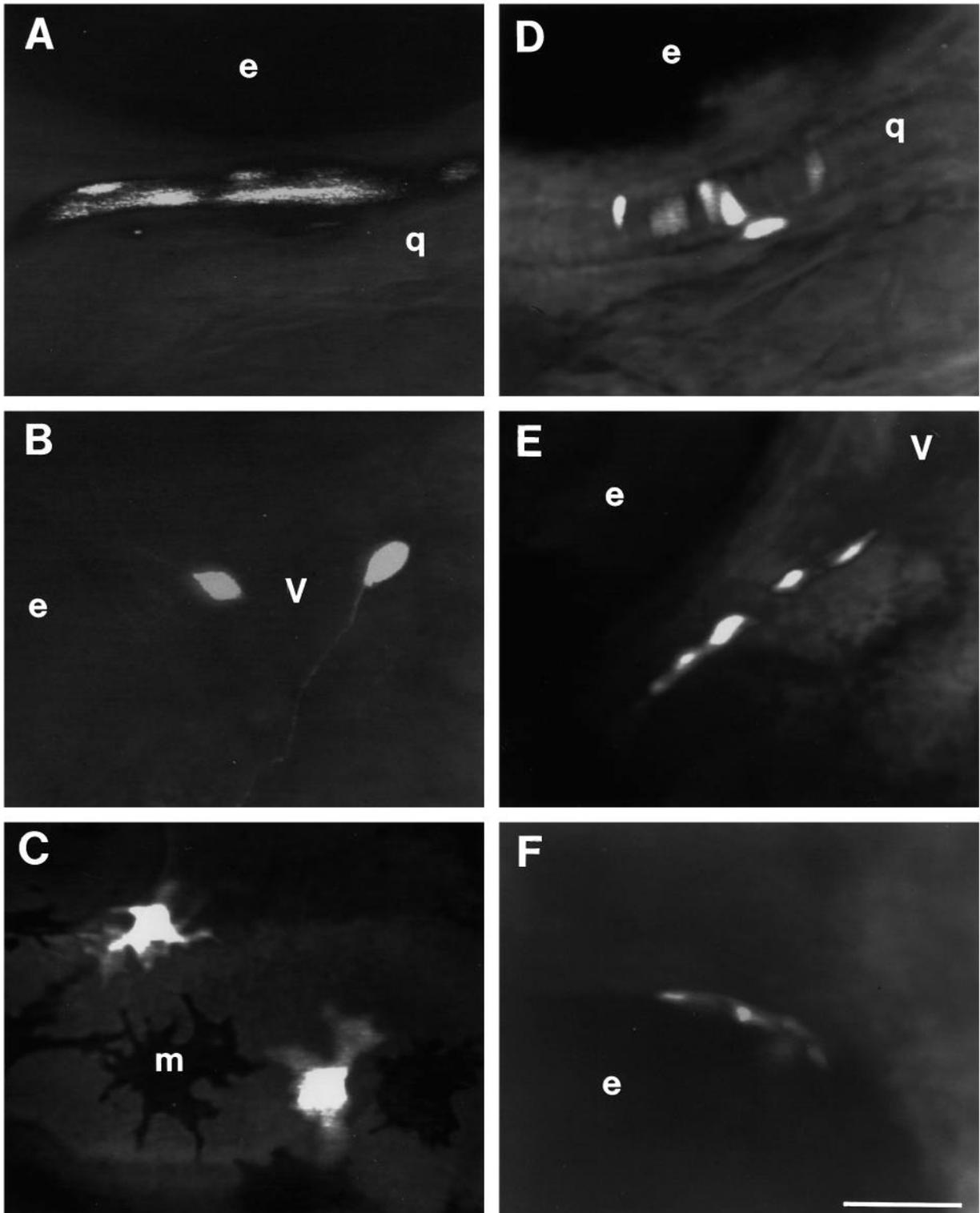
Strikingly, in addition to segment restrictions, nearly all pharyngeal neural crest cells (88%) in the embryonic zebrafish generate progeny of single cell types, demonstrating that at what we interpret to be premigratory stages, many neural crest lineages are restricted. This result is unexpected, given the convincing evidence for unrestricted lineages obtained from single cell studies of trunk (Bronner-Fraser and Fraser, 1988; Fraser and Bronner-Fraser, 1991; Frank and Sanes, 1991; Stemple and Anderson, 1992) and head (Baroffio et al., 1991) neural crest in other systems, with some exceptions (Sieber-Blum, 1989; Ito and Sieber-Blum, 1991, 1993). However, because of dramatic differences between teleost and tetrapod neurulation it is difficult to argue that the crest analyzed here is equivalent to 'pre-migratory' neural crest in the chick. In zebrafish, no obvious neural folds appear and instead of a neural tube, a solid neural keel forms with the central canal forming secondarily.

Fig. 6. Lineages are restricted to single cell types in the mandibular arch. Computer enhanced images of cells labeled intracellularly with rhodamine-dextran have been superimposed upon bright-field, background images. Cell phenotypes were confirmed using Nomarski optics. All images are left side views. (A) Three muscle fibers of the intermandibularis muscle, derived from a single mesodermal cell, lie just dorsal to the quadrate (q) cartilage and ventral to the eye. (B) Two neurons of the trigeminal ganglion (V) extend axons ventral and dorsal to the eye (e) by 30 h. (C) Two pigment cells show characteristic stellate or elongated morphologies at 72 h. (D) Eight cartilage cells show stacked appearance within the developing quadrate (q) cartilage at 72 h. (E) Five Schwann cells are elongated along the mandibular branch of the trigeminal nerve at 72 h. (F) Four connective tissue cells within the dorsal part of the eye capsule at 72 h. Scale bar, 25 μ m.

The cranial neural crest cells are laterally segregated in a pre-migratory mass from which cells migrate several hours later. In addition, clonal analyses of neurulation in zebrafish reveal that even at late gastrula stages (90-100% epiboly) the progeny of single neuroectodermal cells never contribute to both neural tube and neural crest (Schmitz et al., 1993; Schilling, 1993), in contrast to the chick (Bronner Fraser and Fraser, 1988).

A second, novel finding is a fate map for different cell types,

such that cell type-restricted cells are spatially segregated in the pre-migratory mass of neural crest. The results are consistent with the idea that neural crest cell lineage diversification involves early specification events, before they migrate to their ultimate destinations. Whether such specifications would irrevocably commit cells to particular fates needs to be tested by transplanting cells to ectopic environments. They may not, for tissue-restricted cells are present in the early gastrula and trans-



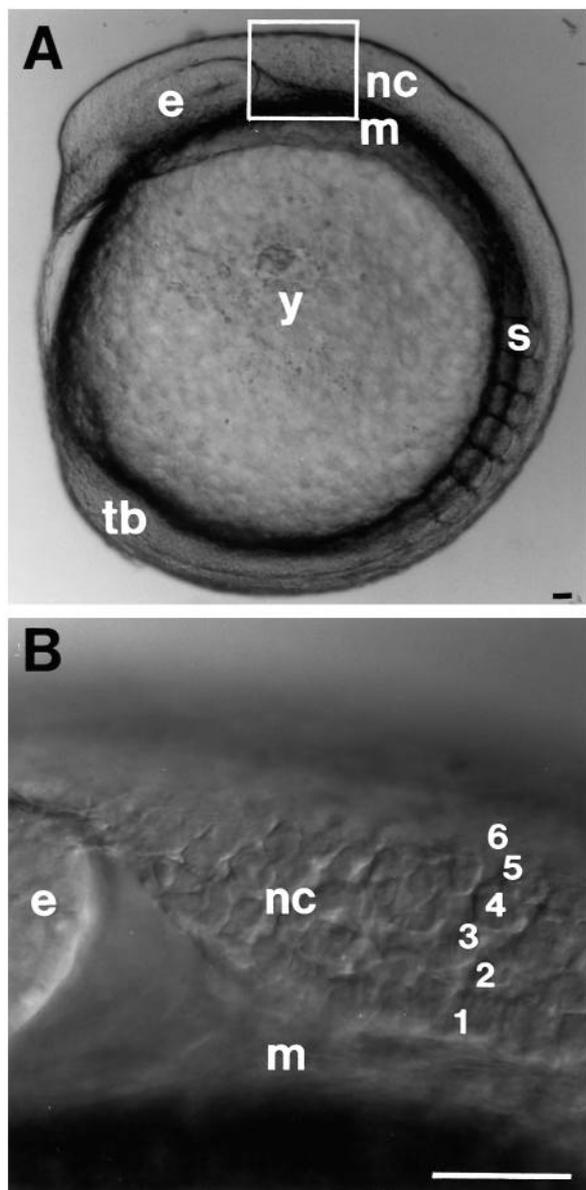


Fig. 7. Mesencephalic and rostral rhombencephalic neural crest cells that will form the mandibular arch map to different medial-lateral positions in the premigratory population. (A) A live embryo was photographed at 12 h using Nomarski optics. The lateral margin of segregating neural crest (nc) is particularly obvious just caudal to the eye and dorsal to the mesoderm (m) as shown at higher magnification in B. Cells at different distances from the margin in this example are designated 1-6 (tiers). Scale bars, 25 μ m.

plantation reveals that the cells remain uncommitted at this stage (Ho and Kimmel, 1993).

Progenitors of neurons and glia

The evidence for cell type-restrictions is perhaps less surprising for the neural lineages where there is some prior evidence for cell type-restrictions. In avian embryos, neurons have occasionally been reported to coexist with other cell types in single crest-derived clones in vitro (Sieber-Blum and Cohen, 1980; Sieber-Blum, 1989; Barroffio et al., 1988) but there are reasons

to suspect that these cell types would have separate progenitors in vivo (Ciment and Weston, 1982). Back transplantation studies have suggested that some committed neuronal and glial precursors exist in the crest (Girdlestone and Weston, 1985; Vogel and Weston, 1988). However, recent evidence from in vitro subcloning experiments in mammalian embryos (Stemple and Anderson, 1992) as well as in vivo clonal analyses using fluorescent lineage tracers or retroviral infections in avian embryos (Frank and Sanes, 1991), demonstrates that there are probably also multipotent precursors for these cell types. Our results, along with those of Raible and Eisen (1994), extend our knowledge to fishes and demonstrate unequivocally that many of their neural crest cells give rise to cell type-restricted neuronal or non-neuronal lineages. However, cranial neural crest cells in the zebrafish, unlike their counterparts in the trunk, never generate progeny of multiple cell types.

Cartilage, connective tissues and pigment cells

Perhaps more surprising was the cell type-restricted nature of most cartilage, connective tissue and pigment lineages. In general, these cell types differentiate later than their neural counterparts. Their clonal relationships have been characterized primarily in vitro (Barroffio et al., 1991) where common progenitors for cartilage, pigment cells, and neural derivatives have been found. The same study, however, also found some cartilage restricted clones. Heterotopic transplantations of fragments of avian cranial neural crest and observations of their migratory behaviors, in vivo, have suggested that the cartilage lineage could segregate early from the other lineages (LeDouarin and Teillet, 1974). Crest cells isolated from avian branchial arches, well after migration, never form pigment derivatives (Ciment and Weston, 1985). Our results suggest that specified precursors for cartilage and melanocytes are generated soon after neural crest segregates from the neuroepithelium and suggest that the same changes that are responsible for restrictions in precursors for neural tissues bring about their type-restrictions.

Premigratory positions may specify cell type-progenitors

The cell type generated by a neural crest cell in the zebrafish can be predicted by its medial-lateral location in the premigratory mass. This leads us to suggest that while in the neuroepithelium there may be pronounced mixing of neural and non-neural producing crest cells, they must mix little during segregation. The fate map roughly corresponds to the timing of crest cell migration (unpublished observations); more laterally located cells migrate first and form neural cell types while medial cells generally migrate later, although this has not been examined in detail. A similar correspondence between migration time and fate has been seen in the zebrafish trunk (Raible and Eisen, 1994). Thus the map may more closely reflect the coordinated migrations, and therefore similar fates, of cells close to each other.

Based on the restricted distributions of precursors for different cell types, the premigratory neural crest can be segregated roughly into three areas, possibly representing three classes of derivatives. The most lateral tiers of cells generate neurons, cells located slightly further medially generate Schwann and pigment cells, while the most medial cells

generate cartilage and connective tissues. A similar classification of crest derivatives has been proposed previously in the chick (Baroffio et al., 1988; Dupin et al. 1990; Baroffio et al., 1991) based on observed frequencies of crest-derived cell types in vitro. The evidence to date only applies to the most superficial cells (~20%) of the segregated mass as these are the cells we could see to label easily; deeper crest cells may behave differently.

Representatives of several major vertebrate classes have now been shown to share both similar fate maps and cell behaviors in cranial tissues. The zebrafish map for neural crest and paraxial mesoderm roughly corresponds to that of Noden (1987) for the avian embryo. The similarities between the zebrafish and chick neural crest fate maps (Couly and LeDouarin, 1990; Noden, 1975, 1988), along with the known contributions of crest to the pharyngeal arches in various other vertebrate groups (Noden, 1983; Langille and Hall, 1988), suggest that such maps are shared primitive features of cephalic ontogenies of all vertebrate radiations (Northcutt, 1990). These initial similarities would account for many of the shared features in the basic organization of the vertebrate head.

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