The *chinless* mutation and neural crest cell interactions in zebrafish jaw development

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SUMMARY

During vertebrate development, neural crest cells are thought to pattern many aspects of head organization, including the segmented skeleton and musculature of the jaw and gills. Here we describe mutations at the gene *chinless, chn*, that disrupt the skeletal fates of neural crest cells in the head of the zebrafish and their interactions with muscle precursors. *chn* mutants lack neural-crest-derived cartilage and mesoderm-derived muscles in all seven pharyngeal arches. Fate mapping and gene expression studies demonstrate the presence of both undifferentiated cartilage and muscle precursors in mutants. However, *chn* blocks differentiation directly in neural crest, and not in mesoderm, as revealed by mosaic analyses. Neural crest cells taken from wild-type donor embryos can form cartilage when transplanted into *chn* mutant hosts and rescue some of the patterning defects of mutant pharyngeal arches. In these cases, cartilage only forms if neural crest is transplanted at least one hour before its migration, suggesting that interactions occur transiently in early jaw precursors. In contrast, transplanted cells in paraxial mesoderm behave according to the host genotype; mutant cells form jaw muscles in a wild-type environment. These results suggest that *chn* is required for the development of pharyngeal cartilages from cranial neural crest cells and subsequent crest signals that pattern mesodermally derived myocytes.

Key words: zebrafish, neural crest, pharyngeal arch, cartilage, *chinless*, jaw

INTRODUCTION

In vertebrates, much of the head skeleton and peripheral nervous system develops from migratory cells of the neural crest. Neural crest cells arise from dorsal regions of the neural tube and form the anterior neurocranium and segmented pharyngeal skeleton, as well as sensory neurons, glia and pigment cells throughout the body (LeDouarin, 1982). The cranial neural crest of the pharyngeal arches, in particular, is thought to produce signals that organize the patterns of surrounding tissues such as mesodermally derived myogenic cells (Noden, 1983). Thus, an important issue in vertebrate development is how these neural crest cells are specified to form the appropriate cell types and relate this spatial information to their neighbors.

Neural crest cells form the pharyngeal skeleton while mesoderm forms the muscles, and these two populations interact extensively with each other and with surrounding epithelia (Platt, 1893; Hall, 1980; Schilling and Kimmel, 1994; reviewed in Hall, 1987; Lumsden, 1988; Noden, 1988; LeDouarin et al., 1994). These eventually form the reiterated bones and muscles of the jaw and branchial arches that function in feeding and breathing. Skeletomuscular patterning has been best characterized in the avian embryo where neural crest cells migrate segmentally and their fates in the skeleton are determined prior to their emigration from the neural tube (Lumsden et al., 1991; Noden, 1988). When transplanted heterotypically, neural crest cells organize ectopic cartilages and muscles specific for their original anterior-posterior positions (Noden, 1983). The transplanted cells themselves form cartilage and connective tissues and these cells reorganize surrounding mesoderm to form muscles with ectopic orientations and attachment sites. These results have led to the proposal that cranial neural crest cells or their skeletal and connective tissue derivatives pattern the ventral head region during normal embryonic development. Thus the regional patterning of the neural crest can now be seen as providing a basis for the establishment of organizing centers that, after migration, generate positional information. The organizing properties of neural crest may be a general feature of vertebrate embryos, since similar contributions of neural crest to cranial cartilage have been described in every vertebrate species that has been examined (reviewed in Smith and Hall, 1990).

Some of the positional cues that specify neural crest cells and their interactions in the arches have been identified but there have been few insights into their genetic or molecular basis. Notable exceptions are relatives of *Antennapedia* and *Bithorax* genes of flies, the *Hox* genes, and their roles in particular head segments and cell types (reviewed in Krumlauf, 1994). Mouse mutants for some *Hox* genes show homeotic transformations of pharyngeal arch segments or lack subsets of neural-crest-derived cell types. For example, the loss of
Hoxa-2 function results in some skeletal elements of the second (hyoid) arch developing characteristics of first (mandibular) arch structures, suggesting that this gene normally specifies the precursors of hyoid cartilages and bones (Rijli, 1993). This has supported the idea that segment identity in the head periphery is transmitted from the neuroectoderm through a Hox code in the neural crest (Hunt et al., 1991a,b).

To learn developmental genetic mechanisms that establish cell fates in the arches, we are using random mutagenesis in the zebrafish to identify genes whose products are necessary for jaw and gill development. In the zebrafish, a mutational approach has led to the identification of genes involved in early embryogenesis (Streisinger et al., 1981; Kimmel, 1989; Hatta et al., 1991; Halpern et al., 1993; Mullins et al., 1994; Solnik-Krezel et al., 1994). Here we describe mutations at the locus chinless (chn\textsuperscript{b146} and chn\textsuperscript{pa20}) that produce embryos lacking pharyngeal cartilages and muscles. We use cell transplantation to show that cells derived from wild-type embryos can autonomously form cartilage in chn mutant hosts and rescue aspects of skeletal patterning. In contrast, mesodermal cells transplanted from mutant donors form muscles in a wild-type environment.
environment, where proper influences from their neural crest neighbors are restored. We suggest that *chn* is required for neural crest signals involved in cartilage and muscle development.

**MATERIALS AND METHODS**

**Embryos**

Zebrafish (*Danio rerio*) from the laboratory colony at the University of Oregon were used in this study. Embryos were obtained either through spontaneous or induced spawns, raised at 28.5°C and staged according to Kimmel et al. (1995).

**Mutagenesis and mutant screens**

The original allele, *chn*b146, was induced by exposure of midblastula embryos to gamma-rays, as described previously (Walker and Streisinger, 1983). Recessive mutations were subsequently found in the haploid, parthenogenetic progeny of the mutagenized females by fertilizing their eggs with sperm rendered genetically impotent by exposure to ultraviolet light (Streisinger et al., 1986). Mutants were identified by visual inspection of the jaw and gills at low magnification (~80×) during the late hatching period (approx. 72 hours after fertilization; Kimmel et al., 1995). A second allele, *chn*pa20, was found by screening diploid, F2 intercross progeny.

**Fig. 3.** *chn* neural crest cells do not differentiate as cartilage in the arches. (A) 12 hour, lateral view. A single neural crest cell, labeled with tetramethylrhodamine-dextran is shown after injection. (B,C) 72 hour, lateral view. Computer-combined bright field and fluorescence images. Clones in the mandibular arch in living wild-type (B) and *chn* mutant (C) embryos, derived from cells located in the largely chondrogenic region of the fate map at early segmentation stages (12-13 hours; Table 1). (B) In the wild type, the clone has contributed several cartilage cells to the palatoquadrate (between arrows). (C) Neural crest cells that migrate into the mandibular arch in *chn*, remain mesenchymal and never form cartilage although surrounding melanocytes, derived from separate unlabeled neural crest lineages develop normally (Schilling and Kimmel, 1994). Abbreviations: e, eye; me, paraxial mesoderm; nc, neural crest; pq, palatoquadrate; y, yolk. Scale bars, 100 μm.

**Fig. 4.** Jaw muscle precursors are specified but do not differentiate in *chn*. (A,C) Wild-type embryos, (B,D) *chn* mutant embryos. Whole-mounted embryos labelled with anti-Eng antibody were photographed with Nomarski optics. (A) 36 hour, lateral view. In wild type, precursors of two mandibular muscles, the levator arcus palatini and dilator operculi, are present in the mandibular arch and express Eng proteins in their nuclei (Hatta et al., 1990). (B) Cells labelled in a similar location are present in *chn* mutants (asterisk). (C) 72 hour. At late hatching the Eng-expressing cells have elongated and striated in the wild type. (D) In *chn* the cells neither elongate nor striate to form contractile muscle fibers. Eng is also expressed in the nervous system at the junction between midbrain and hindbrain in *chn* mutants as well as wild type. Abbreviations: do, dilator operculi; e, eye; h, hyoid arch; hb, hindbrain; mb, midbrain; o, otic vesicle. Scale bar, 200 μm.
Immunohistochemistry and histology

An antibody originally produced against the *Drosophila* Invected protein (4D9) was used to reveal the expression of Engrailed (Eng) proteins in mandibular muscle precursors (Hatta et al., 1990). Briefly, embryos were fixed overnight in 4% buffered parafomaldehyde, frozen in acetone to increase permeability, incubated in primary and secondary antibodies, and reacted using the peroxidase anti-peroxidase method and diaminobenzidine. They were then dehydrated, cleared in methylsalicylate and whole mounted on their sides for photography.

For cartilage staining, embryos were fixed for 2 hours to overnight in buffered 4% formaldehyde, rinsed briefly in distilled water and transferred directly into a 0.1% solution of Alcian blue to stain overnight (Dingerkus and Uhler, 1977). They were then cleared, first by incubating for 1-4 hours in 0.05% trypsin followed by bleaching pigmentation in a 3% hydrogen peroxide solution, cleared in 70% glycerol and whole mounted. For a more general histological overview, we used a ‘trichrome’ stain in which embryos at late hatching stages were immersed in Bouin’s fixative for 24-48 hours, embedded in Paraplast and sectioned in the horizontal plane. Serial sections were cut at 10 μm, treated with HgCl₂ and stained with Mallory’s triple stain (Pantin, 1960). Terminology follows the conventions for zebrafish and other ostriaphyses (Cubbage and Mabee, 1996).

Whole-mount in situ hybridizations

Embryos were fixed in 4% paraformaldehyde dissolved in phosphate-buffered saline. In situ hybridizations were performed with antisense riboprobes as previously described (Thissel et al., 1993). The templates used for synthesizing the probes for dlx2 have been described previously (Akimenko et al., 1994).

Lineage tracer injections

For clonal analysis of neural crest cell fates, single cells were injected in the premigratory neural crest in a series of embryos at early segmentation stages (12-13 hours) as described in Schilling and Kimmel (1994). Embryos were mounted in 1.5% agar and a 5% solution of tetramethylrhodamine-dextran (Molecular Probes) was iontophoresed intracellulary into single neural crest cells. The animals were then removed and allowed to develop for observations at pharyngula (24 hours) and late hatching (72 hours) stages. The clones were visualized using a SIT video camera to amplify fluorescence.

Cell transplantsations

Donor embryos were labeled at the 1-cell stage with yolk injections of tetramethylrhodamine dextran, a non-fixable fluorescent tracer, or a combination of this and a fixable biotin-dextran (Dingerkus and Uhler, 1977). A suction micropipette was used to transplant neural crest cells from future anterior hindbrain and midbrain levels orthotopically to one side of the head of an unlabeled sibling host at early segmentation stages (12-13 hours). At this stage, some of the neural crest cells that will contribute to mandibular and hyoid arches have segregated at the interface between the lateral ectoderm and the neural keel to form a large lateral mass but have not yet begun to migrate (Schilling and Kimmel, 1994). These segregated cells or their unsegregated neighbors in the keel were moved, typically in groups of six to ten cells. Mutants were indistinguishable from their wild-type siblings at the time of transplantation. Thus all experiments were done blindly and individual donors and hosts were kept separate until they could be identified phenotypically 18 hours later. The cases where transplants were performed between wild-type and wild-type embryos, or mutant and mutant, served as controls.

Mesoderms were transplanted earlier, in the blastula or early gastrula periods, but with similar techniques. Blastomeress from donor embryos, labeled as described above, were transplanted into the involuting, marginal zones of unlabeled hosts. Most or all of these cells involuted to join the hypoblast as it converged towards the body midline and only the most dorsal of these formed axial structures (notochord, hatching gland), while most were paraxial, as shown previously in fate mapping studies (Kimmel et al., 1990) of labeled blastomeres. We did not study embryos in which we supposed, by the presence of donor cells in ectodermal derivatives, mesodermal development may have been influenced by the presence of genotypically different ectoderm.

RESULTS

chn mutants lack the pharyngeal arch skeleton and muscles

A gamma-ray-induced mutation chn b146 (Fig. 1) was identified among the parthenogenetic haploid offspring of a female that had been irradiated at the midblastula stage, as part of a systematic F1 screen for mutants that disrupt embryonic zebrafish development (Walker and Streisinger, 1983). The phenotype segregated as a single recessive trait. In crosses between chn/+ heterozygotes, 25% of the progeny showed the phenotype (n=2013), 50% of the haploid progeny of heterozygous females were mutant (n=1256). A second mutation, chn b20, chemically induced with ethylN-nitrosourea, was then recovered in a subsequent screen that had a phenotype indistinguishable from chn b146. The two mutations fail to complement and we infer they are allelic. Transheterozygotes between the two mutations are phenotypically identical to homozygotes. Neither allele shows visible dominant effects in heterozygotes. The mutations are lethal and homozygous mutants die within 5 days of development. Nearly all of the analyses described here were of the original allele, chn b146.

The chn mutant phenotype can first be recognized early in the pharyngula period, around 30 hours, by the small eye size and elongated noses of mutants as compared to wild-type siblings (Fig. 1A,B). By the late hatching period, 72 hours, mutants have reduced heads and lack arch-derived structures of the jaws and gills (Fig. 1C,D). chn mutant embryos lack the entire pharyngeal skeleton, including Meckel’s cartilage and palaquoquadrate of the mandibular arch, the hyosymplectic, ceratohyal, interhyal and basihyal of the hyoid arch, and basibranchials, hypobranchials and ceratobranchials in arches 3-7. All of these cartilages originate, at least in part, from the neural crest (Schilling and Kimmel, 1994). Some cartilage does form in the chn neurocranium, although overall the elements are strongly reduced in size, including the more anterior trabeculae which are thought to originate from the neural crest. Finding neurocranial cartilage development reveals that lack of chn function does not simply block biosynthesis of a key cartilage component and suggests that it is more likely a patterning or specification defect.

As determined further in sectioned material, chn embryos have no cartilage or muscle in the arches and, as a consequence, no protruding jaw (Fig. 1E,F). The mouth opens further ventral and posterior in mutants than in wild type. The phenotype probably is the result of failure of the extension of the jaw and the anterodorsal protrusion of the mouth that characterizes embryos late during the hatching period. Somite-derived trunk and tail muscles are generally well developed in mutants.

Arch precursors are present in chn mutants

Defects in the pharyngeal arches of chn could be the conse-
quence of impaired migration of neural crest. However, mesenchyme that forms the arch primordia appears normal in mutants as determined by inspection, Nomarski optics, utilizing live embryos (not shown) and also by the expression patterns of several genetic markers including *dlx2* (Fig. 2A,B). Three major streams of neural crest that express *dlx2* appear normal in size and position in embryos with 10 somites derived from incursions between heterozygous *chn/+* parents. By the pharyngula period, when the phenotype becomes visible morphologically, pharyngeal arch primordia and early differentiating, neural-crest-derived sensory neurons in the cranial ganglia appear normal in *chn* (Fig. 2B). Hindbrain rhombomeres also appear normal in number and size. We do not know if there are additional defects in other neural crest derivatives such as enteric neurons or in the valves of the heart. However, *chn* mutant embryos have a strong heartbeat and apparently normal gut motility.

To determine if neural crest cells contribute to the mutant mesenchyme, clonal analyses of wild-type and *chn* embryos were compared (Fig. 3; Table 1). In wild-type embryos, single neural crest cells generate clones of cells that all express the same fate and migration occurs in predictable patterns. Hence we can predict the fates of cells by their premigratory positions; cartilage and pigment precursors lie more dorsomedially than neurogenic crest cells (Schilling and Kimmel, 1994). In *chn*, neural crest cells labeled with rhodamine-dextran from the medial, largely chondrogenic, region migrated ventrally into the arches as in wild-type embryos, but remained undifferentiated in positions where cartilage normally forms. By late hatching (72 hours) stages, labeled crest cells in the mutant pharynx had no recognizable differentiated morphology, whereas in wild types many cartilage cells have already differentiated. Other neural crest derivatives normally derived from the same mass of premigratory crest, including glial cells of cranial ganglia and pigment cells, were as commonly observed in mutants as in wild types. Thus we propose that at least some undifferentiated cells in the pharyngeal arches of *chn* mutants are arrested cartilage precursors.

*chn* mutants also lack the segmental array of striated muscles that normally are associated with the arch cartilages, but that stem from mesoderm (Schilling and Kimmel, 1994). To determine if precursors for two jaw muscles were present, we used an antibody that recognizes zebrafish *Eng* homeoproteins. Precursors of two mandibular muscles, the levator arcus palatini and dilator operculi, in wild types express *Eng* in their nuclei beginning during the pharyngula period (30 hours), as they migrate, condense, elongate and form striated muscle fibers by 72 hours (Fig. 4; Hatta et al., 1990) In *chn* mutants, a group of nuclei were labeled in a similar location in the mesenchyme at 30 hours, but did not differentiate as late as 5 days of development, several days after hatching, when the young fry is normally feeding actively. Thus, along with cartilage precursors, at least some muscle precursors derived from the paraxial mesoderm are present in *chn* but do not differentiate.

**Mosaic analysis of *chn* function in the neural crest**

To learn if *chn* mutations block neural crest lineages directly and to define its time of action, we analyzed mosaics, made by transplanting fluorescently labeled wild-type or mutant cells into unlabeled hosts at several different stages (Fig. 5; Table 2). Cells were moved orthotopically into the premigratory neural crest at anterior hindbrain and midbrain levels on only one side of an unlabeled host, of either genotype, and followed until their differentiated phenotypes could be determined.

Wild-type neural crest cells form cartilage in *chn* hosts (Figs 5, 6, 7). When wild-type neural crest cells were transplanted into the chondrogenic region of the premigratory neural crest in embryos derived from crosses between *chn/+* heterozygotes at 12 hours, they formed cartilage in mutant hosts in four of five successful transplants (Table 2), partially rescuing the mutant phenotype. At the time of transplantation the genotype of the host was unknown and was ascertained later by determining if cartilage was present on the unoperated side. By ‘rescue’ we usually mean that some cartilage cells were visible in the mutant host, not that a fully patterned skeletal structure has formed. Typically, cells formed only small clusters of cartilage (Fig. 6) on the transplanted side. However, in one case the cells formed a well developed cartilage bar that was unilateral and stopped abruptly at the ventral midline (Fig. 7). The experiments show that groups of wild-type neural crest cells, transplanted at this stage, differentiate autonomously into cartilage in mutant hosts. The results suggest that cues appropriate to signal cartilage development are present in the mutant environment and that the primary action of the *chn* mutation is directly in the neural-crest-derived cartilage precursor cells.

However, the same analysis showed that adjacent mutant cells also formed cartilage in these mosaics, *chn* cells in this case acting according to the genotypes of their wild-type neighbors (Fig. 5, 6; Table 2). Interestingly, in 100% of rescued embryos, the cartilage contained unlabeled mutant cells in addition to the labeled wild-type cells. Mutant chondrocytes were found as far as ten cell diameters away from the wild-type cells but were always incorporated into a contiguous mass of cartilage. Wild-type cells were always located at the distal ends of rescued masses of cartilage, suggesting that they had migrated through and interacted at close range with differentiating mutant cells. Recruitment of mutant cells into cartilage only occurred in the presence of wild-type cartilage in the mosaic, not other neural-crest-derived cell types (e.g. pigment cells, neurons).

In reciprocal transplants, between *chn* donors and wild-type hosts, mutant cells readily form cartilage (Table 2). In these cases, every mutant cell had a wild-type neighbor. Thus, regardless of the donor or host genotype in mosaics, mutant crest cells could be recruited into cartilage differentiation.

**Table 1. Cell type distribution of cranial neural crest clones in wild type and *chn***

<table>
<thead>
<tr>
<th>Phenotype Position*</th>
<th>Cartilage**</th>
<th>Pigment</th>
<th>Glia</th>
<th>Neuron</th>
<th>Unknown</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type Med. (n=35)</td>
<td>14</td>
<td>8</td>
<td>2</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Lat. (n=36)</td>
<td>0</td>
<td>4</td>
<td>8</td>
<td>25</td>
<td>3</td>
</tr>
<tr>
<td>Total (n=71)</td>
<td>14 (20%)</td>
<td>12 (17%)</td>
<td>10 (14%)</td>
<td>25 (35%)</td>
<td>9 (13%)</td>
</tr>
<tr>
<td><em>chn</em> Med. (n=14)</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>Lat. (n=12)</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>Total (n=26)</td>
<td>0 (0%)</td>
<td>4 (15%)</td>
<td>1 (4%)</td>
<td>9 (35%)</td>
<td>12 (46%)</td>
</tr>
</tbody>
</table>

* Labeled neural crest cells were assigned to the fate map by determining their positions in the premigratory neural crest population at 12 hours (see Schilling and Kimmel, 1994).

** In this and subsequent columns, the number refers to the number of clones that included labeled cells in the indicated structures between 24 and 72 hours. The percentage of total clones with labeled cells is given in parentheses.
Temporal requirements for chn

An interaction that seems to maintain the chondrogenic ability of neural crest cells was revealed by mosaics made only one hour later in development (13 hours) the time when neural crest migration begins. Neither wild-type nor mutant cells form cartilage in mosaics made at that time, while wild type to wild type controls at this same stage often do (Table 2). Whereas transplanted mutant cells migrated successfully into the arch primordium in a wild-type host, they were never incorporated into the developing cartilage (0/10). Crest cells that migrated into other regions in these mosaic embryos formed pigment cells and several neural derivatives. Thus mutant cells, as well as their environment, quickly and specifically lose chondrogenic potential, possibly due to the loss of necessary interactions during migration that were successfully restored in mosaics made earlier at 12 hours.

Surprisingly, when chn embryos were used as hosts in reciprocal transplants at this stage, wild-type cells never formed cartilage. Rather they remained apparently undifferentiated after migrating successfully, for as long as they could be followed (4-5 days). The mesenchymal morphologies of these labeled cells resembled the undifferentiated clones observed in the single cell labeling experiments (see Fig. 3) in chn. Thus

![Fig. 5. Mutant neural crest cells form cartilage in the presence of wild-type neighbors.](image)

(A) 12 hour, lateral view. Neural crest cells from a donor embryo previously marked with lineage tracer dye are shown after transplantation into the premigratory neural crest of an unlabeled host. A number of labelled cells remain in the suction micropipette. (B) 72 hour, ventral view. In a control, wild-type neural crest-derived cartilage is identifiable by its morphology in Meckel's cartilage and the palatoquadrate in a wild-type host. (C) 72 hour, lateral view. A mosaic embryo made by transplanting wild-type neural crest into a chn host. Labelled cartilage cells from the wild-type donor are visible as well as unlabeled cartilage derived from the mutant host (between arrows). (D) 72 hour, ventral view. A mosaic consisting of mutant neural crest transplanted into a wild-type host also shows labelled and unlabelled cartilage. Abbreviations: aa, aortic arch; e, eye; mc, Meckel's cartilage. Scale bars, 100 μm.

![Fig. 6. Wild-type neural crest cells restore patterned cartilage development when transplanted into chn mutants.](image)

72 hour, lateral view. Drawings were traced from computer-combined fluorescence and bright-field images. (A) Wild-type pattern of cartilages and head pigmentation. (B) All pharyngeal cartilages are absent in chn. (C,D) In two examples of mosaic embryos, cartilage derived from wild-type donor neural crest cells (red) contributes to a structure resembling a partial pharyngeal arch, possibly a ceratohyal. Notably, many neighboring, unlabelled, mutant cells have been recruited to differentiate as cartilage.

![Table 2. Tissue distribution of transplanted neural crest in wild type and chn](image)

<table>
<thead>
<tr>
<th>Donor phenotype*</th>
<th>Host phenotype</th>
<th>Medial</th>
<th>Lateral</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cartilage</td>
<td>Pigment</td>
</tr>
<tr>
<td>5 somite‡</td>
<td></td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>wt</td>
<td>wt (n=28)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>wt</td>
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<td>-</td>
</tr>
<tr>
<td>chn</td>
<td>chn (n=10)</td>
<td>-</td>
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</tr>
<tr>
<td>chn</td>
<td>chn (n=6)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1-3 somite</td>
<td></td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>wt</td>
<td>wt (n=25)</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>chn</td>
<td>chn (n=8)</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>chn</td>
<td>chn (n=6)</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>

*Mutant embryos were identified by the lack of jaws and reduced head size at 72 hours.
†Differentiated phenotypes were identified by morphology.
‡Groups of crest cells were transplanted from labeled donors into unlabeled hosts at two different stages as described in Materials and Methods. Transplants were examined soon after grafting to determine their medial/lateral locations. Donor and host were the same age, either 5 somites or 1-3 somites.
the mutant environment had lost the ability to support chondrogenesis.

**Mutant paraxial mesoderm forms muscles in a wild-type environment**

The absence of head muscles in *chn* may be a direct effect of the mutation on paraxial mesoderm, or a secondary effect due to environmental factors, such as the lack of interactions with adjacent neural crest cells. To address this issue, paraxial mesodermal precursors were transplanted between mutant and wild-type embryos (Fig. 8, Table 3). Labeled cells were placed along the involuting, marginal zone of the gastrula so that they later entered the hypoblast that gives rise exclusively to mesodermal and endodermal derivatives (Warga and Kimmel, 1990). Transplants spread extensively along the body axis, contributing to both head and trunk mesodermal tissues or endodermal tissues. We observed that wild-type precursors of the head mesoderm, transplanted at the late blastula stage, do not differentiate into striated pharyngeal muscles in *chn* hosts. In contrast, mutant mesoderm can contribute to head muscles in a wild-type host (Fig. 8). Mutant muscle differentiation did not appear to require adjacent wild-type muscle cells, since, in some cases, mutant cells appeared to constitute entire muscles, such as the adductor mandibulae (am) and intermandibularis posterior (imp) muscles, with no wild-type muscle cells nearby. However, muscles formed from mutant donor cells always neighbored wild-type cartilage and connective tissues.

**DISCUSSION**

We have described recessive, noncomplementing lethal mutations in the zebrafish, *chn* b146 and *chn* pa20, that disrupt the development of pharyngeal arches. Induced by different means both mutations produce similar phenotypes. We infer that both involve a single gene, *chinless*, and that the mutant alleles are severe, perhaps null. *chn* homozygous embryos do not develop neural-crest-derived cartilages or mesoderm-derived muscles in the head. From the early expression of the mutant phenotypes (~30 hours), we infer that *chn* must normally function within the arch precursor populations since differentiation of both of these cell types does not normally occur until mid to late hatching (60-72 hours; Schilling and Kimmel, 1994; Kimmel et al., 1995). Transplanting groups of neural crest cells from wild type into mutants to make mosaic
embryos can partially restore the missing cartilage phenotype. Transplanting wild-type mesodermal cells cannot restore muscles. Based on these results, we suggest that chn normally functions in the specification of chondrogenic neural crest cells, not in mesoderm, and that this specification occurs at an early, premigratory stage.

In addition, at least two types of cell signaling events that involve neural crest appear to be revealed by the mutation. Mutant cells can make pharyngeal cartilage in mosaics, whereas they never do when they develop in mutants, perhaps reflecting 'homeogenetic' induction (Hamburger, 1988) between cartilage precursors, i.e. a developing chondrocyte influences its neighbors to also form cartilage (as discussed further below). Since mutant cells participate productively in this interaction, it is clear that chn does not block the ability of cells to respond, nor does it seem to block the signal. Homeogenetic induction between cartilage cells may be blocked in chn because specified cartilage precursors are not present to initiate it. A second signal, from neural crest to surrounding mesodermally derived myogenic cells, probably occurs later when the two lie together in the pharyngeal primordium (Noden, 1988).

Interestingly, there is an additional temporal aspect of chn action revealed by transplanting neural crest at different stages. The ability for neural crest cells to respond to and probably produce a signal is lost during a narrow window of time as cartilage no longer develops in mosaics, regardless of the host genotype. Thus, at this later stage both the mutant cells and their environment may have lost some aspect of chondrogenic ability. The time period of loss roughly corresponds to the time when neural crest migration begins (Schilling and Kimmel, 1994; Kimmel et al., 1995).

### chn functions in a subset of neural crest derivatives

Cranial neural crest cells participate in the formation of the skeletal components and connective tissue of the face and neck as well as many pigment and neural cell types (D’Amico-Martel and Noden, 1983; Hammond and Yntema, 1964; LeDouarin, 1982; Noden, 1983). The smaller eye size and slightly reduced pigmentation in mutant embryos, along with the cartilage defect, suggests that chn may play some role in the development of the neural crest cells that contribute to these structures. Similarly, eye reduction in the small eye mutation in mice results from defects in midbrain neural crest migraton (Matsuo et al., 1993). It is of particular interest that sensory neurons appear to be specifically unaffected in chn. This specificity is reminiscent of some neural crest mutations, such as Patch (Ph), in the mouse embryo (Morrison-Graham et al., 1992) which similarly spares neuronal derivatives but severely affects crest-derived melanocytes. Ph/Ph mutants have a deletion of the beta-subunit of the receptor for platelet-derived growth factor (Stephenson et al., 1991). This suggests that the function of chn, like Ph, is needed for the normal development of a non-neuronal subset of neural crest derivatives. The absence of the pharyngeal cartilage derivatives of neural crest and not other derivatives in chn, reveals a subtle genetic control over crest cell specification that we propose occurs before migration, as based on our mosaic analyses. By selectively disrupting the development of particular cell types, mutations like chn may also allow us to begin to understand the roles of each cell type in head patterning.

### Multiple cell interactions of the neural crest

The many patterning roles of neural crest in the pharyngeal arches clearly involve a complex network of interactions. However, we can explain the results of our mosaic analyses in a unified way by supposing that the chn phenotype reflects a defect in cartilage specification that results not only in an intrinsic defect in the cells' specification, but also results in mutant cells unable to support subsequent pharyngeal chondrogenesis. This idea is discussed further elsewhere (Schilling and Kimmel, 1994). In the future, by performing heterochronic transplantations, we can distinguish whether this is because mutant cells stop modifying their environment or lose their ability to respond to it.

Taken together, the results suggest that, while the wild-type function of chn may be to autonomously specify pharyngeal chondroblasts, there are several non-autonomous aspects to the mutation, involving both early and late cell signalling. An early specification of a subset of the cranial neural crest might be expected in zebrafish, since cartilage arises from a distinct region of the fate map of premigratory crest and since single cells are clonally restricted to cartilage (Schilling and Kimmel, 1994). Growth factor/receptor systems or extracellular matrix components are obvious candidates for the signals, since they are required for the normal development of subsets of neural crest derivatives in mammalian embryos (Morrison-Graham and Takahashi, 1993). For example, interactions between Steel-factor and c-kit, its receptor, have been demonstrated to influence survival, proliferation and differentiation of melanocytes as well as cells of other tissue types. Members of the TGF-β family of signalling molecules, particularly the bone morphogenetic proteins, are also attractive candidates for mediating such interactions, with known roles in initiation and differentiation of the skeleton in mammals (reviewed in Kingsley, 1994).

### Homeogenetic induction in cartilage

In mosaics made by cell transplantation, chn neural crest cells will only form pharyngeal cartilage in the presence of other wild-type cartilage cells. This suggests that the wild-type pre-cartilage cells homeogenetically induce neighbors, bypassing any deficiency in chn cells. Cartilage precursors may normally interact to provide the necessary specification signals, since wild-type neural crest cells induce differentiation in their mutant neighbors. In the cartilage that forms, mutant cells are not always adjacent to wild-type cells but always have cartilage neighbors. Neural crest cells are known to produce extracellu-
lar matrix material that might mediate such interactions (Weston et al., 1978; Hay, 1981). Similar ‘homeogenetic’ inductive interactions have been observed between floor plate precursors in the CNS (Hatta et al., 1990) and may represent a common mechanism for organizing discrete, coherent groups of cells with similar fates (DeRobertis et al., 1989).

**Muscle induction**

*chn* mesodermal cells can make pharyngeal muscles in a wild-type environment. Because *chn* is required autonomously for cartilage development, we suggest that muscle differentiation may be blocked nonautonomously in *chn* mutants, because necessary cues from surrounding neural-crest-derived cells are absent. In mutants, some mesodermal cells begin to develop along a muscle pathway, as inferred from Eng expression, but then they are blocked. Wild-type neighbors, possibly in the cartilage, may overcome this block, although we have not observed this directly for the Eng-expressing muscles. Since muscles containing mutant cells are always located near wild-type cartilage, we presume that the interactions occur in the pharyngeal arch primordium after neural crest migration.

The results are not unexpected, based on heterotopic transplants of crest cells in avian embryos. In such transplants, ectopic skeletal structures reorganize muscles around them (Noden, 1983). Furthermore, the patterning role of these skeletal and connective tissues may reflect a common type of mesenchymal signal, regardless of their germ layer origins. Experiments on both the limb (Chevallier, 1979; Chevallier and Kienes, 1982; Gumpel-Pinot, 1984) and the head (Noden, 1983) have clearly demonstrated that patterns of myocyte condensation and subsequent muscle growth are dependent upon prepatterns within nonmyogenic connective and skeletogenic tissues. Our results in *chn* are consistent with such a patterning process in the mandibular arch and may add information about its control. Simply the presence of the appropriate neural-crest-derived cells is not sufficient for muscle patterning, but may depend on some crest function that depends on the early function of *chn*.

**REFERENCES**


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