

Jaw and branchial arch mutants in zebrafish I: branchial arches

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SUMMARY

Jaws and branchial arches together are a basic, segmented feature of the vertebrate head. Seven arches develop in the zebrafish embryo (*Danio rerio*), derived largely from neural crest cells that form the cartilaginous skeleton. In this and the following paper we describe the phenotypes of 109 arch mutants, focusing here on three classes that affect the posterior pharyngeal arches, including the hyoid and five gill-bearing arches. In *lockjaw*, the hyoid arch is strongly reduced and subsets of branchial arches do not develop. Mutants of a large second class, designated the flathead group, lack several adjacent branchial arches and their associated cartilages. Five alleles at the *flathead* locus all lead to larvae that lack arches 4–6. Among 34 other flathead group members complementation tests are incomplete, but at least six unique phenotypes can be distinguished. These all delete continuous stretches of adjacent branchial arches and unpaired cartilages in the ventral

midline. Many show cell death in the midbrain, from which some neural crest precursors of the arches originate. *lockjaw* and a few mutants in the flathead group, including *pistachio*, affect both jaw cartilage and pigmentation, reflecting essential functions of these genes in at least two neural crest lineages. Mutants of a third class, including *boxer*, *dackel* and *pincher*, affect pectoral fins and axonal trajectories in the brain, as well as the arches. Their skeletal phenotypes suggest that they disrupt cartilage morphogenesis in all arches. Our results suggest that there are sets of genes that: (1) specify neural crest cells in groups of adjacent head segments, and (2) function in common genetic pathways in a variety of tissues including the brain, pectoral fins and pigment cells as well as pharyngeal arches.

Key words: zebrafish, pharyngeal arch, neural crest, cartilage

INTRODUCTION

Development of the jaw and branchial arches in vertebrate embryos involves a hierarchy of cell movements and interactions between neural crest and mesodermal mesenchyme, and surrounding epithelia (for reviews, see Horstadius, 1950; Noden, 1988; LeDouarin et al., 1994). Cranial neural crest cells arise from dorsal and lateral regions of the neural ectoderm and migrate ventrally to surround the pharynx, eventually forming a reiterated pattern of skeletal, neural and connective tissues in the pharyngeal arches. Surrounding mesoderm forms muscles and endothelia (Noden, 1983, 1988). During development, cells in each arch segment primordium must in some way assess their position and form the appropriate cell type. To understand the molecular mechanisms that specify cell fates in the arches, we are identifying genes whose products are necessary for pattern formation in the jaw and gills of the zebrafish.

The initial specification of arches is linked to early events that establish segmentation in the entire neural ectoderm,

including neural crest. Neural crest-derived sensory neurons and bones are segmentally arranged (Stone, 1922; Goodrich 1930) and their precursors emerge from distinct segmental levels of the hindbrain, presumably conveying segmental information from the ectoderm to the periphery (Hunt et al., 1991a,b; Lumsden et al., 1991; Serbedzija et al., 1992; Schilling and Kimmel, 1994). Evidence for a neural crest contribution to the cranial skeleton has been obtained in members of most vertebrate classes (reviewed by Smith and Hall, 1990). Heterotopic grafting of neural crest in avian embryos has shown that cells are committed to particular segments before they emigrate from the neural tube and can act as signalling centers that organize surrounding tissues, including mesoderm-derived myocytes (Noden, 1983, 1988). During migration, neural crest cells also undergo epithelial-mesenchymal interactions that play important roles in their migration and differentiation (Hall, 1980, 1987). However the nature of the various signals involved and their genetic bases remain largely unknown.

Several genes that function in pharyngeal development have been identified in the mouse embryo. The best studied of these are the *Hox* genes, whose mutant phenotypes include homeotic conversions of arch segments as well as defects in specific subsets of neural crest derivatives. For example, loss-of-function mutations in more anteriorly expressed members of the *Hoxa* cluster, *Hoxa-1* (Lufkin et al. 1991; Mark et al., 1993; Chisaka et al., 1992), *Hoxa-2* (Rijli et al., 1993; Gendron-Maguire, 1993) and *Hoxa-3* (Chisaka and Cappechi, 1991), all disrupt the pattern of arches. In *Hoxa-2* mutants many skeletal elements of the second (hyoid) arch are transformed into first (mandibular) arch structures, suggesting that this gene specifies the identities of neural crest cells that form the hyoid cartilages and bones. Such findings have led to the proposal that patterning of the branchial region of the head is regulated by a combinatorial *Hox* code (Hunt et al. 1991b; Krumlauf, 1994). Additional genes shown to have essential roles in arch patterning include other transcription factors such as *dlx-2* (Qiu et al., 1995) and *msx-1* (Satokata and Maas, 1994), and retinoic acid and its receptors (Lee et al., 1995; Lohnes et al. 1994).

Despite these advances, a limited number of genes involved in development of the vertebrate head have been identified by sequence homology. *Drosophila*, from which most developmental genes were first identified by genetic screening, and other invertebrates have neither a neural crest nor many of the cell types that it forms in the head (Gans and Northcutt, 1983). Therefore, random mutagenesis is an important alternative approach for finding genes involved in head development. Such an approach has only recently become feasible on a large scale with the zebrafish (Kimmel, 1989; Mullins et al., 1994; Solnika-Krezel et al., 1994), where one can screen embryos and larvae for recessive, lethal mutations. Pharyngeal arches are visible in the larval fish at low magnification and can be screened in large numbers. There is a detailed fate map for the arches, and single cells generate progeny restricted to particular segments and cell types (Schilling and Kimmel, 1994). Previous zebrafish screens using haploids have isolated three mutations, *chinless*, *jawless* and *shorttail*, with embryonic arch defects. Lineage tracing of neural crest cells as well as mosaic analyses in some of these mutants have shown that they disrupt early specifications of cartilage precursors and their interactions with surrounding tissues (Schilling, 1993; Schilling et al., 1996; Schilling, unpublished data).

In this study, we describe 59 zygotic, lethal mutations that have their most severe defects in the posterior arches, the hyoid and branchial arches. In the following paper we consider mutants that affect the more anterior arches as well as a large class with defects in chondrogenesis (Table 1). The mutants described here identify at least eleven different genes, including five classes (Table 3). Homozygous mutant fish develop abnormal patterns of differentiated cartilage at 5 days of development, including defects in: (1) several adjacent arches; (2) midline cartilages; (3) brain, fins and arches; and (4) pigmentation and arches. We propose that many of these mutants identify genes that are required during wild-type development to specify the fates of neural crest cells in groups of adjacent segments and, in some cases, in specific neural crest cell lineages.

MATERIALS AND METHODS

Generation of pharyngeal arch mutants

The general methods for growing fish, collecting eggs, raising embryos and making mutants were described previously (Westerfield, 1993; Mullins et al. 1994; Haffter et al., 1996). We screened for recessive, non-conditional mutants with abnormal head morphologies at 5 days and most are larval-lethals. The mutations were generated in the Tübingen wild-type background by mutagenesis of males with

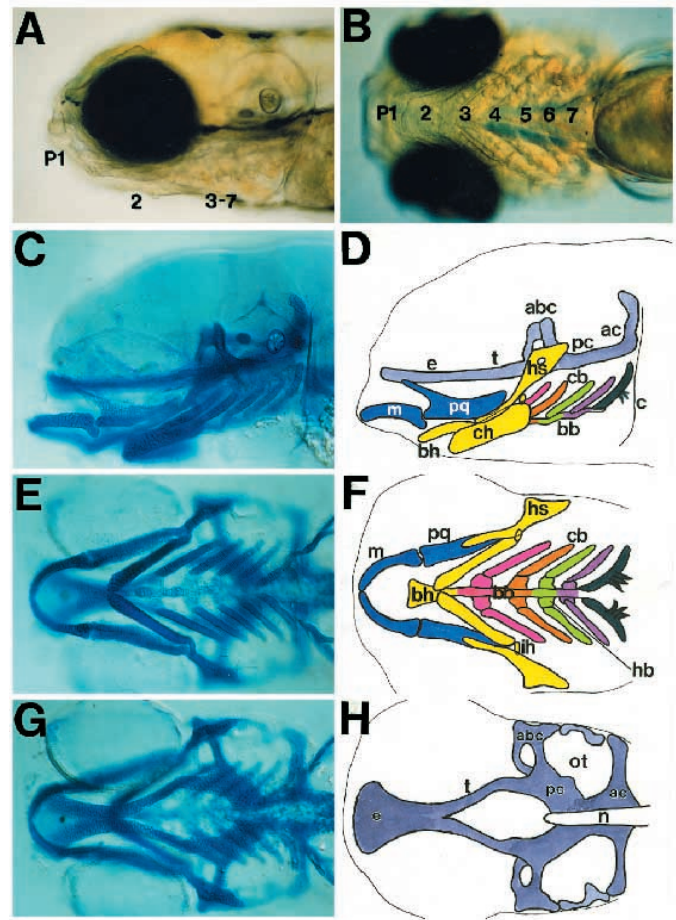


Fig. 1. Anatomy of the pharyngeal arches and head skeleton in wild type. (A) Lateral view of the head of a living larva at approximately 6 days (the stage screened for mutations), showing the extended jaw and branchial arches, P1-P7. (B) Ventral view of the same larva, photographed using Nomarski optics. The lower jaw, P1, and basihyal, P2, are visible anteriorly. Bilateral branchial arch segments, P3-P7, extend posterolaterally from the midline. (C-G) Photomicrographs and corresponding diagrams of the larval pattern of cartilages, stained with Alcian blue and whole-mounted. The dye outlines individual chondrocytes. The pharyngeal skeleton is shown in lateral (C,D), and ventral (E,F) views. The neurocranium is shown in a more dorsal focal plane of the ventral view in E. Cartilages of the same segment share the same color: P1 (mandibular, blue), P2 (hyoid, yellow), P3 (first branchial, pink), P4 (orange), P5 (green), P6 (purple), and P7 (black). The neurocranium is shaded uniformly grey. abc, anterior basicranial commissure; ac, auditory capsule; bb, basibranchial; bh, basihyal; c, cleithrum; cb, ceratobranchial; ch, ceratohyal; e, ethmoid plate; hb, hypobranchial; hs, hyosymplectic; ih, interhyal; m, Meckel's cartilage; n, notochord; ot, otic capsule; pc, parachordal; pq, palatoquadrate; t, trabeculae cranii.

ethylnitrosourea, and identified in the progeny of F₂ families generated from the originally mutagenized founders.

Head morphology was screened under a dissecting microscope at approximately 80× magnification. Anaesthetized larvae (25 on average) from each F₂ cross were first examined laterally, to view the extended jaw and gills, and then ventrally, for a better look at the jaw suspension, the hyoid arch and the ventral midline (Fig. 1A,B). This simple screen is sufficient to detect the position of the lower jaw, the prominent basihyal and ceratohyals, and gill filaments on posterior arches.

Histological and skeletal analyses

Upon recovery in the rescreen, mutant embryos were examined at earlier stages and higher magnification to determine their detailed morphological defects. Embryos were staged according to Kimmel et al. (1995).

For skeletal preparations, we followed the methods of Dingerkus and Uhler (1977) and Kelly and Bryden (1983) using Alcian blue (Sigma), a dye that stains the extracellular matrix associated with chondrocytes. This provides a clear marker of the skeletal pattern beginning at late hatching (approx. 72 hours). Anaesthetized larvae were fixed in 3.7% neutral buffered formaldehyde (pH 7.0) at room temperature for several hours to overnight, and then transferred into a 0.1% solution of Alcian blue dissolved in 80% ethanol/20% glacial acetic acid. After staining in this solution overnight, embryos were rinsed in ethanol and rehydrated gradually into phosphate-buffered saline. Tissues were cleared in 0.05% trypsin dissolved in a saturated solution of sodium tetraborate for 1–3 hours. Pigmentation was then removed by bleaching in 3% hydrogen peroxide/1% potassium hydroxide for several hours. Stained preparations were mounted in 70% glycerol and photographed with a Zeiss Axiophot microscope.

The nomenclature of cartilages and bones used in the present studies was based primarily on descriptions of the close relative of the zebrafish, *Barbus barbus* (Vandewalle et al., 1992) and of Medaka (Langille and Hall, 1988), both of which are adapted from older terminologies including those of DeBeer (1937) and Nelson (1969).

Phenotypic classification and complementation tests

Mutants assigned to the phenotypic class of 'jaw and gill' defects (Haffter et al., 1996) were classified into subgroups based on both their live phenotypes and skeletal preparations. This simplified the complementation analysis, since only mutants with similar phenotypes were initially crossed with each other. Mutants in other classes, for example pigmentation, that also had head defects were subsequently analyzed and classified. Two large categories of mutants were distinguished early on in the screen, 'flatheads' and 'hammerheads', and complementation tests were begun within these groups. To date this is approx. 40% complete for the flathead group and 60% for the hammerhead group.

RESULTS

We isolated 109 mutations that disrupt pharyngeal arch development in the zebrafish. Among them, 59 most severely affect the posterior arches, including the hyoid and five branchial segments that support the gills. Many of the mutants also have defects in the nervous system, pectoral fins or pigmentation. The crossing scheme used to detect zygotic mutations and the proportions of different mutant classes is reviewed elsewhere (Haffter et al., 1996). We screened diploid larvae at 5 days, by

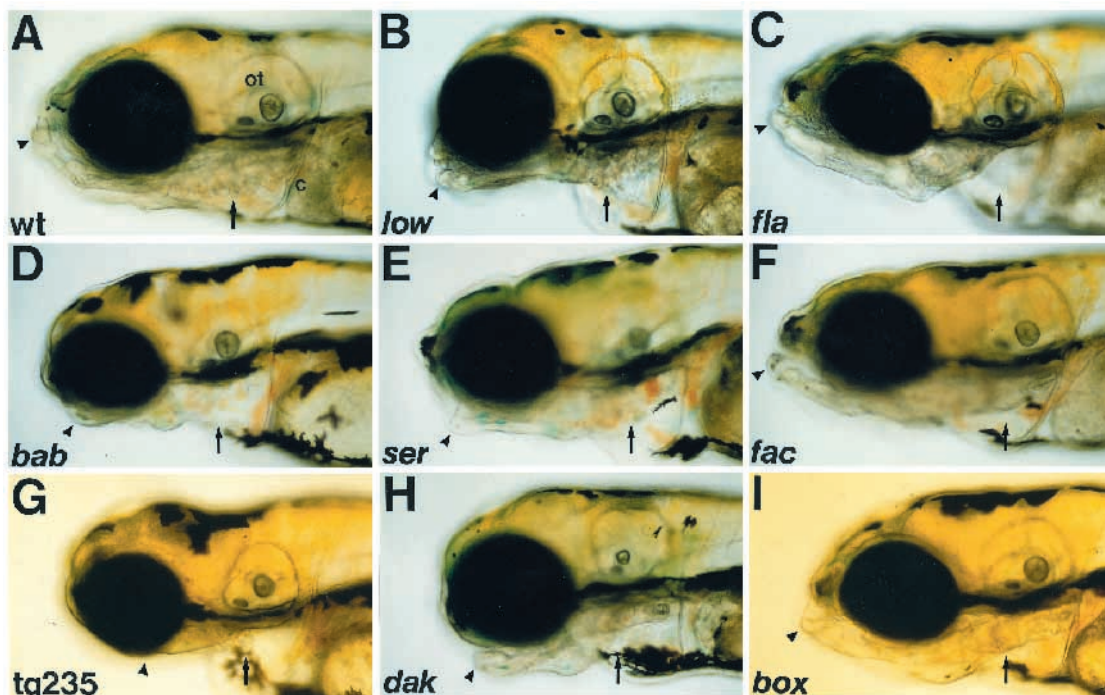


Fig. 2. Mutant phenotypes in living larvae. Lateral views at 6 days. Arrows indicate branchial arches, P3–P7, which are reduced in all of these mutants. Arrowheads indicate the lower jaw. (A) wild type, wt. The jaw extends anterior to the eye and gill filaments of the branchial arches, lined with blood vessels, protrude ventral to the otic vesicle (ot). (B) *low^{ts213}*. The jaw is shifted ventrally and some gill filaments remain. (C) *flat^{21c}*. Gills are severely reduced. (D) *bab^{tb210c}*. The head is severely reduced, the jaw does not extend and gills are absent. (E) *ser^{ak18c}*. The jaw is reduced, the mouth hangs open and gills are slightly reduced. (F) *fac^{uu45b}*. Only gills are reduced. (G) *tq235*. All arches are absent and the mouth is displaced posteriorly. (H) *dak^{l25}*. All arches are shorter and wider than normal and the jaw is displaced posteriorly. (I) *box^{tm70g}*. The jaw hangs slightly and gills are reduced.

which time the head is well developed and the young fry begin feeding (Kimmel et al., 1995). Retardation problems associated with screening for such late phenotypes may have resulted in lower allele frequencies for these mutants than for other classes.

Two large mutant classes emerged initially: the ‘flathead-group’, with reduced eyes, brain and jaw, and the ‘hammer-head group’, with reduced jaws but normal eyes and brain (Table 1). These and other mutations were classified to groups according to their live phenotypes and skeletal preparations. We have used this classification scheme to simplify presentation, although similarities among the phenotypes may be misleading in some cases. Of the 109 mutants, 44 were assigned to 12 complementation groups (including 3 flatheads and 4 hammerheads). An additional 14 mutants had distinct phenotypes. The remaining 51 mutants fell into either the flathead or hammerhead groups; these have such similar phenotypes within a class that complementation among them must be performed randomly. In these cases only a few representative members will be described.

Complementation analysis among the 59 mutant strains with more ‘posterior’ phenotypes, including the flathead group, has defined five complementation groups, *babyface* (*bab*), *flathead* (*fla*), *dackel* (*dak*), *boxer* (*box*) and *pistachio* (*pio*), and seven single mutants with distinct phenotypes: *lockjaw* (*low*), *screamer* (*ser*), *facelift* (*fac*), *howler* (*her*), *compressed* (*cod*), *duckbill* (*dul*), *pinscher* (*pic*). In each case, a description of the live phenotype is followed by a detailed analysis of the head skeleton from Alcian blue preparations and in some cases sectioned material.

Wild-type head skeleton

The chondrocranium in a larval zebrafish (approx. 6 days) consists of seven pharyngeal arches and a neurocranium underlying the brain and surrounding the anterior notochord and otic capsule (Fig. 1; Table 2). This skeleton forms from primordia in the embryo between early and late hatching stages (2-3 days) of development, along with its associated muscles and nerves (Schilling, 1993; Schilling and Kimmel, 1994). Larval and adult skeletal development have been described in detail (Cubbage and Mabee, 1996) and follow a sequence similar to that described in other ostariophysines (Vandewalle et al., 1992) and in Medaka (Langille and Hall, 1987, 1988). Arches are designated P1-P7 in order from anterior to posterior, roughly the same order in which they develop. Only the most posterior arch, P7, appears to differentiate before many of its more anterior branchial arch neighbors (see Discussion). Each of the posterior branchial arches, except perhaps P7, eventually supports a set of filamentous gills.

In wild type, the first (mandibular, P1) arch forms the jaw, consisting of two bilateral cartilages (Fig. 1C-F). Anteriorly, Meckel’s cartilages (m) form single rows of chondrocytes that meet in an arc in the midline underlying the ventral mouth epithelium. These articulate posteriorly with palatoquadrates (pq), which are triangular and up to ten cells wide. Dorsal processes of the palatoquadrates articulate with the ethmoid plate and flank the roof of the mouth. The posterior ends of the palatoquadrates extend beneath the eyes to articulate with the hyosymplectics of the hyoid arch.

The second (hyoid, P2) arch supports the jaw. Ventral cartilages in the hyoid arch include an unpaired basihyal (bh) in the midline, which fans out in its anterior aspect. This chon-

Table 1. Results of the screen for jaw mutants

Phenotypic class	Genes	Unresolved	General description	References
‘flatheads’	<i>fla</i> (5), <i>bab</i> (2), <i>ser</i> (1), <i>her</i> (1), <i>cod</i> (1), <i>dul</i> (1), <i>fac</i> (1), <i>pio</i> (5)	27	Head flattened dorsoventrally, small eyes and brain, loss of post. arches.	a b c
Anterior	<i>stu</i> (2), <i>son</i> (1), <i>sel</i> (1), <i>suc</i> (1), <i>ham</i> (2), <i>hen</i> (3), <i>pek</i> (1), <i>get</i> (2), <i>jeff</i> (1), <i>koi</i> (1), <i>pit</i> (4)	13	Reduction of first two arches. No tissue anterior to the eyes. Cartilage differentiation affected.	d e d f
Other	<i>low</i> (1), <i>box</i> (8), <i>dak</i> (4), <i>dol</i> (1), <i>hot</i> (5), <i>pic</i> (1), <i>vgo</i> (2)	11		a d g h e

References: a, Schilling et al. (1996); b, Furutani-Seiki et al. (1996); c, Kelsh et al. (1996); d, Piotrowski et al. (1996); e, Whitfield et al. (1996); f, Hammerschmidt et al. (1996); g, Trowe et al. (1996); h, van Eeden et al. (1996a).

Table 2. Composition of the cartilaginous pharyngeal skeleton and neurocranium of *Danio rerio*, grouped by region

Region	Cartilages
Pharyngeal skeleton	
Pharyngeal arch 1 (P1)	Meckel’s cartilage
Mandibular	Palatoquadrate
Pharyngeal arch 2 (P2)	Hyosymplectic
Hyoid	Interhyal Ceratohyal Basihyal
Pharyngeal arch 3 (P3)	Ceratobranchial 1
Branchial (gill) arch 1	Hypobranchial 1 Basibranchial 1
Pharyngeal arch 4 (P4)	Ceratobranchial 2
Branchial (gill) arch 2	Hypobranchial 2 Basibranchial 2
Pharyngeal arch 5 (P5)	Ceratobranchial 3
Branchial (gill) arch 3	Hypobranchial 3 Basibranchial 3
Pharyngeal arch 6 (P6)	Ceratobranchial 4
Branchial (gill) arch 4	Hypobranchial 4 Basibranchial 4
Pharyngeal arch 7 (P7)	Ceratobranchial 5
Branchial (gill) arch 5	Basibranchial 5
Neurocranium	
Anterior	Ethmoid plate Trabeculae cranii Anterior basicranial commissure Orbital cartilage
Posterior	Posterior basicranial commissure Basal plate Parachordal cartilage Otic capsule Occipital arch

drifies between the anterior ends of the large ceratohyals (ch), one to three cells in diameter, that extend posterolaterally to contact the interhyals (ih). The small interhyal forms the articulation between the ceratohyal and more dorsally located

hyosymplectic. Ventrally located symplectics, made up of single rows of chondrocytes, are joined with more dorsal, thicker cartilages of the hyomandibular to form an elongated triangle, the hyosymplectic (hs). A hole in the hyosymplectic (the facial foramen) allows passage of branches of facial and lateral line nerves. Dorsally, hyosymplectics articulate with the anterior edge of the auditory capsule and suspend the jaw.

Posterior to the first two pharyngeal arches the skeleton of the branchial arches, P3-P7, is smaller and arranged in a more simple segmented pattern. Homologous cartilages in each segment are largely indistinguishable. Three major elements are present in each of the first four branchial arches, P3-P6, one unpaired in the midline and two bilateral. In the midline, basibranchials (bb) are fused with anterior and posterior neighbors to form continuous medial rows joining groups of segments. These are joined laterally to hypobranchials (hb), which are small, circular groups of chondrocytes. Finally each branchial arch has its ceratobranchial (cb) that extends dorsolaterally. An exception to this reiterated pattern occurs in P7, where only ceratobranchials and a small basibranchial can be distinguished. P7 is also the only arch with pharyngeal teeth, three on each side.

Anteriorly, the neurocranium consists of a large, flat ethmoid plate (e) between the eyes and beneath the forebrain. The ethmoid plate forms as an anterior fusion of bilateral cartilage rods, the trabeculae cranii (t), that remain separate posteriorly beneath the midbrain and anterior hindbrain, surrounding the hypophyseal fenestre. Posteriorly, trabeculae merge into the parachordals (pc) that surround the anterior end of the notochord and form the basal plates. Laterally, the ear becomes surrounded by the occipital arches and auditory capsules (ac). Although not stained with Alcian blue, several ossifications of the dermatocranium are also present at this stage (Cubbage and Mabee, 1996) including a cleithrum (c) that extends dorsoventrally just posterior to P7. Branchiostegals form ventral to P3 and P4 (not shown). A midline parasphenoid and parachordal ossifications are also detectable. Here we only consider the cartilaginous skeleton. Chondrocytes are indistinguishable by morphology, so that only overall shape, size and location can be used as criteria for individual arch identities.

Mutant phenotypes

All of the mutants described are recessive lethals. Homozygous mutants fail to form swim bladders and die within one week of development (Haffter et al., 1996). The trunk and tail as well as internal organs develop normally in mutants unless mentioned otherwise.

Hyoid and branchial deficiencies in *lockjaw*

One gene with a unique phenotype is represented by a single allele designated *lockjaw* (*low^{ts213a}*) (B in Figs 2-6; Tables 3, 4). The *low* phenotype can be distinguished when pigmentation begins, during the pharyngula period (approx. 30 hours) as a slight reduction in the number of melanocytes and a reduced head. Subsequently, mutants develop a ventrally protruding jaw with restricted movements and branchial arches with very few gill filaments (Fig. 2B). The head and eyes remain slightly reduced and melanocytes are reduced throughout the body.

To determine skeletal defects in *low* we compared the pattern of cartilage in mutant larvae with their wild-type siblings using Alcian blue. In lateral view, cartilages of P1 in

low appear normal in size, but displaced. Meckel's cartilages often fuse in the midline and palatoquadrates are shifted posteriorly and dorsally (Fig. 3B). The hyosymplectic is severely reduced or absent in *low* mutants and, in its place, ectopic contacts form between the posterior end of the palatoquadrate and the auditory capsule. We cannot tell if this is merely contact or an ectopic articulation, but there is no fusion, as determined in sections (data not shown). In ventral view the mutant skeleton is broader and obviously less extended than wild type (Fig. 4B). Ventral elements of P2 are reduced and curve posteriorly, ceratohyals fusing with adjacent cartilages both medially and laterally, and interhyals are absent (Fig. 6B). *low* mutants lack basibranchials, hypobranchials and ceratobranchials of subsets of the branchial arches, generally P4-P6, although in some less extreme cases ceratobranchials of P4 are present. Thus the *low* mutation disrupts hyoid and branchial development (P2-P6), yet leaves the morphology of its posteriorly shifted jaw (P1) fairly intact.

In addition to these ventral, pharyngeal defects, the neurocranium is reduced in *low* mutants (Figs 5B, 6B), the ethmoid plate is divided in the midline and there is subtle reduction of the trabeculae and the posterior basicranium. Typically the ossified parasphenoid extends anteriorly through the abnormal gap in the ethmoid plate. Other ossifications develop normally.

flathead mutants lack groups of branchial arches

Within the flathead group, five *flathead* (*fla*) alleles were identified (C in Figs 2-6; Tables 3, 4). *fla* mutants have dorsoventrally compressed heads, small eyes and few to no branchial arches (Fig. 2C). Mutants are first detected at early hatching stages (approx. 60 hours). Temporary, mild midbrain necrosis occurs in mutant embryos at this stage. In all *fla* mutants, P1 and P2 are much less affected than more posterior segments.

Of the various alleles of *fla* only one, *fla^{ta53c}*, shows a slightly less severe skeletal phenotype. Cartilages of P1 and P2 are all present but reduced. In ventral view, the skeleton is narrow and pointed (Fig. 4C), in contrast to *low*. Ventral elements, including Meckel's cartilage and the ceratohyals, narrow and join in a point. Ceratohyals often extend postero-medially, joining the midline close to P3. A basihyal is typically absent. This gives the appearance in living mutant embryos of a fusion medially between the ceratohyals and anteriormost ceratobranchials that resembles the letter X (not shown). Such an apparent fusion is also seen in other members of the flathead group (see below).

Several branchial arches are deleted in *fla*, forming a gap in the arch series (Fig. 4C). Basibranchials, hypobranchials and ceratobranchials in P4-P6 do not form in mutants while single arches anterior (P3 or P4) and posterior (P7) to them always do. Typically, P3 consists only of ceratobranchials in which the more medial end is displaced posteriorly, and often fused with ceratohyals. P7 and some teeth are always present. The neurocranium of *fla* is usually only slightly reduced (Fig. 5C). All other mutations in the flathead group (33 mutants) complement *fla*.

Mutants of the flathead group alter pharyngeal segments and the ventral midline

Similarities among the skeletal phenotypes of the 43 members of the flathead group (including *fla*) indicated that the same

groups of branchial segments were always affected in many different mutants (Table 4). Many flathead group members also lack the ventral, unpaired cartilages including the basihyal and basibranchials associated with P3-P7. These form slightly later than more lateral, paired cartilages and are fused to form continuous columns lying just ventral to the pharyngeal cavity. Absence of midline cartilages correlates with a posterior shift in ceratohyals and ceratobranchials in many of the mutants.

Despite similarities among them, at least six distinct phenotypes were discernible among the flathead group, based primarily on the head skeleton. These will be considered from most to least severe.

babyface (*bab^{tb210}*, *bab^{tw220c}*)

The strong allele of the *bab* mutation, *bab^{tb210}*, along with another similar one, *tm102e*, has the most severe reduction in head and eyes in the flathead group (D in Figs 2-6; Tables 3, 4). In mutants, during the pharyngula period (approx. 30 hours), the midbrain and pectoral fin buds are reduced. The eyes are elliptical, rather than round as in wild type, and by early hatching mutants have less tissue below the eyes. The head remains small and the jaw never extends (Fig. 2D). In a weaker allele, *bab^{tw220c}*, defects are primarily in anterior arches. Meckel's cartilage hangs ventrally and is fused to the palatoquadrate.

Pharyngeal and neurocranial skeletons in *bab^{tb210}* are espe-

cially shortened in the anterior-posterior dimension. In skeletal preparations, all cartilages of the mandibular arch, P1, form and make appropriate contacts, but are half the size of wild-type. Meckel's cartilage protrudes ventrally under the posteriorly located mouth (Fig. 3D). In ventral view, *bab* mutants have small, kinked ceratohyals that join in the midline and point caudally (Fig. 4D). A tiny basihyal is often present. Branchial arches, P3-P7, are typically absent in *bab* although basibranchials or ceratobranchials of the most anterior ones occasionally develop. Single teeth are sometimes found, remnants of P7.

In *bab* mutants the neurocranium is reduced to 60-70% of wild type. The ethmoid plate is very small, with lateral protrusions (Fig. 5D). Behind the ethmoid plate, the trabeculae cranii lie unusually far apart. The posterior basicranium, around and behind the otic capsule, is severely reduced. The anterior end of the notochord extends further anterior to the parachordals than in wild type.

screamer (*ser^{tk18c}*)

Some mutations in the flathead group are unique in having open mouths and lacking caudal gill segments. These include *ser* and at least two others (*tm042d*, *tg239a*; Tables 3, 4) although complementation between them has not been tested (E in Figs 2-6). The *ser* phenotype can be distinguished late in the hatching period (approx. 72 hours) by its smaller head and eyes. The mouth lies further posteriorly in *ser* than wild type

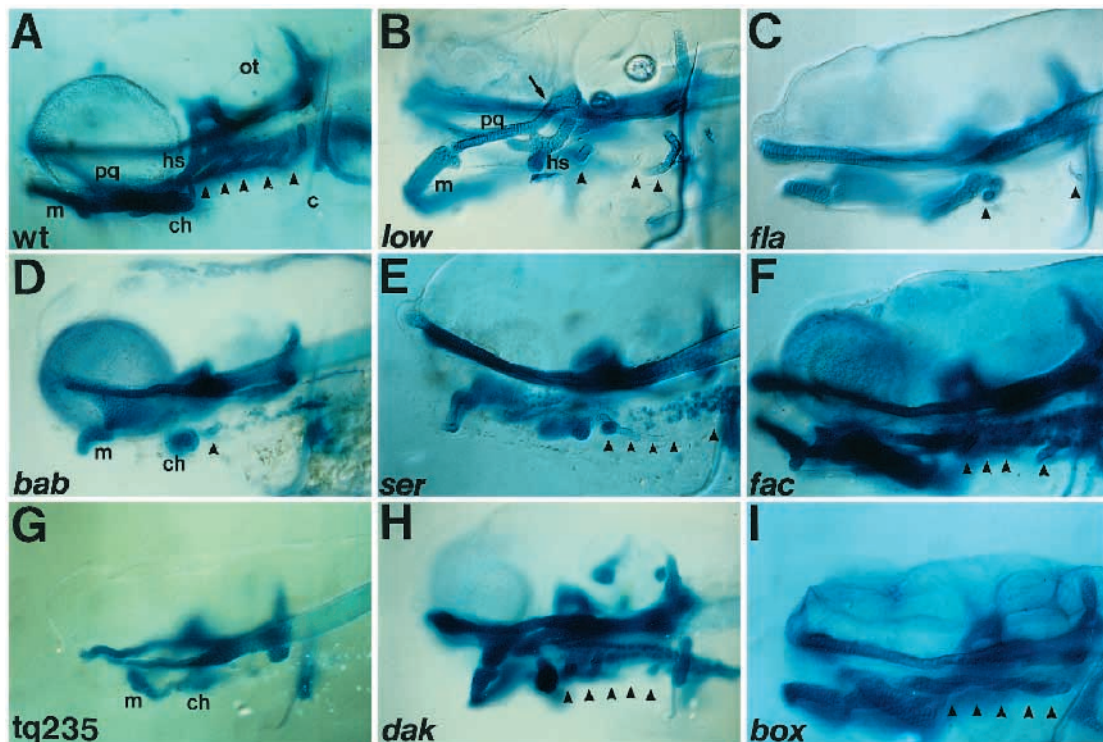


Fig. 3. Skeletal defects in mutants (lateral view). Cartilage in 6-day-old larvae is stained with Alcian blue and whole-mounted. Arrowheads indicate the positions of branchial arches. (A) Wild type, wt. There are five branchial arches, P3-P7. (B) *low*. Two branchial arches, P4 and P5, are absent. Meckel's cartilage hangs ventrally, the hyosymplectic is reduced and the palatoquadrate makes ectopic contact with the neurocranium (arrow). (C) *fla*. Branchial arches P4-P6 are absent. (D) *bab*. All branchial arches are absent except the basibranchial of P3. Meckel's cartilage hangs ventrally and the ceratohyal is reduced. The neurocranium is severely reduced. (E) *ser*. One branchial arch, P6, is absent. The ceratobranchial of P3 and basibranchials of P4 and P5 are present, as is a single tooth of P7. (F) *fac*. P6 is absent. (G) *tq235*. All branchial arches are absent. Anterior arches and the neurocranium are severely reduced. (H) *dak*. All branchial arches are reduced. Meckel's cartilage hangs ventrally. (I) *box*. All branchial arches are reduced.

and tiny Meckel's cartilages hang ventrally (Fig. 2E). Dorsally, palatoquadrates of P1 and hyosymplectics of P2 are reduced. In P2 a basihyal does not form and the small ceratohyal extends posteriorly, joining P3 in an X, as mentioned above.

In contrast to *bab*, however, *ser* mutants develop remnants of branchial arch segments and have a well developed midline (Fig. 3E). P3 is always present and only P6 is absent. Other branchial arches often develop small nodules of chondrocytes (Fig. 6E), remnants of either detached hypobranchials or ceratobranchials. Basibranchials in the ventral midline form well differentiated continuous rows. The neurocranium is slightly reduced, particularly the ethmoid plate, which forms a tripartite anterior extension (Fig. 5E).

howler (*her^{tn14}*)

Like *ser*, *her* mutants (Fig. 6F) have open mouths and reduced brains and eyes, though less extreme than *low* or *bab*. *her* mutants at hatching have small eyes and dorsally curved tails. Bilateral cartilages in the mutant are severely reduced in all arch segments but, like *ser*, the midline is well developed (Fig. 6F; Table 4). Short, thick cartilages make up P1 and P2. There are neither interhyals nor a basihyal in the midline. However, mutants lack all but the midline components of each of the branchial arches (P3-P7), and these are fused as in wild type. The only exception to the complete lack of more lateral branchial arch structures is the formation of small teeth on P7. The neurocranium in *her* shows only a slight reduction in size and narrowing of the ethmoid plate.

facelift (*fac^{tu45b}*)

Among the less severe phenotypes in the flathead group is *fac*, first detected at late hatching (approx. 72 hours) by its slightly small eyes and lack of an obvious basihyal element in ventral view, and later by reductions in gills (F in Figs 2-5, 7A). Skeletal preparations reveal a basihyal that protrudes dorsally to contact the neurocranium (Fig. 3F). This dorsal displacement is accompanied by displacements of the ceratohyals dorsally. Otherwise, branchial arches P5-P7 are reduced and only P6 is absent. The neurocranium in *fac* mutants develops normally (Fig. 5F).

compressed (*cod^{tj13c}*)

Homozygous *cod* mutants typically show arch reductions but do not lack whole cartilage elements (Fig. 7B; Table 4). These pharyngeal defects are detected during day 4 of development after much differentiation has occurred. However *cod* can be distinguished from other flatheads at early hatching, by its dull iridophores. The skeletal phenotypes of *cod* mutants indicate that hypobranchials are missing in every branchial segment (P3-P7). Ceratobranchials are also reduced, most strongly in posterior arches. Mandibular and hyoid arches are only mildly reduced, as is the neurocranium.

duckbill (*dul^{th34c}*)

dul mutants are distinct among the flathead group in having reduced pectoral fins and heart defects at late hatching stages (Fig. 7C; Tables 3). The entire head skeleton is reduced. In ventral view, the pattern of pharyngeal arches resembles

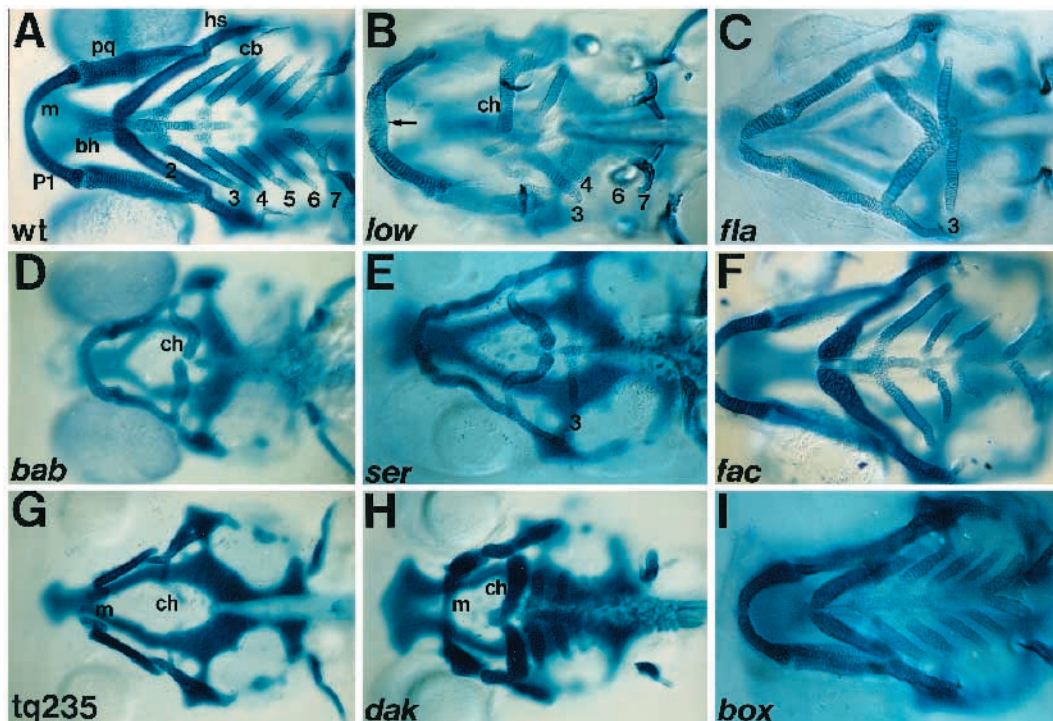


Fig. 4. Skeletal defects in mutants (ventral view; pharyngeal arches). (A) Wild type, wt. The seven arch segments contain 13 bilateral and 6 midline cartilages. (B) *low*. The ceratohyal and ceratobranchials are reduced, P5 is absent. P7, including teeth, is well developed. Meckel's cartilages fuse in the midline (arrow). (C) *fla*. All arches are reduced. Meckel's cartilages are narrow and pointed. Ceratohyals point posteriorly and contact ceratobranchials of P3. There are single teeth on P7. (D) *bab*. Branchial arches are absent except the basibranchial of P3. Anterior arches are reduced, ceratohyals kinked and posteriorly displaced. (E) *ser*. Ceratobranchials of P4-P7 are absent; basibranchials are present. Anterior arches are reduced, ceratohyals kinked and posteriorly displaced. (F) *fac*. Branchial arches, P4-6, are reduced. (G) *tq235*. Branchial arches are absent. Anterior arches are severely reduced. (H) *dak*. All arches are abnormally short and wide, narrowing medially. (I) *box*. All arches are shorter and wider than wt.

Table 3. Overview of mutants that affect branchial arches

Gene name	Alleles	Day*	Arch phenotypes	Other phenotypes	References
I. Ventrally displaced mouth, loss of gills					
<i>lockjaw (low)</i>	<i>ts213</i>	1	Reduced hyoid, some branchial arches absent	Melanophore differentiation, reduced neurocranium	a
II. Flat head, loss of gills					
<i>flathead (fla)</i>	<i>ta53c, tf21c, th5b, ty76a, tu255e</i>	2	Ant. reduced, post. reduced or absent; ch extends post. and dorsally	Small eyes and brain, slight midbrain necrosis; reduced ethmoid plate	b
<i>babyface (bab)</i>	<i>tb210c, tw220c</i>	2	Ant. severely reduced, post. reduced or absent	Small eyes and brain; reduced neurocranium; reduced pec. fins	
<i>screamer (ser)</i>	<i>tk18</i>	3	Ant. reduced, post. reduced or absent	Small eyes and brain	
<i>howler (her)</i>	<i>tn14</i>	3	Ant. reduced, post. reduced or absent	Small eyes and brain	
<i>facelift (fac)</i>	<i>tu45b</i>	3	Ant. reduced, post. reduced or absent	Small eyes and brain	
<i>compressed (cod)</i>	<i>tj13c</i>	2	Post. reduced; ch kinked; no bh	Small eyes and brain; iridophore differentiation	a
<i>duckbill (dul)</i>	<i>th34c</i>	3	Ant. reduced, post. reduced or absent	Small eyes and brain; short pectoral fins; heart edema	
<i>pistachio (pio)</i>	<i>tb202, tc276c, tk17, to75a, ts41</i>	2	Ant. reduced, post. reduced or absent	Small eyes and brain; xanthophore and iridophore differentiation	a
Unresolved:	<i>ty103a</i>	1	Ant. and post. reduced	Small eyes and brain; tail curls up; heart edema	
	<i>ta42</i>	2	Post. reduced; ch extend dorsally	Small eyes and brain	
	<i>tv60c</i>	2	Ant. and post. reduced	Small eyes and brain	
	<i>tm102e</i>	2	Ant. reduced, post. absent	Small eyes and brain; reduced melanophores	
	<i>tm9d</i>	3	Ant. reduced; post. reduced or absent	Small eyes and brain	
	<i>tm83c</i>	3	Ant. and post. reduced	Small eyes and brain	
	<i>tm88e</i>	3	Ant. reduced; post. reduced or absent	Small eyes and brain	
	<i>tp46</i>	3	Ant. reduced; post. reduced or absent; ch kinked	Small eyes and brain	
	<i>tw4</i>	3	Ant. reduced; post. reduced or absent; ch extend dorsally	Small eyes and brain; heart edema	
	<i>ta85d</i>	4	Ant. and post. reduced	Small eyes and brain	
	<i>tl17a</i>	4	Ant. and post. reduced; ch kinked	Small eyes and brain; short pec. fins	
	<i>tm42d</i>	4	Hyoid reduced; post. arches absent	Small eyes and brain; heart edema	
	<i>tm20c</i>	4	Post. arches reduced or absent; ch extend dorsally; no bh	Small eyes and brain	
	<i>ty5</i>	4	Post. reduced or absent; ch kinked; no bh	Small eyes and brain	
	<i>th9</i>	5	Post. reduced or absent; ch extend dorsally; no bh	Small eyes and brain; heart edema	
	<i>tx238f</i>	5	Ant. and post. reduced	Small eyes and brain	
	<i>ty7c</i>	5	Ant. reduced; post. reduced or absent	Small eyes and brain	
	<i>ty17b</i>	5	Post. reduced or absent; ch kinked	Small eyes and brain	
III. Flat head, loss of gills, midbrain necrosis					
Unresolved:	<i>tc294a</i>	2	Ant. reduced; post. reduced or absent	Small eyes and brain; midbrain necrosis	b
	<i>tg239a</i>	2	Ant. reduced; post. reduced or absent	Small eyes and brain; midbrain necrosis	b
	<i>tg279</i>	2	Ant. reduced; post. reduced or absent	Small eyes and brain; midbrain necrosis	b
	<i>tj229</i>	2	Ant. reduced; post. reduced or absent	Small eyes and brain; midbrain necrosis	b
	<i>tm46b</i>	3	Ant. and post. reduced; ch inverted	Small eyes and brain; midbrain necrosis	b
	<i>tp230</i>	2	Post. reduced or absent; ch inverted	Small eyes and brain; midbrain necrosis	b
	<i>tq235</i>	2	Ant. reduced; post. absent	Small eyes and brain; midbrain necrosis	b
	<i>tt227c</i>	2	Ant. reduced; post. reduced or absent	Small eyes and brain; midbrain necrosis	b
	<i>ty224</i>	2	Ant. reduced; post. reduced or absent	Small eyes and brain; midbrain necrosis	b
IV. Jaw not extended, pectoral fin and retinotectal defects					
<i>dackel (dak)</i>	<i>tf205, to273b, tw25e, to79c</i>	2	All arches short and thick and strongly reduced	No pectoral fins; tail often curls up	c d
<i>boxer (box)</i>	<i>te242, tg308c, tm4, tm70g, tm317c, to232, tp67z, tw24</i>	4	All arches slightly shorter and thicker than wild type	Short pectoral fins	c d
<i>pinscher (pic)</i>	<i>to216z</i>	2	All arches short and thick and reduced	No pectoral fins	c
V. Arches reduced and pigmentation defects					
<i>gossamer (ger)</i>	<i>tc11c</i>	2	Necrosing jaw and arches	Melanophores thin, elongate or spot-like	a
<i>puzzle (puz)</i>	<i>tp49c</i>	2	Retarded jaw and arches	Pigmentation; ear	a,e
<i>sunbleached (sbl)</i>	<i>to4</i>	2	Retarded jaw and arches	Pigmentation; eye	a,f
Other					
	<i>to273c</i>	4	All arches hang; ch inverted	Heart edema	

*Day indicates the stage (days postfertilization) at which the phenotype is first detected. All mutants are recessive. All pharyngeal mutations that were not assigned to a complementation group ('unresolved', see Table 1) are listed under the appropriate phenotypic class.

ant., anterior; post., posterior; pec., pectoral; ch, ceratohyal; bh, basihyal.

References: a, Kelsh et al. (1996); b, Furutani-Seiki et al. (1996); c, Trowe et al. (1996); d, van Eeden et al. (1996b); e, Whitfield et al. (1996); f, Heisenberg et al. (1996).

Table 4. Pharyngeal arch segments affected in mutants

Mutant name	Segment affected						
	P1	P2	P3	P4	P5	P6	P7
<i>tq235</i>	++	++	X	X	X	X	X
<i>tb210c (bab)</i>	++	++	X	X	X	X	++
<i>tm102e</i>	++	++	X	X	X	X	++
<i>tn14 (her)</i>	++	++	X	X	X	X	++
<i>tg279</i>	++	+	X	X	X	X	+
<i>ty17b</i>	+	+	++	X	X	X	++
<i>ts213a (low)</i>	+	++	++	X	X	X	+
<i>ta53c, etc. (fla)</i>	+	+	+	X	X	X	++
<i>th34c (dul)</i>	+	+	+	X	X	X	++
<i>tp230</i>	+	+	+	X	X	X	++
<i>to75a, etc. (pio)</i>	+	+	+	X	X	X	+
<i>tm88e</i>	+	+	+	X	X	X	+
<i>tc294a</i>	+	+	+	X	X	X	+
<i>tt227c</i>	+	+	+	X	X	X	+
<i>th9b</i>	+	++	+	+	X	X	X
<i>tw4</i>	+	+	+	++	X	X	++
<i>tg239a</i>	+	+	++	++	X	X	+
<i>tm42d</i>	+	+	+	+	X	X	+
<i>tk18 (ser)</i>	++	++	++	++	++	X	++
<i>tj229g</i>	+	+	+	++	++	X	+
<i>tu45b (fac)</i>	+	+	+	+	++	X	+
<i>ty7c</i>	+	++	+	+	++	X	+
<i>tm9d</i>	+	+	+	+	++	X	+
<i>tn20c</i>	+	+	+	+	++	X	+
<i>tp46</i>	+	+	+	+	++	X	+
<i>ty5a</i>	+	++	+	+	++	X	+
<i>ty224</i>	+	+	+	+	++	X	+
<i>tw25e, etc. (dak)</i>	+	+	+	++	++	++	++
<i>to216z (pic)</i>	+	+	+	+	++	++	++
<i>tm83c</i>	+	+	+	+	+	++	++
<i>tj13c (cod)</i>	+	+	+	+	+	++	+
<i>to273c</i>	+	+	+	+	+	+	++
<i>ta42</i>	+	+	+	+	+	+	+
<i>ta85d</i>	+	+	+	+	+	+	+
<i>tc11c (ger)</i>	+	+	+	+	+	+	+
<i>tl17a</i>	+	+	+	+	+	+	+
<i>tm4a, etc. (box)</i>	+	+	+	+	+	+	+
<i>tm46b</i>	+	+	+	+	+	+	+
<i>to4 (sbl)</i>	+	+	+	+	+	+	+
<i>tp49c (puz)</i>	+	+	+	+	+	+	+
<i>tv60c</i>	+	+	+	+	+	+	+
<i>tw220c (bab)</i>	++	+	+	+	+	+	+
<i>tx238f</i>	+	+	+	+	+	+	+
<i>ty103a</i>	+	+	+	+	+	+	+

X, segment absent; ++, segment severely reduced; +, segment reduced.

flathead, although each element is smaller. Meckel's cartilages are narrow and ceratohyals are reduced and often point posteriorly. Branchial arches P4-P6 are consistently missing. Teeth are often found alone, in the position of P7 and apparently without associated chondrocytes. The neurocranium is also narrow.

Mutants with pharyngeal defects and midbrain necrosis

Many members of the flathead group are recognizable at early hatching by region-specific cell death in the brain, primarily restricted to the midbrain and cerebellar primordia. The brain defects in these mutants are described elsewhere (Furutani-Seiki et al., 1996). Two examples of these phenotypes are described.

tq235

As in other tectum necrosis mutants, *tq235* mutant larvae have

small eyes with protruding lenses, and reduced pharyngeal arches at all anterior-posterior levels (G in Figs 2-5, 7D). At early hatching, cells die in the developing tectum and cerebellar folds, melanophores are often expanded and the tail tends to curve dorsally. By late hatching and at later stages, neither jaw nor branchial arches are detected (Fig. 2G).

The skeletal phenotype of *tq235* mutants is among the most severe of the flathead group, lacking the ventralmost cartilages in all seven pharyngeal arches (Fig. 3G). Many more dorsal elements, including the palatoquadrate of P1 and the hyosymplectics of P2, are relatively less affected. In ventral view, disconnected remnants of Meckel's cartilage and ceratohyals are visible (Fig. 4G). The neurocranium of *tq235* is severely reduced, with a narrow ethmoid plate and small auditory capsule.

tt227c

A second type of tectum necrosis mutation develops a jaw phenotype that resembles a more typical flathead group member, lacking groups of branchial arches. In *tt227c* mutants (Tables 3, 4) early brain necrosis appears confined to the tectum and cerebellum and the eyes are small. In larvae, gills are reduced or absent in mutants and the jaw is strongly reduced. Skeletal preparations of this mutant reveal defects in all arches and lack of P4-P6, similar to *fla*.

Mutants with arch and pigmentation defects

In addition to the head skeleton, which we have focused on, the neural crest also forms chromatophores and we have looked closely among the pigment class of mutants for cartilage defects (Kelsh et al., 1996). *low* has reduced melanocytes and *cod* has dull iridophores. In addition, we found several mutants with specific defects in specific chromatophores and cartilage formation. The particular combinations of phenotypic traits makes these mutants easier to classify.

pistachio (pio^{to75a})

Five alleles of *pio* are all characterized by a strong reduction in the head, eyes and arches as well as xanthophore pigmentation. Other pigment cell types are also affected (Kelsh et al., 1996). The phenotype is detected at pharyngula stages (approx. 30 hours) as pigmentation begins, but head morphology at this stage is normal. In mutant larvae, skeletal preparations reveal defects in all pharyngeal arches, P1-P7 (Tables 3, 4). P4-P6 do not form, as in *fla*. Also like *fla*, Meckel's cartilages are variably reduced and meet in the midline at an acute angle.

In three additional mutants with distinct phenotypes, *gossamer (ger^{tl1c})*, *puzzle (puz^{tp49c})* and *sunbleached (sbl^{to4})*, both pigmentation and pharyngeal arch development are affected. These appear to be associated with general necrosis of the animal. In all three mutants, melanophores degenerate and other chromatophores are also less prominent (Kelsh et al., 1996). In mutant larvae, the jaw and branchial arches are variably reduced or absent.

Mutants with simultaneous defects in pharyngeal arches, pectoral fins and retinotectal projections

In a few cases, groups of mutations representing different genes all have similar phenotypes in several seemingly unrelated body regions. This indicates that these genes may be involved in common genetic pathways. One example is the set

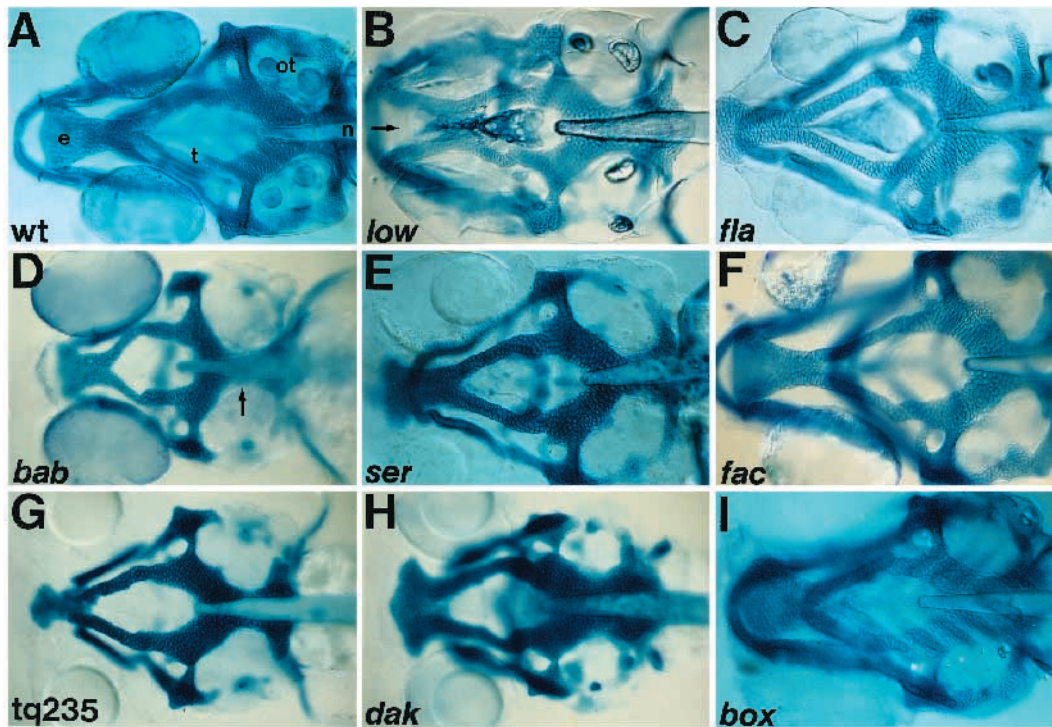


Fig. 5. Skeletal defects in mutants (ventral view; neurocranium). A more dorsal focus of the animals shown in Fig. 4. (A) Wild type, wt. (B) *low*. The ethmoid plate is split in the midline (arrow). (C) *fla*. The ethmoid plate is narrow. (D) *bab*. All cartilages are severely reduced, including parachordal cartilages (arrow). (E) *ser*. (F) *fac*. (G) *tq235*. All cartilages are severely reduced, particularly the ethmoid plate. (H) *dak*. All cartilages short and thick. (I) *box*.

of three mutations, *boxer*, *dackel*, *pinscher*, that disrupt pharyngeal morphogenesis, pectoral fin formation (van Eeden et al., 1996b) and topographic mapping of retinal axons on the optic tectum (Trowe et al., 1996).

dackel (*dak*)

Among this class, *dak* shows the most severe pharyngeal defects (H in Figs 2-5, 7E) and all alleles appear similar. In contrast to members of the flathead group, the eyes and brain are normal size in *dak*. Mutant embryos are detected at early hatching by the reduced head and lack of pectoral fins. Often the postanal tail curls dorsally. The mouth does not extend as far anteriorly as wild type and hangs open slightly. Gills are reduced (Fig. 2H).

The head skeleton in *dak* is strongly reduced though all cartilages are present (Fig. 3H). Each cartilage is shorter and wider. In ventral view, Meckel's cartilages and ceratohyals taper from lateral to medial in mutants, often four to five cell diameters wide laterally but only one to two cells wide medially. A midline basihyal is generally absent but basi-branchials are present posteriorly. Ceratobranchials become progressively smaller posteriorly and only a few chondrocytes are present in P6 and P7 (Fig. 4H).

The neurocranium in *dak* is severely reduced as well as misshapen (Fig. 5H). The ethmoid plate spreads further laterally than in wild type and has a characteristic trilobed appearance. Thickened trabeculae surround a hypophyseal fenestre of approximately normal size. The posterior cartilages are differentially affected, thickened in the anterior, parachordal regions but narrowed around the auditory capsule.

boxer (*box*)

In contrast to *dak*, the heads of *box* mutants are nearly normal in size. The nose is slightly less extended anterior to the eyes and the front of the head appears smooth (I in Figs 2-5, 7F). The phenotype is first detected at late hatching when the lower jaw protrudes abnormally in mutants. Mutants have short pectoral fins and reduced motility. Differences in head shape in *box* appear to result from defects in the anterior neurocranium, the posterior neurocranium is virtually unaffected. All pharyngeal arches are slightly reduced, with each element slightly thicker than wild type. Chondrocytes in each arch are also smaller and misshapen.

pinscher (*pic*^{to216z})

Homozygous *pic* mutants are first detected at 5 days by their thickened jaw and branchial arches, somewhat similar to *dak*. All arches are reduced in *pic*, but most severely P5 and P6 (Tables 3, 4).

DISCUSSION

We have identified at least 12 genes required for development of the posterior pharyngeal arches in zebrafish. Despite similarities in the live phenotypes of many mutants, they differ in their skeletal defects, particularly in which segments they disrupt. Mutations in *low* disrupt the hyoid and branchial arches, P2-P6; in *fla* defects are confined to P4-P6; in other flathead-like mutants, such as *bab*, all branchial arches are

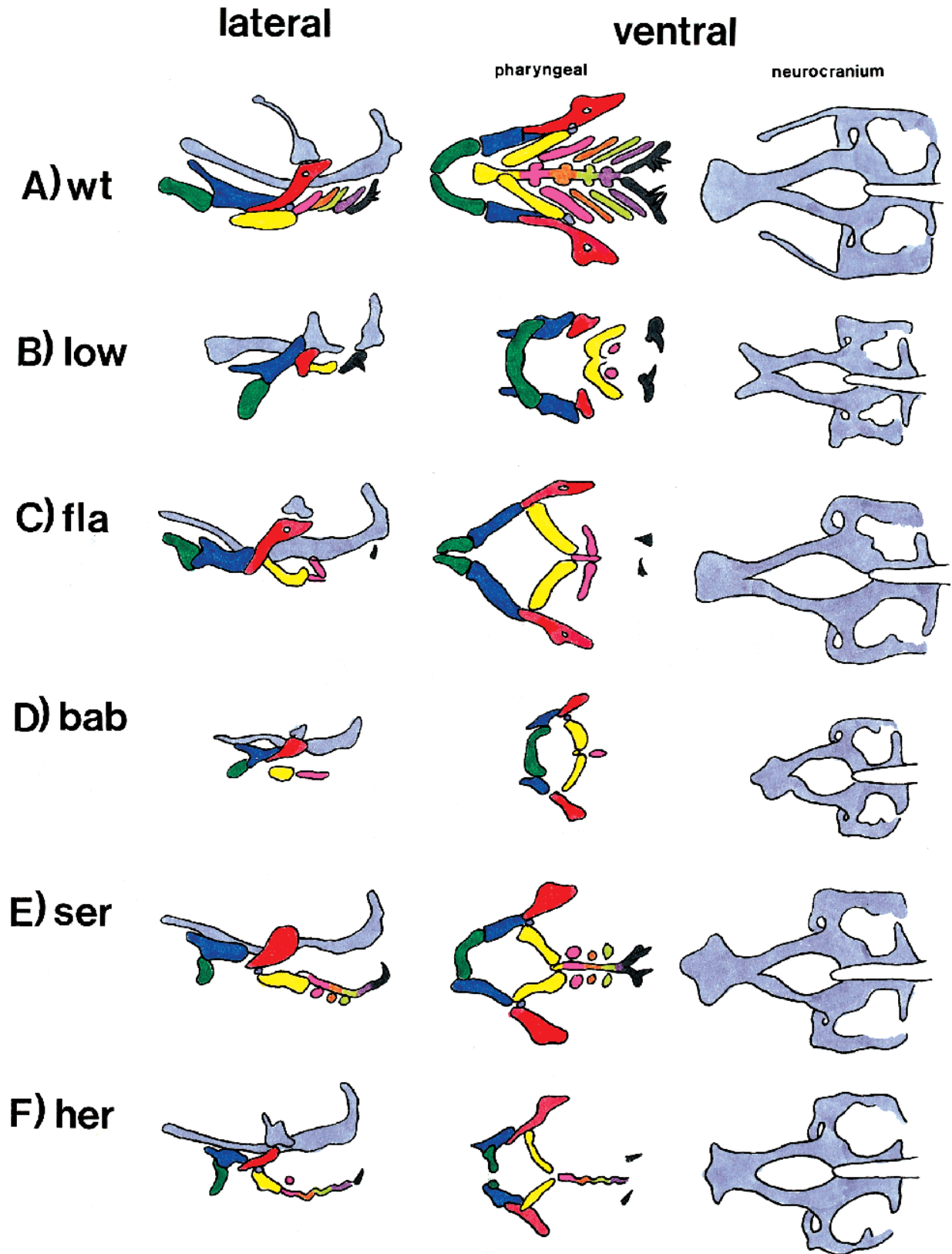


Fig. 6. Camera lucida drawings of skeletal defects in mutants. Three vertical columns from left to right show lateral views and ventral views of the pharyngeal skeleton and the neurocranium. (A) Wild type, wt. (B) *low*. (C) *fla*. (D) *bab*. (E) *ser*. (F) *her^{ml4c}*. Individual cartilages are color-coded: Meckel's cartilage (dark green); palatoquadrate (dark blue); ceratohyal and basihyal (yellow); interhyal (light blue); hyosymplectic (red); P3 including ceratobranchial, hypobranchial and basibranchial (pink); P4 (orange); P5 (light green); P6 (purple); and P7, including teeth (black). The neurocranium is colored grey.

absent and anterior arches are reduced. We suggest that these sets of mutants with similar phenotypes reveal genes that normally function in common pathways to specify neural crest cells in groups of segments (Fig. 8). Similarly mutants such as *box*, *dak* and *pic* may reveal common genetic pathways in arch, fin and retinotectal development. These are hypotheses that we can now test with standard genetic analyses. The results are considered in the context of what is known about arch morphogenesis and its genetic control.

Genes identified by these mutations might normally function in early developmental events, such as neural crest specification, or later in differentiation. Our data suggest that at least some act early because: (1) their phenotypes are detected early (e.g. *low*, *fla*, *her* or mutants with tectal necrosis; Table 3), at least a day before chondrogenesis; and (2) cartilage develops normally in other body regions of mutants, such as the pectoral girdle and fins. For comparison, mutants that disrupt cartilage differentiation (hammerhead group) are only detected later, as larvae, most with corresponding reductions in the pectoral skeleton (Piotrowski et al., 1996).

Genes specify subsets of posterior arches

Mutants in the flathead group fall into one of three classes: affecting all of the arches (e.g. *tq235*, *her* and *ser*), deleting only P3-P7 (e.g. *bab*) and deleting only P4-P6 (e.g. *fla*, *pio*, *tt27c*). The arch primordia can, consequently, be divided into concentric fields containing groups of adjacent segments (Fig. 8; Table 4). The scheme combines our observations of mutant defects with the known origins and movements of neural crest cells determined in fate mapping studies (Schilling and Kimmel, 1994; Schilling, 1993). Similarly, in the anterior class of jaw mutants the first and second arch are always affected together (Piotrowski et al., 1996). One attractive idea is that genes specify neural crest cells in continuous stretches of segments, reminiscent of the gap genes in *Drosophila* (Nüsslein-Volhard and Wieschaus, 1980). Strikingly, the most posterior branchial arch, P7, with teeth, remains even in the most severe mutants, a difference that may reflect the early differentiation of this segment (Vandewalle et al., 1992; Langille and Hall, 1988) and possibly its independent mesodermal origins.

Mutations that affect broad anterior-posterior regions of the arches might have been expected, based on previous descriptions of gene expression patterns. *Hox* genes are expressed in overlapping domains of the neural tube, including all posterior rhombomeres and their corresponding neural crest, while unique genes are expressed anteriorly (reviewed by Krumlauf, 1994). During neural crest migration, *dlx-2* in zebrafish is expressed in three major streams of cells corresponding to a mandibular, hyoid and a large posterior domain that appears to form all of the branchial arches, P3-P7 (Akimenko et al., 1994). With time, this posterior expression domain subdivides into individual arches. Fate mapping has shown that the branchial arches also appear to be derived from a common precursor mass. Neural crest cells are not segmentally restricted in these arches, unlike cells in anterior arches (Schilling and Kimmel, 1994). The condensed development of the posterior arches may represent a secondary reduction in zebrafish and its relatives. The pectoral endoskeleton of zebrafish is thought to have undergone such a reduction (Coates, 1994).

The number of arches that develop in mutants may depend on morphological constraints, including the amount of available mesenchyme for prechondrogenic condensations. Cartilage that forms last may be the most easily lost, as has been proposed for digit formation in the limb (Alberch, 1989), if a mutation generally reduces the extent of neural crest formation or migration into the appropriate chondrogenic region. Similarly, within the same segment, ceratobranchials may be present but not basibranchials, possibly because basibranchials simply develop later. Exceptions include *her*^{tn14}, which has a complete midline skeleton but no ceratobranchials. Thus, midline cartilages can develop independent of bilateral elements, and there are separate genes that act in midline and bilateral skeletal development.

Hyoid defects in *low*

The hyoid arch is severely reduced in *low*, particularly the hyosymplectic cartilage. The hyosymplectic is the most posterior and dorsal element of the hyoid arch and articulates with the auditory capsule. In its absence, the palatoquadrates of the first arch take its place, extending further posteriorly and dorsally than in wild type and contacting a similar location of the capsule. This resembles normal features of the reptilian skeleton, where the jaw articulates via the pterygoquadrate. An attractive explanation is that *low* normally specifies the locations of palatoquadrate and hyosymplectic, and by simply losing *low* function this region of the head skeleton adopts characteristics of a more derived state in vertebrate evolution. One possible source of this positional information is the overlying epithelium, which is known to specify where, when and how much skeletal tissue will form in the mandible (Hall, 1980, 1987). Mosaic analyses, by transplanting cells between mutant and wild types, in future studies can determine which cell types, epithelia or neural crest for example, are affected directly in *low*.

Common genetic pathways for pharyngeal cartilage and other cell types

(a) Brain, fins and cartilage

Most of the flathead group, as well as *box*, *dak* and *pic*, have defects in the brain and/or pectoral fins. The reduced midbrain and eyes in flatheads could result from an early defect in the neural tube including neural crest that forms the branchial arches. A similar problem is found in *small eye* (*Sey*), a mutation in *pax6* in mice, where midbrain neural crest migration is impaired (Matsuo et al., 1993; Hogan et al., 1988). Consistent with this idea, flathead mutants with smaller brains have smaller eyes and fewer cartilages in branchial arches. However, it is difficult to understand the association between early midbrain necrosis and a failure of posterior arches to develop, as seen in 66% of the flatheads. Mesencephalic neural crest cells populate only the most anterior arch, P1, and much of the anterior neurocranium (Langille and Hall, 1988; Schilling and Kimmel, 1994) and do not migrate posteriorly. However, brain necrosis in these mutants may extend into regions that generate posterior arch neural crest.

Mutations in at least three genes simultaneously disrupt pharyngeal arch, pectoral fin and retinotectal development (Trowe et al., 1996; van Eeden et al., 1996b). The phenotypes differ in severity of their pharyngeal defects. Two of them,

dak and *pic*, severely impair arch morphogenesis, shortening the length of all cartilages in the pharyngeal arches and neurocranium while all eight alleles of the third, *box*, do so to a lesser extent. Interestingly, *box* also shows less severe pectoral fin defects (van Eeden et al., 1996b). How might their roles in the arches relate to roles in other affected areas? The same genes may regulate both the outgrowth and morphogenetic movements of mesenchyme in the arches and fins and target recognition by growth cones of retinal axons. Many of the same molecules that are known to regulate limb development (e.g. sonic hedgehog or BMPs) are also expressed in the brain and/or pharyngeal arches and are likely to have similar functions in many of these regions (Wall and Hogan, 1995). Future genetic analyses will determine if the genes act in a common molecular pathway in these different tissues.

(b) Pigment cells and cartilage

In addition to cranial cartilage, the neural crest forms chromatophores, as well as a variety of other cell types (Lopashov, 1944; LeDouarin, 1982). Mutations that disrupt cartilage and chromatophore development, such as *low*, *pio* and *cod*, could identify genes that have similar functions in more than one neural crest derivative. Most of these mutants appear to disrupt differentiation or survival factors in chromatophores (Kelsh et al., 1996). This may also be the case in cartilage, since *pio* and *cod* are detected after chondrogenesis is well underway and often show retardation or necrosis of arch structures. An alternative explanation, particularly for early mutations like *low*, might be that the mutant disrupts the specification of a subset of neural crest cell lineages. Cartilage and pigment cell precursors arise from a similar region of the neural crest fate map, which is distinct from the neurogenic region (Schilling and Kimmel, 1994). Pigment cells and cartilage cells may also experience similar environments, not dependent on their lineages, that are disrupted. It is now important to determine if other neural crest cell lineages, sensory neurons for example, are affected in these mutants.

(c) Neurocranium and arches

Nearly all of the mutants examined have reductions in the neurocranium, particularly in anterior elements including the ethmoid plate and trabeculae. These often appear narrower than in wild type. Posterior cartilages tend to be less affected. This observation is interesting in light of the fact that anterior neurocranium is derived from neural crest while the posterior, parachordal region is mesodermal, at least in tetrapods (Noden, 1988; LeDouarin, 1982). Furthermore, when the posterior neurocranium is affected, as in *box*, *dak*, and many of the hammerheads (Piotrowski et al., 1996), other mesodermally derived skeletons such as those of the pectoral fins are also affected.

Arch mutants and segmentation

Pharyngeal arches in all vertebrates are thought to develop as reiterations of the same fundamental segmental unit. During their early development arches show similar patterns of morphogenesis (Schilling and Kimmel, 1994) and clear segmental homologues can be recognized in the skeleton. Many of our zebrafish mutants disrupt segmental homologues in most or all arch segments. The larger anterior segments, mandibular and hyoid, are thought to represent evolutionary modifications of the more primitive posterior arch pattern. Genetic evidence for

the idea that the anterior arches are segments comes from the homeotic transformations of arches observed in *Hox* mutants, such as *Hoxa-2* (Rijli et al., 1993), where the hyoid arch takes on characteristics of the mandibular. However, mutations have not been found that transform the anterior arches to resemble more posterior arches as one might expect, and we have not found them here.

Few of the mutants from our screen have defects confined to particular arch segments or particular neural crest derivatives. This probably reflects a requirement for several genes to combine in patterning a given segment or cell type. In addition, early specific defects may be compensated for by other tissues, and neural crest cells are only one in a series of interactions in arch development (Hall, 1987). Early segmental patterning may be relatively normal in many mutants, since we have looked at early molecular markers in a few including *low*, *bab*, *ser*, *her* and *cod*. They do not appear to have gross changes in segmentation of the anterior hindbrain and some of its associated neural crest, as determined by the expression of *krx-20* in rhombomeres 3 and 5 (data not shown; Oxtoby and Jowett, 1993).

It is difficult to distinguish one arch skeleton from another, other than by size and position. Thus, although we found no evidence for homeotic transformations of arch segments in these mutants, these might be difficult to recognize. Alternatively, several genes may be required to combine in patterning an arch, so that inactivation of a single component is not sufficient to generate a complete transformation. Support for this notion comes from studies of murine *Hox* genes; for example, *Hoxb-4* and *Hoxd-3* mutants alter complementary aspects of development of the second cervical vertebrae (Krumlauf, 1994). Curiously, we found no phenotype equivalent to the *Hoxa-2* knock-out (Rijli et al., 1993; Piotrowski et al., 1996), suggesting that either this phenotype in fish differs from that in mouse or it was missed.

Limitations of the screen for head mutants

The simplicity and clarity of developing zebrafish have allowed screening for head defects at low magnification, but this has limited detection of mutants to those with severe phenotypes. Probably more than most other mutant classes, many additional essential genes could be found with a more sensitive screening method, such as skeletal staining. Further limitations were imposed by screening at 5 days, after extensive differentiation had already occurred. Mutations that showed general necrosis or retarded organs, by this stage, were often discarded. Fish that are retarded in development often show arch defects, many of them flathead-like, and these are difficult to separate from the specific ones (Mullins et al., 1994; Solnica-Krezel et al., 1994). An argument against retardation accounting for the mutants that we have analyzed is the observation that all alleles of a particular mutation show the same phenotype, and the phenotypes are distinct from one another. Surprisingly, all of the mutants are lethal, possibly reflecting the requirement for a well developed pharyngeal region in breathing and feeding in the larva.

Many of the mutants are only detectable close to or after the completion of embryogenesis. Thus many are probably late differentiation defects. In at least one group, the hammerheads, the morphologies of individual chondrocytes are abnormal (Piotrowski et al., 1996). In contrast, abnormal cell morphologies are rarely observed in the flathead group; rather these mutants eliminate or reduce entire cartilages, supporting the

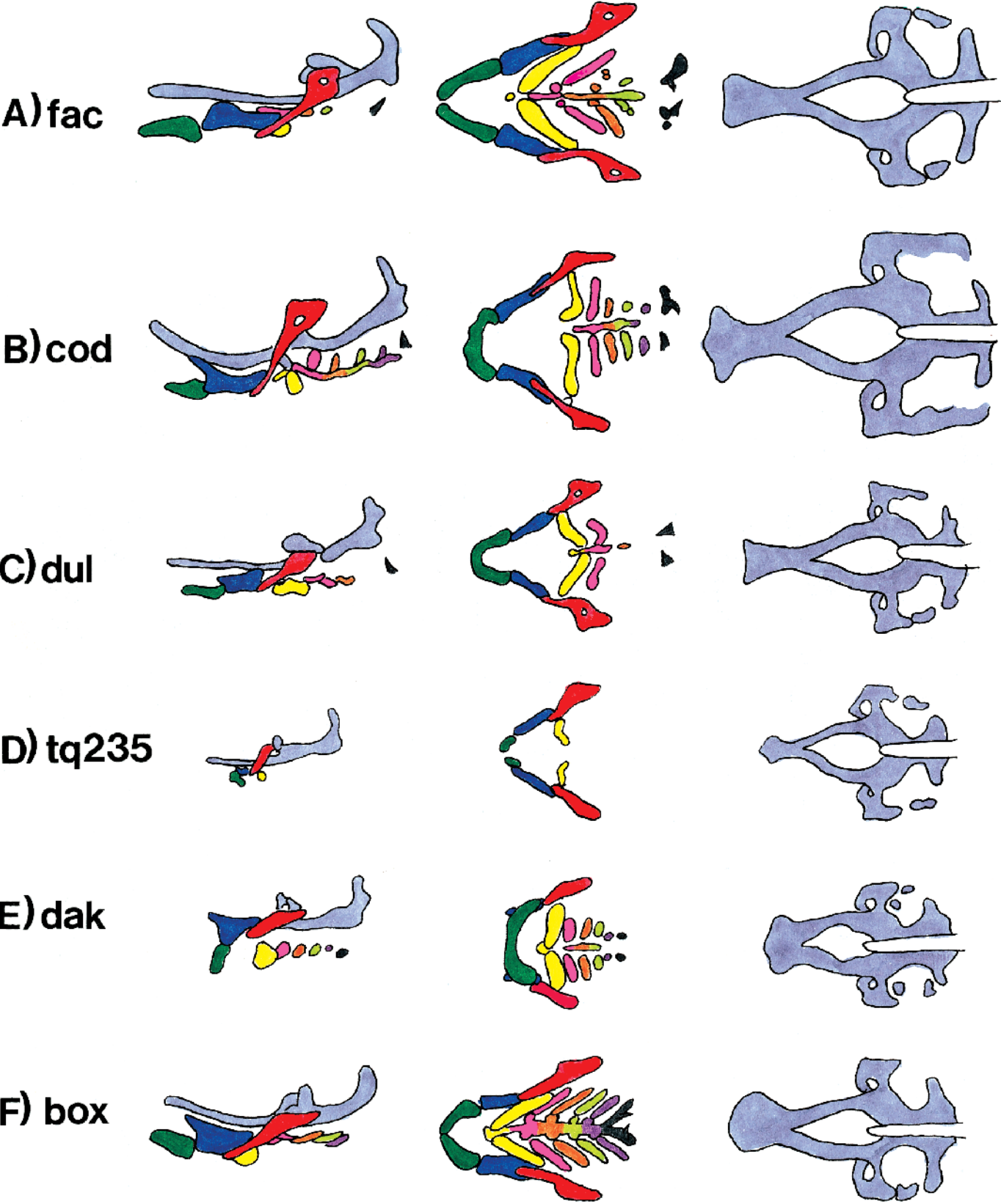


Fig. 7. Camera lucida drawings of skeletal defects in mutants. (A) *fac*. (B) *cod*. (C) *dul*. (D) *tq235*. (E) *dak*. (F) *box*.

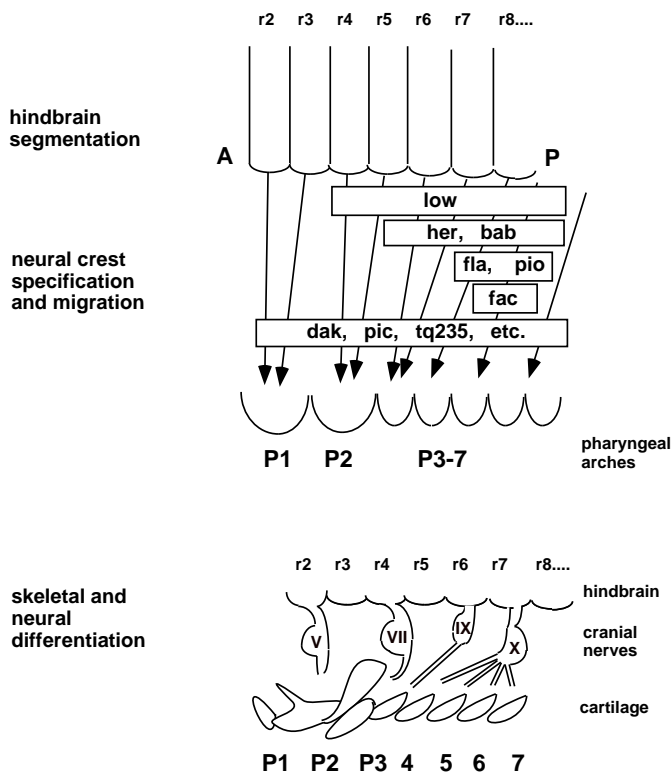


Fig. 8. Genes may specify neural crest cells in groups of adjacent pharyngeal arches. Schematic representations of lateral views of the hindbrain rhombomeres, migrating neural crest cells (arrows) and pharyngeal arches (P1-P7) at two stages: early segmentation (approx. 13 hours) and late hatching (approx. 3 days). The figure illustrates the spatial domains affected in mutants and their relationship to the final pattern of cranial nerves, ganglia and cartilages in the pharyngeal arches.

idea that these genes may specify neural crest cells. Jaw mutants that have been found previously in zebrafish are detectable by early hatching. At least one of these, *chinless*, clearly identifies a gene involved in neural crest specification, particularly in the chondrogenic lineage (Schilling, 1993; Schilling and Kimmel, 1994; Schilling et al., 1996). None of the mutations in our screen are obvious candidates to be alleles of these Oregon mutants, possibly because they have been classified differently based on other aspects of their phenotypes (i.e. pigmentation, brain morphogenesis etc.) or because they were discarded due to their more general defects in older embryos.

Some of the craniofacial abnormalities that we have observed in mutant zebrafish resemble malformations in spontaneously occurring mutations in humans as well as deformities induced by teratogenic agents (reviewed by Poswillo, 1988). In particular, mutants that disrupt morphogenesis of the first and second pharyngeal arches have been described in some detail in mice, as well as those with defects in cartilage differentiation, and the underlying genetic defects in some mutants have been identified.

All of the mutants that we have described share the feature that the neural crest-derived head skeleton develops abnormally. This might result either from a requirement for a given gene within neural crest cells themselves or in their environments. One flathead, for example, could result from a primary

defect in cartilage precursors, another in surrounding epithelia. These two possibilities can be distinguished, in future studies, by mosaic analyses. Thus, while the specific molecular defects underlying these mutant phenotypes will require the cloning of the genes affected, these mutants will provide invaluable tools for studying lineage-dependent and -independent mechanisms of neural crest cell specification.

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