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Jaw and branchial arch mutants in zebrafish II: anterior arches and cartilage differentiation

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SUMMARY

In a large scale screen for mutants that affect the early development of the zebrafish, 109 mutants were found that cause defects in the formation of the jaw and the more posterior pharyngeal arches. Here we present the phenotypic description and results of the complementation analysis of mutants belonging to two major classes: (1) mutants with defects in the mandibular and hyoid arches and (2) mutants with defects in cartilage differentiation and growth in all arches. Mutations in four of the genes identified during the screen show specific defects in the first two arches and leave the more posterior pharyngeal arches largely unaffected (*schmerle, sucker, hoover* and *sturgeon*). In these mutants ventral components of the mandibular and hyoid arches are reduced (Meckel's cartilage and ceratohyal cartilage) whereas dorsal structures (palatoquadrate and hyosymplectic cartilages) are of normal size or enlarged. Thus, mutations in single genes cause defects in the formation of first and second arch structures but also differentially affect development of the dorsal and ventral structures within one arch.

In 27 mutants that define at least 8 genes, the differentiation of cartilage and growth is affected. In *hammerhead* mutants particularly the mesodermally derived cartilages are reduced, whereas *jellyfish* mutant larvae are characterized by a severe reduction of all cartilaginous elements, leaving only two pieces in the position of the ceratohyal cartilages. In all other mutant larvae all skeletal elements are present, but consist of smaller and disorganized chondrocytes. These mutants also exhibit shortened heads and reduced pectoral fins. In homozygous *knorrig* embryos, tumor-like outgrowths of chondrocytes occur along the edges of all cartilaginous elements. The mutants presented here may be valuable tools for elucidating the genetic mechanisms that underlie the development of the mandibular and the hyoid arches, as well as the process of cartilage differentiation.

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

INTRODUCTION

The development of the vertebrate skull and its pivotal role in vertebrate evolution have been investigated by vertebrate biologists for over a century. In contrast to the trunk skeleton, the craniofacial mesenchyme of vertebrates is derived from both neural crest and mesoderm and differentiates into three components. The neurocranium protects the brain and the sensory organs of the head and originates from both sources of mesenchyme, whereas the pharyngeal skeleton includes the jaw and the branchial arches and is solely derived from neural crest cells (reviewed by Hörstadius, 1950; Le Douarin, 1982; Langille, 1988). The dermatocranium comprises the bony elements which, later in development, incorporate the two other components of the skull.

The fact that neural crest cells are only present in vertebrates and contribute to most of the structures in the head reflects their importance in the evolution of the vertebrate head (Northcutt and Gans, 1983; Gans and Northcutt, 1983). Neural crest cells represent an initially pluripotent population of migratory cells, which arise from dorsal areas of the neural tube and give rise to a wide array of adult tissue types (Hörstadius, 1950; Le Douarin, 1982; Langille, 1988). Cephalic neural crest cells, which originate from the embryonic midbrain and hindbrain, migrate ventrally into the pharyngeal arches and into more dorsal regions of the head to form cartilage, connective tissue, sensory neurons

of the peripheral nervous system and pigment cells. In order to contribute to the formation of these structures, neural crest cells have to undergo extensive migration. During their migration, neural crest cells come into contact with extracellular matrix molecules of changing distribution, concentration and composition. Several authors have shown that these proteins are capable of guiding the neural crest cells (Weston, 1980; Newgreen and Gibbins, 1982). Neural crest cells also interact with adjacent epithelia along their way, and experimental evidence exists to indicate that these mesenchymal-epithelial interactions are necessary for the induction of chondrogenesis (Tyler and Hall, 1977). At their destination neural crest cells form condensations in which differentiation commences, providing the condensations reach a critical size. Thus neural crest cells can be used for studies of the relationship between autonomous and non-autonomous determination of cell fate.

Neural crest cells that are important for pattern formation of the vertebrate head arise from axial levels corresponding to distinct rhombomeres (Lumsden et al., 1991; Schilling and Kimmel, 1994; Sechrist et al., 1993). In heterotopic grafting experiments between chick and quail embryos, Noden has shown that neural crest cells are specified according to their axial position largely prior to the onset of migration (Noden, 1983, 1988). He transplanted first arch neural crest from quail embryos into the position of presumptive second arch neural crest of chicken embryos and observed that grafted cells developed in a donor-site specific manner and formed first arch structures in the location of the second arch. It has been proposed that this specification of neural crest cells is under the control of *Hox* genes. The *Hox* genes are expressed combinatorially in the rhombomeres; their expression pattern is retained by the migrating neural crest cells, and at later stages appears in the neural crest-derived cranial ganglia, mesenchyme and overlying ectoderm (Hunt et al., 1991a,b; Hunt and Krumlauf, 1991; Couly and Le Douarin, 1990). Thus, neural crest cells convey positional information from the hindbrain to the pharyngeal arches. (Hunt et al., 1991a,b; Lumsden et al., 1991). Experimental evidence for the importance of combinatorially expressed *Hox* genes has been provided by targeted mutations affecting *Hox* genes in mice, which result in severe perturbations in the development of the branchiorhombomeric area (Lufkin et al., 1991; Chisaka et al., 1992; Mark et al., 1993; Gendron-Maguire et al., 1993; Rijli et al., 1993). For instance, the disruption of the *Hoxa-2* gene leads to the disappearance of mesenchymal structures that are specific for the second arch, and causes transformations of these elements into first arch elements (Gendron-Maguire et al., 1993). The *Hoxa-2* gene, which possesses the anteriormost expression pattern of *Hox* genes, is only expressed in rhombomere 2 (r2) and more posterior rhombomeres, and not in the neural crest that migrates from r2 into the first arch. Since *Hox* gene expression has not been found in r1 or in the neural crest cells that contribute to the first arch, the formation of this arch is probably

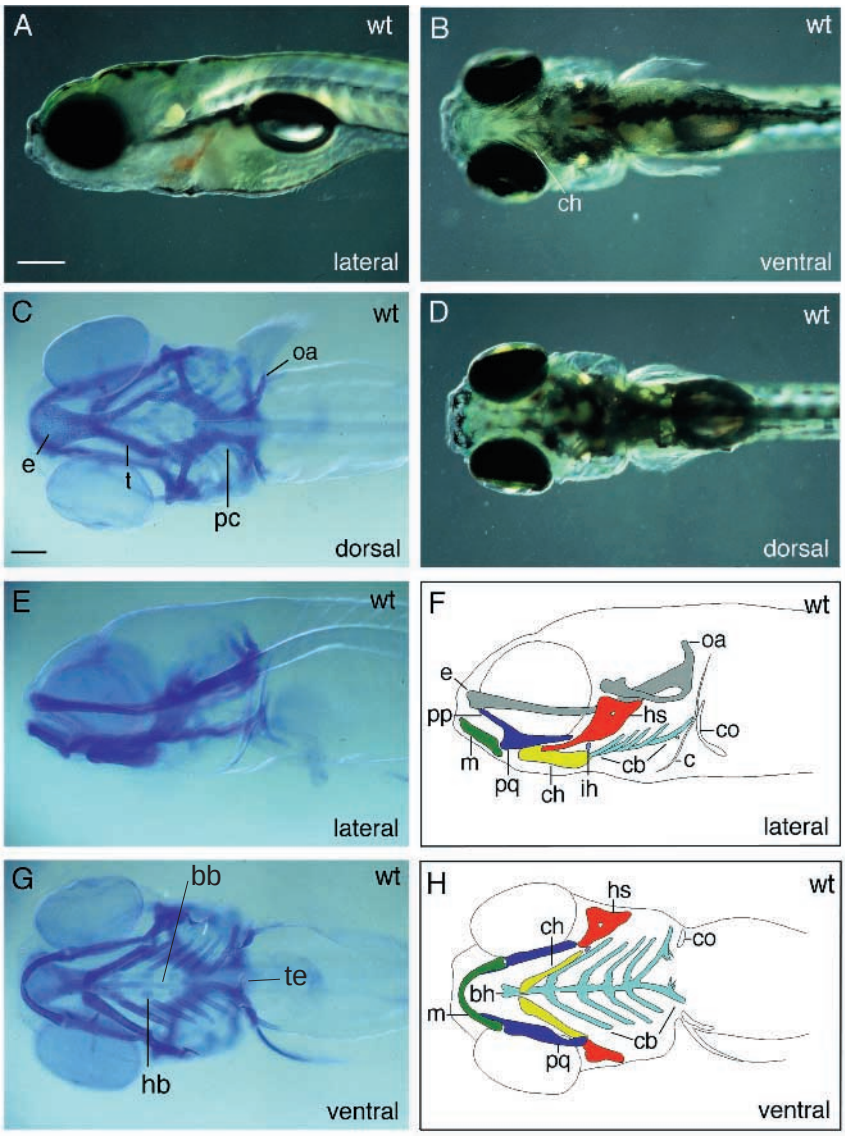

Fig. 1. Photomicrographs and schematic drawings of live (A,B and D) and Alcian blue-stained (C,E,G) wild-type larvae. Color-coded digrams (F,H) of the stained larvae shown in E and G depict distinct cartilaginous elements of the head and pectoral girdle. In H the neurocranium is omitted. C and D present dorsal views, whereas B and G show ventral views of live and stained larvae, respectively. A,E and F show lateral views of these larvae. Abbreviations used throughout all figures: bb, basibranchial; bh, basihyal; ch, ceratohyal; co, coracoid of pectoral girdle; cb, ceratobranchial; c, cleithrum; e, ethmoid plate; ff, facial foramen; hb, hypobranchial; hs, hyosymplectic; m, Meckel's cartilage; oa, occipital arch; op, opercle; pc, parachordal; pp, pterygoid process of the palatoquadrate; pq, palatoquadrate; t, trabeculae; te, teeth. Color code used in all diagrams: green, Meckel's; blue, platoquadrate; red, hyosymplectic; yellow, ceratohyal; light blue, interhyal; light green, arches 3-5; grey, neurocranium or ectopic cartilages; white, coracoid of pectoral girdle. Scale bars: A,B,D, 200 μm; C,E-H: 100 μm.

controlled by a mechanism that is independent of *Hox* genes and may be regulated by other anteriorly expressed genes. The phenotype of retinoic acid receptor null mutant mice also supports this view (Lohnes et al., 1994). Retinoic acid has been shown to be capable of regulating *Hox* genes. In mutant mice all neural crest-derived elements of the viscerocranium showed gross malformations, whereas the mandibular arch structures were largely unaffected. *Hox* gene-independent patterning mechanisms may involve head-specific homeobox genes like *Otx* and *Emx* (Simeone et al., 1992). These genes represent the vertebrate homologs of the *Drosophila orthodenticle (otd)* and *empty spiracles (ems)* genes and are expressed in the midbrain and forebrain.

In this paper we describe the phenotypes and results of complementation analysis of mutants in the zebrafish in which the anterior mandibular and hyoid arches are affected, mutants with impaired tissue interactions or muscle physiology of the jaw, and also 27 mutants that define at least eight genes that exhibit defects in cartilage differentiation and growth. We call this latter group 'hammerhead-like' mutants because they are characterized by a lack of tissue anterior to the eyes. The accompanying paper focuses on mutant classes with defects in the more posterior pharyngeal arches (Schilling et al., 1996). The phenotypes of the mutants with defects in the anterior arches suggest that the mechanisms underlying the formation of the mandibular and hyoid arches are linked and that dorsal and ventral structures respond differently to the same genes. Mutations in genes that cause alterations in chondrocyte morphology might disturb different processes involved in cartilage differentiation and growth, for example the composition of extracellular matrix molecules.

MATERIALS AND METHODS

The methods used for the performance of the large-scale saturation screen for mutations affecting the early development in zebrafish embryos have been described (Mullins et al., 1994; Driever et al., 1994; Haffter et al., 1996). Procedures concerning fish raising and maintenance are described by Brand et al. (1996a).

Whole-mount skeletal stainings

5-day-old larvae were killed by an overdose of 0.2% ethyl-m-aminobenzoate methanesulfonate and subsequently fixed in 3.7% neutral-buffered formaldehyde at room temperature for several hours to overnight. Specimens were stained for cartilage following the modified method of Dingerkus and Uhler (1977). The larvae were transferred into a 0.1% Alcian blue solution dissolved in 80% ethanol/20% glacial acetic acid for 6 to 8 hours. After staining, the larvae were rinsed in ethanol and rehydrated gradually into phosphate-buffered saline. They were then transferred into a solution of 1% KOH/3% H_2O_2 for about 1 hour to bleach all pigment. Subsequently, the tissue was cleared in a 0.05% trypsin solution (Sigma) dissolved in saturated sodium tetraborate for another hour. The specimens were stored in 50% glycerol/50% phosphate-buffered saline to which a few crystals of thimerosal were added to prevent fungal growth. The specimens were then photographed with a Zeiss Axiophot microscope.

Conventions

Some confusion over the nomenclature of the arches exists in the literature. In this paper and the accompanying study we refer to the mandibular and hyoid arches as first and second pharyngeal arches, respectively. The arches that lie posterior to the hyoid arch and are the ones that bear gills in teleosts are called the third to seventh pharyngeal arches, respectively. The terminology of the other cartilaginous elements is adopted from studies by de Beer (1937), Daget (1964) and Langille and Hall (1987).

RESULTS

During the screen, 109 mutations that cause defects in the development of the jaw and the pharyngeal arches were isolated. Complementation analysis has shown that these mutations fall into at least 26 genes. All mutations described in this paper are homozygous lethal and result in death within 6 or 7 days of hatching. The classification of these mutants is detailed in Table 1 and is described by Schilling et al. (1996).

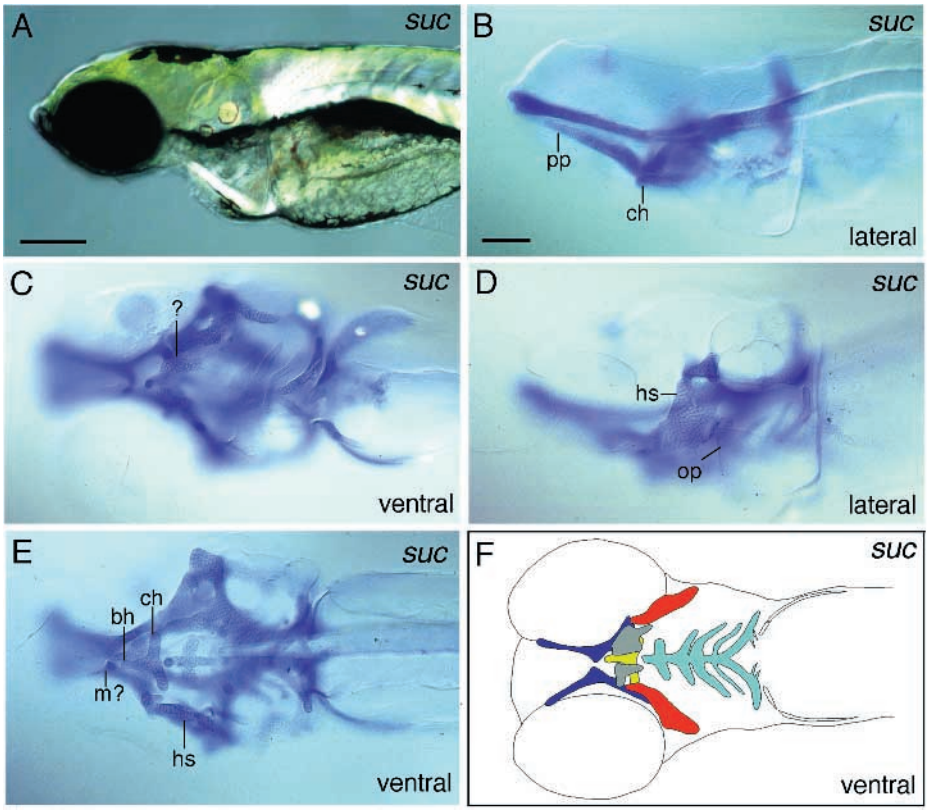

Fig. 2. Photomicrographs of lateral views (A,B,D) and ventral views (C,E,F) of *sucker* mutant embryos reveal that Meckel's cartilage (lower jaw) is strongly reduced or absent. The diagram (F) represents a composite of elements photographed in C and E. The neurocranium is omitted. The ceratohyal is reduced and fused to the basihyal (E). Although the posterior arches are present, they are reduced. Ventral to the ceratohyal additional unidentified plate-like cartilaginous elements can be detected (C,F). Scale bars: 200 μm (A); 100 μm (B-F).

Here we describe mutations in four genes that particularly affect the formation of the mandibular and the hyoid arches: *sucker (suc), schmerle (she), hoover (hoo)* and *sturgeon (stu)* (Figs 2-4), one mutation that also causes defects in the posterior arches and the ear: *van gogh (vgo)*, two mutations that affect tissue interactions or muscle physiology: *dolphin (dol)* and *hanging out (hot)* (Figs 4, 5) and 27 'hammerhead-like' mutants in which the morphology of chondrocytes is altered (Figs 6-8). The 'hammerhead-like' mutants can be further subdivided into five groups characterized by: (1) kinked elements and shortened bodies; (2) severe reduction and loss of cartilage; (3) defects in extracellular matrix; (4) slightly reduced elements; and (5) tumor-like growth of chondrocytes. These 'hammerhead-like' mutations can be assigned to at least eight complementation groups, based on complementation analysis or their unique phenotypes, whereas the mutations that affect the formation of the anterior arches define four genes. 60 mutants with defects in the posterior arches are described in the accompanying paper (Schilling et al., 1996). All mutants described here, in addition to mutants from the screen that show abnormal jaw development but are classified according to other phenotypes, are listed in Table 2.

Live larvae were screened for jaw and pharyngeal arch defects on day 5 of development. At this stage, most of the cartilaginous elements have differentiated and defects in their formation are easily detectable. In order to study the skeletal phenotypes in more detail the larvae were treated with Alcian blue, which stains both neural crest-derived and mesodermally derived cartilage.

Skeletal elements in the zebrafish larva

In 5-day-old wild-type larvae the skeleton consists of a cartilaginous neurocranium and the pharyngeal skeleton. Elements that belong to the neurocranium and surround the sensory organs and the brain are the ethmoid plate (e), the trabeculae (t), the parachordals (pc) and the occipital arches (oa) (Fig. 1C,F). The pharyngeal skeleton is composed of the elements of the jaws and the more posterior gill-bearing pharyngeal arches. The first pharyngeal arch (mandibular arch) forms the jaw and consists of two elements. The palatoquadrate (pq) represents the upper jaw (dorsal element), and Meckel's cartilage (m) forms the lower jaw (ventral element) (Fig. 1E-H). The second (hyoid) arch is composed of the dorsal hyosymplectic (hs) and the ceratohyal (ch) more ventrally (Fig. 1E-H). The hyosymplectic possesses a facial foramen through which the hyomandibular trunk of the facial nerve and some rami of the anterior lateral line nerves run (Fig. 1F). The ceratohyal does not articulate directly with the hyosymplectic but contacts the small interhyal (ih) dorsally, which in turn articulates dorsally with the hyosymplectic (Fig. 1F). The more posterior gill bearing arches consist of ceratobranchials (cb) and hypobranchials (hb) (Fig. 1F-H). The small hypobranchials lie medial to the ceratobranchials and connect these elements with the unpaired basibranchials, which form at the ventral midline (Fig. 1G,H). The basihyal (bh) is the most rostral midline structure of the pharyngeal skeleton and lies dorsal to the ceratohyal (Fig. 1H). The only elements of the dermatocranium that have formed at this stage are the parasphenoid bone, located in between the trabeculae (t) (not illustrated), and the cleithrum (c) of the pectoral girdle (Fig. 1E,F). Other bony elements that have formed at this stage are the opercle (op), which articulates with the hyosymplectic and branchiostegal rays posterior to the ceratohyal (not illustrated). Since most of the dermatocranium and the axial skeleton have not formed before the larvae die, the development of these structures cannot be analyzed in the mutants discussed in the paper.

The development of the head skeleton in teleost fishes is described, elsewhere (e.g. de Beer, 1937; Langille and Hall, 1987; Miyake and Hall, 1994; Cubbage and Mabee, 1996; and Schilling and Kimmel, manuscript in preparation).

Mutant phenotypes

Mutations that affect the anterior arches

Mutations in four genes (*sucker*, *schmerle*, *hoover* and *sturgeon*) affect the two anterior arches. Live homozygous mutant larvae exhibit reduced lower jaws and their mouth opening forms a 'v' with an apex at the lower lip on day 3 (Fig. 2A). In these mutants the eyes, pigmentation, gut, liver, ear and trunk are unaffected, which indicates that the defects in the first two arches are not caused by general retardation of the embryos. Skeletal preparations reveal that Meckel's cartilage is shorter and comes to lie much further to the posterior than in wild-type embryos (Figs 2E, 3A-D, 4A,D). In most larvae the ventral components of the first two arches (Meckel's and ceratohyal cartilages) are reduced and fused to the dorsal elements (palatoquadrate and hyosymplectic cartilages) (Fig. 4A,D,F). In contrast, the dorsal components are normal or even enlarged, as is the case for the hyosymplectic cartilage in *sucker* and *schmerle* mutant embryos (Figs 2D, 3B,D). The mesodermally and neural crest-derived neurocranium is unaffected in all five genes. Although in all five genes the anterior arch elements are affected, skeletal preparations reveal that their skeletal phenotypes are distinct.

Table 1. Results of the screen for jaw mutants

Mutant class	Genes (number of alleles)	Unresolved	General description	References
'flatheads'	*fla (5), bab (2), ser (1), her (1), cod (1), dul (1), fat (1), pio (5)*	27	Head flattened dorsoventrally, small eyes and brain, loss of posterior arch elements.	a, b
Anterior	*suc (1), she (2), hoo (1), stu (2)*	0	Reduction of first two arches	c
'hammerheads'	*ham (2), hen (3), pek (1), get (2), jef (1), koi (1), ppt (4)*	13	No tissue anterior to the eyes. Cartilage differentiation affected	c, d
Other	*low (1), box (8), dak (3), dol (1), hot (1), pic (1), vgo (2)*	11		a, c, e, f, g

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

Table 2. Overview of mutants which affect the anterior arches and cartilage differentiation

Gene name	Alleles	Visible on day	Arch phenotypes	Other phenotypes	Other reference
Anterior arches reduced					
sucker (suc)	tf216b	3	Anterior arches reduced		
schmerle (she)	th210, tg203e	3	Anterior arches reduced	Variably shorter pectoral fins,	a
sturgeon (stu)	td204e, tg419	3	Anterior arches reduced		
hoover (hoo)	tn213	3	Anterior arches reduced	Short pectoral fins	a
Defects in several arches					
van gogh (vgo)	tm208 tu285	1	Meckel's inverted ch reduced post. arches reduced.	Small ear	b
dolphin (dol)	ti230g	4	Ant. ethmoid plate reduced tissue missing dorsal to jaw.		
hanging out (hot)	tq213d, tl20b, tv49b, tl17d, to273e	4	Open mouth, ceratohyal points ventrally		
Cartilage differentiation					
(1) Kinked elements and short body					
hammerhead (ham)	to16, te296c	3	Cartilage differentiation affected, no tissue ant. to eyes	Short pectoral fins, short body	a
head on (hen)	tq251f, tt209b, tu248	3	Cartilage differentiation affected, no tissue ant. to eyes	Short pectoral fins, short body	a
pekinese (pek)	td14	3	Cartilage differentiation affected, no tissue ant. to eyes	Short pectoral fins, short body	a
pipe tail (ppt)	ta98, te1c, tc271b, ti265	3	Cartilage differentiation affected, no tissue ant. to eyes	Shorter body axis, undulated notochord	c
Unresolved	tx239a	3	Cartilage differentiation affected, no tissue ant. to eyes	Short pectoral fins, short body	
(2) Severely reduced cartilage					
jellyfish (jef)	tw37	3	All cartilage highly reduced	Short pectoral fins	a
(3) Extracellular matrix affected					
geist (get)	ti240, ty111	3	Cartilage differentiation affected do not stain well	Short body	
Unresolved	tv42b	3	Cartilage differentiation affected. do not stain well	Short pectoral fins	a
	to119e	3	Cartilage differentiation affected, no tissue ant. to eyes	Short pectoral fins	a
	ty22e	3	Cartilage differentiation affected. do not stain well	Short pectoral fins	a
	ty118a	3	Cartilage differentiation affected. do not stain well	Short pectoral fins	a
	ti236	3	Cartilage differentiation affected, do not stain well	Short pectoral fins	a
(4) Slightly reduced elements					
	tc4	3	Cartilage differentiation affected, no tissue ant. to eyes		
	to259b	3	Cartilage differentiation affected, no tissue ant. to eyes		
	tx224	3	Cartilage differentiation affected, no tissue ant. to eyes		
(5) Tumor-like chondrocytes					
knorrig (koi)	tl226d	4	Tumor-like chondrocytes		
'hammerhead-like' Unresolved	td207	3	Cartilage differentiation affected, no tissue ant. to eyes		
	th05d	3	Cartilage differentiation affected, no tissue ant. to eyes		
	tl205b	3	Cartilage differentiation affected, no tissue ant. to eyes		
	tu259	3	Cartilage differentiation affected, no tissue ant. to eyes		
Others					
	te292d	3	Lower jaw protrudes		
	tx238c	3	Tip of mc points ventrally		
	tt209c	3	bh points ventrally		
	to274	3	mc points ventrally		
	tq221a	3	ch points ventrally		
	tq253g	3	Olfactory pits displaced medially		
	tg212	3	Meckel's points ventrally		
	tr206c	3	Open mouth		
	tn17a	3	Meckel's lies further posterior		
	tj214	3	Meckel's points dorsally		
Other mutant classes					
chameleon (con)	tf18b, th6, tm15a, ty60, tu214	2	Defective ventral neurocranium	Ventral brain defects	d
schmalspur (sur)	ty68b	2	Defective ventral neurocranium	Brain midline defects	d
iguana (igu)	tm79a, ts294e	2	Defective ventral neurocranium	Brain midline defects	d
you-too (yot)	ty17a, ty119	2	Defective ventral neurocranium	Brain midline defects	a
detour (dtr)	ts269, te370a, tm 276b	2	Defective ventral neurocranium	Brain midline defects	d
silberblick (slb)	tx226, tz216	0	Defective ventral neurocranium	Ventral forebrain defect	e

References: a, van Eeden et al. (1996); b, Whitfield et al. (1996); c, Hammerschmidt et al. (1996); d, Brand et al. (1996b); e, Heisenberg et al. (1996).
ch, ceratohyal; post, posterior; ant, anterior; bh, basihyal; mc, Meckel's cartilage.

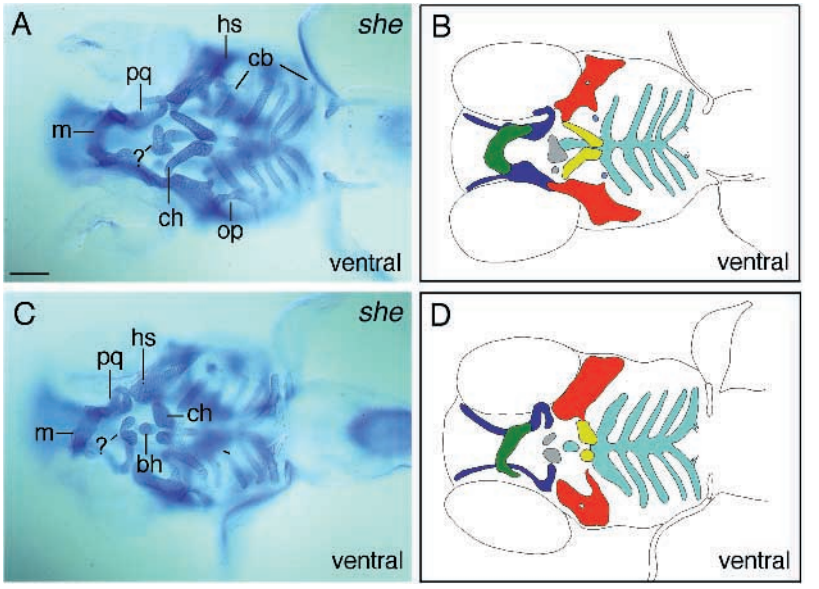

Fig. 3. Photomicrographs (A,C) and diagrams (B,D) of ventral views of *schmerle (th210)* larvae, showing reductions of elements of the first two arches. B and D depict the elements of larvae shown in A and C. Larvae of one egg lay show variable reductions of elements (A,C). In the less-affected larva (A,B) the ceratohyal articulates with the hyosymplectic further to the anterior, but points posteriorly, whereas in the more severely affected specimens (C,D) these elements are reduced to two roundish structures. In both embryos additional pieces of cartilage are located in the vicinity of the basihyal indicated by (?) (C,A). Scale bar, 100 μm.

A mutation in *sucker* causes the most severe reductions of the lower jaw, as revealed by a lateral view of the live larva (Fig. 2A). Skeletal preparations show that within the first arch the lower jaw (Meckel's cartilage) is severely reduced (Fig. 2B,E,F). The palatoquadrate (upper jaw) is also affected and only its pterygoid process seems to remain (Fig. 2B). In contrast, the dorsal part of the hyosymplectic (second arch) is broader than in wild-type larvae (Fig. 2D), whereas the ceratohyal cartilage is severely reduced (Fig. 2E,F). Ventral to the ceratohyal a plate-like shaped piece of cartilage is present which does not articulate with other elements and therefore cannot be identified in skeletal preparations (Fig. 2C,F). At this point, it is not possible to determine whether these structures represent additionally formed cartilages or whether they are transformations. All the posterior pharyngeal arches are present, although the third and fourth ones in particular are reduced in length (Fig. 2E-F).

In *schmerle* mutant larvae the reduction of the lower jaw is not as severe as in *sucker* mutant larvae, but the second arch is affected to the same degree. Particularly affected are the palatoquadrate and ceratohyal of the first and second arches, respectively. The posterior pole of the palatoquadrate curves either medially or laterally (Fig. 3B,D) and the ceratohyal cartilage is reduced to two small elements (Fig. 3C,D). In a few larvae, the ceratohyal is less reduced and retains its elongated shape (Fig. 3A,B) but the ceratohyal points posteriorly instead of anteriorly. Additional small elements can be found close to the basihyal (Fig. 3, grey elements). At this point, it is not clear whether these structures represent parts of the basihyal or if they develop from independent condensations of chondroblasts. All ceratobranchials are present, although the third and fourth onwards show a reduction in length (Fig. 3B,D).

Mutations in the genes *hoover* and *sturgeon* cause milder phenotypes. The basihyal cartilage of the first arch consists of more cells than in wild-type larvae and is more irregularly shaped (Fig. 4A,B,D) and additional small pieces of cartilage can be found posterior to Meckel's (Fig. 4B, arrow). In some homozygous *hoover* larvae the ceratohyal of one side consists of two pieces (Fig. 4D).

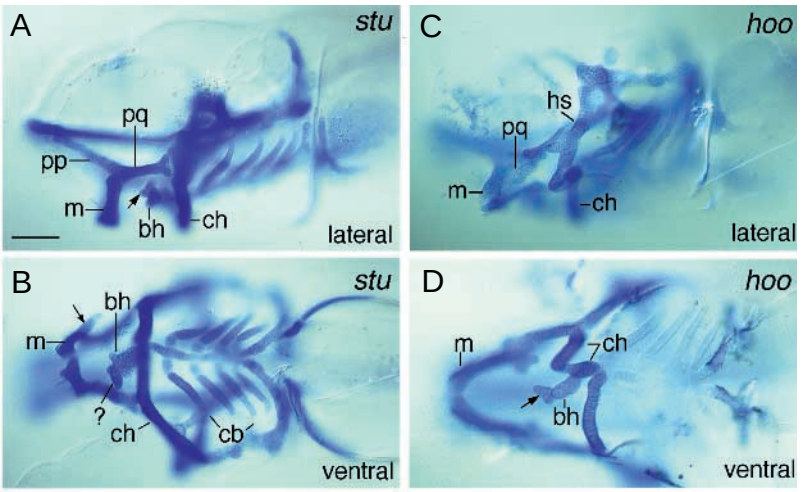

Fig. 4. Photomicrographs of *sturgeon* (td204e) (A,B) and *hoover* (C,D) mutant larvae in which Meckel's (first arch) is fused with the palatoquadrate. Lateral (A,C) and ventral (B,D) views of stained *sturgeon* and *hoover* larvae reveal that the ceratohyal is fused to the hyosymplectic as well. Additional unidentified pieces of cartilage occur in the vicinity of the basihyal and caudal to Meckel's (A,B,D, arrows). In *hoover* mutant larvae the ceratohyal cartilage is sometimes divided into two pieces (D). Scale bar, 100 μm.

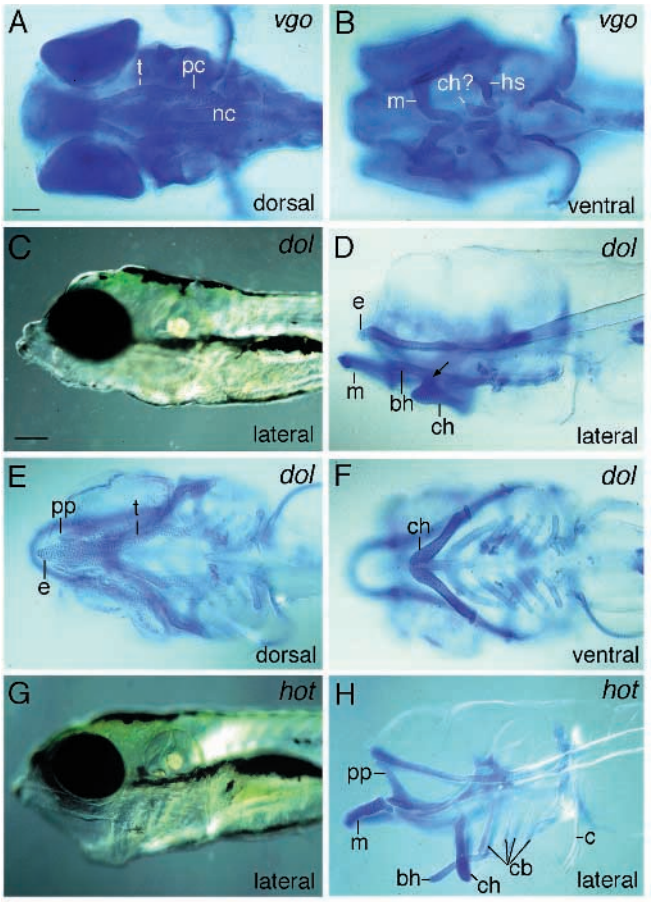

Fig. 5. Photomicrographs of *van gogh* (tm208) (A,B), *dolphin* (C-F) and *hanging out* (G,H) mutant larvae. In *van gogh* mutant embryos all elements are severely malformed. A ventral view (A) reveals that Meckel's is inverted and the ceratohyals are not clearly identifiable any more. The posterior arches are reduced or absent. The ear capsules are misshapen, as can be seen from a dorsal view (A). Live *dolphin* mutant larvae are characterized by a lack of tissue dorsal to the ethmoid plate (C). The ethmoid plate is arrow-like in shape and consists of fewer chondrocytes anteriorly (E). Lateral (D) and ventral (F) views of stained larvae show that the the ceratohyals fuse dorsally with each other and the overlying basihyal. In larvae homozygous for *hanging out*, the mouth always stays open (G) and the pharyngeal skeleton points ventrally (H). Scale bars: 200 μm (C and G) and 100 μm (A,B,D,E,F and H).

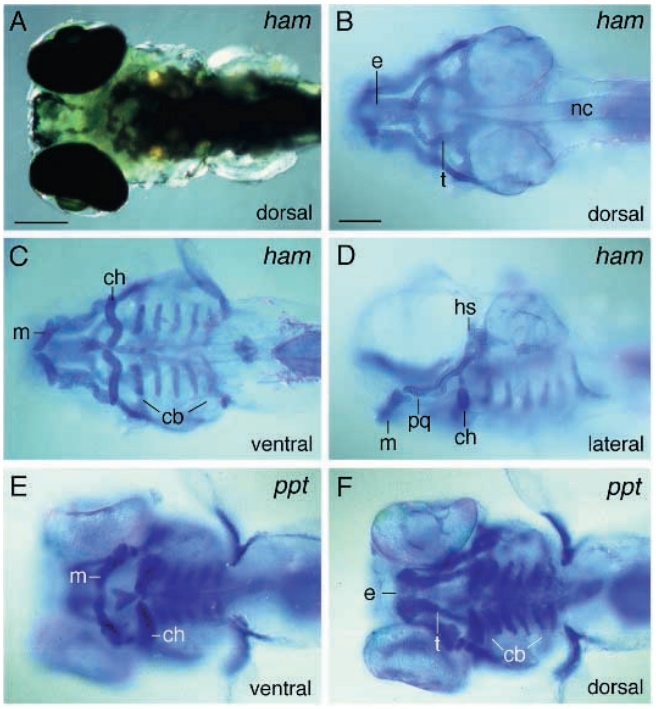

Fig. 6. Photomicrographs of live (A) and Alcian blue-stained (B-F) larvae belonging to the 'hammerhead-like' group. The live larvae are characterized by a lack of tissue anterior to the eyes, as can be seen from a dorsal view (A). *Hammerhead* mutant larvae (A-D) are characterized by a shortened head and kinked elements. All elements are present but consist of smaller and less organized chondrocytes (B-D). The pharyngeal skeleton of *pipe tail* (ti265) mutant embryos (E,F) is reduced in length but appears thicker, especially the ceratohyal (E). Meckel's cartilage is located further to the posterior, at about the level of the center of the eyes. The neurocranium is also reduced in length and the ethmoid plate is less differentiated (F). Scale bars: 200 μm (A) and 100 μm (B-F).

Mutations that cause defects in several arches

Homozygous mutant larvae for the *van gogh* mutation show defects in the formation of the entire pharyngeal skeleton (Fig. 5A-B). In addition, the larvae are characterized by a malformation of the ear, described in the paper by Whitfield et al. (1996).

Skeletal preparations reveal that Meckel's cartilage is inverted and points posteriorly and ventrally but is not reduced in size (Fig. 5B). The palatoquadrate of the first arch is reduced, and the ceratohyal of the second arch cannot be identified with certainty any more (Fig. 5B). It is possibly represented by a piece of cartilage located medially to the symplectic cartilages, but does not articulate with it any more. The ceratobranchials are also highly reduced or even absent (Fig. 5B). The ear capsules are malformed and reduced and the mesodermally derived parachordals lie further to the posterior and leave the tip of the notochord free of cartilage (Fig. 5A). Therefore, in *van gogh* mutant embryos both the neural crest-derived viscerocranium and the mesodermally derived parts of the neurocranium are affected. Some of the mutants described in the accompanying paper also affect several arches but never exhibit such strong defects in the first arch as in *van gogh* mutant embryos (Schilling et al., 1996).

From a lateral view of live *dolphin* mutant embryos the upper and lower jaw protrude and resemble a beak-like structure (Fig. 5C). This appearance is caused by a lack of tissue dorsal to the upper jaw, possibly forebrain, and is first visible on day 4 of development. At the distal end of the ceratohyal dorsal outgrowths of chondrocytes fuse with the basihyal, which passes dorsally (Fig. 5D, arrow). The ceratobranchials, in contrast, are normally shaped whereas the ethmoid plate of the neurocranium is reduced at its anterior pole and pointy instead of roundish (Fig. 5E). The combination of defects in the ethmoid plate and the tissue that lies dorsal to it suggest that interactions between these two populations of cells are necessary to induce cartilage differentiation in the ethmoid plate. Other components of the neurocranium,

such as the mesodermally derived parachordals, are reduced, whereas the occipital arches are unaffected.

Embryos homozygous for the *hanging out* mutation are also characterized by an open mouth (Fig. 5G), but all cartilaginous elements appear normal. Skeletal preparations reveal that Meckel's and the ceratohyal point ventrally, as during lower jaw depression while feeding (Fig. 5H). Possibly, the fixed position of the skeletal element is caused by permanent contractions of muscles involved in lower jaw depression.

Mutants with abnormal chondrocyte morphology

27 of the 'hammerhead-like' mutants, which can be subdivided into five groups, exhibit defects in cartilage formation and are characterized by a lack of tissue anterior to the eyes. Despite similarities in their external phenotype the mutants belong to at least eight different complementation groups and show distinct defects in the formation of their chondrocranium. The mutant larvae die at around 9 days of development.

(1) Mutants characterized by kinked elements and shortened bodies

Larvae homozygous for the *hammerhead (ham)* mutation are characterized by a reduction in head length of approximately 20%, shortened body length and smaller pectoral fins. The eyes form the most anterior structures in the head (Fig. 6A).

Skeletal preparations reveal that all elements of the pharyngeal skeleton are present but smaller and severely kinked, independently of their location along the A-P axis (Fig. 6B-D). They consist of smaller chondrocytes, which are not aligned in single cell rows as in wild-type embryos but seem to be randomly arranged within the elements. The prechordal neurocranium is shorter than in wild-type embryos but retains the same width and the trabeculae are oriented perpendicular to the ethmoid plate and thus contribute to the shortening of the head (Fig. 6B). The mesodermally derived components of the neurocranium, such as the parachordals and the occipital arches, are more reduced than the neural crest-derived neurocranium or are even absent.

The phenotypes of *head on (hen)* and *pekinese (pek)* larvae are very similar to that of *hammerhead* larvae, with the exception that the occipital arches and parachordals are not as severely reduced (not illustrated). Following day 5 of development in *pek* mutant larvae all fins start to necrose.

As in other *hammerhead-like* mutants, all cartilaginous elements of *pipe tail (ppt)* mutant embryos are reduced in size but consist of even smaller and more numerous chondrocytes (Figs 6E-F, 8E). Although the ceratohyal is also reduced in size it is strikingly thicker than the other elements (Fig. 6E). In addition, Meckel's cartilage is located further to the posterior and points ventrally. Reductions can also be observed in the length of the ethmoid plate (Fig. 6F). *Pipe tail* mutants are also characterized by defects in tail formation which are described in an accompanying paper by Hammerschmidt et al. (1996).

(2) Severe reduction and loss of cartilage in *jellyfish* mutant larvae

Live homozygous *jellyfish* (*jef)* larvae can be identified by a lack of tissue anterior to the eyes (Fig. 7A) and by a slight decrease in body length. Skeletal preparations reveal that mutant embryos homozygous for this mutation hardly possess any cartilaginous elements, with the exception of two paired pieces in the approximate position of the ceratohyals and sometimes remnants of Meckel's and the palatoquadrate (Fig. 7B). The neurocranium and cartilaginous elements of the pectoral girdle, like the coracoid, are also completely lacking. In contrast, the cleithrum, a bony element of the pectoral girdle, is present, as is the parasphenoid bone at the base of the cranium. Teeth can also be observed, although no seventh ceratobranchial can be seen in the skeletal preparations. In these mutants it is striking that both neural crest and mesodermally derived cartilages are lacking although dermal bones start to form. This indicates that this gene is not required for dermal bone development but is necessary for the differentiation of both neural crest and mesodermal mesenchyme into cartilage.

(3) Mutations that affect the extracellular matrix

5-day-old homozygous *geist* (*get)* larvae show an approx. 20% reduction in body length. The cartilaginous elements of the viscerocranium are all present but are composed of less orderly aligned cells (Fig. 7C,D). The extracellular matrix of these cells stains only faintly with Alcian blue (Fig. 8F), which indicates that the composition of the matrix might be altered.

In five other mutants (*tv42b*, *to219*, *ty22e*, *ty118a* and *ti236*, not illustrated) the cartilaginous elements are also less stainable with Alcian blue but their body length is not reduced. In addition, they show reductions in Meckel's cartilage, which is not true for *geist* mutant larvae. The cartilaginous elements are severely reduced in size and consist of smaller and randomly organized chondrocytes. Nevertheless, the elements appear to have the same thickness as wild-type structures. All mutant larvae are characterized by shorter pectoral fins (van Eeden et al., 1996). Based on preliminary complementation analysis, there appear to be at least four genes affected in these mutants.

(4) Mutants with reduced or malformed elements

In contrast to the mutants described above, in *tc4* mutant larvae the cartilaginous elements stain normally (not illustrated). As in *tv42b* and *to219* homozygous larvae Meckel's cartilage lies further to the posterior but is slightly kinked, as is the ceratohyal. Nevertheless, the two elements are not as severely malformed as in *hammerhead*, *head on* and *pekinese* mutant larvae.

In mutant larvae homozygous for *to259b* (not illustrated), Meckel's cartilage is reduced in length and the palatoquadrate and ceratohyal consist of smaller and less organized cells. The ceratobranchials appear wild-type in shape, whereas the neurocranium is also reduced in length.

Homozygous *tx224* larvae (not illustrated) show a subtle reduction of the length of Meckel's cartilage, the palatoquadrate and the ceratobranchials, but stain normally. The anterior pole of the neural crest-derived ethmoid plate is irregularly shaped and the trabeculae are a slightly kinked.

(5) Tumor-like growth of chondrocytes

In larvae homozygous for *knorrig* (*koi*), all elements are smaller and thicker but the individual chondrocytes are normal-sized (Figs 7E-F, 8D). In addition, the edges of the elements are not clearly defined and tumor-like outgrowths of chondrocytes occur along the margin of every cartilaginous element in the head, including the neurocranium (Fig. 8D). In contrast, the pectoral fin cartilage seems to be unaffected.

DISCUSSION

Genes that function in the development of the first two arches

Mutations in five genes (*sucker, schmerle, hoover, sturgeon, gaping mouth*) cause reductions in the anterior arch elements but leave posterior ones mostly unaffected. In contrast to the 'hammerhead-like' mutants, the morphology of the chondrocytes appears normal, which suggests that differentiation is not affected. We hypothesize that the neural crest cells that normally migrate into the two anteriormost arches are affected by these genes. It has been shown in transplantation experiments between urodeles and anurans, and between chick and quail (Andres, 1949; Wagner, 1949; Noden, 1983), that neural crest cells are specified with respect to the axial level they originate from and that they carry information of the species-specific shape of skeletal elements. Since the cartilaginous structures of the first two arches are misshapen in our mutants, this could indicate that the neural crest cells that give rise to these elements are misspecified. Further studies will be necessary to determine if this is the case.

The mutations described here do not exclusively affect the anteriormost arches, but also consistently cause defects in the second (hyoid) arch. This implies that the mechanisms that pattern the first and the more posterior arches are somehow linked and possibly regulate each other, as proposed by Rijli et al. (1993). In the adult animal the mandibular and hyoid arches also form a functional unit in the process of lower jaw depression during feeding (Westneat, 1990), which may reflect the common mechanism underlying their development. Likewise, in mutants in which the posterior pharyngeal arches are affected we observe reductions or absence of several adjacent segments instead of individual ones (Schilling et al., 1996).

The dorsal and ventral structures within the first two arches are affected to different degrees. For example, the ceratohyal, which represents the ventral part of the hyoid arch, is reduced in size whereas the hyosymplectic, which is the corresponding dorsal component of the hyoid arch, is enlarged. This suggests that genes which pattern the anterior arches have different effects on dorsal and ventral structures, respectively. Such differences have also been proposed for the action of *Hox* genes in the spinal cord of mice (Graham et al., 1991).

Among the mutations that affect the pharyngeal arches, none were found to exhibit obvious transformations of skeletal elements, as has been described for mouse embryos homozygous for the *Hoxa-2* mutation. In these embryos a transformation of second arch structures into first arch structures takes place (Rijli et al., 1993, Gendron-Maguire et al., 1993). There are at least two explanations for the fact that we did not find the strong transformation we would expect from a *Hoxa-2* mutant. Either the absence of this gene causes different phenotypes in lower and higher vertebrates and therefore was not recognized, or mutations in the *Hoxa-2* gene have not been identified in zebrafish. For example, our mutations could be alleles of other anteriorly expressed genes like *Otx* amd *Emx*. Mutations in such genes might not cause transformations of skeletal elements, but rather deletions of anterior structures as was shown in *Drosophila* (Finkelstein and Perrimon, 1990; Cohen and Jürgens, 1990).

Although none of our phenotypes resembles the one caused by a mutation in the *Hoxa-2* gene, it is possible that the ectopic cartilages that we observe in *sucker*, *schmerle*, *hoover* and *sturgeon* mutant larvae represent homeotic transformations. Nevertheless, the identity of these cartilages remains hypothetical until more detailed studies of the identity of rhombomeres, the innervation of tissues surrounding these elements and the specification of neural crest cells that migrate into these arches have been carried out. Additionally, lineage tracings of neural crest cells and studies of gene expression in these cells are necessary to determine whether their fate is transformed or if only specific subpopulations of the neural crest are affected.

The dolphin phenotype could be caused by impaired tissue interactions

While the phenotype of the mutants with defects in the anteriormost arches might be explained by a misspecification of neural crest cells, the defect in the anterior ethmoid plate in *dolphin* mutant embryos is possibly caused by the lack of tissue anterior to the eyes. Although we have not yet investigated which dorsal tissues are absent we believe that interactions with these tissues are necessary for the differentiation of cartilage in the ethmoid plate. Some of the curly tail mutants (*chameleon, schmalspur, iguana, you-too* and *detour*) and one forebrain mutant (*silberblick*) that exhibit ventral brain defects also affect the underlying neurocranium (Brand et al., 1996b; Heisenberg et al., 1996; and our unpublished results). In these mutants, however, inhibiting signals from ventral brain tissues may be absent, since neural crest cells migrate further ventrally and end up closer to the midline. Thus paired elements of the neurocranium, like the trabeculae, sometimes even fuse. The phenotypes of *dolphin* and the curly tail mutants suggest that the brain emits both inducing and inhibiting signals which play a role in patterning the neural crest-derived neurocranium (Brand et al., 1996b).

The mutations that affect anterior arch structures might enable us to elucidate the genetic mechanisms which underlie the patterning of these arches. To further characterize these mutants, detailed investigations of the central and peripheral nervous system are necessary. Neurons and axonal tracts serve as markers for segment identity and will also provide us with more information on how distinct subpopulations of neural crest cells are affected by particular genes.

Hammerhead-like mutants show defects in cartilage differentiation

As soon as the neural crest cells leave the neural tube they are subjected to factors which control their migration and initiate differentiation at their destination. For example, several studies have shown that migrating neural crest cells have to undergo epithelial-mesenchymal interactions before they are capable of differentiating (Hörstadius, 1950; Hall, 1980).

Chondroblasts reaching their target site, form condensations in which differentiation commences, given that these condensation sites reach a certain size (Thorogood, 1983; Hall, 1984). The subsequent growth of differentiated chondrocytes is controlled by both intrinsic and extrinsic factors such as the secretion of extracellular matrix, cell enlargement, interstitial cell division and apposition from the perichondrium (reviewed by Hinchliffe and Johnson, 1983).

Although we have not studied the mutants described here in

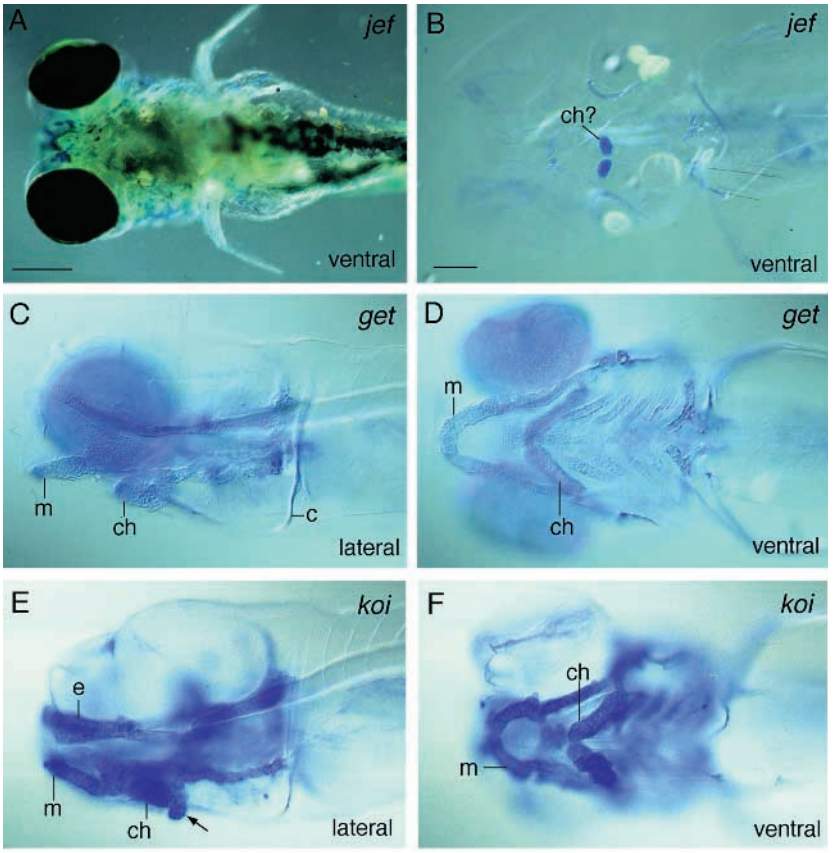

Fig. 7. Photomicrographs of several members of the hammerhead-like group stained with Alcian blue. Mutant *jellyfish* embryos (A,B) can be identified by a severe lack of tissue anterior to the eyes (A, ventral view) and strongly reduced or loss of cartilage. Two elements are always present at the level of the ceratohyals (B, ventral view). In *geist* (ti240) mutant larvae (C,D) the elements hardly stain with Alcian blue and consist of less organized chondrocytes. Tumor-like outgrowths of chondrocytes are characteristic for *knorrig* mutant larvae (E,F). From a lateral view (E), additional chondrocytes can be seen, particularly ventral to the ceratohyal (arrow). A ventral view (F) reveals that none of the elements possesses well defined edges. Scale bars: 200 μm (A) and 100 μm (B-F).

sufficient detail, we believe that those mutations influencing cartilage differentiation might affect distinct regulatory events during chondrogenesis. We have isolated 27 mutants that both lack tissue anterior to the eyes and exhibit defects in cartilage differentiation. In Alcian blue-stained embryos all elements are affected to the same degree and no change of shape that is specific to particular elements is observed.

Genes that affect cartilage formation in the head and fins, as well as trunk development

Mutants that belong to the first subclass (*hammerhead, head on* and *pekinese*) are characterized by a severe reduction in body length, highly kinked cartilaginous elements and shortened pectoral fins. Because the individual chondrocytes are smaller and appear to be surrounded by less extracellular matrix it is likely that these genes affect cartilage growth. The combination of phenotypic traits of these mutants resembles the phenotypes described for naturally occuring mutations in mice, which are characterized by dwarfism, shortened head and limbs and altered morphology of chondrocytes, e.g. *shorthead, cartilage anomaly, phocomelia, achondroplasia, stubby, brachymorphic* and *cartilage matrix deficiency* (Fitch, 1961; Lane and Dickie, 1968; Sisken and Salome, 1959; Johnson and Wise, 1971; Rittenhouse et al., 1978). It has been shown that *cartilage matrix deficiency* is caused by a deletion in the aggrecan gene (Watanabe et al., 1994). Based on the similarity between the phenotypes observed in mice and zebrafish it is possible that these phenotypes are caused by mutations in homologous genes.

It is not yet known why these mutants show shortened

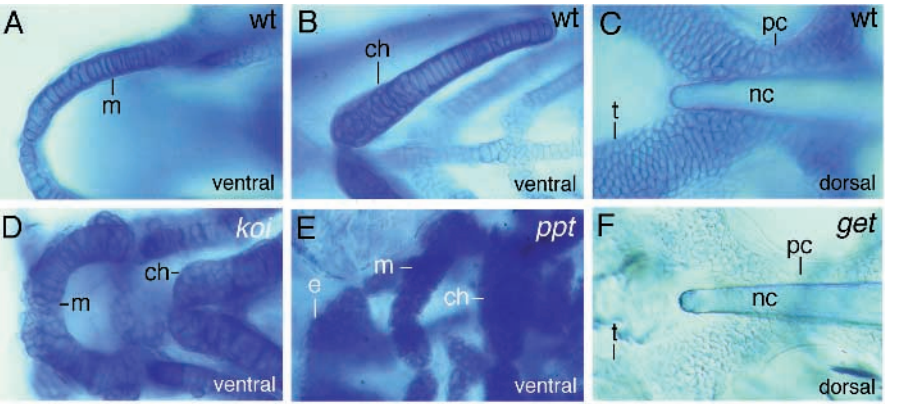

Fig. 8. Photomicrographs of cartilaginous elements of wild-type (A-C), *knorrig* (D), *pipe tail* (E) and *geist* (F) mutant larvae. (A,D,E) show ventral close-up views of Meckel's and the ceratohyal. In *knorrig* mutant larvae (D), Meckel's is smaller but thicker and chondrocytes grow along the edges. In *pipe tail* mutant embryos, Meckel's lies further to the posterior and all elements consist of smaller but more numerous cells. The ceratohyal is relatively bigger than the other elements. Dorsal views of the neurocranium (C,F) indicate that chondrocytes of *geist* mutant larvae hardly stain with Alcian blue.

bodies in addition to their cartilage phenotype. In the zebrafish mutants at least, the axial skeleton has not formed at this stage and therefore it seems that these genes also function in other tissues besides chondrogenic mesenchyme.

In the neurocranium of *hammerhead*, *head on* and *pekinese* mutants the mesodermally derived parachordals and the occipital arches are absent or are more severely reduced than the neural crest-derived elements. This suggests that these two mesenchymal tissues might respond differently to these particular genes.

In *pipe tail* mutant embryos the cartilaginous elements are composed of much smaller but seemingly more numerous chondrocytes (Figs 6E,F, 8E). There are a number of possible explanations for this phenomenon. For example it is feasible to imagine that more neural crest cells migrate ventrally and differentiate into chondrocytes or that the length of the chondrocyte cell cycle is decreased, resulting in a higher number of chondrocytes. Possibly the neural crest cells carry information about the approximate size of the elements and therefore upregulate cell division to compensate for the decrease in cell size. Biochemical analysis is needed before we can assess which processes in cartilage differentiation or growth are affected by the *pipe tail* gene. As discussed by Hammerschmidt et al. (1996), this gene is also involved in proper anterior-posterior outgrowth of the body axis early in development. This interpretation is supported by the shortened neurocranium in these mutants.

The *jellyfish* gene might be involved in the differentiation of both neural crest cells and mesodermal mesenchyme into chondrocytes

In contrast to the mutants described above, *jellyfish* mutant larvae are characterized by an almost complete loss of cartilage, with the exception of some highly reduced elements. This phenotype could be explained by defects in neural crest cells, but since mesodermally derived mesenchyme is also reduced, this gene is probably involved in cartilage differentiation in general. Dermal bones like the cleithrum of the shoulder girdle and the parasphenoid bone are present, which indicates that different genes are responsible for the formation of cartilage and dermal bones. In addition, the presence of teeth shows that tooth formation is independent of cartilage differentiation.

Genes that affect the extracellular matrix

Skeletal stainings of *geist* and five other mutants show that cartilaginous elements in these mutants only stain faintly with Alcian blue. Since Alcian blue stains extracellular matrix molecules like glycosaminoglycans, this suggests that the composition of the matrix has changed. In contrast to the *geist* gene, the other five genes are not involved in the development of tissues in the trunk.

The *knorrig* gene controls cell growth

In *knorrig* mutant embryos the growth of chondrocytes is severely disturbed. Along every cartilaginous element, including the neurocranium, tumor-like outgrowths of chondrocytes can be observed. It is possible that *knorrig* is not directly involved in cartilage differentiation but belongs to a group of tumor suppressor genes or affects the cell cycle of these cells.

Biochemical studies are necessary to further analyze the defects of the mutants that affect cartilage differentiation and growth. The development of an *in vitro* organ culture system for the development of fish skeletal tissues by Miyake and Hall (1994) might be a helpful technique for investigating these processes. Thus the mutations that affect cartilage differentiation and growth provide us with valuable tools for studying craniofacial development and might eventually help us to learn more about the causes of craniofacial malformation in humans.

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