Musculoskeletal patterning in the pharyngeal segments of the zebrafish embryo

Thomas F. Schilling[1],* and Charles B. Kimmel[2]

[1]Molecular Embryology Laboratory, Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, UK
[2]Institute of Neuroscience, University of Oregon, Eugene, Oregon 97403-1254, USA

*Author for correspondence (e-mail: schillin@icrf.icnet.uk)

SUMMARY

The head skeleton and muscles of the zebrafish develop in a stereotyped pattern in the embryo, including seven pharyngeal arches and a basicranium underlying the brain and sense organs. To investigate how individual cartilages and muscles are specified and organized within each head segment, we have examined their early differentiation using Alcian labeling of cartilage and expression of several molecular markers of muscle cells. Zebrafish larvae begin feeding by four days after fertilization, but cartilage and muscle precursors develop in the pharyngeal arches up to 2 days earlier. These chondroblasts and myoblasts lie close together within each segment and differentiate in synchrony, perhaps reflecting the interdependent nature of their patterning. Initially, cells within a segment condense

and gradually become subdivided into individual dorsal and ventral structures of the differentiated arch. Cartilages or muscles in one segment show similar patterns of condensation and differentiation as their homologues in another, but vary in size and shape in the most anterior (mandibular and hyoid) and posterior (tooth-bearing) arches, possibly as a consequence of changes in the timing of their development. Our results reveal a segmental scaffold of early cartilage and muscle precursors and suggest that interactions between them coordinate their patterning in the embryo. These data provide a descriptive basis for genetic analyses of craniofacial patterning.

Key words: branchial arch, neural crest, segmentation, zebrafish

INTRODUCTION

Vertebrates develop a precise network of musculoskeletal connections. In the head, these are subdivided along the anterior-posterior (AP) axis into reiterated segments, the pharyngeal arches, and a dorsal neurocranium. In all vertebrates that have been examined the pharyngeal skeleton is derived embryonically from neural crest, while muscles are derived from mesoderm (LeDouarin, 1982; Noden, 1983b; Schilling and Kimmel, 1994). Pharyngeal segmentation is patterned, in part, by the segmental migration of neural crest and this correlates with rhombomeric organization of the hindbrain (Lumsden et al., 1991; Schilling and Kimmel, 1994; Koentges and Lumsden, 1996). However, how this translates into a segmentally organized set of cartilages, bones and muscles is unclear.

It is difficult to distinguish cranial neural crest and mesoderm by morphology alone. Thus, little is known about the spatial relationships of skeletal and muscle precursors in any vertebrate embryo. However, some evidence suggests that patterning is coordinated between the two. In the chick, cartilages and connective tissue muscle attachments in the same segment are derived from neural crest that share the same rhombomeric origins (Noden, 1983a,b; Koentges and Lumsden, 1996). In mouse embryos, these neural crest cells initially surround a central core of mesoderm in each pharyngeal arch (Trainor and Tam, 1995). In both frog and chick embryos, cranial neural crest cells can reorganize skeletal and

muscle patterns when grafted to ectopic AP levels (Horstadius and Sellman, 1946; Noden, 1983a). Members of the *Hox* family of homeodomain transcription factors specify AP identity both in the hindbrain and in the neural crest-derived skeleton, and possibly mesoderm (reviewed in Krumlauf, 1994; Rijli et al., 1993).

There are seven pharyngeal arches in the zebrafish embryo, each with distinct dorsal and ventral sets of cartilages and muscles (Schilling and Kimmel, 1994; Schilling et al., 1996b). Both sets within a segment may be subdivided from a common arch primordium. Studies in teleost embryos both by Bertmar (1959) of the chondrocranium, and Edgeworth (1935) of muscles, have hypothesized such a progressive subdivision based on histological analyses. However, molecular evidence to support this idea is available only for zebrafish: as pointed out by Miyake et al. (1992), the dorsal subdivision of the putative mandibular muscle plate, constrictor dorsalis mandibularis, is specified by Engrailed (Eng) homeoprotein expression and splits into two muscles that continue expressing Eng in the larva (Hatta et al., 1990). This suggests that other subsets of bones or muscles are specified by their own unique molecular identities.

The early specification of jaw muscle precursors by Eng expression also suggests that many muscles may be organized by specialized pioneer or founder cells, as has been observed in insects (Ho et al., 1983; Bate, 1990) or, to some extent, in developing somites (Felsenfeld, 1991; Devoto et al., 1996). Like

somites, cranial skeletal muscles in vertebrates are derived from paraxial mesoderm (Noden, 1983a,b; Schilling and Kimmel, 1994), and express a stereotyped sequence of myogenic regulatory genes that mark differentiating myoblasts. Among these *myf-5* and *myoD* are expressed earliest in the mammalian somite and, along with other basic helix-loop-helix proteins, are essential for muscle differentiation (Rudnicki et al., 1993). In the zebrafish somite these mark early differentiating muscle pioneers and adjacent adaxial cells (Weinberg et al., 1996; Thisse et al., 1993). Following early specification by myogenic genes, structural genes such as *tropomyosin* (Thisse et al., 1993) and myosins (Devoto et al., 1996) are expressed to give muscles their contractile functions. The exact sequence of myogenic gene activation and requirements for these genes in cranial muscles have not been examined.

The large anterior arches, mandibular and hyoid, of jawed vertebrates are thought to have evolved from a more simple, segmental set of arches in their jawless ancestors (reviewed by Forey and Janvier, 1993). Thus it is important to determine segmental homologies within the arches, a complex task requiring a more detailed developmental analysis. There are numerous studies of cranial bones and muscles in gnathostome fishes, encompassing development (Edgeworth, 1935; Bertmar, 1959; Langille and Hall, 1987; Vandewalle, 1992) comparative anatomy and phylogenetic analyses, including several for zebrafish (Pashine and Marathe, 1974; Miyake et al., 1992; Miyake and Hall, 1994; Cubbage and Mabee, 1996) and other ostariophysines (Tewari, 1971; Pashine and Marathe, 1977). Most have focused on larval and adult stages, providing the basis for a detailed look at the embryonic period in zebrafish.

Zebrafish also provide the opportunity for a genetic and molecular dissection of pharyngeal patterning. Fate maps for both neural crest and paraxial mesoderm have revealed segment and cell type-restricted lineages of pharyngeal precursors, suggesting that pharyngeal arches are lineage-restricted compartments (Schilling and Kimmel, 1994). A large collection of

mutants that disrupt the neural crest-derived skeleton are available (Driever et al., 1996; Haffter et al., 1996; Neuhauss et al., 1996; Piotrowski et al., 1996; Schilling et al., 1996a,b) and can now be used to dissect genetic pathways underlying segmentation and musculoskeletal patterning. A mosaic analysis of mutations in the gene *chinless (chn)*, using cell transplantation, has shown that the wild-type gene is required autonomously for formation of pharyngeal cartilage and non-autonomously for formation of cranial muscles (Schilling et al., 1996a).

In this study we (1) describe the craniofacial anatomy of the zebrafish, including segmental homologies in the arches, and (2) analyze the segregation and differentiation of cartilage and muscle using Nomarski optics as well as molecular markers. We focus on the embryonic period, during jaw elongation, when the pattern is established, and it is this pattern that is disrupted by all of the mutations now available. A detailed knowledge of the

Table 1. Relationship of jaw elongation to head length at different temperatures in the zebrafish

Group*	HL†	YL‡	D§	h(27)¶	h(28.5)**
6	430	105	0.1	48	[44]
7	435	100	0.1	49	[45]
6	440	115	0.2	50	[46]
3	440	120	0.3	51	[47]
2	480	180	0.3	{55}	50
5	560	300	0.4	{58}	53
1	600	420	0.6	65	[60]
6	610	400	0.5	69	[63]
6	610	450	0.7	76	[70]
6	625	485	0.8	78	[72]
4	640	540	0.9	{81}	74
6	695	620	1.0	96	[89]
6	720	700	1.1	100	[92]

*Seven separate groups of staged siblings were examined.
†Head length in μm.
‡The distance from the anterior end of the yolk to the lower lip in μm.
§The fraction of the eye's diameter that the jaw has extended.
¶Parentheses indicate the calculated age at 27°C based on measurements at 28.5°C.
**Brackets indicate the calculated age at 28.5°C based on measurements at 27°C.
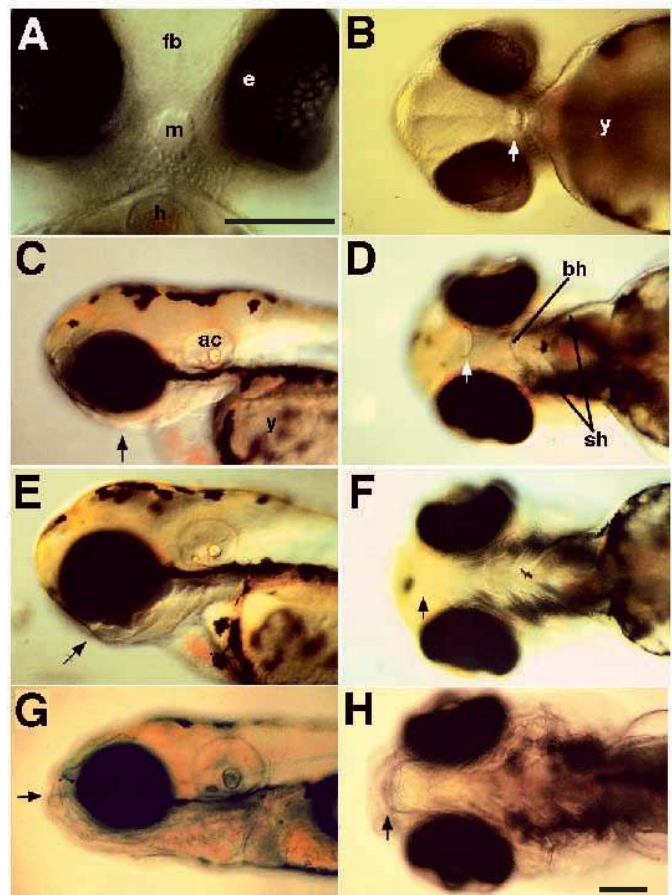

Fig. 1. Head morphology and jaw extension during the hatching period. (C-H) Left side and ventral views of the same embryo at each stage. The mouth is indicated by an arrow. (A,B) Ventral views at 50 h (0.1 D). (C,D) 65 h (0.6 D). The auditory capsule is rounded. The ventral boundary of the hyoid arch (the developing basihyal) is visible in ventral view just anterior to the heart. (E,F) 77 h (0.8 D). Four arches are visible in ventral view. The auditory capsule is more rectangular. The heart has receded posteriorly with the yolk cell. (G,H) 120 h (1.3 D). The mouth lies anterior to the eyes. Seven pharyngeal arches are visible in ventral view. ac, auditory capsule; bh, basihyal; e, eye; fb, forebrain; h, heart; m, mouth; sh, sternohyal; y, yolk. Scale bars, 100 μm.

differentiation pattern will allow us to test models proposed by Noden (1983a), regarding interactions between neural crest and mesoderm in AP patterning, and by Edgeworth (1935) and Bertmar (1959) as to the subdivision of early pharyngeal primordia into individual cartilages and muscles. In support of these hypotheses, we describe cases where more than one cartilage or muscle arises from a single primordium. We propose a scheme of segmental homologies and suggest that differences between segments can be explained by heterochronic changes in their development. Furthermore, premyogenic cells differentiate in close synchrony with their cartilage neighbors, perhaps reflecting interdependency between the two tissue types.

MATERIALS AND METHODS

Embryos and staging

Embryos were collected after pair matings from stocks of wild-type or *golden* mutant, *gol^{b1}*, zebrafish, maintained at 27°C or 28.5°C and staged according to Kimmel et al. (1995). Embryos homozygous for *gol^{b1}* were used for most histological preparations and stainings because of their reduced pigmentation. Control experiments comparing Alcian labeling showed that this mutation does not change the rate of cartilage development. Embryonic stages are designated in hours postfertilization at 28.5°C (h) or from the beginning of jaw elongation (approx. 50 h) by the distance that the lower jaw has extended (Table 1). Jaw extension was measured in micrometers (μm), and this was converted to eye diameters (D), representing the fraction of the AP length of the eyes that the elongating jaw primordium has reached. For this measurement animals were anaesthetized in tricaine, mounted ventral side up in 3% methyl cellulose, and examined with a Zeiss microscope fitted with an ocular micrometer. This distance can be estimated quickly by comparing the position of the lower lip with the lens size and position, which is approximately 0.25 eye diameters and in the exact eye center.

Histology and skeletal preparation

Several staged series of embryos and larvae, some from single pair matings, were fixed every 1-3 hours in 10% buffered formalin between 48 and 96 h, and stained for developing cartilage with Alcian blue or green, as described previously (Schilling et al., 1996). We stained siblings raised in identical conditions to control for individual age variations (Cubbage and Mabee, 1996). In zebrafish, Alcian-stained preparations can be used for describing individual cartilages from as early as 50 h. We use the terms 'chondrification' or 'cartilage differentiation' to mean detectable Alcian labeling. In contrast, precartilage condensations that do not stain with Alcian can be identified earlier in sections or in whole mounts with Nomarski optics. Some embryos were fixed with 5% trichloroacetic acid, instead of formaldehyde, which improved visualization of cartilages with Nomarski optics.

Several staged series of plastic and paraffin sections were cut in horizontal, transverse and parasagittal planes. For plastic sections, tissue was fixed in Bouin's, dehydrated and embedded in Epon. Sections, 7.5 μm in thickness, were cut on glass knives, dried down on droplets of water and stained with Azure II, Methylene blue and Basic Fuchsin (Humphrey and Pittman, 1974). Stained sections were air dried and mounted in Permount.

Immunohistochemistry and visualization of muscle innervation

For visualizing differentiated myofibers, embryos were labeled with the anti-myosin antibody 1025 (kindly provided by Dr S. Hughes), which recognizes several myosin subtypes. Cranial nerves were labeled with an antibody that recognizes acetylated tubulin. Specimens fixed overnight in 4% paraformaldehyde were permeabilized in 0.1% trypsin, dissolved in a saturated solution of sodium tetraborate, for 1-5 hours depending on age, and 'cracked' in cold acetone (−20°C) for 10 minutes. Whole-mount immunostaining used a peroxidase-antiperoxidase complex and was visualized by diaminobenzidine following the method described previously (Westerfield, 1994). Stained preparations were then dehydrated in an ethanol series, cleared in methyl salicylate and mounted on slides in Permount. To visualize muscle innervation, embryos were cleared in 80% glycerol and viewed at high magnification with polarized light to reveal muscle striations.
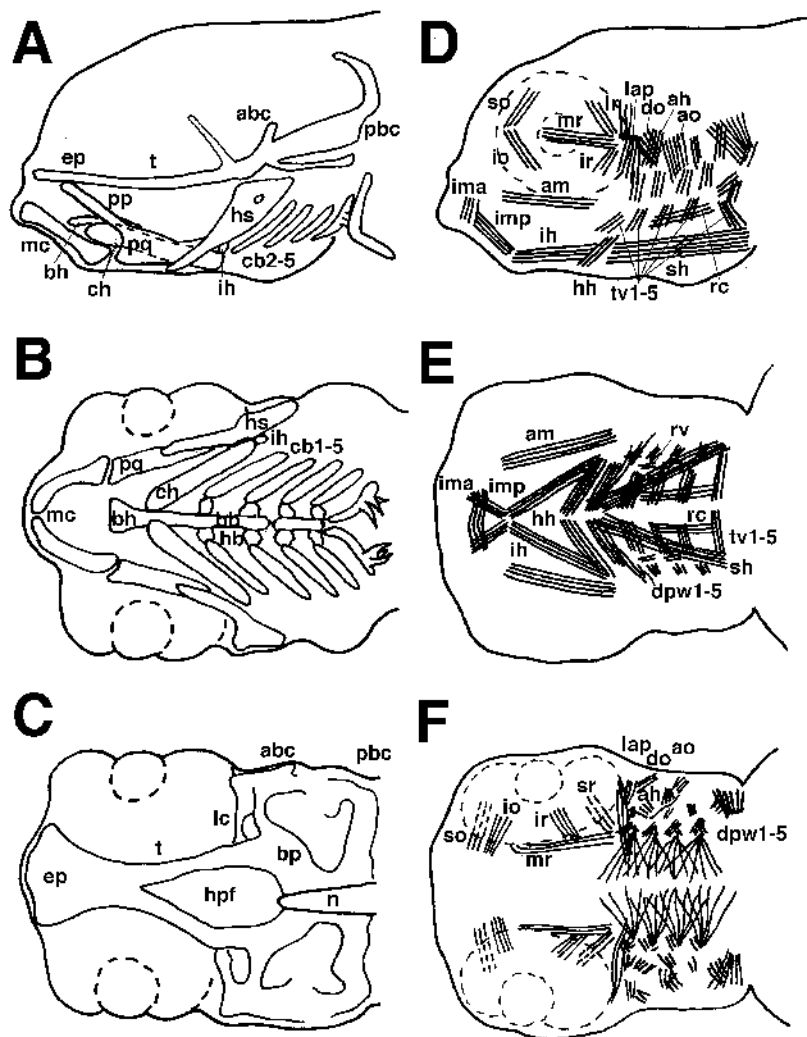

Fig. 2. Camera lucida drawings of whole-mounted specimens stained with Alcian blue (A-C) or an anti-myosin antibody (D-F), left side and ventral views at 120 h (1.3 D). (A) Left side view of the skull and pharyngeal cartilages. (B) Ventral view of pharyngeal arches. (C) Ventral view of the neurocranium, a more dorsal focus of B. (D) Left side view of cranial muscles. (E) Ventral view of pharyngeal muscles. (F) Ventral view of dorsal muscles, a more dorsal focus of E. hpf, hypophyseal fenestre; n, notochord. For abbreviations for skeletal and muscle elements see Table 5.

Whole-mount in situ hybridization

Embryos were fixed overnight in 4% paraformaldehyde, rinsed in PBS, dehydrated in methanol and stored at −20°C. In situ hybridization was performed essentially as described previously (Thisse et al., 1993). Probes for *myoD* (Weinberg et al., 1996) and *tropomyosin* (Thisse et al., 1993) RNA have been described previously.

RESULTS

During the hatching period in zebrafish, 48-72 h, the jaw extends rapidly (Fig. 1). To determine the extension rate and morphological changes in pharyngeal arches during this period, we examined live embryos with Nomarski optics. The mouth first appears as a hole in the ventral surface ectoderm, in a mid-ventral position near the posterior edges of the eyes at 45-48 h (Fig. 1A,B). From 48-60 h the mouth moves anteriorly and the pharyngeal arches elongate in a ventral-anterior direction, becoming progressively more visible as the head shifts anteriorly relative to the heart (Fig. 1C-F). The ventral end of the hyoid arch extends in synchrony with the mouth, maintaining a constant distance behind it of 110 μm. The jaw extends at approximately 20 μm/hour during early stages, slowing slightly after 68 h. Embryos can be staged easily by the mouth's position between the eyes (D, see Materials and Methods; Table 1). By 5 days of development the lower jaw has shifted dorsally and

Table 2. Cranial cartilages and their sequence of appearance in the zebrafish

Region and cartilage	Time of appearance*	References and comments†
Mandibular arch		
Meckel's cartilage	55	1-7
palatoquadrate	53	1-7
Hyoid arch		
basihyal		1-7
ceratohyal	54	1-7
interhyal	68	1-7
hyosymplectic	57	1-7
Branchial arches		
basibranchials	68	1-7
hypobranchials	74	1-7
ceratobranchial 1	56	1-7
ceratobranchial 2	60	1-7
ceratobranchial 3	64	1-7
ceratobranchial 4	68	1-7
ceratobranchial 5	64	1-7
Neurocranium		
trabeculae	45	1,2,4,6,7
ethmoid plate	52	1,2,4,6,7
polar cartilages	52	1,2,4,6,7
anterior basicranial commissure	55	1,2,6,7
lateral commissure	89	1,2
parachordals	50	1,2,4,6,7
posterior basicranial commissure	60	1,2,6,7

References: [1]deBeer (1937). [2]Bertmar (1959). [3]Pashine and Marathe (1974). [4]Langille and Hall (1987). [5]Kimmel et al. (1995). [6]Cubbage and Mabee (1996). [7]Schilling et al. (1996b).

*Time is reported in hours postfertilization at 28.5°C, and first appearance refers to the stage when alcian labeling is first observed.

†References include the major sources for cartilage identification and zebrafish work, and are not meant to be comprehensive. For a more thorough literature coverage see Cubbage and Mabee (1996).

the mouth lies directly anterior to the eyes. Gill filaments lined with blood vessels protrude from posterior arches (Fig. 1G,H).

The skeletal pattern

We examined how jaw extension and pharyngeal arch elongation reflect cartilage and skeletal muscle differentiation. Embryos were labeled with a variety of histological, immuno-chemical or molecular markers and examined primarily in whole-mounts. We first summarize the larval pattern assembled from these observations and then deal with different markers individually (Figs 2, 3; Tables 2, 3). The cartilage pattern has been described previously (Cubbage and Mabee, 1996; Schilling et al., 1996b; Piotrowski et al., 1996).

There are seven pharyngeal arches (Fig. 2A-C). Both mandibular and hyoid arches contain two large bilateral cartilages, one ventral and one dorsal. In the mandibular arch, Meckel's cartilages form the U-shaped lower jaw. At their posterior ends they articulate with dorsally located palato-quadrates, which develop pterygoid processes that articulate with the ethmoid plate of the neurocranium. Ventral elements of the hyoid skeleton are an unpaired basihyal in the midline and large paired ceratohyals. Ceratohyals articulate posteriorly with tiny interhyals and large, triangular hyosymplectics. Hyosymplectics, like the palatoquadrates in the mandibular arch, are the most dorsal hyoid cartilages. By larval stages the hyoid arch has grown posteriorly to form the opercles that eventually cover more posterior arches (Fig. 3).

Both dorsal and ventral cartilages can also be recognized in each of the five posterior, branchial arches. The simple, and presumed primitive, branchial pattern includes a ventral midline, basal component ([e.g.] basibranchials 1-3, fused together and a separate basibranchial 4), and paired ventrolateral components (hypobranchials 1-4, ceratobranchials 1-5). Teeth, three on each side at 5 days, form only on the fifth branchial arch, attached to the enlarged fifth ceratobranchial. Many specializations observed in this segment are probably related to its unique role in feeding.

The anterior neurocranium consists of two longitunal rods of chondrocytes, the trabeculae, that fuse across the midline to form the ethmoid plate (Fig. 2C). Polar cartilages lie at their posterior ends. Trabeculae fuse posteriorly with basicapsular commissures and parachordal cartilages underlying posterior regions of the brain. Lateral, anterior and posterior basicapsular commissures form in the neurocranium, the first two anterior and the last posterior to the auditory capsule (deBeer, 1937).

The muscle pattern

Dorsal and ventral muscle groups in each arch contract or expand the pharyngeal cavity or, in the case of the fifth branchial arch, process food (Fig. 2D-F; Table 3). It is difficult to determine which cranial muscles in embryos correspond to adult muscles since they rearrange and grow, becoming associated with dermal bones that are not formed at stages that we consider. Therefore, to confirm our muscle identifications we examined innervation patterns in larvae stained with an anti-acetylated tubulin, which labels early cranial nerve axons (Fig. 4). Antibody-labeled nerves, viewed under polarized light to highlight muscle striations, could be followed nearly to their endings at neuromuscular junctions.

Five bilateral muscle pairs form in the mandibular arch. Two

Table 3. Cranial muscles and their sequence of appearance in the zebrafish

Region and muscle	Time of appearance*	Innervation	References**
Extraocular			
superior oblique	58	III	2,5,6,7
inferior oblique	62	IV	2,5,6,7
superior rectus	58	III	2,5,7
inferior rectus	58	III	2,5,7
medial rectus	53	III	5,7 (internal rectus of 2)
lateral rectus	58	VI	2,5,7
Mandibular arch			
intermandibularis ant.	62	mandibular (V)	2,3,4 (intermandibularis of 1,6)
intermandibularis post.	62	mandibular (V)	2,3 (geniohyoideus of 1,4; protractor hyoidei of 6)
adductor mandibulae	53	mandibular (V)	1-4,6
levator arcus palatini	62	maxillomand. (V)	1-4,6
dilatator operculi	62	maxillomand. (V)	1-4,6
Hyoid arch			
interhyal	58	hyoides (VII)	
hyohyal	58	hyoides (VII)	1,2,3,4,6 (hyohyoidei abductores + h. inferioris + h. adductores of 2,6; h. inferior + h. superior of 4)
adductor hyomandibulae	68	hyomand. (VII)	2,4,6 (adductor hyomandibularis of 1,3)
adductor operculi	68	hyomand. (VII)	1-4,6
levator operculi	85	hyomand. (VII)	1-4,6
Branchial arches			
transversus ventralis	62	posttrematic (IX, X)	2,4,6 (transversi ventralis anterior of 1)
rectus ventralis	85	posttrematic (X)	4,6 (obliqui ventrales of 1, obl. laterales of 3, subarcuales recti of 2)
pharyngeal wall (branchial levator)	72	posttrematic (IX,X)	(levators int. of 6; lev. arcuum branchialium of 2; lev. arc. interni of 3; lev. arc. branch. int. of 1)
rectus communis	85	posttrematic (X)	4,6 (pharyngohyoideus of 1, subarcualis communis of 2, pharyngoarcualis of 3)
sternohyoideus	53	occipitospinals	1,3,4,6 (rectus cervicis of 2)
Dorsal posterior			
protractor pectoralis	72		

References: [1]Edgeworth (1935); [2]Allis (1917); [3]Harder (1964); [4]Miyake et al. (1992); [5]Oliva and Skorepa (1968); [6]Winterbottom (1974); [7]Easter and Nicola (1996).

*Time is reported in hours postfertilization at 28.5°C, and first appearance refers to the stage when striations and myosin protein expression is first observed.

**References include the major sources for muscle identification and zebrafish work, and are not meant to be comprehensive. For a more thorough literature coverage see Winterbottom.

dorsal pairs, levator arcus palatini and dilator operculi, are conical in shape and lie laterally between the neurocranium and dorsal cartilages. These muscles originate lateral to the basicapsular commissures and insert along the dorsal hyosymplectic and the dorsolateral face of the opercle, respectively (Fig. 3). Thus they insert on the hyoid skeleton despite being derived from the mandibular primordium, and innervated by the trigeminal nerve (V; Fig. 4F). A third muscle pair, the adductor mandibulae, forms along dorsolateral surfaces of the palatoquadrate cartilages. These muscles originate from this surface, insert on Meckel's cartilage, and function as jaw 'closers' acting in antagonism with sternohyals and other jaw 'openers'. The mandibular ramus of V passes close to and innervates the lateral muscle surface (Fig. 4A). The ventral muscles, intermandibularis anterior and posterior, form a triangle that points posteriorly between the eyes, behind the lower jaw. Transverse fibers of intermandibularis anterior insert in the midline, and pass laterally between Meckel's cartilages to overlap the anterior ends of the intermandibularis posterior muscles. The latter connect anterior ends of the ceratohyals to the lower jaw. The mandibular ramus of V extends small side branches into intermandibularis anterior and then curves posteriorly to innervate intermandibularis posterior (Fig. 4E).

The five muscle pairs of the hyoid arch are restricted to its dorsal and ventral extremes (Fig. 2, 3; Table 3). Dorsally the adductor hyomandibulae pull the anterior basicapsular commissures towards the dorsomedial faces of the hyosymplectics. A second dorsal muscle pair, the adductor operculae, lie further posteriorly and move the auditory capsules with the dorsomedial faces of the opercles. In apposition to this force, the levator operculae open the operculum (Fig. 3). Dorsal hyoid muscles are innervated by small, posterior branches of the facial nerve (VII; Fig. 4F). Ventral hyoid muscles include interhyals and hyohyals which insert in the ventral midline, the latter extending from the ceratohyals to branchiostegal rays that ossify in the surrounding dermis by this stage (Cubbage and Mabee, 1996). These muscles are innervated by the hyoides ramus of VII (Fig. 4D).

There is a similar set of 2-4 muscle pairs in each of the five branchial arches. Like the cartilages, fifth branchial arch muscles are specialized for feeding. The basic larval set includes dorsal pharyngeal wall muscles (tentatively identified as branchial levators), along the dorsal ceratobranchials (Fig. 2, 3; Table 3), and tranversi ventrales, found in all five arches but larger in branchial arches 1 and 5, which originate on the ceratobranchials and insert at the ventral midline on a median raphe. Between these muscles, in the centers of branchial arches 1-3, the first few fibers of the rectus ventralis muscles
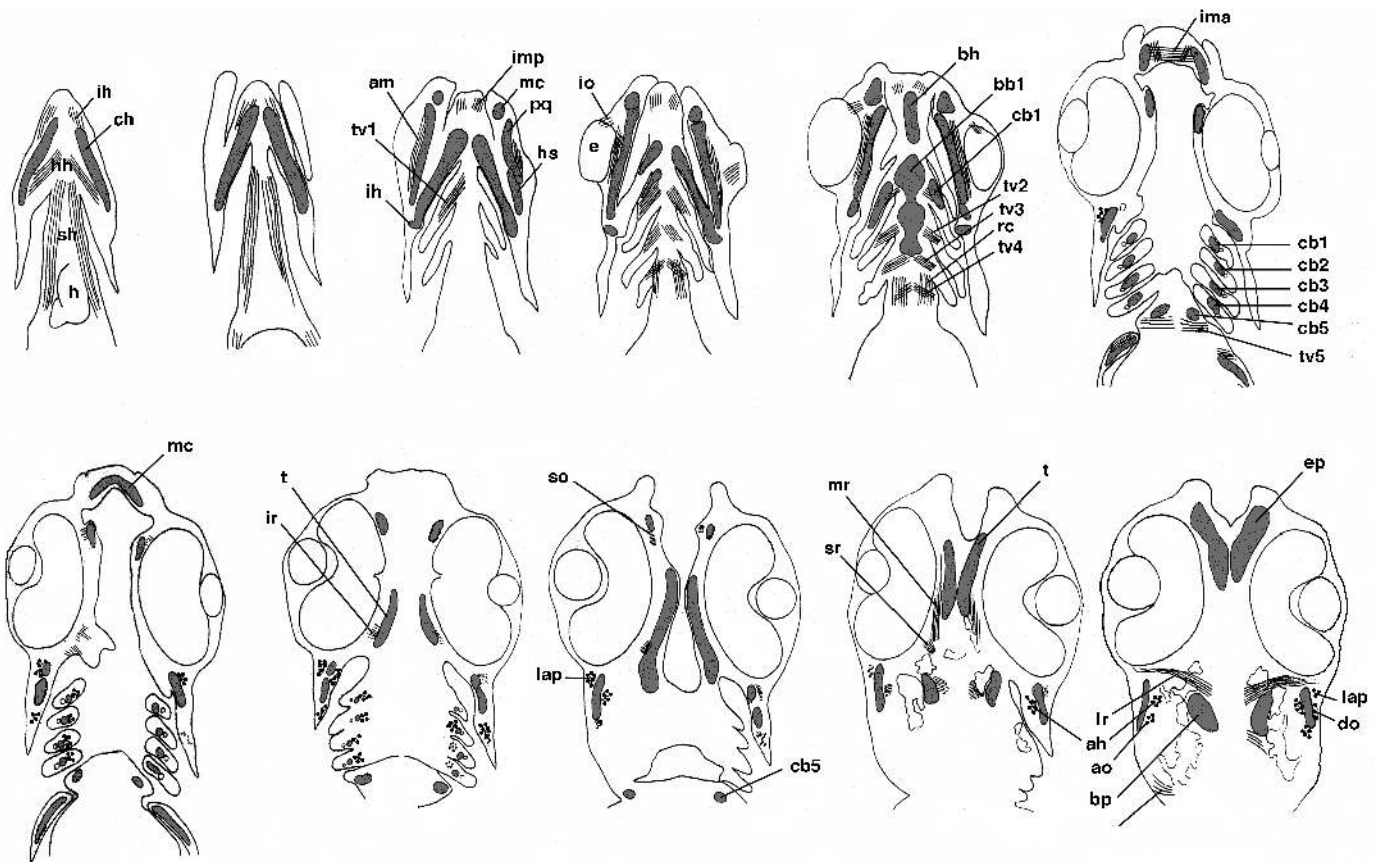
Fig. 3. Camera lucida drawings of horizontal sections at 120 h (1.3 D). Sections proceed from ventral in the upper left-hand corner to dorsal in the lower right. Cartilage is shaded and muscles are represented by parallel lines or clustered dots when shown in cross-section. For abbreviations see Table 5.

join ceratobranchials of 1 to 2 and 2 to 3. A longitudinal rectus communis muscle, variable among teleosts, spans the three most posterior arches in the entire series (3-5). Thin, fan-shaped muscles radiate through the dorsal pharyngeal wall from lateral points of origin in the first four branchial arches. Branchial muscles are innervated by posttrematic branches of the glossopharyngeal (IX; arch 3) and vagus nerves (X; arches 4-7; Fig. 4C). Ventral to the branchial muscles, sternohyals stretch from the cleithrum and coracoid of the pectoral girdle to the hyoid arch. These muscles consist of three subdivisions. The subdivisions may correspond not to pharyngeal segments, but to three anterior somites from which this muscle is derived. Corresponding to their presumed origin, sternohyals are innervated by anterior branches of occipito-spinal nerves (Fig. 4G).

Six pairs of extraocular muscles move the eyes in a pattern highly conserved in all vertebrates, including 2 obliques anteriorly and 4 recti posteriorly. Three motor nerves innervate extraocular muscles, as described previously (Oliva and Skorepa, 1968; Winterbottom, 1974; data not shown). These nerves can be traced to their target muscles at least as early as 72 h, close to the stage when eye movements begin (Easter and Nicola, 1996). The oculomotor nerve (III) innervates four pairs of muscles: superior oblique, superior, inferior and medial recti. The other two are innervated individually by the trochlear (inferior oblique; IV) and abducens (lateral rectus; VI) nerves (Table 3).

Early cartilage formation

Cranial cartilage differentiation, as revealed with Alcian blue or green, begins before jaw elongation near the end of the pharyngula period. Initial Alcian labeling marks a rapid developmental transition in cells that we term 'chondrification' or cartilage 'differentiation'. Staining is preceded by precartilage condensations, which can be observed in sectioned material, in unstained living embryos with Nomarski optics (see Fig. 6), and after labeling them with molecular markers including *dlx2* (Akimenko et al., 1994) and *col2a1* (Yan et al., 1995).

The earliest cartilages are paired rudiments of trabeculae, which stain in some but not all staged embryos fixed at 45 h (Table 2). As few as 2-3 adjacent cells stain on each side of the midline, between the eyes. Within 2 hours trabeculae label more intensely and elongate rapidly to acquire their definitive columnar shapes. A similar general pattern of differentiation was observed for all head cartilages, i.e. weak and variable labeling initially followed by more intense staining and rapid enlargement.

Parachordal cartilages appear next, just lateral to the anterior notochord at 50 h, and these expand to form a broad basal plate positioned medial to the auditory capsules. By the same stage anterior ends of the paired trabeculae reach the midline, where within 2-3 hours they fuse medially to form the ethmoid plate. Their posterior ends elongate and, by 53 h, fuse with anterior extensions of the parachordal cartilages. This fusion occurs
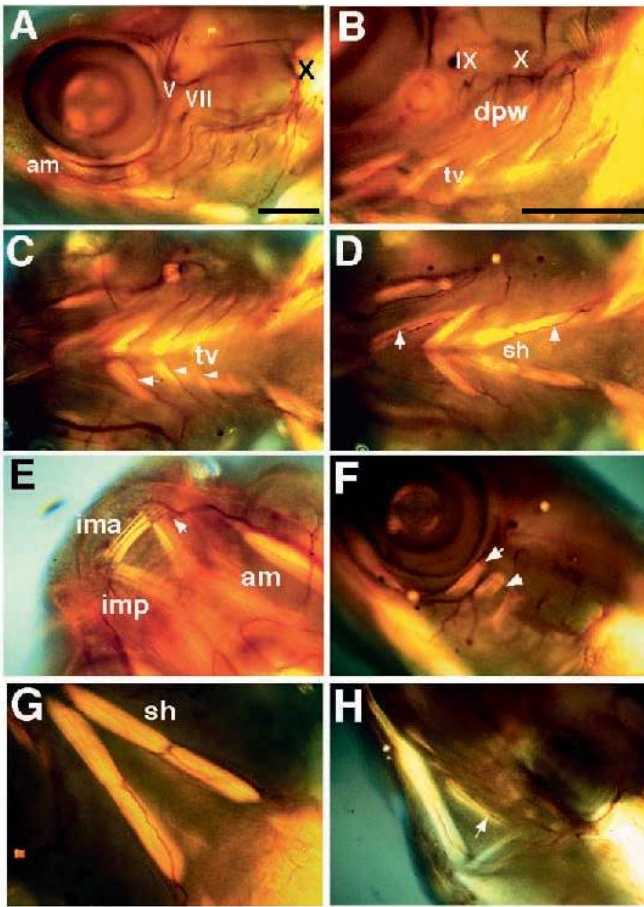
Fig. 4. Cranial muscle innervation at 120 h (1.3 D). Nerves were labeled with anti-acetylated tubulin and muscles visualized by their birefringence with polarized illumination. (A) Left side view. Major nerve branches include the mandibular branch of the trigeminal (V), hyomandibular branch of the facial (VII), glossopharyngeal (IX) and multiple branches of the vagus (X). (B) Ventrolateral, left side view of the branchial region. (C) Ventral view of branchial arch muscles. Posttrematic branches of IX (arrow) and X (arrowheads) project to transversus ventralis muscles. (D) Ventral view of hyoid arch and hypobranchial muscles, a more ventral view of C. The hyomandibular branch of VII (arrow) innervates interhyoideus and hyohyoideus muscles. Anterior spinal axons innervate sternohyoideus (arrowhead). (E) Ventral view of mandibular arch muscles. Axons of the mandibular branch of V (arrow) project to the intermandibularis anterior muscles, while the main branch curves posteriorly to innervate intermandibularis posterior. (F) Left side view of dorsal mandibular and hyoid muscles. A small branchlet of the mandibular branch of V innervates the levator arcus palatini (arrow). A larger branchlet, from a similar position along the hyomandibular branch of VII, innervates the adductor operculi and levator operculi (arrowhead). (G) Ventral view of the sternohyals. A spino-occipital nerve extends along posterior regions of the muscle bundles. (H) Left side view of hypobranchial muscles. A posterior branch of the vagus innervates the rectus communis (arrow). For abbreviations see Table 5. Scale bars, 100 μm.

well lateral to the midline, and cartilage around the fusion remains distinctive as the polar cartilage. The ethmoid, trabeculae and basal plate complex form a continuous scaffold beneath the brain, surrounding the medial, cartilage-free hypophyseal fenestra (Fig. 5B).

At 53 h, faint labeling is detected at an anterior-lateral site of the primordial auditory capsule, forming a knob where it will articulate with the hyosymplectic. This is the first indication that a burst of differentiation in anterior pharyngeal cartilages is beginning, which will continue for 2-4 hours (Fig. 5A). Individual embryos fixed together from a single staged batch between 53 and 57 h vary in the number of these cartilages that are stained. However, such a batch of embryos can be reliably sorted into substages by inspecting the cartilages themselves. This reveals that although development occurs asynchronously, the differentiation sequence is almost invariant. Palatoquadrates appear first, followed in turn by ceratohyals, Meckel's cartilages, ceratobranchials 1 and hyosymplectics.

We used Nomarski optics, with whole-mount preparations, fixed with 5% trichloroacetic acid and stained with Alcian green, to examine this sequence in detail, enhancing contrast by capturing and processing black and white video images. This method revealed that the earliest sites of cartilage differentiation generally occur adjacent to future articulations, and within larger precartilage condensations that were not distinctive by staining, but by their dense mesenchymal cell packing. As illustrated for the mandibular arch (Fig. 6), more than one cartilage can arise from a single condensation, without prior splitting of the condensation. At first, within a large condensation on each side of the midline, palatoquadrates alone begin to chondrify, as flat sheets, always 1-2 cells wide (Fig. 6A,B) and located just proximal to where the joints with Meckel's cartilages will form. Chondrification includes cell enlargement and increased refractility of cell boundaries, the latter presumably due to rapid matrix deposition. Meckel's cartilages then chondrify in a similar fashion, within the same condensations as the palatoquadrates, and just distal to the same joints (Fig. 6B,D). As development continues these chondrifications enlarge by expanding away from the joint, which itself remains Alcian-negative (Fig. 6F).

We observed a similar situation in the hyoid arch. In this case there are three separate sites of chondrification during the 53-57 h interval, within a single hyoid precartilage condensation on each side of the midline. These correspond to the future ceratohyals, symplectic regions of the hyosymplectics (Fig. 6A,C), and hyomandibular regions of the hyosymplectics (not shown). Later, around 68 h, interhyals chondrify between the hyosymplectics and ceratohyals, apparently again within the single hyoid condensations.

After their initial appearance each element enlarges in a distinctive manner. Meckel's cartilage and the ceratohyal elongate as thick bars towards the ventral midline. Meckel's cartilage forms a joint with its contralateral counterpart by 74 h. Within the same time period, symplectic and hyomandibular chondrifications expand to join one another at 68 h. In this case an Alcian-negative joint does not persist at the junction and the two elements fuse to form the hyosymplectic. The palatoquadrate acquires its complex shape differently than the hyosymplectic. Here, at 64 h, well after initial chondrification, a distinctive protrusion of stained cells appears secondarily, just medial to the joint with Meckel's cartilage. This protrusion is the incipient pterygoid process, and it elongates towards the ethmoid, forming a joint with it much later (90 h).

Table 4. Segmental homologues among the cartilages and muscles

Cartilage

Pharyngo-	Epi-	Cerato-	Hypo-	Basi-
Mandibular				
–	Palatoquadrate	Meckel's	–	–
Hyoid				
–	Hyosymplectic	Ceratohyal	–	Basihyal
Branchials 1-4				
Pharyngobranchial	Epibranchial	Ceratobranchial	Hypobranchial	Basibranchial
Branchial 5				
–	–	Ceratobranchial	–	–

Muscle

Dorsal	Ventral closer	Ventral opener
Mandibular		
Adductor mandibulae	Intermandibular anterior	Intermandibular posterior
Levator arcus palatini		
Dilator operculi		
Hyoid		
Adductor hyomandibulae	Hyohyal	Interhyal
Adductor operculi		
Levator operculi		
Branchials 1-5		
Dorsal pharyngeal	Transverse ventral	Rectus ventralis
		(rectus communis)

1 The interhyal is not included in this scheme. An interhyal is generally present in ray-finned fishes, including primitive members of the group like the bichir and sturgeon, but not found in other fishes, such as sharks (deBeer, 1930). Hence we assume it is not present in a generalized arch of a primitive gnathostome, but is a derived speciality of the hyoid arch in Actinopterygians.

2 A hypohyal-like element appears fused to the ventromedial end of the ceratohyal. A separate hypohyal cartilage is present in other teleosts such as Salmo (deBeer, 1930).

3 The anterior basal fusion, or 'copula', includes the primitive basihyal and basibranchials 1-3 (i.e. four segments). There is also a basibranchial 4 that is typically separate.

4 The sternohyoid is thought to be a hypobranchial 'interloper' into the pharynx, not part of the series we consider here. Its precursor cells migrate into the pharynx from the anterior-most somites (data not shown).

5 In our scheme the dorsal muscle is primitively a 'closer', attaching to a ceratobranchial and pulling it dorsally, to close the joint between the ceratobranchial and epibranchial. The derived pattern in the mandibular and hyoid arches include several muscles that function either as closers (e.g. adductor mandibulae) or openers (e.g. levator arcus palatini).

6 The rectus communis projects only to branchial arch 3, but in attaching to the fused basal elements its contraction would serve to retract all of the anterior gill arches.

More posterior, branchial cartilages as well as the posterior neurocranium chondrify more slowly (Fig. 5C-G). Cerato-branchial 2 first stains at 60 h, coinciding with differentiation of the anterior basicranial commissure surrounding the anterior border of the auditory capsule. Ceratobranchials 3 and 5 arise nearly simultaneously, beginning at 64 h, and coinciding with differentiation of the occipital arch and posterior basicranial commissure, such that the basicapsular foramen is now completely surrounded by cartilage. The last ceratobranchial to develop is ceratobranchial 4, which stains first at 68 h. The same stage marks initial differentiation of ventral midline cartilages. Adjacent but separate patches of Alcian labeling appear simultaneously along the midline corresponding to the basihyal and basibranchials 1-3. Six hours later (by 74 h) these patches have largely fused and paired hypobranchial chondrifications have appeared beside them. The last cartilage to chondrify during the interval we examined is the lateral commissure, at 89 h. In contrast to anterior and posterior basicranial commissures, which grow laterally from the basal plate around the auditory capsule, chondrification of the lateral commissure begins laterally at

Table 5. List of abbreviations for cranial skeletal and muscle elements of *Danio rerio*

	Skeletal		Muscle
abc	anterior basicranial commissure	ah	adductor hyoideus
		am	adductor mandibulae
bb	basibranchial	ao	adductor operculi
bh	basihyal	do	dilator operculi
bp	basal plate	dpw	dorsal pharyngeal wall
cb	ceratobranchial	hh	hyohyoideus
ch	ceratohyal	ih	interhyoideus
ep	ethmoid plate	ima	intermandibularis anterior
hs	hyosymplectic	imp	intermandibularis posterior
ih	interhyal	io	inferior oblique
lc	lateral commissure	ir	inferior rectus
mc	meckel's cartilage	lap	levator arcus palatini
pbc	posterior basicranial commissure	lr	lateral rectus
		mr	medial rectus
pq	palatoquadrate	rc	rectus communis
pp	pterygoid process	rv	rectus ventralis
t	trabeculae	sh	sternohyoideus
te	teeth	so	superior oblique
		sr	superior rectus
		tv	tranversus ventralis

the auditory capsule, and grows medially. This commissure elongates toward the polar cartilage and encloses the facial foramen that lies just posteriorly.

Cranial muscle development

Myosin labeling

Muscles differentiate slightly later than cartilage within a given region, as determined by Nomarski optics and expression of molecular markers such as myosin (Fig. 7; Table 3). The antibody 1025 recognizes several myosin proteins found in every differentiating muscle fibers beginning at 58 h. The first labeled muscles include the medial rectus of the extraoculars, the adductor mandibulae in the mandibular arch, and hypobranchials such as sternohyals (not shown). The developmental sequence of extraocular muscle development has been described previously (Easter and Nicola, 1996). By 65 h the antibody labels ventral muscles of both mandibular and hyoid arches (Fig. 7A). These muscles form an hourglass pattern, capped by intermandibularis anterior and with intermandibularis posterior and interhyoideus meeting in an X in the midline. Two dorsal muscles, levator arcus palatini and dilator operculi, also express myosins by 65 h (Fig. 7B). Slightly later, by 66 h, two of three dorsal hyoid muscles, adductor hyomandibulae and adductor operculi (Fig. 7B), and both pairs of ventral hyoid muscles, interhyoideus and hyohyoideus, are labeled.

Myosin expression is first detected in anterior branchial muscles at 68 h and then progressively in more posterior arches. Transverse ventrals differentiate first at this stage, followed closely by dorsal pharyngeal wall muscles by 78 h (Fig. 7C,D). As observed for cartilages, muscles of the most posterior, fifth branchial arch differentiate slightly before those of the fourth. Expression of myosins persists in the larvae and, at 89 h, gives a detailed look at the morphology of individual myofibers that are difficult to image with polarized light (Fig. 7E,G,H). In ventrolateral view the ventral muscles hang below the eyes (Fig. 7E). There is one transverse ventral and one dorsal pharyngeal muscle in each branchial arch.

The ventral view at 89 h is more revealing of the segmental pattern radiating from the midline, with most muscles extending in a posterolateral direction, except for intermandibularis posterior (Fig. 7G,H). The hourglass pattern of anterior ventral muscles has moved forward, following jaw elongation. All ventral muscles, with the exception of the rectus communis, insert at or near the midline (Fig. 7G). Hyohyal muscles, in particular, contain only a small number of loosely associated fibres inserting just anterior to the sternohyals. Staining reveals identical transverse ventral muscles in four branchial arches, while those in the fifth are larger and extend perpendicular to the midline.

myoD expression in myogenic condensations

To investigate how the initial pattern of cranial muscles is established, and its relationship to cartilage patterning we examined earlier markers of myogenesis, such as *myoD*. In the zebrafish trunk, *myoD* RNA is expressed in skeletal muscle precursors several hours before myosin expression. In cranial muscles it is expressed up to 7 hours before myosins.

Transcripts of *myoD* are first detected at 50 h in precursors of the medial and inferior rectus extraocular muscles, the adductor mandibulae and sternohyals (Fig. 8A). A similar
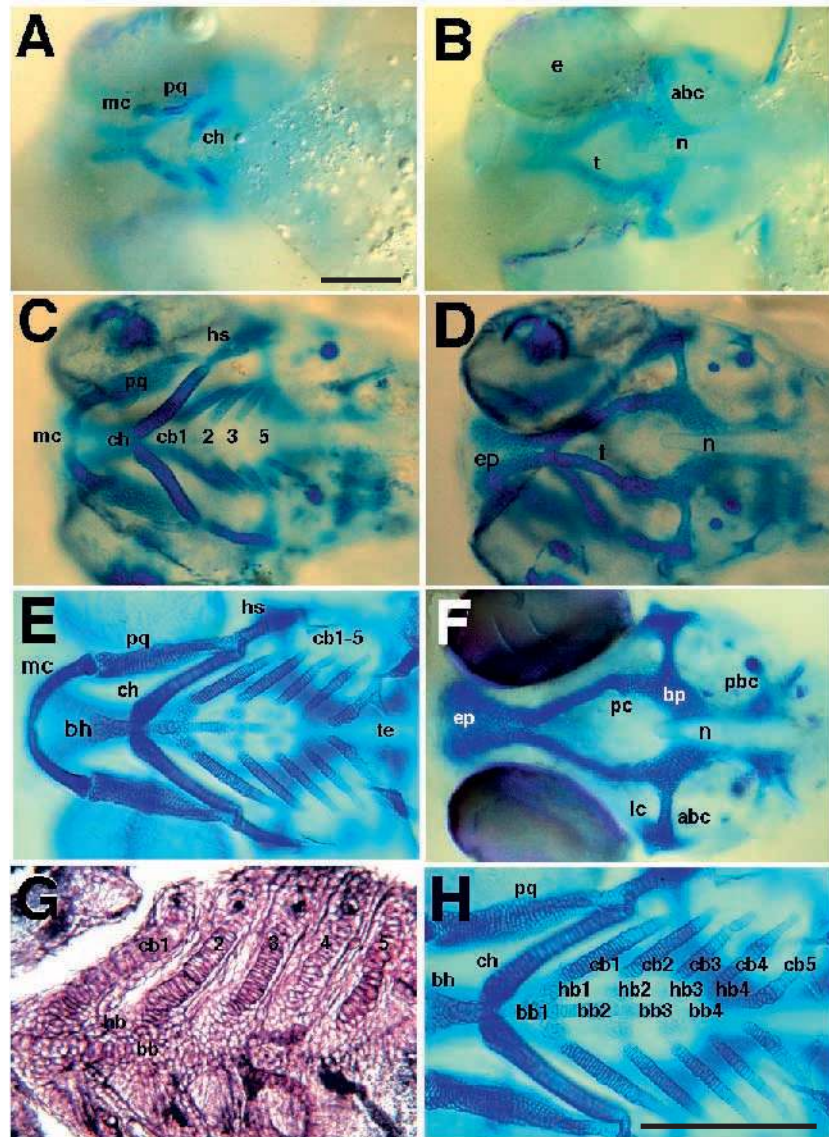

Fig. 5. Cartilage differentiation during jaw elongation. Ventral views of pharyngeal arches and a more dorsal focus on the neurocranium are shown at each stage, stained with Alcian blue. (A) 52 h (0.3 D). Ventral view of pharyngeal cartilage. Three bilateral elements are stained just anterior to the heart. (B) Ventral view of the neurocranium, a more dorsal focus of A. (C) 68 h (0.6 D). Pharyngeal cartilage. (D) Neurocranium, dorsal focus of C. (E) 96 h (1.2 D). Larval pharyngeal cartilages. (F) Neurocranium, dorsal focus of E. (G) Horizontal section through branchial cartilages, showing the ventralmost elements. Chondrocytes form stacks within hyaline cartilage. (H) Higher magnification of E showing basibranchials and hypobranchials. For abbreviations see Table 5. Scale bars, 100 μm.

general pattern was observed for all head muscles; expressing cells are rounded when expression begins, followed by rapid elongation (Fig. 8B). Two groups of myoblasts label in each pectoral fin bud. Initially, bilateral groups of 5-10 *myoD*-expressing cells, precursors of the adductor mandibulae, lie in rounded clusters just behind the eyes. By 55 h, two additional clusters are detected just anterior to the yolk sac, intermandibularis and interhyals, in the distal mandibular and hyoid arch primordia and, slightly later, in dorsal muscle precursors until 55 h (Fig. 8D). By 58 h the ventral clusters have elongated ventroanteriorly, while more dorsal myogenic condensations of the arches remain rounded (Fig. 8E,F). The ventral clusters complete elongation by 65 h and the hourglass pattern emerges in the mandibular arch, composed anteriorly of intermandibularis anterior and intermandibularis posterior, and posteriorly of interhyal muscles (Fig. 8G). The adductor mandibulae lie laterally, beneath the eyes. There are separate precursors for each transversus ventralis muscle of the branchial arches (Fig. 8H). *myoD* expression diminishes in later embryonic and larval stages, when levels of myosin protein remain high.
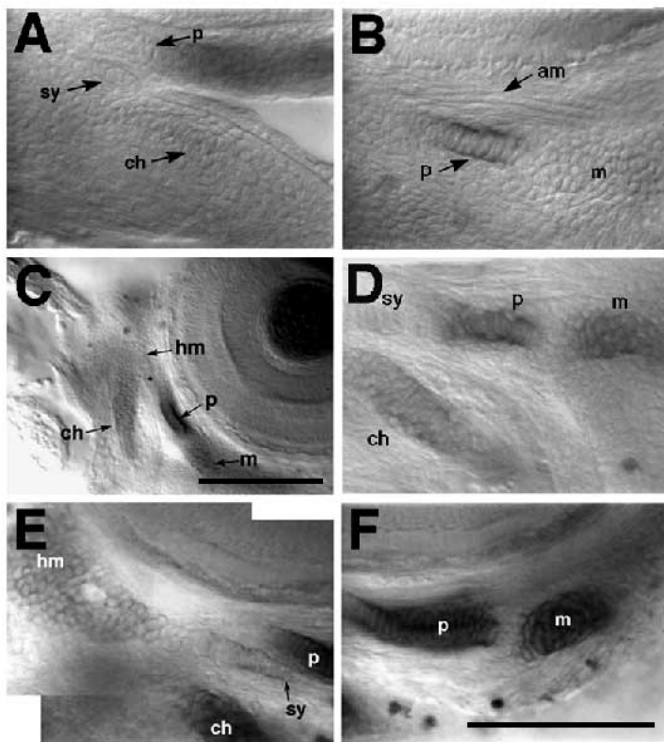

Fig. 6. Precartilage condensations and differentiation in mandibular and hyoid primordia, ventrolateral views. Each photomicrograph shows an Alcian-labeled preparation of a different individual, fixed from one of several sets of siblings and viewed with Nomarski optics. (A) 53 h (0.3 D). Unstained clusters of chondrogenic cells can be identified in the positions of the future palatoquadrate (p), symplectic (sy) and ceratohyal (ch). (B) 53 h, staining in the palatoquadrate, adjacent to the adductor mandibulae muscle. (C) 53 h, lower magnification showing positions relative to the eye. (D) 53 h, labeling includes the ceratohyal and Meckel's cartilage. (E) 60 h, early labeling of the hyosymplectic. (F) 60 h, mandibular chondrification. Abbreviations: am, adductor mandibulae; ch, ceratohyal; hm, hyomandibular; m, Meckel's cartilage; p, palatoquadrate; sy, symplectic. Scale bars, 100 μm.

Tropomyosin expression

Like early differentiating adaxial muscle precursors in the somites (Thisse et al., 1993), cranial muscles begin to express tropomyosin as they elongate and striate. Transcripts are first detected at 53 h, 2-3 hours later than *myoD*, but before myosin labeling, in a similar pattern in developing extraocular muscles, adductor mandibulae and pectoral fin buds (Fig. 9A). In ventral aspects of the arches three pairs of muscle precursors express tropomyosin. The anterior ones will form the adductor mandibulae. Two groups of expressing cells are found posteriorly, one medial to and aligned with the other, probably precursors of sternohyal muscles. Slightly later (58 h) interhyal and hyohyal muscles are labeled (Fig. 9B). Ventral hyoid muscles express tropomyosin prior to those of the mandibular arch. Precursors of dorsal muscles are also detected at this stage (Fig. 9C).

By 72 h many ventral arch and hypobranchial muscles express high levels of tropomyosin (Fig. 9D) as do all extraocular muscles (Fig. 9F). Initially (approx. 70 h), expression is restricted to three of five branchial arches (Fig. 9H). Dorsal muscles in the first two arches are labeled by 72 h (Fig. 9E). Transverse ventral and dorsal pharyngeal wall muscles are labeled by 85 h (Fig. 9G).

DISCUSSION

We have analyzed the spatial and temporal patterns of cranial cartilage and muscle development in the zebrafish embryo. As the jaw elongates, pharyngeal cartilages and muscles arise from a small and stereotyped set of precursors located in seven segments. Separate cartilages or muscles in one arch (i.e. mandibular) develop by subdivision of a single segment primordium, and groups of early myoblasts lie adjacent to the first regions of chondrogenesis, perhaps reflecting their coordinated patterning (Noden, 1986). We also argue for a scheme of segmental homologies in the arches suggesting that certain segments have evolved differences in the number and size of elements due to changes in developmental timing. These hypotheses can now be tested using the many craniofacial mutants available (Schilling et al., 1996b; Piotrowski et al., 1996; Neuhauss et al., 1996).

Coordinated segregation and differentiation of cartilage and muscle

Alcian labeling of cartilage and myosin labeling of muscle would indicate that these tissues develop nearly simultaneously within a given pharyngeal arch (Fig. 10), as exemplified by the mandibular arch. The dorsal palatoquadrate and ventral Meckel's cartilage develop from a common primordium, with the dorsal cartilage differentiating first. At the same stage (53-55 h), the dorsal adductor mandibulae muscle of this arch differentiates before the ventral intermandibularis anterior, and these derive from a common primordium that also divides at 58 h. Chondrification proceeds toward the ventral midline, and differentiation of ventral midline muscles follows.

The pattern in more posterior arches is similar to the first. The interhyal muscle develops together with the ceratohyal cartilage in the hyoid arch, and ventral transverse muscles and ceratobranchial cartilages form approximately in synchrony in the branchial segments. Development is relatively early (see

below) in the last of these segments, the fifth, which is specialized for mastication rather than respiration. Still, in this arch, early chondrogenesis is correlated with early myogenesis. Expression of *myoD* is visible in the fifth transversus ventralis before the fourth, just as cartilage at this stage (65 h) forms in the fifth branchial arch before the fourth. A possible exception is that the large dorsal elements of the fifth branchial arch form later than more anterior dorsal muscles. However, this change is not very informative as there are no such dorsal muscles in the third or fourth branchial arches.

What are the cellular interactions underlying such coordinated development? Paraxial mesoderm might impart early AP patterning onto the premigratory neural crest (Itasaki et al., 1996), which then takes on the main organizing role. In the avian embryo, paraxial mesoderm forms the core of the developing branchial arch and is surrounded by a shell of postmigratory neural crest (Trainor and Tam, 1995). Assuming the same positional relationships exist in the zebrafish, one attractive idea is that neural crest cells impart spatial and temporal information to the more centrally located mesoderm during this period. This idea stems from work revealing the organizing properties of neural crest cells (Noden, 1983a) and from studies of chimaeric limbs, where the region-specific organization of muscle depends on the connective tissue (Chevallier, 1979). Mutation of *chn* in zebrafish causes a loss of both cartilage and muscle, but only neural crest development is effected cell-autonomously. The muscle defect might be a secondary consequence of this neural crest defect, due to a loss of cell-cell interactions (Schilling et al., 1996).

Regionalization and specification of muscle precursors

How do founders of particular muscles arise and become arranged into appropriate patterns? All cranial striated muscle fibers, including the extraocular and arch muscles derive from paraxial mesoderm (Edgeworth, 1935; Hatta et al., 1990; Kimmel et al., 1991; Noden, 1983b; Schilling and Kimmel, 1994). This mesoderm first segregates into AP subdivisions, perhaps segmental (Martindale et al., 1987). Each subdivision or primordium is then thought to subdivide into discrete muscle plates. Edgeworth (1935) illustrated the migration and growth of at least three major muscle plates in fishes: mandibular, hyoid and branchial. Initially compact, these plates extend, both ventrally and dorsally in the mandibular and hyoid plates, and ventrally only in the branchial arches. Further subdivisions of these plates ultimately generate discrete groups of pre-

cursors for each individual muscle. During these rearrangements, splitting of muscle primordia and directional growth of myofibers was observed. Such splitting also has been well described in the limb (Chevallier and Kieny, 1982).

Support for the idea of progressive subdivision of the mandibular muscle plate has come from observations of Eng-expression in the zebrafish (Hatta et al., 1990; Miyake et al.,
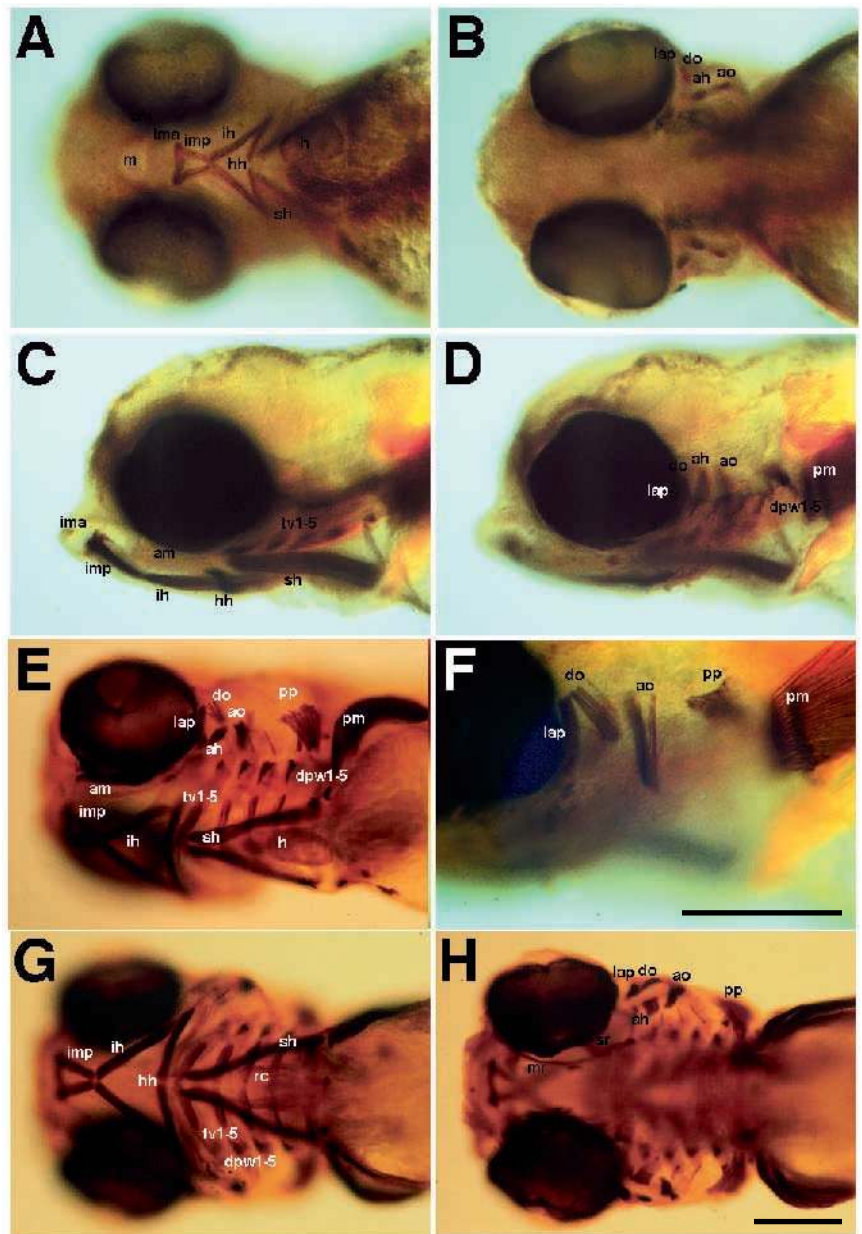

Fig. 7. Myosin expression in cranial muscles. Ventral views at 65 h (A,B) and lateral or ventral views at 120 h (C-H) of embryos labeled with the 1025 antibody. (A) Ventral view of pharyngeal and hypobranchial muscles. The hourglass-shaped pattern of ventral mandibular and hyoid muscles lies posterior to the mouth. Adductor mandibulae muscles are stained but obscured by the pigmented eye epithelium. (B) Ventral view of dorsal muscles. Three bilateral cell groups are labeled posterior to the eyes. (C) Lateral view of ventral pharyngeal and hypobranchial muscles. (D) Lateral view of dorsal muscles. (E) Ventrolateral view of the larval muscle pattern. (F) 65 h, higher magnification, lateral view of dorsal muscles. (G) Ventral view of ventralmost pharyngeal and hypobranchial muscles. (H) Ventral view of dorsal muscles. For abbreviations see Table 5. Scale bar, 100 μm.

1992). Expression begins in a single group of dorsal mesodermal cells at 28 h, 15 hours earlier than the stages considered here. Expression then continues in two dorsal muscles, the levator arcus palatini and dilator operculi. The presumed homologues of these muscles also express Eng in the lamprey velum (Holland et al., 1993) and in mandibular mesenchyme of tetrapods. Thus Eng expression defines the constrictor dorsalis subdivision, proposed by Edgeworth (1935) to subdivide to form these two muscles. Our data suggest that they divide at 52-55 h, when the myogenic precursors express *myoD* and before they begin to elongate. *myoD* expression slightly precedes division, revealing both this dorsal subdivision and a ventral masticatory subdivision. The ventral subdivision later develops into the intermandibularis anterior and intermandibularis posterior. Because we have not followed labeled muscle precursors in the same embryo, we cannot distinguish between de novo formation of separate muscles and splitting, either with Eng or *myoD*. However, the case for Eng as a lineage tracer is stronger since, unlike *myoD*, only these two muscles express the gene.

Our observations of the pattern of *myoD* and *tropomyosin* expression in the hyoid and branchial muscle plates suggest that most muscles in these regions arise by de novo differentiation, rather than splitting. Individual muscles arise from small groups of precursors already located in their final positions (Fig. 8). Thus Edgeworth's muscle plate model may only apply to the mandibular arch, as can be solved by lineage tracing.

Generally, the numbers of myoblasts that prefigure the formation of each muscle are small. On average, a given muscle begins with 5-10 *myoD*-expressing cells, and subsequently 5-20 *tropomyosin*/myosin-expressing cells. Smaller muscles, like those of the branchial arches, start with only 2 or 3 founders. Thus, at later stages there must be an orderly and restricted cell fusion to form the adult muscle or stem cells within each group of founders that produce later growth. This problem too can only be resolved by lineage tracing experiments. The number of fibers in each muscle increases steadily as the fish grows; for example the number of Eng-expressing muscle fibers in the mandibular arch triples by five weeks of age (Hatta et al., 1991).

Hypobranchial muscles, the sternohyals, form unusually by a fusion of three sets of founders, apparently derived from ventral ends of the first few spinal myomeres (2nd to 5th in *Salmo*; Winterbottom, 1974; Goodrich, 1930). The founders migrate anteriorly beneath the branchial arches, join end to end, and attach to the hyoid arch.

Progressive subdivision of cartilage precursors

Cartilage patterning, like muscle, in zebrafish may also reflect progressive subdivision of common primordia. This idea follows those of Bertmar (1959) who examined chondrocranial

development in a variety of actinopterygians, as well as dipnoi and elasmobranchs, and described 'prochondral and protochondral' stages of tight cell packing prior to cartilage differentiation, resembling those that we have observed in whole-mounted zebrafish with Nomarski optics. Bertmar argued that the skeleton of a single arch initially 'consists of a continuous blastema', and that similar blastemae form in each arch. Subsequently, as we have observed in zebrafish, protrusions from these condensations then develop that prefigure individual skeletal elements and contain separate sites of chondrification. Thus, we observed the first Alcian-positive chondrocytes of the ceratohyal, symplectic and hyomandibular cartilages at separate sites within a single hyoid condensation. These observations suggest that common primordia at so-called blastemal or prochondral stages are shared among actinopterygians, and
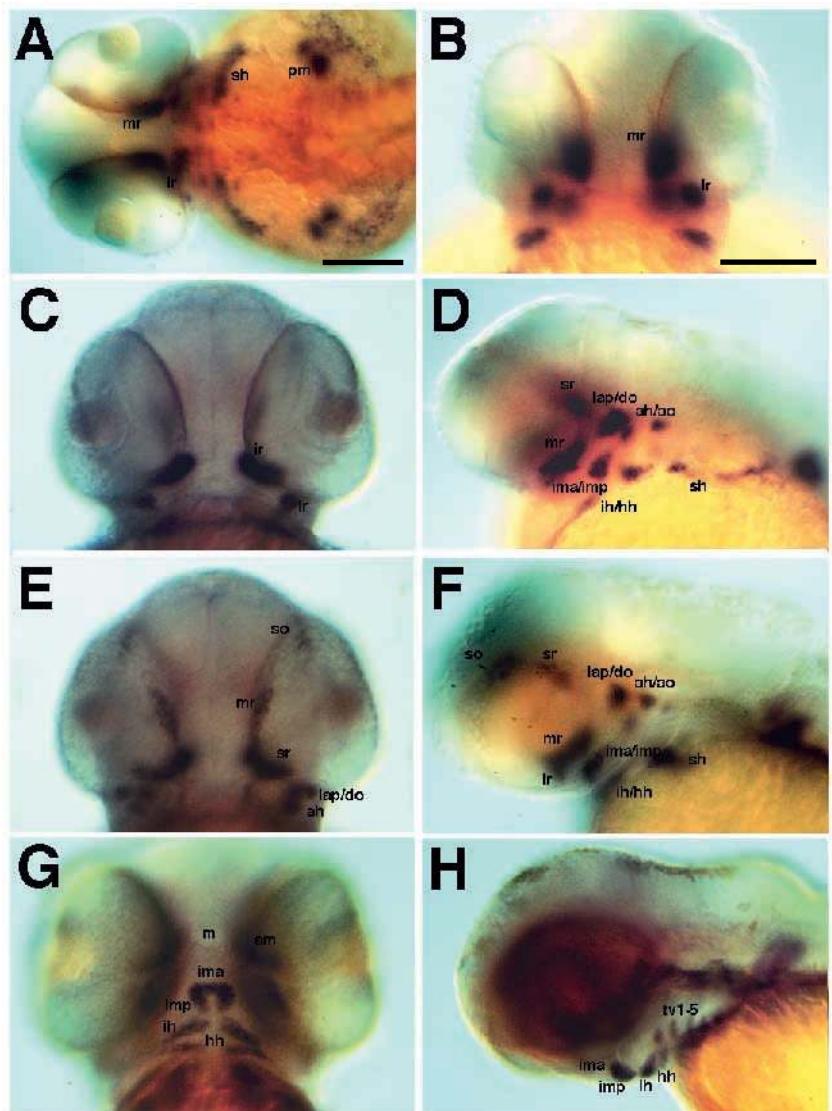

Fig. 8. Localization of *myoD* transcripts by in situ hybridization during early stages of jaw elongation (50-65 h). (A) 50 h, dorsal view showing developing extraocular, hypobranchial and pectoral fin muscles. (B) 52 h, ventral view. (C-H) Ventral and left side views of the same embryos. (C,D) 55 h. Muscle precursors are clustered at dorsal and ventral ends of pharyngeal arches. (E,F) 58 h. (G,H) 65 h. For abbreviations see Table 5. Scale bars, 100 μm.

suggest that modifications in how the primordia subdivide may have led to the present variation among species.

Segmental homologies in the pharyngeal arches

Teleosts, though highly derived fishes, retain many features of the early segmental pattern of head development thought to have been present in early ancestral gnathostomes (deBeer, 1937). Fig. 11 and Table 4 portray our scheme for segmental patterning of cartilages and muscles, and the derived states in each arch of the zebrafish larva. Branchial arches 1-4, which bear gills, retain, nearly intact, the cartilage pattern we suppose primitive: two dorsal and two ventral, bilaterally paired cartilages and a single unpaired cartilage in the ventral midline. The dorsal cartilages, epibranchials and pharyngobranchials, develop more than a week later than stages described here (Cubbage and Mabee, 1996). Dorsal and ventral constrictor muscles, branchial levator muscles and transverse ventral muscles develop in all four arches. Longitudinally oriented ventral muscles include the segmentally arranged recti ventrales and a rectus communis spanning several segments and arising either as a fusion of muscles that were primitively separate or as an outgrowth of branchial segments 4 (Nelson, 1967) or 5 (Edgeworth, 1935). Unpaired ventral midline cartilages that primitively seemed to be segmental (deBeer, 1937) are represented by two fused elements in zebrafish, an anterior element representing four segmental elements fused together (hyoid and branchials 1-3) and a posterior basibranchial 4.

Homologies in the fifth branchial arch, the only tooth-bearing arch, can be understood as modifications through loss of elements of the basic pattern. Gills are not present, and only a single cartilage develops, required to support the teeth. Likewise, the enlarged constrictor muscles are presumably adaptive for food processing. If the fifth branchial arch primordium simply fails to subdivide, the single cartilage may be considered a 'branchial 5 cartilage', serially homologous to the entire set of cartilages present in other arches, but not to any one (Oster et al., 1988). Alternatively, since the first cells that chondrify in gill-bearing arches develop as ceratobranchials, the fifth arch cartilage can be considered a ceratobranchial; it also resembles other ceratobranchials in its anatomy – position, size, orientation, shape and associations with muscles (at least in early larval stages). We treat the cartilage as a ceratobranchial homologue, which becomes important in considering how segment-specific differences come about.

Meckel's cartilage in the mandibular arch and the ceratohyal in the hyoid arch may also be in the ventral series that includes ceratobranchials. A single unpaired midline element

(basi-) and the ventralmost bilateral elements (hypo-) have been lost in the mandibular arch. The palatoquadrate and hyosymplectic have similar dorsal positions and shapes which suggests that they are homologues in a dorsal series, a supposition supported by molecular genetic study of their putative homologues in mice (Rijli et al, 1993). However, both cartilages are larger and shaped differently than their supposed homologues in the gill arches, the epibranchials (and/or pharyngobranchials). Assigning muscle relationships in the mandibular and hyoid arches is tentative, and our scheme differs from previous ones (Edgeworth, 1935; Miyake et al., 1992). Dorsal modifications in
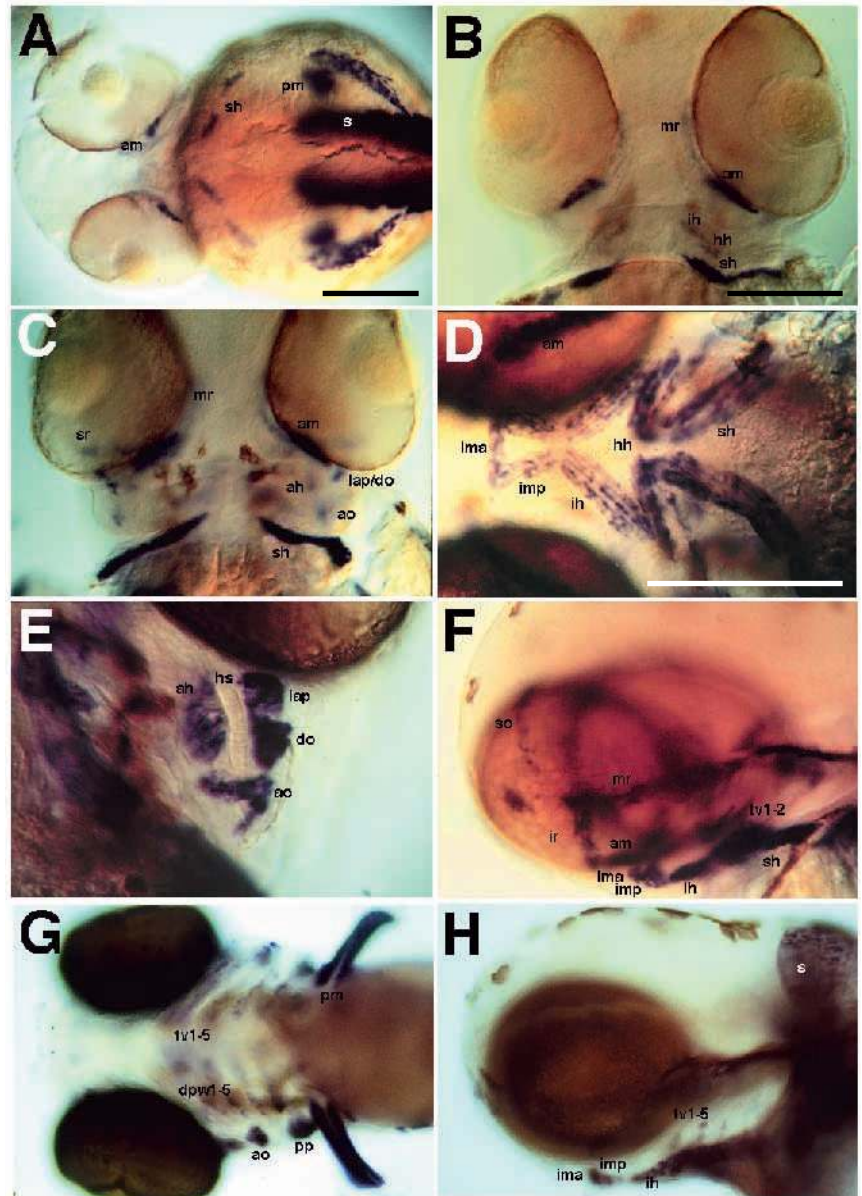

Fig. 9. Localization of *tropomyosin* transcripts during jaw elongation. (53-72 h). (A) 53 h, dorsal view showing developing mandibular, hypobranchial and pectoral fin muscles. (B) 56 h, ventral view. (C) 54 h, ventral view. (D) 72 h, ventral view, anterior to the left. (E) 72 h, ventral view, anterior to the top, of dorsolateral muscles of mandibular and hyoid arches. (F) Left side view, through the eye showing the pattern of extraocular muscles. (G) 72 h, ventral view showing both dorsal and ventral series of branchial muscles. (H) 72 h, left side view. For abbreviations see Table 5. Scale bars, 100 μm.
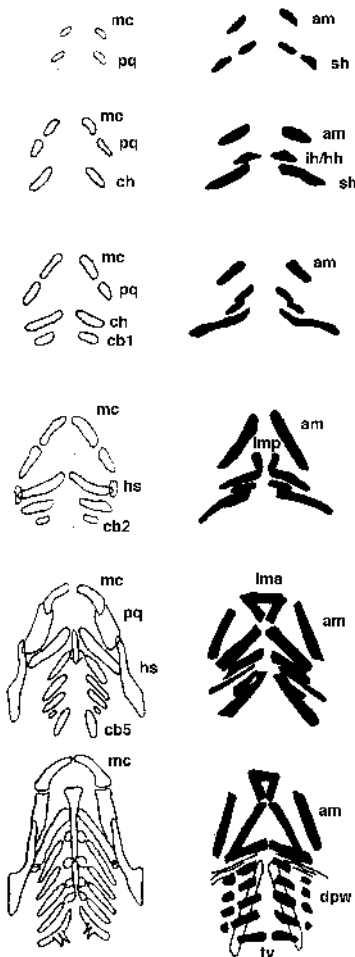
Fig. 10. A comparison of cartilage and muscle development during jaw elongation. Camera lucida drawings of ventral views, anterior to the top. Drawings of cartilage are derived from Alcian blue stained preparations while muscle outlines are based on a combination of *tropomyosin* and myosin expression patterns. For abbreviations see Table 5.

Fig. 11. Summary of segmental homologies in cranial cartilages and muscles of the zebrafish larva. (A) Schematic left side view of the pharyngeal skeleton at 96 h. Homologues are colored similarly: basi – blue; hypo – black; cerato – purple; epi – red. (B) Cranial muscles: Dorsal – brown; middle – green; ventral – yellow; putative dorsal – orange. (C) A and B combined. For abbreviations see Table 5.

Developmental sequences

Differences in timing may play an important role in how segment-specific features of the pharyngeal arches arise, just as changes in developmental timing during evolution are associated with modifications (heterochrony; Gould, 1977). We propose specifically that 'acceleration' of development, or early initiation of differentiation, promotes development of larger cartilages. Both cartilage size (e.g. Fig. 5E,H) and the time of initiation of chondrogenesis follow a general AP sequence through the series of arch segments. However, exceptions to the sequence are telling. The ceratohyal in the second arch differentiates before Meckel's, its homologue in the first. Correlated with this exception to the AP sequence, the ceratohyal is larger than Meckel's. Similarly, ceratobranchial 5 differentiates before, and is larger than, ceratobranchial 4. Precocious chondrification correlates with the fact that this arch alone bears teeth, an unusual condition among teleosts, but a shared derived feature of cyprinids. Ossification of the fifth ceratobranchial is also accelerated (Cubbage and Mabee, 1996). Thus there may well have been adaptive selection for an acceleration for the hardening of this arch. These associations suggest that cartilage size is a function of when chondrogenesis is initiated and, accordingly, that control of size of a particular element might be accomplished by accelerating or retarding when differentiation begins. The same rule might

these segments are clearly pronounced. In particular we consider most changes in anterior arches to be additions of dorsal musculature to the primitive pattern, including the huge mandibular adductor as a 'dorsal' mandibular muscle. Dorsal mandibular muscles are peculiar in that they function as openers (not constrictors as usual) and connect to the hyosymplectic cartilage and opercular bone of the hyoid arch.

Our scheme of serial homologies between jaws and gill arches in zebrafish, along with cell lineage studies showing that arches are compartmentalized and derived from similar rhombomeric levels as those of tetrapods (Schilling and Kimmel, 1994; Koentges and Lumsden, 1996), suggest that segmental patterning mechanisms have been conserved throughout vertebrate evolution. This has important evolutionary implications for the origins of jaws, as can now be tested by comparison with visceral arch organization in jawless vertebrates (Forey and Janvier, 1993; Ahlberg, 1997). For example, Eng is expressed in a dorsal subset of first arch muscles in lampreys, supporting the notion that it is the homologue of the mandibular arch of gnathostomes (Holland et al., 1993).
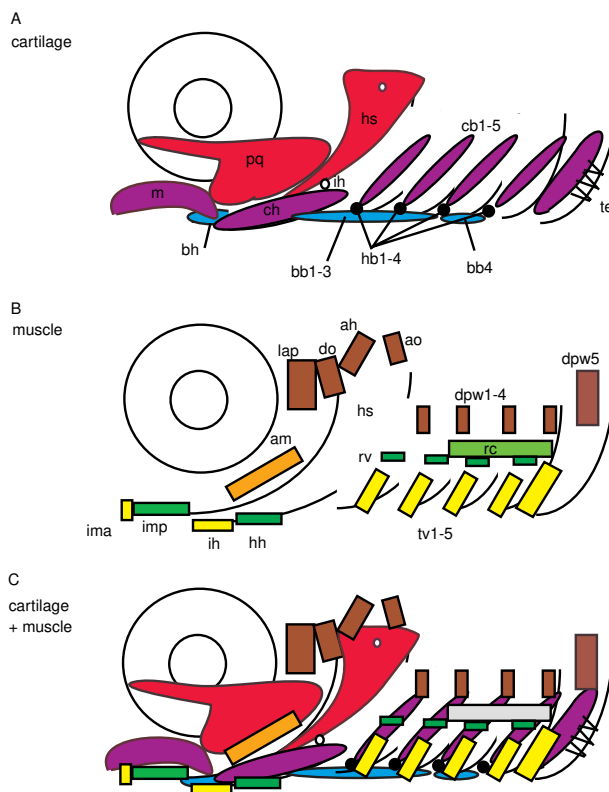
hold for muscles since, as we have emphasized, cartilages and their muscles develop together, and larger cartilages tend to be associated with larger muscles. Coordinated timing changes might ensure proper size relationships between the two tissues.

Development of the dorsal pharyngeal cartilages provides perhaps the most dramatic illustration of acceleration. Epibranchials and pharyngobranchials are small (Cubbage and Mabee, 1996), and the last to differentiate in the dorsal series. In contrast, their anterior counterparts, the palatoquadrate and hyosymplectic in the mandibular and hyoid arches respectively, are among the first pharyngeal cartilages to develop and among the largest. They support the jaw and operculum, and their accelerated development might account for the size differences between these arches and the branchial arches, as would be important in jaw-opercular evolution. How developmental control of timing sequences is achieved, and why acceleration promotes large cartilage size might be resolved by mutational analysis.

A genetic approach to craniofacial patterning

A sound knowledge of wild-type development is necessary to understand the defects generated by genetic mutation and makes important predictions for the types of mutant phenotypes that may occur. The zebrafish provides the opportunity to examine large numbers of randomly generated, lethal mutations that affect a particular embryonic process of interest, and large mutant screens have recently isolated over a hundred with defects in craniofacial development (Driever et al., 1996; Haffter et al., 1996; Neuhauss et al., 1996; Piotrowski et al., 1996; Schilling et al., 1996). Although only skeletal defects have been analyzed thus far in most existing mutants, many have cartilage defects in subsets of pharyngeal segments (e.g. *flathead* and a large number of mutants of the flathead-like class disrupt branchial arches 2-4) or in dorsal or ventral subsets of elements (e.g. *sucker* deletes ventral mandibular and hyoid cartilages). One might expect these mutants to have defects in both cartilages and muscles in the same segments, if their patterning is interdependent. If correlated skeletal and muscle defects are found, we can use mosaic analysis to determine which cells are affected directly, as has been done for *chn* (Schilling et al., 1996a), which lacks cartilage and muscles in all arches but only disrupts cartilage directly (i.e. autonomously). Muscles in *chn* lack necessary signals from their environments, perhaps the cartilage precursors themselves. In the future, similar studies should allow us to order genes into pathways as they are eventually defined at the molecular level with cloning of the mutant genes.

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