Segmental relationship between somites and vertebral column in zebrafish

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SUMMARY

The segmental heritage of all vertebrates is evident in the character of the vertebral column. And yet, the extent to which direct translation of pattern from the somitic mesoderm and de novo cell and tissue interactions pattern the vertebral column remains a fundamental, unresolved issue. The elements of vertebral column pattern under debate include both segmental pattern and anteroposterior regional specificity. Understanding how vertebral segmentation and anteroposterior positional identity are patterned requires understanding vertebral column cellular and developmental biology. In this study, we characterized alignment of somites and vertebrae, distribution of individual sclerotome progeny along the anteroposterior axis and development of the axial skeleton in zebrafish. Our clonal analysis of zebrafish sclerotome shows that anterior and posterior somite domains are not lineage-restricted compartments with respect to distribution along the anteroposterior axis but support a 'leaky' resegmentation in development from somite to vertebral column. Alignment of somites with vertebrae suggests that the first two somites do not contribute to the vertebral column. Characterization of vertebral column development allowed examination of the relationship between vertebral formula and expression patterns of zebrafish Hox genes. Our results support co-localization of the anterior expression boundaries of zebrafish *hoxc6* homologs with a cervical/thoracic transition and also suggest Hox-independent patterning of regionally specific posterior vertebrae.

Key words: Anteroposterior, Compartments, Hox expression, Resegmentation, Sclerotome, Segmentation, Somite, Vertebral column, Zebrafish

INTRODUCTION

The vertebral column, the hallmark of vertebrate species, retains through adulthood evidence of embryonic segmentation and regional specificity along its anteroposterior (AP) axis. Despite the importance of the vertebral column in defining the overall vertebrate body plan, we still lack a comprehensive understanding of the genetic and cellular interactions that control the size, shape and number of its elements. Investigating sclerotome development from its somitic origins through its differentiation into intricately patterned axial skeleton derivatives provides insight into mechanisms underlying vertebrate segmentation and AP patterning, as well as evolutionary variation among vertebrate species.

The vertebral column and ribs that make up the post-cranial axial skeleton have a metameric organization. The functional segmental unit of the vertebral column, the vertebra, is composed, in the simplest terms, of the vertebral body or centrum that develops around the embryonic notochord, dorsally extending neural arches and spines, ventrally extending hemal arches, attachment sites for ribs, and various other decorations of these regions (Fig. 1A). These vertebral column elements are distributed in a region-specific manner along the AP axis. Based on such attributes, vertebrae can be grouped into common types, the relative number of each type providing the so-called vertebral or axial formula for a given vertebrate. For example, tetrapods generally have a characteristic number of cervical, thoracic, lumbar, sacral and caudal vertebrae; however, in other vertebrates the vertebral types are less easily categorized (see Romer, 1956).

Characterizing the patterning mechanisms that define distinct regions along the AP axis is an important goal for both developmental and evolutionary biology. The presence or absence of region-specific vertebral elements has been crucial for understanding the action of genes, including but not limited to Hox genes, involved in patterning along the vertebrate AP axis. In a landmark study, Burke and colleagues (Burke et al., 1995) demonstrated that the anterior limit of expression of specific Hox genes correlates with particular AP regional landmarks in a variety of vertebrate species, rather than with specific enumerated segments. Thus, although at very different somite levels in the chick and mouse, the anterior limit of *Hoxc6* expression correlates with the transition from cervical-type vertebrae to thoracic-type vertebrae and with the forelimb/brachial plexus region. Similarly, Hox paralog group 9 gene anterior limits correlate with the transition from thoracic-type vertebrae to lumbar-type vertebrae (Burke et al., 1995). Expression of zebrafish *hoxc6* homologs relative to the forelimb/brachial plexus region (Burke et al., 1995) and anterior vertebral types (Prince et al., 1998a) is consistent with mouse and chick (Burke et al., 1995). However, a more thorough examination of the relationship of Hox expression boundaries and vertebral types in a non-tetrapod embryo is
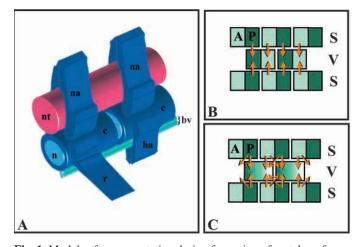
Fig. 1. Models of resegmentation during formation of vertebrae from somites. (A) Primary elements of the axial skeleton include the centrum (c) or vertebral body, which develops around the notochord (n); neural arches (na), paired dorsal elements that together with the dorsal margin of the centrum enclose regions of the neural tube (nt); hemal arches (ha), paired ventral elements that extend from the centrum adjacent to midline blood vessels (bv); and ribs (r), which extend ventrolaterally from the centrum or hemal arch. Viewed from anterolateral with anterior towards the left and dorsal towards the top. Resegmentation models (B,C) describe development of vertebrae from somites. Views of a generic vertebrate from the dorsal aspect. (B) Compartmental resegmentation model proposes that somites (S) are comprised of anterior (A) and posterior (P) halves. Each vertebra (V) develops by recombination of posterior half-sclerotomes from one somite pair with anterior half-sclerotomes from the next posterior somite pair (yellow arrows). In this strict model, half-sclerotome derivatives are restricted to one-half of the vertebra as well. This model resembles the contribution of parasegmental anterior and posterior compartments to segments in fly integument, in which patterning is maintained by lineage restriction (reviewed by Dahmann and Basler, 1999). (C) Leaky resegmentation model in which sclerotome cells from one somite (S) contribute to (yellow arrows) two adjacent vertebrae (V) in a manner that is not strictly dependent upon the A or P domain of origin in the somite.

crucial for drawing meaningful conclusions about the similarities and differences in Hox gene function in different model vertebrates. For example, Prince and colleagues (Prince et al., 1998a) and Amores and colleagues (Amores et al., 1998) have suggested that the compression of anterior Hox family expression boundaries in zebrafish may reflect a less diversified vertebral column than tetrapods. This issue has significance for understanding the developmental role of Hox genes in patterning the vertebral column and for understanding the evolution of differences in absolute and regional vertebral numbers across vertebrates (Richardson et al., 1998).

The post-cranial axial skeleton is derived from somitic mesoderm but the nature of the segmental relationship between these structures remains obscure. The vertebral column develops from sclerotome, a mesenchymal cell population derived from ventral somite. Sclerotome cells that will contribute to the vertebral column move to surround axial midline structures, condense and differentiate as chondrocytes, thus forming a cartilaginous skeletal framework that later is replaced by bone. The somites themselves are segmentally repeating units of paraxial mesoderm. The segmental register of the 'somite column' and vertebral column are offset, a fact recognized from the early days of modern embryology (see Remak, 1855) (reviewed by Brand-Saberi and Christ, 2000). Experimental studies of somite contribution to the vertebral column has been confined to avian embryos in which sclerotome comprises a major part of the somite. The overall picture that has emerged is that each somite contributes, essentially without significant AP dispersal, to adjacent body structures including sclerotome-derived vertebral components (Fig. 1B) (Beresford, 1983; Aoyama and Asamoto, 1988; Bagnall et al., 1988; Lance-Jones, 1988; Ewan and Everett, 1992; Aoyama and Asamoto, 2000; Huang et al., 2000a). The original boundary between somites ultimately aligns near the midline of the adjacent vertebral segment, a phenomenon commonly referred to as resegmentation (reviewed by Brand-Saberi and Christ, 2000; Christ et al., 2000; Saga and Takeda, 2001).

Recent work has begun to elucidate the events of paraxial mesoderm segmentation and somitogenesis (reviewed by Christ et al., 2000; Holley and Nüsslein-Volhard, 2000; Stickney et al., 2000; Stockdale et al., 2000; Maroto and Pourquié, 2001; Saga and Takeda, 2001). It is now clear that anterior (A) and posterior (P) domains exist within each segmental unit and are established prior to somite epithelialization. Evidence of A and P domains within the somite and the offset segmental register of somites and their vertebral derivatives underlie comparisons between somite/vertebral development and segmentation in *Drosophila melanogaster* (Christ et al., 1998; Christ et al., 2000; Huang et al., 2000a; Stern and Vasiliauskas, 2000). In fact, the textbook 'resegmentation' presentation of development from somites to vertebral column (Fig. 1B) coincides closely with the current model of segmental development in fly integument. A prominent feature of early metamerism in *D. melanogaster* is the repeating anterior and posterior subdivisions of the elemental segmental unit – the parasegment (Martinez-Arias and Lawrence, 1985). The register of parasegments is offset from that of the segments, which become morphologically evident later in development. These AP subdivisions are lineage-based ectodermal compartments (García-Bellido et al., 1973), a feature that is thought to be important for formation and maintenance of developmentally relevant boundaries (reviewed by Dahmann and Basler, 1999). In vertebrates, single cell labels in chick segmental plate, prior to somite formation, have ruled out lineage-restricted compartments for formation of somites or their AP domains (Stern et al., 1988). Half-somite transplant experiments in avian embryos (Aoyama and Asamoto, 2000), however, appear consistent with a strict resegmentation (Fig. 1B) and suggest anterior and posterior lineage-restricted compartments after somite formation.

In contrast to avian embryos, many other vertebrate embryos, including representatives in fish (Swaen and Brachet, 1899; Swaen and Brachet, 1901; Sunier, 1911; Morin-Kensicki and Eisen, 1997) and frogs (reviewed by Keller, 2000), develop sclerotome as a relatively minor somite component. How these minor-component sclerotome cells distribute along the AP axis has not been described, although it is clear that significant cell movement is associated with sclerotome development (Morin-Kensicki and Eisen, 1997). Thus, the generality of restricted anterior and posterior sclerotome contribution to vertebrae needs

to be explored in other vertebrate embryos, in addition to avians. For example, scant sclerotome production might support increased dispersal along the AP axis as schematized in Fig. 1C, which depicts a 'leaky' resegmentation model in which sclerotome contribution to the vertebral column is not strictly dependent upon the anterior or posterior somite domain of origin.

Two outstanding issues motivated us to analyze vertebral column development in zebrafish. First, understanding the true developmental sequence leading from somites, in which patterning genes such as those of the Hox complex are expressed, to vertebrae, the structures in which we typically identify evidence of altered patterning and homeosis, provides insight into patterning along the AP body axis. Second, understanding how somite and vertebral numbers relate illuminates evolutionary variation of the vertebrate body plan. In this work, we explore the validity of current views on the segmental relationship of somites and vertebral column to zebrafish development and we perform the critical test of AP somite domain distribution at the single cell level. We find that while the distribution of sclerotome along the AP axis is consistent with a leaky resegmentation, the anterior and posterior sclerotome domains clearly are not lineage-restricted with respect to future vertebrae. We also describe a somites/vertebrae alignment that suggests the two anterior-most somites do not contribute to the zebrafish vertebral column. We characterize the development of the zebrafish vertebral column and relate the AP regional character of the axial skeleton to Hox gene expression patterns. These results support co-localization of anterior expression boundaries of *hoxc6* homologs with the cervical/thoracic transition zone in zebrafish, as described for tetrapods. Finally, we find that patterned posterior vertebral types fall outside the domain of Hox family anterior expression boundaries, suggesting alternative patterning mechanisms for these regions.

MATERIALS AND METHODS

Zebrafish maintenance

Zebrafish (*Danio rerio*) embryos were collected from spontaneous spawnings of AB or *AB strains and reared at 28.5°C. During the first day of development, embryos were staged by counting somites and converting to standard hours (h) or days (d) of development at 28.5°C (Hanneman and Westerfield, 1989; Kimmel et al., 1995). Older embryos were staged by days of development post-fertilization. To maximize the rate of development, embryos were reared singly or in groups of five or fewer.

Cell labeling

Embryos of 16-22 h had chorions removed, were anesthetized in dilute tricaine methanesulfonate (TMS, Sigma) in physiological saline (Westerfield et al., 1986) and mounted in 1.2% agar on a microslide (Eisen et al., 1989). Individual sclerotome or presumptive myotome cells were injected with fluorescent dextrans (Molecular Probes) as described by Morin-Kensicki and Eisen (Morin-Kensicki and Eisen, 1997). Because somite cells that will contribute to myotome remain within their somite of origin (DeVoto et al., 1996; Morin-Kensicki and Eisen, 1997), one or more cells of the myotome in a more anterior somite also were labeled to serve as reference points for following sclerotome migration. Data from somites 5-17 were included in this study. For analysis of the cell distribution from anterior and posterior sclerotome domains, only those labeled cells in anterior-most or posterior-most positions within the somite were considered. Early

migration data were included from some of the labeled sclerotome cells by Morin-Kensicki and Eisen (Morin-Kensicki and Eisen, 1997).

Sclerotome progenitors were labeled at 18 h and clone size assayed at 3 d. The fluorescent label often became faint and punctate, precluding a rigorous analysis of sclerotome cell proliferation, although clone size appeared to range between two to six cells. We recorded the distribution of labeled sclerotome cell progeny along the dorsoventral axis in 13 embryos. From a total of 55 cells counted at 3 d, 44 were located dorsal to the notochord or dorsal or lateral to the neural tube, nine were located ventral to the notochord and two were located lateral to the notochord.

Videomicroscopy

Embryos were mounted either in agar or between bridged coverslips (Myers et al., 1986) and examined using a Leitz 50× water immersion objective on a Zeiss Universal microscope. Low light level fluorescent images were obtained through a Dark Invader (Night Vision Systems) and Koyo camera. Images, contrast-enhanced and averaged to remove noise with an Argus 10 image processor (Hamamatsu), were stored on an optical disc recorder (Panasonic). Fluorescent cells were viewed first 5 to 20 minutes after labeling and then at 24 h and 48 h and again between 3-5 d. Zebrafish remain optically clear at these stages allowing three-dimensional localization of labeled cells in live fish by the *z*-series record at the light microscope. We collected positional data on every labeled cell of each clone in this manner (Morin-Kensicki and Eisen, 1997). Somites were counted in 24 h embryos as described by Kimmel et al. (Kimmel et al., 1995). In 4 d fish, the number of somites was inferred from the number of myotomes.

Visualizing vertebrae and myotomes

The early vertebral cartilaginous framework was visualized by in situ hybridization using an α-*coll2a1* riboprobe (a gift from Y.-L. Yan) (Yan et al., 1995) as described by Thisse et al. (Thisse et al., 1993) with the following modifications for larger tissue: fixation, soak and wash times were increased approx. threefold; proteinase K concentration was doubled; tissue was rinsed in 2 mg/ml sodium borohydride for 30 minutes prior to prehybridization; and prehybridization and hybridization buffer contained 1% SDS.

Sclerotome cells injected with fluorescein dextran were visualized in frozen sections at 10 d according to the methods of DeVoto et al. (DeVoto et al., 1996) with an alkaline phosphatase conjugated anti-fluorescein antibody according to manufacturer's recommendations (Roche). To visualize vertebrae in these sections, tissue was rinsed twice for 10 minutes in PBS at pH 8.3 and incubated overnight at 4°C in Xylenol Orange (Rahn and Perren, 1971) (Sigma, X0127) at 1 mg/ml in PBS at pH 8.3. The tissue was then rinsed five times for 10 minutes in PBS at pH 8.3, coverslipped and visualized on an Axioplan microscope (Zeiss) with a rhodamine filter set. Images were captured with a MicroMAX 1300Y5M digital camera (Princeton Instruments) using MetaView software (Universal Imaging Corporation).

Myotome cells injected with fluorescein dextran were visualized by antibody staining in 12-13 d whole-mount zebrafish after 24 hour/4°C fixation in 4% paraformaldehyde and careful removal of the skin. Fish were post-fixed and then washed extensively prior to staining in Alizarin Red (see below). Embryos were stored in a 0.5% solution of KOH in water.

Histological staining

To visualize developing vertebrae, Alcian Blue, 8GX (CI 74240), a common marker for non-mineralized cartilage matrix, and Alizarin Red S (CI 58005), a stain for mineralized cartilage and bone matrix were used. Zebrafish from 3-30 d were immobilized in ice water, anesthetized in dilute TMS, then fixed for 12-72 hours in buffered 4% paraformaldehyde. After washing, fish were placed in either 0.1 mg/ml Alcian Blue in 1:4 glacial acetic acid:95% ethanol (pH 4.5) for 12 hours, or 0.1 mg/ml alizarin red in 0.5% KOH for 2-5 hours. Alcian Blue-stained zebrafish were either transferred to Alizarin Red or

Fig. 2. Developmental sequence and regional character of zebrafish axial skeleton. Zebrafish skeletal matrix stained with Alcian Blue and/or Alizarin Red at representative stages between 7 d and adult. Anterior towards the left; dorsal towards the top. (A) Matrix foci (arrows) of the anterior-most centra dorsal to the notochord at 7 d. (B) At 8 d matrix of the developing centra form rings around the notochord in anterior trunk. (C) Matrix surrounds chondrocytes (arrows) at the base of the neural arches of vertebrae (V) 3 and 4 visualized here at 13 d. (D-J) The vertebral column is composed of classes of vertebrae based on developmental and morphological features characteristic of AP position. Examples of each category are visualized at 16 d (F,I,J), 21 d (D,E), or 2 years (G,H). (D,F) Cervical vertebrae comprise two unique and relatively small vertebrae with neural arches (arrowheads in D) that are lost in adults. (E-G) The number of rib-bearing vertebrae (arrows indicate ribs in G) was variable. The first two (V3, V4) had distinctly large neural arches (arrowheads in E) and modified ribs. V3-V5, in conjunction with cervical vertebrae (V1, V2) formed the series (F) that later contributes to the Weberian ossicles. Rib- and hemal arch-bearing vertebrae were found in most but not all fish; after 20 d the final one or two ribs sometimes had hemal arch attachment sites (H, arrows). Hemal arch-bearing vertebrae began with a series of three or four progressively longer hemal arches (arrows in I). The tail set (J) included four vertebrae with unique morphology such as a dorsal bend of the penultimate centrum (arrow) and no neural arch on the last vertebra. Centra were also sometimes decorated with dorsoposterior projections (black arrowheads in G,H) and ventroposterior projections (H, white arrowhead) with a characteristic distribution along the AP axis. Scale bars: 15 μm in A-C; 50 μm in D-F,I-J; 400 μm in G,H.
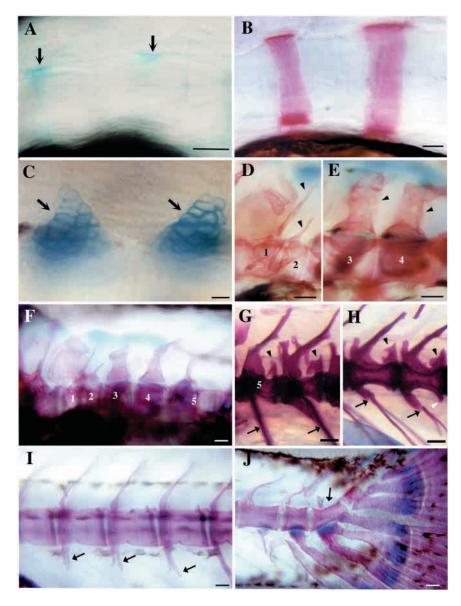
dehydrated in 100% ethanol for 1 hour and cleared in methyl salicylate. Alizarin Red-stained zebrafish were dehydrated in 100% methanol, cleared in methyl salicylate and mounted between bridged coverslips in Permount (Fisher Scientific). Procedures for staining adult zebrafish were similar, but fixation, staining and dehydration times were each 1 week.

A modification of a Phloxine-Methylene Blue-Thomas Method staining procedure (Humason, 1972) was used to visualize boundaries between successive myotomes and successive vertebrae. After fixation for 12-72 hours in buffered 4% paraformaldehyde, 15 d zebrafish were immersed in a solution of Methylene Blue (CI 52015), Azure B (CI 52010) and borax, each at 1.25 mg/ml, in water for 2 hours. Fish were then destained in 0.2% aqueous acetic acid for 20 minutes followed by 30 minutes each in 95% then 100% ethanol, cleared in methyl salicylate and mounted between bridged coverslips in Permount.

RESULTS

Axial skeleton development

Histological staining revealed that the zebrafish axial skeleton developed in a stereotyped sequence. We characterized primary elements of the axial skeleton, including the centrum, neural and hemal arches, and ribs. Because the rate at which zebrafish larvae developed was quite variable, the age at which various stages in the sequence of vertebral development were reached also varied considerably. Thus, ages given below represent the earliest age at which each stage in vertebral development was observed. The anterior end of the zebrafish notochord stains with Alizarin Red, ossifies and is surrounded by the developing basioccipital bone of the cranium (Cubbage and Mabee, 1996). We found that anterior notochord was marked by matrix stains in concert with development of other elements of the cranium (data not shown). These events initiated approximately 3 days prior to the onset of stainable matrix in the post-cranial axial skeleton (Cubbage and Mabee, 1996) (data not shown). Therefore, given both developmental timing and position within the embryo, we classify the matrix surrounding the anterior-most portion of the notochord as a head element as do Cubbage and Mabee (Cubbage and Mabee, 1996) and Kimmel et al. (Kimmel et al., 2001), but in contrast to a recent report by Du and colleagues (Du et al.,

Table 1. Zebrafish axial skeleton characteristics and axial formula

Vertebrae	Average±s.d.	Minimum	Maximum	Mode*	$n^\dagger$
Total	31.8±0.8	29	33	32	305
Cervical	2.0±0.0	2	2	2	179
Rib bearing	10.2±1.0	8	12	10	179
Rib and hemal arch bearing	1.0±0.9	0	2	2	179
Hemal arch bearing	14.5±1.2	12	18	14	179
Tail fin set	4.0±0.1	3	4	4	179

*Combined modes from each vertebral-type category gives the most common axial formula.

†Number of fish observed. Data set with 305 individuals composed of fish ages 13-31 d stained with Alcian Blue or Alizarin Red, or both. Data set of 179 individuals is a subset that includes only fish ages 18-31 d stained with both Alcian Blue and Alizarin Red.

2001) in which this region is labeled as the first element of the vertebral column. We found that the three most anterior vertebral centra were the first to develop cartilaginous matrix. Stained foci were evident dorsal to the notochord at 6 d with Alizarin Red (data not shown) and at 7 d with Alcian Blue (Fig. 2A). Later development of the two anterior-most centra lagged behind development of the remaining trunk and tail centra, a phenomenon also noted by Du and colleagues (Du et al., 2001). Additional foci arose ventrally and eventually rings of smoothly stained matrix surrounded the notochord (Fig. 2B) (Fleming et al., 2001). Development of the centra proceeded in an anterior to posterior sequence (Du et al., 2001), apparent with both Alcian Blue and Alizarin Red, and was complete along the length of the fish at 9 d. Neural and hemal arches on individual vertebrae arose synchronously in an anterior to posterior sequence first visible at 8 d by α-coll2a1 expression (data not shown) and at 11 d with Alcian Blue staining. In contrast to the smooth appearance of the stain surrounding the notochord, the chondrocytes of the developing neural arches were readily apparent (Fig. 2C). The general anterior to posterior sequence of development was retained for the centra, neural and hemal arches, and rib development. At 21 d, the axial skeleton was essentially complete with evidence of all adult components. Adult-specific axial skeleton characteristics included apparent fusion of centra and loss of the neural arches of the first two vertebrae, formation of a complete rib-case frequently including hemal arch attachment sites for the last one to two ribs (Fig. 2G), a complete set of elaborate dorsal and ventral posterior processes from the centrum (Fig. 2G,H) and apparent fusion of the two posterior-most centra (data not shown).

The total number of vertebrae in the column and the number of region-specific vertebral types were variable. An average of 32 total vertebrae were divisible into distinct categories based on features characteristic of their AP position (Fig. 2, Table 1) as follows: (1) cervical (Fig. 2D,F); (2) rib-bearing (Fig. 2E-G); (3) rib and hemal arch-bearing (Fig. 2H); (4) hemal arch-bearing (Fig. 2I); and (5) tail fin set (Fig. 2J). Specific vertebrae (V) in the series also had dorsal (V4-6 to V27-29; $n=42$) and/or ventral (V14-18 to V27-28; $n=28$) posterior processes from the centrum (Fig. 2G,H). Following the order of these categories, the axial formula most frequently observed was 2:10:2:14:4 (Table 1). The invariant first five vertebrae contributed to a striking specialization termed the Weberian ossicles, characteristic of Ostariophysan fishes (Fink and Fink, 1981).

This structure, comprised of the two cervical vertebrae and the first three rib-bearing vertebrae with rib specializations of the third and fourth vertebrae, is thought to transmit vibrations from the anterior swim bladder to the inner ear (Fink and Fink, 1981). The tail fin set nearly invariably comprised four distinct vertebrae. By contrast, the number of mid-column vertebrae at the junction between rib-bearing and hemal arch-bearing was variable. Thus, while most fish had two vertebrae that bore both a rib and a hemal arch, others had vertebrae in this region with neither a rib nor a hemal arch (Table 1).

The occurrence of 'anomalous' vertebrae correlated positively with extremes in total vertebral number. Anomalous vertebrae with duplicate or missing neural arches, duplicate hemal arches and missing or short centra were found posteriorly, usually just anterior of or within the tail fin set of 'short' fish with only 30-31 total vertebrae. Of short fish, 16/60 (26.6%) showed such anomalies compared with 6/119 (5.0%) of 'long' fish with 32-33 total vertebrae. Similarly, the presence of an anomalous vertebra with no hemal arch just anterior to the tail fin set was observed in 10/119 (8.4%) of long fish, but never in short fish ($n=60$).

Relationship between somites and vertebrae

In zebrafish, mesenchymal cells derived from a ventromedial cell cluster of the somite migrate to positions adjacent to the notochord and neural tube (Morin-Kensicki and Eisen, 1997), as expected for sclerotome contribution to connective tissue and vertebral cartilages. To show these mesenchymal cells contribute to the developing vertebral column, we labeled cells of the ventromedial cluster with vital fluorescent dye and processed fish to visualize the label at stages when the developing vertebral column was apparent. We were able to visualize both labeled cells and vertebral components in sectioned material from eight fish. In each case, some of the labeled cells contributed to developing vertebral components that labeled with Xylenol Orange (Rahn and Perren, 1971) (Fig. 3) verifying that they derived from sclerotome, and thus, that sclerotome makes the expected contributions in zebrafish.

To understand somite contribution to the vertebral column we needed first to characterize the morphological relationship of zebrafish somites and vertebrae. The myotome in zebrafish retains a permanent record of original somite segmentation and allows an unambiguous assessment of the vertebral column segmental register to that of the somites. Although the contour of the myotome was complex at ages when vertebrae could be visualized, at the medial surface, boundaries between myotomes traversed the anterior region of each centrum (Fig. 4). In effect, a single myotome spanned the posterior three-quarters of one vertebral centrum and the anterior one-quarter of the next posterior centrum. To define which somites are adjacent to which developing vertebrae in the series, we labeled somite 3 or 5 myotome progenitors at 18 h and then visualized them at 12 or 13 d when the vertebrae could be stained with Alizarin Red. At 12 d, labeled somite 5 cells were found in myotome 5 in three out of three fish. Similarly, at 13 d, labeled somite 3 and somite 5 cells were found in myotomes 3 and 5 respectively in six out of six fish. In each case, the medial boundaries of myotome 5 spanned the centra of the second and third vertebrae, thus we could align somites with vertebrae along the AP axis (Fig. 5). Curiously, however, we identified no clear relationship between an observed variable total somite (Kimmel et al., 1995) or

myotome number, counted at 24 h and 4 d respectively, to the variable total vertebral number counted at 15 days in 29 fish followed as individuals (data not shown).

Sclerotome cells can contribute progeny to two consecutive vertebrae regardless of their initial AP position within the somite. The progeny of fluorescently labeled cells originating from either anterior-most or posterior-most positions within the sclerotome were analyzed by segmental location defined by myotome boundaries on or after 3 d (Table 2). Some labeled cells were followed to the outer limits of fluorescence detection around 7 d; there was no significant change in segmental location between 3 and 7 d (data not shown). Vertebral components are not marked at these stages; however, some of these cells contribute to the developing vertebrae (Fig. 3F,G) and some of these cells will contribute to other sclerotome derivatives, including meninges, connective tissue and blood vessel endothelia (Lance-Jones, 1988; Bagnall et al., 1988; Bagnall, 1992; Morin-Kensicki and Eisen, 1997).

Because the myotome spans two consecutive developing vertebrae, the anterior myotomal border is aligned with one vertebra and the posterior myotomal border is aligned with the next posterior vertebra. By relating sclerotome cell position to myotomal boundaries, we found that sclerotome originating from positions anterior in the somite tended to migrate to the location of the more anterior of the two adjacent developing vertebrae. Similarly, those sclerotome cells originating from positions posterior in the somite tended to migrate to the location of the more posterior vertebra. Nonetheless, individual anterior and posterior sclerotome cells were each capable of contributing progeny to either anterior or posterior vertebral positions and in some cases to both positions (Table 2, Fig. 3A-C).

DISCUSSION

The vertebral column is an intricately patterned structure with repeating units displaying anterior to posterior regionally specific form. The mode by which the overall segmental nature and regional character develops from embryonic patterning events remains unclear. When addressing this issue, it is important to recognize 'segmentation' and 'regionalization' as two distinct features of development. Here, segmentation refers to development of repeated units along the AP axis, whereas regionalization refers to development of dissimilar regions along the AP axis. In segmented structures, regionalization is revealed when segments are similar but not identical to one another.

Sclerotome distribution pattern in zebrafish supports leaky resegmentation but not lineage-restricted compartments

Models describing development of the vertebral column from somites, usually referred to as resegmentation, have relied on partitioning of the somite into anterior and posterior domains. The shift in register observed between somites and vertebral column is posited to occur when the posterior sclerotome of
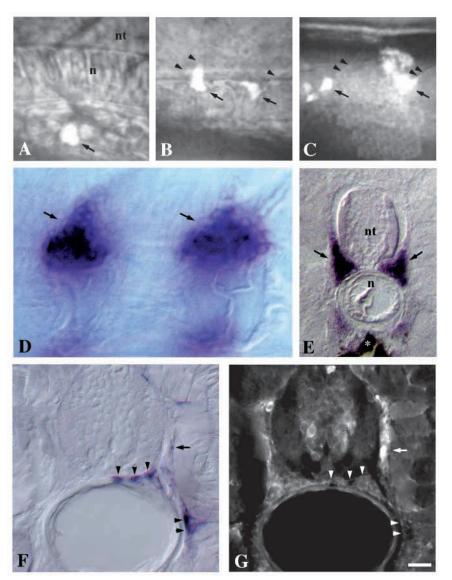

Fig. 3. Mesenchymal cells derived from ventral somite contribute to vertebrae. Cells of the ventromedial cell cluster of each somite migrate dorsally and spread along the AP axis (Morin-Kensicki and Eisen, 1997). (A) Shown is a single cell (arrow) labeled at 18 h and the distribution of its progeny (arrows) at 24 h (B) and 48 h (C). Arrowheads mark myotomal boundaries. (D,E) Serving as an early marker for vertebral column components, α-coll2a1 is localized to anterior neural arches (arrows) by 10 d in the side view in D (compare with Fig. 2C) and in transverse section (E). (F,G) Progeny derived from a single, labeled ventromedial cluster cell of an anterior trunk somite contributed to vertebral components when visualized at 10 d. (F) Cells visualized with an antibody to fluorescein;. The arrow indicates a cell that is incorporated into a neural arch and the arrowheads indicate cells in other regions of the developing vertebra. (G) The same section as in F labeled with Xylenol Orange to reveal developing bone; the cells indicated in F are also positive for this bone marker. Asterisk in E marks pigment cells. n, notochord; nt, neural tube. Scale bar: 10 μm in A; 15 μm in B,D,F,G; 20 μm in C; 35 μm in E.

Fig. 4. Segmental patterns of somites and vertebral column are out of register. A region of the trunk of a 15 d zebrafish stained to reveal vertebral (arrows) and myotomal (broken line) boundaries. Viewed from the side with anterior towards the left, dorsal towards the top. Myotomes retain the original segmental pattern of the somites. Adjacent to the axis, a single myotome spans the posterior three-quarters of one vertebral centrum and the anterior quarter of the next posterior centrum. Scale bar: 45 µm.

Table 2. Distribution along AP axis of sclerotome from anterior or posterior somite domains

	Number of progenitors contributing progeny to specific segmental positions relative to somite of origin		
Origin in somite	Next anterior	Anterior and posterior	Next posterior
Anterior sclerotome (n=17 single cells)	12	4	1
Posterior sclerotome (n=15 single cells)	5	1	9

one somite pair recombines with the anterior sclerotome of the next posterior somite pair. The compartmental version of resegmentation holds that the anterior and posterior domains are lineage-restricted compartments. This conception of resegmentation has a direct correlate in the anterior and posterior compartments that subdivide ectodermal parasegments in *D. melanogaster*. But does this model describe how the vertebral column is derived from somites in vertebrate embryos?

Compartmental resegmentation in vertebrates has only recently been tested experimentally (Aoyama and Asamoto, 2000). Previous work had shown by several methods that a single avian somite contributes to more than one vertebra (Beresford, 1983; Aoyama and Asamoto, 1988; Bagnall et al., 1988; Lance-Jones, 1988; Ewan and Everett, 1992; Huang et al., 2000a) but the relative contribution of individual anterior or posterior domains had not been assessed. Aoyama and Asamoto (Aoyama and Asamoto, 2000) showed that in

chick-quail chimeras, individual anterior or posterior half-somite transplants had progeny restricted to one half of one adjacent vertebra in a manner consistent with strict compartmental resegmentation (Fig. 1B). Here, we show that cells derived from individual anterior or posterior sclerotome cells in zebrafish do not distribute along the AP axis in a manner strictly dependent upon their anterior or posterior origin. In fact, clonal analysis (Table 2) indicates that individual cells can contribute progeny to both adjacent vertebral locations, a result that is incompatible with the idea of lineage-restricted compartments. Our results in zebrafish are thus more consistent with a leaky resegmentation model (Fig. 1C).

Recent genetic evidence indicates that AP subdivisions within paraxial mesoderm relate to morphogenesis of the vertebral column. Disruption of gene pathways important for establishing future somite AP subdivisions in presomitic mesoderm (reviewed by McGrew and Pourquié, 1998; Stickney et al., 2000;
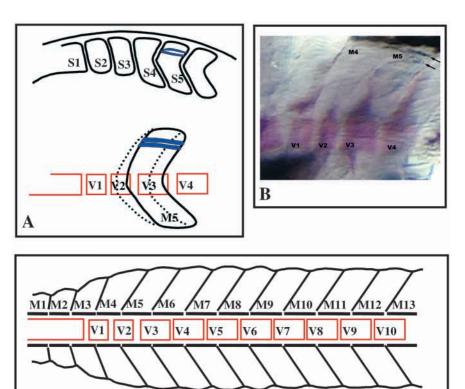
Fig. 5. Relationship of somites or myotomes and vertebral column in zebrafish. To determine the alignment of the somite series with the vertebral column, future myotome cells in somite 5 (S5) were labeled during the first day of development and then visualized in conjunction with Alizarin Red staining of the vertebral column at 12 d as shown schematically in A. Cells originally labeled in S5 were found in the fifth myotome (M5) at 12 d (arrows in B). M5 was adjacent to vertebra 2 (V2) and vertebra 3 (V3), as shown schematically in A and in a representative fish in B. The resulting relationship between somite and vertebral alignment is shown in a schematized dorsal view in C.
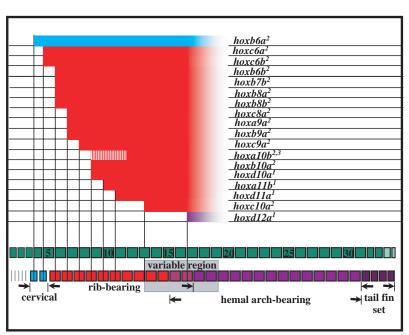
Fig. 6. Schematic representation of relationships between somites, anterior expression boundaries of some Hox genes and vertebrae. Anterior expression boundaries of zebrafish Hox genes [names according to Amores et al. (Amores et al., 1998)] are primarily as in [1]van der Hoeven et al. (van der Hoeven et al., 1996) [2]Prince et al. (Prince et al., 1998a) and [3]Sordino et al. (Sordino et al., 1996). Differing anterior boundaries indicated by stripes have been reported (Sordino et al., 1996; Prince et al., 1998a) for *hoxa10b*. Zebrafish *hoxc6* homologs, with anterior expression boundaries at somite 5, align with the transition between cervical and thoracic (rib-bearing) vertebrae as in chick and mouse (Burke et al., 1995). By contrast, the anterior expression boundaries of zebrafish Hox paralog group 9 genes fall within the anterior thoracic domain, as apposed to Hox paralog group 9 alignment with the thoracic/lumbar transition zone in chick and mouse (Burke et al., 1995). The *hoxd12a* anterior expression boundary is the posterior-most described and yet also falls within the region that will contribute to rib-bearing vertebrae. Posterior somites are indicated by stripes because the resolution of total somite and vertebral numbers remains unclear.

Christ et al., 2000; Pourquie, 2001; Saga and Takeda, 2001) results in perturbed somite and vertebral column segmentation (Evrard et al., 1998; Sparrow et al., 1998; Zhang and Gridley, 1998; Schubert et al., 2001) notably without disturbing differentiation of somite into sclerotome and dermamyotome or disrupting the broad pattern of anteroposterior regional identity (e.g. thoracic versus lumbar). Perhaps AP distinctions exist in sclerotome important for vertebral segmentation that are maintained via cell interactions rather than lineage restrictions. In *D. melanogaster*, the sharp boundary of *engrailed* expression stripes is maintained by cell interactions, despite movement of individual cells away from the boundary and their coincident loss of *engrailed* expression (Vincent and O'Farrell, 1992). In addition, distinct domains in the developing leg (Brook and Cohen, 1996; Jiang and Struhl, 1996; Johnston and Schubiger, 1996; Penton and Hoffman, 1996; Theisen et al., 1996) and body wall (Diez del Corral et al., 1999; Calleja et al., 2000) are maintained by cell interactions and not lineage restriction. Moreover, cell-labeling experiments in chick embryo hindbrain indicate that while the majority of cells respect rhombomere boundaries (Fraser et al., 1990), some cells cross those boundaries (Birgbauer and Fraser, 1994) in a manner inconsistent with lineage-restricted compartments, as we have described here for zebrafish somite domains. Thus, while *D. melanogaster* may use lineage-restricted anterior and posterior compartments as a patterning mechanism in the segmented ectoderm, at a more general level both flies and vertebrates may rely on fundamental cell interactions for refining and maintaining boundary information in the establishment of distinct domains with subsequent patterning roles.

Alignment of somites and vertebral column in zebrafish reveals possible occipital somites and aberrant vertebrae

The question of the involvement of somites in head skeleton development has a long history (Jeffs and Keynes, 1990). Experimental manipulation in chick (Couly et al., 1993; Huang

et al., 2000b) indicates that a specific number of somites contributes cells to formation of the posterior-most bones of the skull. In mouse, manipulating Hox gene expression via retinoic acid (Kessel and Gruss, 1991), creation of transgenics (Kessel et al., 1990) or mutation (Chisaka and Capecchi, 1991; Lufkin et al., 1991; Chisaka et al., 1992) also supports this notion. In zebrafish the alignment of the myotome derived from somite 5 with the second and third vertebrae provides evidence that the first two somites and perhaps part of the third may not contribute to the vertebral column. Whether these anterior somites contribute sclerotome cells to formation of posterior skull bones as documented in chick (Couly et al., 1993; Huang et al., 2000b) is currently an unanswered question that can be addressed by future lineage-labeling studies.

One possible relationship between somites and vertebrae is shown in Fig. 6. However, our comparison of total somite number with total vertebral number indicates that ultimate resolution of somites and vertebrae during development remains obscure. We found that long fish often have an extra vertebra that lacks a hemal arch just before the more rigidly patterned tail fin set, while short fish have various patterning defects in the size, shape and presence, absence or duplication of various vertebral elements, primarily in posterior regions. Finding that anomalous vertebrae are more frequently associated with extremes of total vertebral number suggests that the mechanisms underlying patterning are not entirely able to compensate for extreme paucity or excess of material.

Hox expression boundaries align with trunk, but not tail vertebral types in zebrafish

To gain a better understanding of the role of Hox genes in AP patterning, we have aligned zebrafish Hox expression domains (Sordino et al., 1996; van der Hoeven et al., 1996; Prince et al., 1998a; Prince et al., 1998b) with the somite series and vertebral column (Fig. 6). The anterior expression boundaries of zebrafish *hoxc6* homologs align with the cervical/thoracic transition in zebrafish, as in tetrapods, although the thoracic (rib-bearing)

nature of V3 and V4 is somewhat masked by the ostariophysin specialization of the Weberian apparatus (Fink and Fink, 1981). Burke et al. (Burke et al., 1995) previously showed a similar correspondence between zebrafish and tetrapods in that the zebrafish *hoxc6* anterior expression boundary aligned with the pectoral fin (forelimb) plexus region. Prince et al. (Prince et al., 1998a) also pointed out *hoxc6* anterior expression boundary alignment with vertebral type transition in zebrafish. By contrast, the anterior expression boundaries of zebrafish paralog group 9 Hox genes fall within somite regions that will contribute to anterior rib-bearing vertebrae, consistent with a derived role for Hox paralog group 9 genes in marking the thoracic/lumbar transition in tetrapods (Burke et al., 1995).

The anterior expression boundaries of all known zebrafish Hox genes fit within the region encompassed by trunk somites [somites 1-17 as defined by Kimmel et al. (Kimmel et al., 1995)]. We show here that this region corresponds to somites forming the cervical and rib-bearing vertebrae (Fig. 6). Zebrafish *hoxd12a* with the posterior-most described anterior expression boundary at somite 17 (van der Hoeven et al., 1996) may correspond to the posterior end of the trunk and the transition from rib-bearing to hemal arch bearing vertebrae. The anterior expression boundaries of zebrafish *hoxc10a* (somite mid-13) (Prince et al., 1998a) and *hoxd12a* fall within the region of the zebrafish vertebral column that shows a variable distribution of vertebral types (Fig. 6). It would be interesting to learn if any variability exists in anterior expression boundaries of these Hox genes that might predict a specific vertebral formula outcome in individual fish.

The tail shows patterned vertebrae but no 'Hox code'

The vertebrae of the zebrafish tail (with origins posterior to somite 17) showed regional distinctions. These included differences in neural arch, hemal arch and centrum size, presence of dorso- and ventroposterior extensions from the centrum and unique shapes of the tail set vertebrae. Such region-specific tail vertebrae morphologies may indicate a tail-specific AP regional patterning mechanism distinct from but similar to the 'Hox code' of the trunk. Alternatively, these characteristic morphologies may reveal unique local patterning mechanisms. Using intracellular labeling techniques, Müller and colleagues (Müller et al., 1996), showed that the first 13-14 somites form during gastrulation in zebrafish but that somites more posterior to this arise from the tail bud. This mechanistic transition also coincides with the transition to somites contributing to the axial skeleton variable region. It is intriguing that the region of variability may coincide with a region of transition between distinct mechanisms of defining AP regional specificity, that is, trunk- and tail-specific patterning. While a similar distinction between somites formed by primary and secondary gastrulation noted by Christ and colleagues (Christ et al., 2000) does not appear to correlate with Hox gene expression domains in chick (Burke et al., 1995; Christ et al., 2000), our results lend support to the concept that the tail region develops with mechanisms that are in some ways distinct from those employed for head and trunk patterning (Kanki and Ho, 1997; Griffin et al., 1998; Ahn and Gibson, 1999; Kimelman and Griffin, 2000). Thus, in zebrafish, AP patterning along the trunk vertebral column may be influenced by Hox gene expression patterns but the regional identity of tail vertebrae may be defined by different mechanisms.

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