Development 124, 159-167 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
DEV4777

Sclerotome development and peripheral nervous system segmentation in embryonic zebrafish

Elizabeth M. Morin-Kensicki and Judith S. Eisen*

Institute of Neuroscience, 1254 University of Oregon, Eugene OR 97403-1254, USA

*Author for correspondence (e-mail: eisen@uoneuro.uoregon.edu)

SUMMARY

Vertebrate embryos display segmental patterns in many trunk structures, including somites and peripheral nervous system elements. Previous work in avian embryos suggests a role for somite-derived sclerotome in segmental patterning of the peripheral nervous system. We investigated sclerotome development and tested its role in patterning motor axons and dorsal root ganglia in embryonic zebrafish. Individual somite cells labeled with vital fluorescent dye revealed that some cells of a ventromedial cell cluster within each somite produced mesenchymal cells that migrated to positions expected for sclerotome. Individual somites showed anterior/posterior distinctions in several aspects of development: (1) anterior ventromedial cluster cells produced only sclerotome, (2) individual posterior ventromedial cluster cells produced both sclerotome and muscle, and (3) anterior sclerotome migrated earlier and along a more restricted path than posterior sclerotome. Vital labeling showed that anterior sclerotome colocalized with extending identified motor axons and migrating neural crest cells. To investigate sclerotome involvement in peripheral nervous system patterning, we ablated the ventromedial cell cluster and observed subsequent development of peripheral nervous system elements. Primary motor axons were essentially unaffected by sclerotome ablation, although in some cases outgrowth was delayed. Removal of sclerotome did not disrupt segmental pattern or development of dorsal root ganglia or peripheral nerves to axial muscle. We propose that peripheral nervous system segmentation is established through interactions with adjacent paraxial mesoderm which develops as sclerotome in some vertebrate species and myotome in others.

Key words: ablation, dorsal root ganglion, motoneuron, neural crest, patterning

INTRODUCTION

Vertebrate embryos develop a number of segmented body structures in the trunk and tail that show a related metameric pattern. Somites, reiterated blocks of paraxial mesoderm arranged on either side of the neural tube and notochord, are the first structures to display an overt segmental pattern. Later, peripheral nervous system (PNS) elements such as dorsal root ganglia (DRGs) and nerves to limb and axial muscle develop a one-to-one segmental alignment with the somites. It is unclear how these proper segmental relationships are established.

Several lines of evidence from work in avian embryos indicate that paraxial mesoderm is responsible for establishing PNS segmentation. First, ablation of the entire somite results in loss of segmentation of DRGs and peripheral nerves to the limbs (Lewis et al., 1981; Tosney, 1987, 1988); this is also the case in amphibian embryos (Lehmann, 1927; Detwiler, 1934). In addition, migrating neural crest cells (Rickmann et al., 1985), developing DRGs (Remak, 1855; Lallier and Bronner-Fraser, 1988) and the proximal portion of limb peripheral nerves (Remak, 1855; Keynes and Stern, 1984; Rickmann et al., 1985) are restricted primarily to the anterior half of each avian somite. Surgical manipulation shows that this restricted pattern of neural crest cell migration and limb peripheral nerve outgrowth is redirected by the adjacent paraxial mesoderm following 180° reversals of regions of the segmental plate (Keynes and Stern, 1984; Bronner-Fraser and Stern, 1991) or early somites (Aoyama and Asamoto, 1988); neural crest cells, peripheral axons, and DRGs still associate with the original anterior half of each somite although it is located posteriorly after rotation. Finally, differences between anterior and posterior regions of each somite are required for development of segmentation of limb peripheral nerves (Stern and Keynes, 1987) and DRGs (Kalcheim and Teillet, 1989). Thus, surgical formation of regions composed solely of multiple anterior half-somites or posterior half-somites has dramatic effects. Segmentation of the PNS is lost within anterior half-somite regions although axons extend into the periphery and unsegmented DRGs form, much as with somite ablation. In posterior half-somite regions, peripheral axons fail to extend normally and only small, dorsally-located DRGs form. Taken together, these results suggest that in avian embryos, the anterior of the somite is permissive for formation of PNS structures while the posterior of the somite is relatively non-permissive, thus establishing a segmentation of the PNS that shows a one-to-one relationship to the segmentation of the somites.

Short-range interactions from medial somite to extending

axons and migrating neural crest cells have been implicated in directing this PNS patterning in avian embryos. The avian somite can be divided into two distinct regions, a dorsolateral dermamyotome and a ventromedial sclerotome. By performing partial and complete somite ablations and observing subsequent segmentation in peripheral nerves and DRGs, Tosney (1987, 1988) established that the medial region of the somite nearest each developing PNS structure is sufficient for proper segmentation. First, removal of dermamyotome alone has no effect on the segmental pattern of DRGs or limb motor nerves (Tosney, 1987). Second, DRG segmentation is disrupted only by removal of the dorsal part of the somite and peripheral nerve segmentation is disrupted only by removal of the ventral part (Tosney, 1988). Thus, it appears that the medial region of the somite, the sclerotome, establishes PNS segmentation in avian embryos.

These studies in avian embryos have led to a model in which an anterior/posterior (A/P) alternation of properties within the sclerotome is primarily responsible for patterning DRGs and limb peripheral nerves. Since removal of sclerotome alone has not been reported, this model is derived by a process of elimination, comparing experiments in which either dermamyotome alone or entire somites were manipulated. While sclerotome seems involved in patterning avian PNS segmentation, several questions remain. First, is sclerotome required for proper PNS patterning? Second, does sclerotome play a similar role in vertebrates, such as fish and amphibians, where it constitutes a much smaller proportion of the somite (Swaen and Brachet, 1899, 1901; Sunier, 1911; Kielbowna, 1981; Youn and Malacinski, 1981)? Finally, does sclerotome exert the same sort of patterning influence on the segmentation of peripheral nerves to axial muscle that it does on nerves to limbs?

In this study, we characterized sclerotome development in embryonic zebrafish and directly tested its role in PNS patterning. We ablated sclerotome precursors and observed subsequent development of DRGs, axons of identified motoneurons and peripheral nerves to axial muscle which are composed of the axons of these identified motoneurons as well as the axons of later developing motoneurons (Pike et al., 1992). If sclerotome plays a critical role in PNS segmentation in embryonic zebrafish, then in the absence of sclerotome we would expect to see loss of segmentation of peripheral axons and DRGs. We found that following sclerotome ablation, axonal outgrowth of identified motoneurons was essentially normal, although delayed in some cases. DRG and peripheral nerve segmentation appeared entirely normal. These results indicate that sclerotome is not required for proper patterning of the these PNS structures in embryonic zebrafish.

Portions of this work constitute part of the Ph.D. dissertation of E. M.-K. (Morin-Kensicki, 1994).

MATERIALS AND METHODS

Embryos

Zebrafish, *Danio (Brachydanio) rerio,* embryos were collected from spontaneous spawnings and incubated at 28.5°C. During the first day of development, embryos were staged by counting somites and converting to standard hours of development at 28.5°C (h; Hanneman and Westerfield, 1989; Kimmel et al., 1995). During the second day, embryos were staged according to the position of the posterior lateral line primordium and also converted to standard hours of development (Metcalfe, 1985; Kimmel et al., 1995). Older embryos were staged by days of development postfertilization at 28.5°C (d).

Cell labels

Embryos were anesthetized in dilute tricaine methanesulfonate (TMS; Sigma Chemical Company) in physiological saline (Westerfield et al., 1986) and mounted in 1.2% agar on a microslide as described by Eisen et al. (1989). Individual cells in somites 5 through 17 were labeled by injection with fluorescent dextrans (Molecular Probes) as previously described (Raible et al., 1992); labeling of single cells was confirmed by inspection immediately after labeling. For analysis of anterior and posterior sclerotome development, only individual cells located in distinctly anterior or posterior positions within a somite were considered; cells in mid-segment positions were excluded. In addition, one or more cells of the myotome in a more anterior somite were also labeled to serve as reference points for following sclerotome migration.

Videomicroscopy and image processing

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). Image processing was performed on a Macintosh IIci using Photoshop (Adobe) and an early version of AxoVideo (Axon Instruments, Inc.; Myers and Bastiani, 1991) software. Techniques used include contrast enhancement, combining white light and fluorescent images and combining images from multiple focal planes.

Sclerotome ablation

Embryos between 16 h and 20 h were prepared for ablation as described by Eisen and Pike (1991). Micropipettes were pulled on a Brown-Flaming electrode puller (Sutter Instruments) and broken manually to a tip diameter of about 20 μm. A pipette was inserted several segments anterior to the experimental segments and moved through the embryo between skin and somites until 1-2 segments anterior to experimental segments, then moved to the level of medial somite. Prospective sclerotome was ablated by gentle aspiration in three consecutive segments. Embryos were removed from agar and allowed to develop in physiological saline at 28.5°C for about 2 hours. Embryos were then anesthetized in TMS, remounted in agar and scored for success of ablation by observing them using Nomarski DIC optics. Embryos that appeared normal in segments bordering the experimental region but lacked mesenchymal cells in the region of the ablation were used for further analysis. Excluded from analysis were those embryos in which mesenchyme was present within the region of the ablation or in which obvious scarring or damage to the notochord had occurred. Embryos were allowed to develop to an age appropriate for immunocytochemistry or for vital labeling of motoneurons. Data on PNS element development were collected only from the middle segment of the three-segment region of ablation.

Whole-mount in situ RNA hybridization with a probe against a zebrafish homolog of the *Drosophila melanogaster twist* gene was used to assay the efficiency of ablation. This probe recognizes both prospective sclerotome and other cell types in the zebrafish embryo (see Halpern et al., 1995). The pattern of *twist* expression following sclerotome ablation was used to corroborate the microscopic observation of ablation efficiency. Sclerotome was ablated in 9 embryos and ablation success assessed using Nomarski DIC optics as described above. Whole-mount in situ RNA hybridization was then performed as described by Thisse et al. (1993) using a single strand digoxigenin-labeled RNA probe (gift from C. Thisse) prepared from a zebrafish cDNA isolated by sequence similarity to the *D. melanogaster twist*

gene (gift from B. Riggleman and D. Grunwald) and the results of the two techniques compared.

Immunocytochemistry

The monoclonal antibody znp-1 (Trevarrow, 1990) labels the axons and growth cones of identified motoneurons from early stages in axogenesis (Melancon, 1994), in addition to other cells. To visualize the effects of sclerotome ablation on the peripheral axons of two identified primary motoneurons, CaP and MiP, surgically manipulated embryos between 27 to 30 h were processed as described below.

DRGs and peripheral nerves to axial muscle can be labeled with the monoclonal antibody zn-5 (Trevarrow, 1990) from about 48 h of development. To analyze the effect of sclerotome ablation on DRG segmentation and peripheral nerve formation, 3 d to 5 d embryos were processed as described below.

Processing of zebrafish embryos for immunolabeling is described here briefly. More detailed methods are in Eisen et al. (1989). Embryos fixed in 4% paraformaldehyde at 4°C had yolks removed and then were permeabilized by exposure to acetone at –20°C. Embryos were soaked in normal goat serum (Sigma) followed by the primary antibody. After several buffer washes, embryos were placed in peroxidase-conjugated goat anti-mouse secondary antibody (Sternberger Monoclonal), washed, and placed in mouse peroxidase anti-peroxidase (Sternberger Monoclonal). Labeled structures were then visualized using a peroxidase-based diaminobenzidine reaction. Embryos were mounted in 1:1, phosphate-buffered saline: glycerol, or were dehydrated in an ascending ethanol series, cleared in methyl salicylate and mounted in Permount between coverslips separated by teflon tape.

RESULTS

Zebrafish sclerotome development

Classical studies of somite development describe separation of epithelial somites from more posterior paraxial mesoderm followed by differentiation of each somite into component parts: dermamyotome and sclerotome. The sclerotome portion of a somite is defined as the ventromedial region which loses its initial epithelial character to become mesenchymal and which ultimately contributes to connective tissue and vertebral cartilages (see Arey, 1946). Avian embryos have served as the primary model of somite development (Fig. 1). Somite development has not been as well-studied in most other species. In the work described here, we observed somite development in live zebrafish embryos and used the technique of vital cell marking to characterize sclerotome development.

Zebrafish sclerotome formed at the ventromedial region of each somite, as observed in other teleosts (Swaen and Brachet, 1899, 1901; Sunier, 1911). Each somite initially separated from the remaining paraxial mesoderm as an epithelial envelope containing rounded cells (Fig. 2A). We observed the formation of an epithelial cell cluster (Fig. 2A) at the ventromedial region of each somite approximately 2-3 hours after the somite formed (Fig. 3). The ventromedial cell cluster appeared in an anterior to posterior sequence along the length of the embryo, consistent with the anterior to posterior development of somites in general (Hanneman and Westerfield, 1989). Later, cells within the cluster exhibited protrusive activity, followed by an L-shaped distortion of the cluster as anterior-most cells began to migrate dorsally (Fig. 2B). The position of this cluster and the behavior of its cells suggest that it is the sclerotomal portion of the somite.

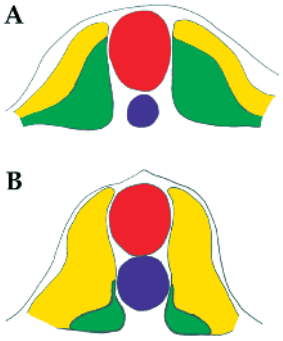

Fig. 1. Schematic transverse sections through trunk region of avian and zebrafish embryos. (A) Avian embryo. Sclerotome (green) constitutes a significant portion of the somite, directly adjacent to the neural tube (red) and notochord (blue), while dermamyotome (yellow) comprises a much smaller portion. (B) Zebrafish embryo. Sclerotome constitutes a very small portion of the somite, located only at the ventromedial edge.

To determine whether all cells of the ventromedial cell cluster were sclerotomal, we labeled individual cells with vital fluorescent dyes and followed their subsequent development. As expected from previous work (Devoto et al., 1996), ventral somite cells located lateral to the cluster differentiated as muscle (459/459; data not shown). Within the cluster, some labeled cells migrated dorsally to populate regions of the embryo adjacent to neural tube and notochord as expected for sclerotome contributing to vertebral cartilages and connective tissue (n=55; Fig. 2C-E). Labeled cells of the cluster also contributed to segmental blood vessels and blood vessels ventral to the notochord (data not shown), consistent with sclerotomal endothelial contributions previously described in avian embryos (Beresford, 1983; Bagnall et al., 1988; Bagnall, 1992).

Not all cells of the ventromedial cell cluster formed sclerotome. Analysis by initial location of cells within the cluster showed that essentially all anterior cells of the cluster became mesenchymal, thus we define these cells as anterior sclerotome. However, only 1/3 of the posterior cells became mesenchymal. We define these mesenchymal cells as posterior sclerotome. Surprisingly, most remaining posterior cells developed into muscle (Table 1). In addition, a few individual posterior cells produced both sclerotome and muscle progeny (Fig. 2F-H; Table 1). Thus, the ventromedial cell cluster is not synonymous with sclerotome, although sclerotome arises from it. Moreover, the anterior and posterior portions of the cluster may constitute separate populations since the entire anterior region gives rise to sclerotome cells while the posterior region gives rise to a mixture of sclerotome cells and muscle cells.

Sclerotome from anterior and posterior regions of the cluster in each somite migrated at different times and traversed different territories. To learn when sclerotome migration began, the onset of sclerotome migration was observed in segments 7 through 16 (Fig. 3). Anterior cells migrated first, producing a characteristic L-shaped distortion of the ventromedial cell cluster (Fig. 2B). We defined the onset of anterior sclerotome migration as the time when mesenchymal cells reached the ventral extent of the hypochord. Because posterior sclerotome migrates later than anterior sclerotome, it is more

Table 1. Fate distribution of cells from the ventromedial cell cluster

Position of cell in cluster	Cell fate		
	Sclerotome	Sclerotome and muscle	Muscle
Anterior (*n*=23)	22	0	1
Posterior (*n*=47)	11	4	32

difficult to observe. Thus, we defined the onset of posterior sclerotome migration as the time when dye-labeled posterior cells became mesenchymal in appearance. Sclerotome cell migration onset occurred in an anterior to posterior sequence along the length of the embryo. In addition, within a segment, anterior cells began to migrate 1 to 2 hours after formation of the ventromedial cell cluster and 3 to 4 hours prior to the onset of posterior cell migration (Fig. 3).

To determine sclerotome migration patterns, we followed the migration of individual labeled sclerotome cells and their descendants through 3 to 5d. Only singly labeled cells, as confirmed by inspection immediately after labeling, were used. Anterior cells migrated dorsally along a path between the notochord and myotome, midway between anterior and posterior somite boundaries, until they reached the level of the neural tube, after which they spread out along the axis (Fig. 4A-C). Posterior cells migrated over a broader region that included the pathway taken by anterior cells. However, even within the ventral portion of the somite, posterior cells showed a greater anterior-posterior spread, spanning the boundary between the myotomal region of the same somite and the next posterior somite (Fig. 4D-F). In addition, posterior sclerotome cells showed a greater tendency to remain ventral to the notochord (anterior, 1/22; posterior, 8/15).

PNS segmentation

The distinctions between anterior and posterior regions of sclerotome within each somite raise the possibility that zebrafish sclerotome plays a role in PNS segmentation much like that described for avian embryos. Thus, through an A/P alternation of properties, zebrafish sclerotome might contribute to a PNS segmental pattern that develops in a one-to-one alignment with the somite segmental pattern. In this study, we characterized the spatiotemporal relationship of zebrafish sclerotome and PNS elements including migrating neural crest cells that may later contribute to DRGs (Raible et al., 1992) and axons of identified primary motoneurons. To learn if sclerotome plays a patterning role for these PNS elements and for peripheral nerves to axial muscle, we ablated sclerotome and observed subsequent PNS development.

Anterior sclerotome has the opportunity to influence the pattern of migrating neural crest cells and extending primary motor axons. By 18 h, many primary motoneurons of the trunk have peripheral axons extending along a path midway between somite boundaries (Eisen et al., 1986). At the same time, some trunk neural crest cells have begun to migrate ventrally along the same path (Raible et al., 1992). Since this is the path taken by dorsally migrating sclerotome cells, it is likely that all three cell types encounter one another during migration or axonal extension. To examine this more directly, we labeled individual cells of each population and followed their subsequent development. An individual primary motoneuron or neural crest cell was labeled with a fluorescent vital dye of one color,

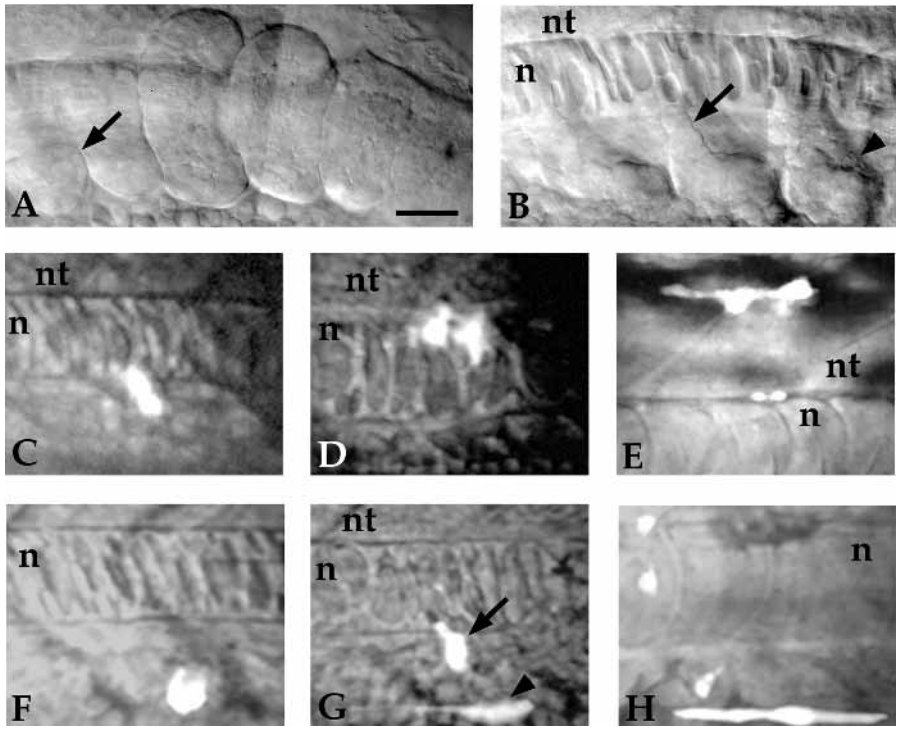

Fig. 2. Sclerotome origin in embryonic zebrafish. Anterior to the left and dorsal to the top in all panels. A and B show Nomarski (DIC) images of a living 18 h embryo. (A) The last five somites and segmental plate. Somites mature in an anterior to posterior sequence; the last-formed somite appears as an epithelial envelope containing rounded cells. As development continues, somite shape changes include formation of the ventromedial cell cluster (arrow). (B) More anterior somites. These more mature somites are showing the characteristic 'L-shaped' distortion of the ventromedial cell cluster (arrow) as anterior sclerotome begins to migrate dorsally. Prior to migration, cluster cells show protrusive activity (arrowhead). (C-E) Combined bright-field and fluorescence images of a single labeled anterior sclerotome cell (C) and its progeny at 24 h (D) and 48 h (E) in a living embryo showing migration from ventral somite to regions adjacent to neural tube and notochord. (F-H) Combined bright-field and fluorescence images of a single labeled posterior sclerotome cell (F) and its progeny at 24 h (G) and 3 d (H) in a living embryo. The progeny of the labeled cell formed both sclerotome (arrow) and muscle (arrowhead). We followed labeled cells until fluorescence was obscured due to fading or autofluorescence of the embryo. Because chondrogenesis in the trunk has not been observed before 7 d (E. M.-K., unpublished), we did not ascertain whether the labeled cells contributed to cartilagenous structures. n, notochord; nt, neural tube. Scale bar, 20 μm.

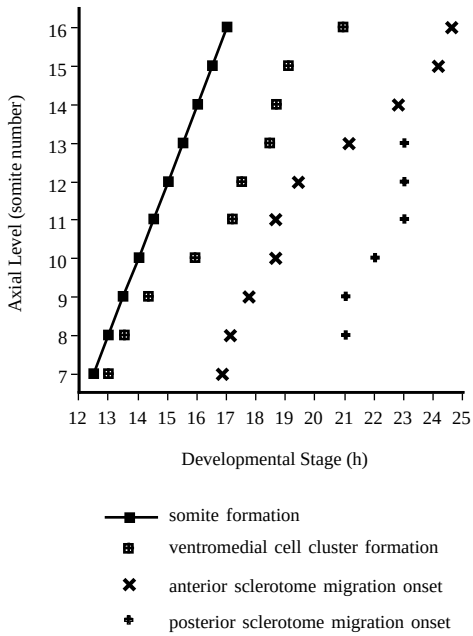

Fig. 3. Time of ventromedial cell cluster formation and sclerotome migration. Data for somite formation are from Hanneman and Westerfield (1989). Each data point represents from 3-33 observations. The beginning of ventromedial cell cluster formation was defined by the most posterior segment in which a cluster was visible. See text for details defining onset of anterior and posterior sclerotome migration.

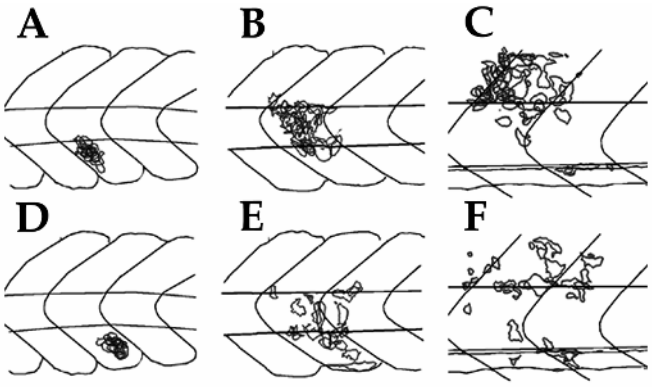

Fig. 4. Migration territories of anterior and posterior sclerotome cells. For analysis of sclerotome migration, combined fluorescent and bright-field images were reduced to cell or cell cluster outlines and superimposed on a composite, stage-specific, standardized schematic by aligning somite and notochord borders; embryo size differences were compensated for by scaling the size of the images. Parallax error due to slight but variable rotation of embryos was uncompensated. Individual labeled anterior (A-C; *n*=17) and posterior (D-F; *n*=13) sclerotome cells and their progeny were observed at three time points, 18 h, 24 h, and 48 h; some cells are represented here at two time points. Anterior cells showed a restricted migration pathway adjacent to the notochord and midway between somite boundaries (A,B), but later the cells spread out along the A/P axis (C). Posterior cells showed a broader migration territory (E), a greater tendency to remain ventral to the notochord (F), and a broader spread along the A/P axis (F). Scale bar, 40 μm.

and an anterior sclerotome cell was labeled with a fluorescent dye of a different color. Labeled cells were found to coincide in time and space along the path adjacent to the notochord, midway between somite boundaries (Fig. 5). Thus, zebrafish sclerotome, despite constituting a relatively small proportion of the somite, appears to be in an appropriate position to influence the segmental pattern of developing motor axons and migrating neural crest cells.

We ablated zebrafish sclerotome to learn whether it plays a role in the development of PNS segmental pattern. To accomplish this, we aspirated the ventromedial cell cluster and tested the efficiency of ablation by examining changes in the pattern of expression of a zebrafish homolog of the *D. melanogaster* gene *twist*, which is expressed by cells of the ventromedial cell cluster (Fig. 6A,B). Thus, we compared changes in *twist* expression with our assessment in living embryos of cell cluster ablation efficiency. In all 9 embryos from which the ventromedial cell cluster was removed in 3 consecutive somites, the extent of *twist*-positive cells was consistent with the absence of mesenchymal cells observed in the live embryo (Fig. 6C). Occasionally, following sclerotome ablation, mesenchymal cells observed in the live embryo were unlabeled by the probe. It is probable that these cells were migrating neural crest cells for which we have no good marker. Thus, aspiration of the ventromedial cell cluster resulted in ablation of sclerotome and the efficiency of ablation was reliably scored in living embryos.

Table 2. Appearance of primary motoneurons, peripheral nerves and dorsal root ganglia following sclerotome ablation

Peripheral nervous system element	Abnormal	Normal
Dorsal root ganglion	0	15
Peripheral nerves	0	21
MiP axon	0	11
CaP axon*	11	19
		Abnormal/normal late
CaP axon†		4

*Antibody label at 27-30 h.
†Vital fluorescent dye label followed through 36-40 h.

Sclerotome ablation does not prevent proper patterning of PNS elements. We analyzed the effect of sclerotome ablation on three elements of the PNS: axons of the identified primary motoneurons CaP and MiP, DRGs and peripheral nerves to axial muscles. Primary motor axons were entirely normal in the path of outgrowth (Table 2; Fig. 7A,B) following sclerotome ablation. In some cases, however, the CaP axon was shorter than normal and was unusually branched when analyzed at 30 h in fixed tissue (Table 2). To learn whether sclerotome removal caused permanent stunting of some CaP axons, after sclerotome ablation, we labeled individual CaP motoneurons with a vital dye and followed their subsequent development. In all four cases labeled this way in which CaP axons were abnormally short at 26-30 h, CaP morphology appeared normal by 36-40 h. Thus, even in the absence of sclerotome, both MiP and CaP were able to extend axons along their normal paths. In all cases, the development of DRGs and peripheral nerves to axial muscle appeared unaffected by sclerotome ablation (Table 2; Fig. 7C,D).

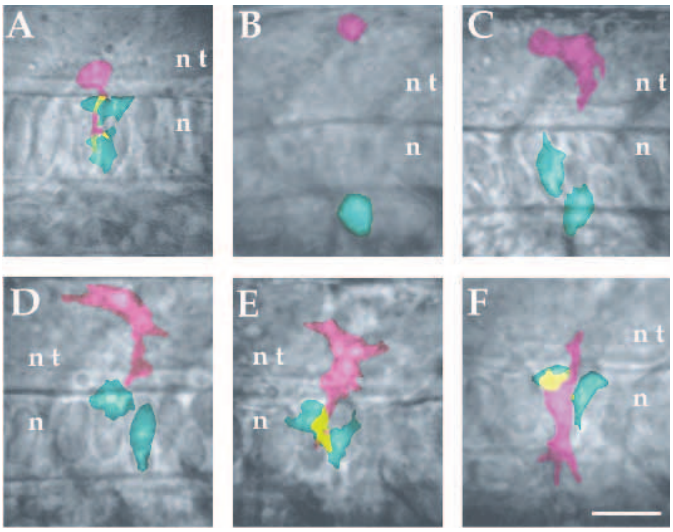

Fig. 5. Anterior sclerotome cells share a pathway with primary motor axons and migrating neural crest cells. Individual sclerotome cells were labeled with fluorescein dextran (green) and individual primary motoneurons and neural crest cells were labeled with rhodamine dextran (magenta); yellow indicates regions of overlap within one focal plane. Cells were followed over time in living embryos; labeled sclerotome cells divided as they migrated dorsally. (A) The CaP axon overlapped extensively with migrating sclerotome cells in segment 10 of this 21 h embryo. (B-F) Labeled neural crest cells and sclerotome in segment 10 at (B) the time of labeling (18.5 h); (C) 2 hours after labeling, (D) 4 hours after labeling, (E) 5 hours after labeling, (F) 6 hours after labeling. n, notochord; nt, neural tube. Scale bar, 21 μm.

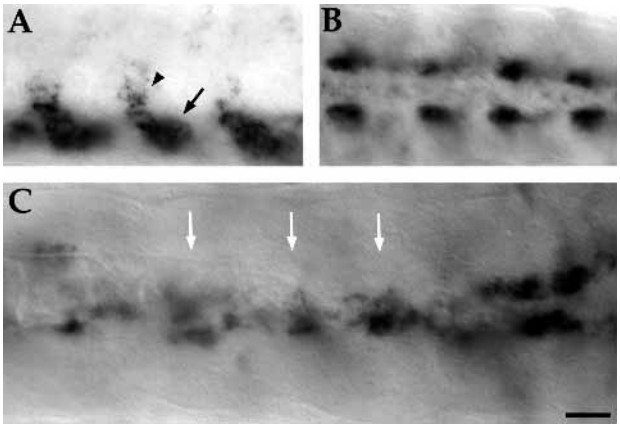

Fig. 6. Aspiration of ventromedial cell cluster removes sclerotome. (A) Lateral view of 18 h embryo processed for in situ RNA hybridization with a probe for a zebrafish *twist* homolog; this probe labels the ventromedial cell cluster (arrow) and migrating sclerotome cells (arrowhead). (B) Ventral view showing bilateral sclerotome expressing *twist.* (C) Ventral view showing that in the 3 somites from which sclerotome was removed by aspiration (arrows), *twist* expression is absent, while it is still present on the contralateral side. Scale bar, 18 μm.

DISCUSSION

Our study of sclerotome and PNS development in embryonic zebrafish provides evidence for A/P distinctions within the sclerotome and for PNS association with anterior sclerotome, and yet shows that PNS segmentation develops normally in the absence of sclerotome. We discuss here modification of a favored model for the development of PNS segmental pattern that incorporates both results from work in avian embryos and our results from zebrafish embryos.

A new model for peripheral nervous system patterning

The current model for the development of a segmented PNS in vertebrate embryos posits an A/P alternation of properties within the sclerotome portion of the somites that directs the segmental arrangement of peripheral nerves and DRGs. A model consistent with the development of PNS segmental pattern in diverse vertebrate embryos may need to consider distinct somite morphologies in different species. Avian (see Williams, 1910; Bellairs, 1963), mammalian (see Butcher, 1929) and reptilian (see Werner, 1971; Winchester and Bellairs, 1977) somites have large numbers of sclerotome cells positioned adjacent to ventral neural tube and notochord (see Fig. 1A). In contrast, as in other fish (Swaen and Brachet 1899, 1901; Sunier, 1911) and some amphibians (see Kielbowna, 1981; Youn and Malacinski, 1981), we have shown that zebrafish embryos have a small number of sclerotome cells originating ventral to the notochord (see Fig. 1B).

Sclerotome may play the patterning role in avian embryos simply because of its position, adjacent to the neural tube. This possibility is supported by evidence that PNS patterning information is contained within the entire avian somite. For example, reversal of segmental plate by 180° about the A/P axis redirects host limb motor axons and migrating neural crest cells to donor segmental pattern without regard to the mediolateral orientation of the grafted tissue (Keynes and Stern, 1984; Bronner-Fraser and Stern, 1991). Thus, the more lateral region of the segmental plate that normally would have formed dermamyotome also contains the original A/P patterning information, even though, under the conditions of these experiments, it may be respecified mediolaterally (see Aoyama and Asamoto, 1988).

Short range signals (see Tosney, 1988) may be more important than tissue type in providing a patterning influence. In zebrafish, despite the fact that motor axons and neural crest cells share a pathway with anterior sclerotome, at the earliest stages of motor axon outgrowth and neural crest cell migration, sclerotome is too ventral to provide short-range signals. However, myotomal precursors are in precisely the right position to have such an influence. Therefore, we propose a modified model for PNS segmentation in vertebrate embryos. In this model, peripheral nervous system segmentation is established through interactions with the adjacent paraxial mesoderm, which may differentiate as sclerotome in some species and as myotome in others. Predictions of this model can be further tested in zebrafish by surgical or genetic manipulation of somitic, and specifically myotomal segmental pattern.

Comparison of sclerotome in zebrafish and avian embryos

Although the experiments we used to test the role of sclerotome in PNS patterning are different from the experiments used in avian embryos, these differences seem unlikely to account

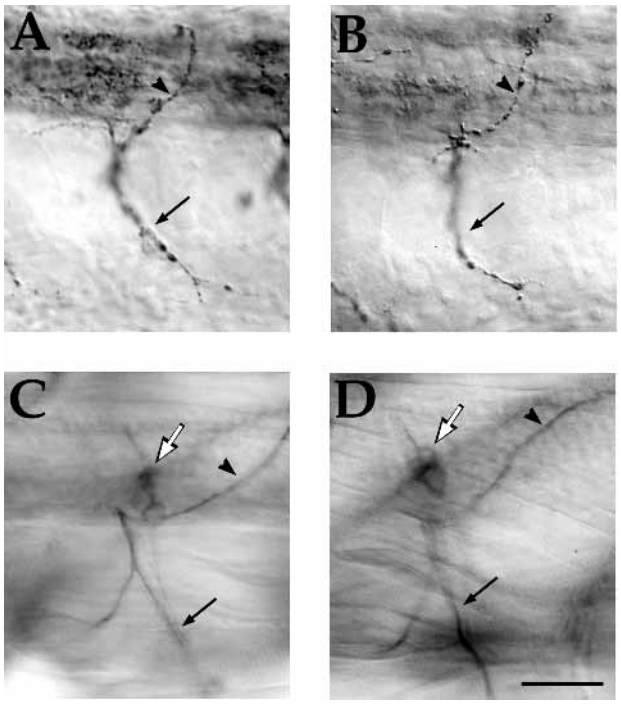

Fig. 7. Primary motor axons, peripheral nerves, and DRGs appear normal following sclerotome ablation. Antibody-labeled motor axons in control (A) and sclerotome-ablated (B) somites of 28 h embryos. The CaP (arrow) and MiP (arrowhead) axons in the experimental somite are essentially indistinguishable from those in the control somite. Antibody-labeled ventral (arrow) and dorsal (arrowhead) peripheral nerves in control (C) and sclerotome-ablated (D) somites of 4 d embryos showing that peripheral nerves were unaffected by the ablation. DRGs (white arrow) were also located in their normal positions (Raible et al., 1992), just medial of the somite border in control (C) and sclerotome-ablated (D) somites of the same embryos. Scale bar, 19 μm in A,B; 28 μm in C,D.

for the contrasting results. In avian embryos, the proximal region of motor nerves to limbs form the major portion of trunk peripheral nerves; nerves to body wall muscle are relatively small. In zebrafish, the major peripheral nerves of the trunk and tail are composed largely of motor nerves to axial muscle (Westerfield et al., 1986) while axons to the pectoral fins exit from only a few anterior nerve roots (Myers, 1985). Thus, we examined the development of peripheral nerves to axial muscle while most reports from experiments in avian embryos consider nerves composed primarily of motor axons that will extend to limbs. The effect of somite manipulation on the segmental pattern of nerves to axial muscle in avian embryos has not been specifically investigated, although Tosney (1987) and Phelan and Hollyday (1990) have demonstrated a dependence upon the presence of the dermamyotome for normal formation of the dorsal ramus, the nerve extending to epaxial muscle. It seems likely that the same influences would pattern the segmentation of nerves to limb and axial muscle since the axons extending to both regions initially form a single nerve tract, the ventral root. However, because we found normal DRG development in the absence of sclerotome which also is in contrast to results from work in avian embryos, it is probable that there are real differences in the patterning role played by sclerotome in the two species.

Zebrafish and avian embryos are comparable in showing distinctions between anterior and posterior sclerotome and somite regions and an association of anterior sclerotome with developing PNS elements. Avian somites, and those of other vertebrate embryos (reviewed in Tam and Trainor, 1994), show functional (Keynes and Stern, 1984; Bronner-Fraser and Stern, 1991) and molecular (Norris et al., 1989) evidence of distinct A/P subdivisions. In zebrafish, early A/P pattern is suggested by the expression of the *snail1* gene which is differentially expressed in anterior and posterior somite regions and appears in a similar banding pattern within the segmental plate (Thisse et al., 1993). We have demonstrated additional A/P asymmetry within the zebrafish somite including a greater tendency for sclerotome to form in anterior regions and the production of sibling myotome and sclerotome cells in posterior regions. Although we have yet found no evidence for molecular differences analogous to those described in avian embryos within the sclerotome itself (EMK, unpublished), we demonstrated A/P differences in the time of sclerotome migration onset and in the territories traversed by migrating sclerotome within single somites. It is apparent that A/P differences within each somite in both avian and zebrafish embryos arise early in development and are not restricted to the sclerotome region. The specific relationship between these A/P regions and PNS segmental pattern remains unclear.

The association of PNS elements with anterior sclerotome provides another remarkable correspondence between avian and zebrafish morphology. In avian embryos, motor axons, migrating neural crest cells and DRGs are aligned with anterior sclerotome. In zebrafish, as anterior sclerotome migrates dorsally adjacent to the notochord, due to the chevron shape of the somites it is soon positioned midway between somite boundaries. The dye-labeled cells observed in this study and previous work (Eisen et al., 1986; Raible et al., 1992) reveal that three cells types, motoneurons, neural crest cells and anterior sclerotome cells, restrict their migration or axon extension to this same mid-segment path. Thus, we find in these embryos a comparable association of PNS elements with anterior sclerotome. It is unclear if a similar correspondence of anterior sclerotome and PNS elements can be found in all vertebrate embryos, although Keynes and Stern (1984) cite examples from each vertebrate class. Furthermore, it is apparent that we can not yet assign a functional significance to this association.

In addition to the similarities between the association of anterior sclerotome and PNS elements in avian and zebrafish embryos, there is one clear difference. In avian embryos, posterior sclerotome is never associated with motor axons or migrating neural crest cells. However, in zebrafish at least some posterior sclerotome cells migrate along the same pathway as anterior sclerotome, motor axons and neural crest cells, although they migrate later than anterior sclerotome cells. That any posterior sclerotome cells migrate along this pathway provides additional support for differences in the patterning roles of avian and zebrafish sclerotome for PNS elements.

In zebrafish, the ventromedial cell cluster does not define sclerotome. Despite being a readily identifiable structure within each somite and displaying gene expression distinct from the rest of the somite, the cell cluster is not homogeneous. The A/P differences in cell behavior and fate tendency found within the cluster hint at a more complex pattern to the somite.

For example, the finding of sibling myotomal and sclerotomal cells in posterior regions of the cluster is unique. This relationship has not been described for other vertebrate embryos, despite the apparent plasticity of early somites (see Aoyama and Asamoto, 1988). These A/P differences may reflect a difference in environmental signals (see Mauger, 1972), in the rate at which these regions develop (see Kaehn et al., 1988; Neff et al., 1989), or in the developmental potential of anterior and posterior cells. These possibilities might be distinguished in the zebrafish by transplanting individual cells to new locations within the somite, or by analysis of mutations affecting sclerotome differentiation.

Role of target cells in motor axon extension

The removal of potential target cells of the myotome may account for the abnormally short axons observed for the CaP motoneuron. From our cell fate analysis of the ventromedial cell cluster, we know that aspiration of the cluster removes sclerotome but also some myotome, the target of the CaP motoneuron (Westerfield et al., 1986). Possibly then, a loss of potential target cells following ablation of the cluster caused some CaP axons to extend more slowly resulting in a stunted appearance at 27-30 h. A similar effect on neuron axonal outgrowth following loss of target cells has been previously described (see Eisen, 1994). When CaP motoneurons with abnormally short axons were followed past 36 h, all had recovered and appeared entirely normal, consistent with an early influence of target reduction that is overcome with normal growth of the somite. In addition, the axons of MiP motoneurons, which have a dorsal target (Westerfield et al., 1986), were never found to be stunted or delayed in outgrowth following ablation of the ventromedial cell cluster.

We thank numerous colleagues, especially Charles Kimmel, James Weston, David Raible, Ellie Melancon, Sue Pike and Christine Gatchalian for helpful comments on drafts of the manuscript, the staff of the University of Oregon Zebrafish Facility for excellent support services, Bob Riggleman and David Grunwald for their generous gift of the *twist* cDNA, and Christine and Bernard Thisse for the *twist* RNA probe. Supported by NS23915, HD22486, NS01476, HD07348, GM070257.

REFERENCES

Aoyama, H. and Asamoto, K. (1988). Determination of somite cells: independence of cell differentiation and morphogenesis. *Development* **104,** 15-28.

Arey, L.B. (1946). *Developmental Anatomy*. pp. 372, 526. W.B. Saunders Co., Philadelphia.

Bagnall, K. M. (1992). The migration and distribution of somite cells after labelling with the carbocyanine dye, DiI: the relationship of this distribution to segmentation in the vertebrate body. *Anat. Embryol.* **185,** 317-324.

Bagnall, K. M., Higgins, S. J. and Sanders, E. J. (1988). The contribution made by a single somite to the vertebral column: experimental evidence in support of resegmentation using the chick-quail chimaera model. *Development* **103,** 69-85.

Bellairs, R. (1963). The development of somites in the chick embryo. *J. Embryol. exp. Morphol*. **11,** 697-714.

Beresford, B. (1983). Brachial muscles in the chick embryo: fate of individual somites. *J. Embryol. exp. Morphol.* **77,** 99-116.

Bronner-Fraser, M. and Stern, C. D. (1991). Effects of mesodermal tissues on avian neural crest migration. *Dev. Biol.* **143**, 213-217.

Butcher, E. O. (1929). The development of the somites in the white rat (Mus norvegieus albinus) and the fate of the myotomes, neural tube, and gut in the tail. *Am. J. Anat.* **44,** 381-439.

Detwiler, S. R. (1934). An experimental study of spinal nerve segmentation in amblystoma with reference to the plurisegmental contribution to the brachial plexus. *J. Exp. Zool.* **67**, 395-441.

Devoto, S. H., Melançon, E., Eisen, J. S. and Westerfield, M. (1996). Identification of separate slow and fast muscle precursor cells in vivo, prior to somite formation. *Development* **122**, 3371-3380.

Eisen, J. S. (1994). Development of motoneuronal phenotype. *Ann. Rev. Neurosci.* **17**, 1-30.

Eisen, J. S., Myers, P. Z. and Westerfield M. (1986). Pathway selection by growth cones of identified motoneurones in live zebrafish embryos. *Nature* **320**, 269-271.

Eisen, J. S. and Pike, S. H. (1991). The *spt-1* mutation alters segmental arrangement and axonal development of identified neurons in the spinal cord of the embryonic zebrafish. *Neuron.* **6**, 767-776.

Eisen, J. S., Pike, S. H. and Debu, B. (1989). The growth cones of identified motoneurons in embryonic zebrafish select appropriate pathways in the absence of specific cellular interactions. *Neuron* **2**, 1097-1104.

Halpern, M. E., Thisse, C., Ho, R. K., Thisse, B., Riggleman, B., Trevarrow, B., Weinberg, E. S., Postlethwait, J. H. and Kimmel, C. B. (1995). Cell-autonomous shift from axial to paraxial mesodermal development in zebrafish *floating head* mutants. *Development* **121**, 4257-4264.

Hanneman, E. and Westerfield, M. (1989). Early expression of acetylcholinesterase activity in functionally distinct neurons of the zebrafish. *J. Comp. Neurol.* **284**, 350-361.

Kaehn, K., Jacob, H.J., Christ, B., Hinrichsen, K. and Poelmann, R. F. (1988). The onset of myotome formation in the chick. *Anat. Embryol.* **177**, 191-201.

Kalcheim, C. and Teillet, M. A. (1989). Consequences of somite manipulation on the pattern of dorsal root ganglion development. *Development* **106**, 85-93.

Keynes, R. J. and Stern, C. D. (1984). Segmentation in the vertebrate nervous system. *Nature* **310**, 786-789.

Kielbowna, L. (1981). The formation of somites and early myotomal myogenesis in *Xenopus laevis*, *Bombina variegata*, and *Pelobates fuscus. J. Embryol. exp. Morphol*. **64,** 295-304.

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. and Schilling, T. F. (1995). Stages of embryonic development in the zebrafish. *Dev. Dyn.* **203,** 253-310.

Lallier, T. E. and Bronner-Fraser, M. (1988). A spatial and temporal analysis of dorsal root and sympathetic ganglion formation in the avian embryo. *Dev. Biol.* **127**, 99-112.

Lehmann, F. E. (1927). Further studies on the morphogenetic role of the somites in the development of the nervous system of amphibians. *J. Exp. Zool.* **49**, 93-131.

Lewis, J., Chevallier, A., Kieny, M. and Wolpert, L. (1981). Muscle nerve branches do not develop in chick wings devoid of muscle. *J. Embryol. Exp. Morphol.* **64**, 211-232.

Mauger, A. (1972). Rôle du mésoderme somitique dans le développement du plumage dorsal chez l'embryon de poulet. II. Régionalisation du mésoderme plumigène. *J. Embryol. exp. Morphol.* **28,** 343-366.

Melançon, E. F. (1994). *The role of muscle pioneers in pathfinding of primary motoneurons in the embryonic zebrafish.* Master's Thesis. Department of Biology, University of Oregon. Eugene, Oregon.

Metcalf, W. K. (1985). Sensory neuron growth cones comigrate with posterior lateral line primordial cells in zebrafish. *J. Comp. Neurol.* **238**, 218-224.

Morin-Kensicki, E. M. (1994). *Metameric Relationship of the Somitic Sclerotome to Elements of the Peripheral Nervous System and the Vertebral Column During Zebrafish Development.* Doctoral dissertation. Department of Biology, University of Oregon. Eugene, Oregon.

Myers, P. Z. (1985). Spinal motoneurons of the larval zebrafish. *J. Comp. Neurol.* **263**, 555-561.

Myers, P. Z. and Bastiani, M. J. (1991). NeuroVideo: a program for capturing and processing time-lapse video. *Comput. Programs Methods Biomed.* **34**, 27-33.

Myers, P. Z., Eisen, J. S. and Westerfield, M. (1986). Development and axonal outgrowth of identified motoneurons in the zebrafish. *J. Neurosci.* **6**, 2278-2289.

Neff, A. W., Malacinski, G. M. and Chung, H.-M. (1989). Amphibian (Urodele) myotomes display transitory anterior/posterior and medial/lateral differentiation patterns. *Dev. Biol.* **132,** 529-543.

Norris, W. E., Stern, C. D. and Keynes, R. J. (1989). Molecular differences between the rostral and caudal halves of the sclerotome in the chick embryo. *Development* **105,** 541-548.

Phelan, K. A. and Hollyday, M. (1990). Axon guidance in muscleless chick

wings: the role of muscle cells in motoneuronal pathway selection and muscle nerve formation. *J. Neurosci*. **10**, 2699-2716.

Pike, S. H., Melancon, E. F. and Eisen, J. S. (1992). Pathfinding of zebrafish secondary motoneurons in the absence of normal pioneer axons. *Development* **114,** 825-831.

Raible, D. W., Wood, A., Hodsdon, W., Henion, P. D., Weston, J. A. and Eisen, J. S. (1992). Segregation and early dispersal of neural crest cells in the embryonic zebrafish. *Dev. Dynam.* **195**, 29-42.

Remak, R. (1855). *Untersuchungen über die Entwicklung der Wirbeltiere.* Berlin: Reimer.

Rickmann, M., Fawcett, J. W. and Keynes, R. J. (1985). The migration of neural crest cells and the growth of motor axons through the rostral half of the chick somite. *J. Embryol. Exp. Morphol.* **90,** 437-455.

Stern, C. D. and Keynes, R. J. (1987). Interactions between somite cells: the formation and maintenance of segment boundaries in the chick embryo. *Development* **99**, 261-272.

Sunier, A. L. (1911). Les premier stades de la différentiation interne du myotome et la formation des éléments sclérotomatiques chez les acraniens, les Sélaciens, et les Téléostéens. *Tijdsch. Nederlansche Dierkundige Vereniging.* **12,** 75-181.

Swaen, A. and Brachet, A. (1899). Étude sur les premières phases du dévelopment des organes dérivés du mésoblaste chez les poissons Téléostéens. *Arch. Biol. Paris.***16,** 173-311.

Swaen, A. and Brachet, A. (1901). Étude sur les premières phases du développement des organes dérivés du mésoblaste chez les poissons Téléostéens. *Arch. Biol. Paris* **18,** 73-190.

Tam, P. P. L. and Trainor, P. A. (1994). Specification and segmentation of the paraxial mesoderm. *Anat. Embryol.* **189,** 275-305.

Thisse, C., Thisse, B., Schilling, T. F. and Postlethwait, J. H. (1993). Structure of the zebrafish *snail1* gene and its expression in wild-type, *spadetail* and *no tail* mutant embryos. *Development.* **119**, 1203-1215.

Tosney, K. W. (1987). Proximal tissues and patterned neurite outgrowth at the lumbosacral level of the chick embryo: deletion of the dermamyotome. *Dev. Biol.* **122**, 540-558.

Tosney, K. W. (1988). Proximal tissues and patterned neurite outgrowth at the lumbosacral level of the chick embryo: partial and complete deletion of the somite. *Dev. Biol.* **127**, 266-286.

Trevarrow, B., Marks, D. L. and Kimmel, C. B. (1990). Organization of hindbrain segments in the zebrafish embryo. *Neuron.* **4,** 669-679.

Werner, Y. L. (1971). The ontogenetic development of the vertebrae in some gekkonoid lizards. *J. Morph.* **133,** 41-92.

Westerfield, M., McMurray, J. V., and Eisen, J. S. (1986). Identified motoneurons and their innervation of axial muscles in the zebrafish. *J. Neurosci.* **6**, 2267-2277.

Williams, L. W. (1910). The somites of the chick. *Am. J. Anat.* **2,** 55-100.

Winchester, L. and Bellairs, A. d'A. (1977). Aspects of vertebral development in lizards and snakes. *J. Zool., Lond.* **181,** 495-525.

Youn, B. W. and Malacinski, G. M. (1981). Comparative analysis of amphibian somite morphogenesis: cell rearrangement patterns during rosette formation and myoblast fusion. *J. Embryol. exp. Morphol*. **66,** 1-26.

(Accepted 7 October 1996)