Contribution of somitic cells to the avian ribs

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Abstract

The traditional view that all parts of the ribs originate from the sclerotome of the thoracic somites has recently been challenged by an alternative view suggesting that only the proximal rib derives from the sclerotome, while the distal rib arises from regions of the dermomyotome. In view of this continuing controversy and to learn more about the cell interactions during rib morphogenesis, this study aimed to reveal the precise contributions made by somitic cells to the ribs and associated tissues of the thoracic cage. A replication-deficient lacZ-encoding retrovirus was utilized to label cell populations within distinct regions of somites 19–26 in stage 13–18 chick embryos. Analysis of the subsequent contributions made by these cells revealed that the thoracic somites are the sole source of cells for the ribs. More precisely, it is the sclerotome compartment of the somites that contributes cells to both the proximal and distal elements of the ribs, confirming the traditional view of the origin of the ribs. Results also indicate that the precursor cells of the ribs and intercostal muscles are intimately associated within the somite, a relationship that may be essential for proper rib morphogenesis. Finally, the data from this study also show that the distal ribs are largely subject to resegmentation, although cell mixing may occur at the most sternal extremities.

Keywords: Avian; Somites; Sclerotome; Dermomyotome; Ribs; Resegmentation; Intercostal muscles; Skeletal muscle; Vertebrae

Introduction

The ribs form the majority of the thoracic skeleton of birds and mammals, except for narrow mid-dorsal and mid-ventral strips, which are formed by the vertebral column and the sternum, respectively. The ribs articulate with a corresponding thoracic vertebra dorsally and, in most cases, also attach to the sternum ventrally. A typical rib presents a vertebral extremity, a sternal extremity, and an intermediate body or shaft. The vertebral extremity consists of a head, neck, and tubercle and can be referred to as the proximal rib. The costal body of the rib has both a vertebral and sternal portion, based on anatomical position and has often been termed the distal rib. Intercostal spaces lie between adjacent ribs cranially and caudally and are spanned by intercostal muscles, which are involved, in part, in the process of ventilation.

The question of where the thoracic ribs originate, embryologically, has been the focus of debate for some time. Controversy has existed since at least the 1960’s, when Seno (1961) challenged the work of Straus and Rawles (1953), who had demonstrated, using carbon particle labeling, that both the somitic and lateral plate mesoderm gave rise to parts of the thoracic ribs. Using similar techniques, Seno (1961) showed that in fact all parts of the ribs were somite-derived, a conclusion subsequently confirmed by a number of other workers using a variety of techniques, including coelomic grafting, foil barriers, and chick-quail chimeras (Chevallier, 1975; Christ et al., 1974; Pinot, 1969; Sweeney and Watterson, 1969). Although it is now generally agreed that the ribs originate from the somites, the question of which somitic compartment gives rise to the ribs remains open. Traditionally, it has been thought that all parts of the ribs are derived from the sclerotome, the mesenchymal ventral portion of the developing somite (Christ and Wilting, 1992; Huang et al., 1994, 1996). More recent data, however, have suggested that the dermomyotome also makes a significant contribution to the formation of the ribs. Using experimental ablation and chick-quail grafting techniques, Kato and Aoyama (1998) demonstrated that only the...
Fig. 1. Schematic illustration showing the appearance of thoracic somites in cross section at somite stages (ss) I and X. All injections were made into somites at stages within this range.

Fig. 2. LacZ-encoding retroviral injections made into somites at stage 1. (A) Illustration of injection into the somitocoele of a stage I somite. (B) Labeled hypaxial muscles within the wing and scapular region following injection of virus made into somite 19. (C) Labeled chondrocytes within the distal element of the sixth rib after an injection into somite 25. (D) β-Gal-positive labeling of dermis (arrowed) overlying the fifth and sixth ribs after an injection into somite 24.
proximal parts of the ribs derive from the sclerotome and that the distal rib instead originates from the dermomyotome, the epithelialized dorsal portion of the somite. Huang et al. (2000b) have subsequently challenged this alternative postulation based on another series of transplantation assays and experimental extirpations. The results of these studies conflict with those of Kato and Aoyama (1998) and reaffirm the classical idea that the ribs are exclusively derived from

Fig. 3. Somitic origin of the ribs. (A) Lateral view of the skeleton of a 10-day chick embryo, stained with Alcian blue, showing the axial skeleton, including the ribs at the thoracic level. (B) Superior view of the fourth thoracic vertebra and associated rib, stained with Alcian blue and Alizarin red (v, vertebra; ch, costal head; ct, costal tubercle; vb, vertebral body; sb, sternal body). (C) Schematic illustration of a typical rib of a chick embryo. The ribs are divided into the proximal rib, including the costal head and tubercle (hatched shading), and the distal rib. (D) β-Gal labeling within the cranial rim of the fourth rib (arrowed) and the cranial rim of the fifth rib (arrowhead) following an injection of virus into somite 23. (E) β-Gal-positive cells (arrowed) within the caudal rim of the seventh rib after injection into somite 26.
types. Both recent genetic mutations that present various rib phenotypes have also become a pertinent issue in light of a number of findings, however, did reveal that rib formation is somewhat dependent on the interaction between the dermomyotome and the sclerotome.

The failure to resolve the question of where ribs originate has also become a pertinent issue in light of a number of recent genetic mutations that present various rib phenotypes. Both Myf-5- and Pax-3-deficient mice, for example, exhibit severe distal rib defects. Myf-5 null mutants display truncated ribs, a phenotype that results in perinatal death (Braun et al., 1992), while Splotch mutants present rib fusions and bifurcations (Dickman et al., 1999; Henderson et al., 1999; Tremblay et al., 1998). In contrast, other mutations present proximal rib defects. Where Pax-1 or Uncx4.1 has been targeted, for example, mutant mice exhibit normal distal rib formation, while the proximal ribs are either reduced or totally missing (Wallin et al., 1994; Leitges et al., 2000; Mansouri et al., 2000). The expression patterns of these genes within the distinct somitic compartments during somitogenesis, and also the interactions between the various signals, have led to a number of suggestions being put forward as to how these defects arise; however, these are largely based on assuming the exact origin of the proximal and distal rib primordia. It is therefore widely agreed that the exact basis for these rib mutations cannot really be determined until the definitive embryological origins of the ribs have been established.

In view of the continuing controversy over the origins of the ribs and to learn more about the cell interactions during rib morphogenesis, I decided to follow the fate of cells arising from the thoracic somites, this time using an alternative technique. A replication-deficient lacZ-encoding retrovirus was utilized to label cell populations within distinct regions of somites 19–26 in the avian embryo and to analyze the subsequent contribution of these cells to the developing ribs. Overall, the resulting data clearly indicate that the sclerotome compartment of all thoracic somites contributes cells to both the proximal and distal elements of the ribs and that the distal parts of the ribs are largely subject to resegmentation during their development. A preliminary report of some of this work has been published in abstract form (Evans, 2001).

### Material and methods

**Viral preparation**

CXL replication-deficient retrovirus, encoding for the lacZ marker gene, was obtained from D17 packaging cells, a fibroblastic cell line transfected with pCXL DNA (Mikawa et al., 1991). Virus was harvested from Dulbecco’s modified Eagle’s Medium (DMEM), containing 7% fetal calf serum and 1% penicillin/streptomycin, following an overnight incubation over near confluent monolayers of D17 cells. Following clarification by centrifugation, 100 μg/ml of polybrene (Hexadimethrine Bromide; Sigma) was added to the culture supernatant prior to the embryonic injections. All culturing and harvesting procedures were based on those previously described by Mikawa et al. (1991). Primary cultures of embryonic chick limb mesenchymal cells were used to test the concentration of CXL virus, which was found to be approximately 10^6 CFU/ml. Similar cultures were used to continually test the replication incompetency of the virus, and at no stage were helper viruses detected.

**Avian embryo injection procedure**

Fertilized White Leghorn chicken eggs (Gallus gallus domesticus) were obtained from Henry Stewart and Company Limited (Lincolnshire, UK) and incubated on their sides at 38°C in a humidified incubator. All injections were performed on stage 13–18 embryos, staged according to the criteria of Hamburger and Hamilton (1951). Following the removal of approximately 1 ml of thin albumin, eggs were windowed and embryos stained with a minimal amount of 0.5% neutral red (BDH)/Hank’s balanced salt solution to aid in subsequent visualization. After carefully recording the

### Table 1

Summary showing the overall pattern of labeling in thoracic ribs following injection of lacZ-encoded retrovirus into somites 19–26 of chick embryos

<table>
<thead>
<tr>
<th>Somite injection</th>
<th>Thoracic rib number</th>
<th>Rib 1</th>
<th>Rib 2</th>
<th>Rib 3</th>
<th>Rib 4</th>
<th>Rib 5</th>
<th>Rib 6</th>
<th>Rib 7</th>
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<tr>
<td></td>
<td></td>
<td>Cr</td>
<td>Ca</td>
<td>Cr</td>
<td>Ca</td>
<td>Cr</td>
<td>Ca</td>
<td>Cr</td>
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<td>19 (n = 3)</td>
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<td>20 (n = 2)</td>
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<td>+</td>
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<td>21 (n = 4)</td>
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<td></td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>22 (n = 3)</td>
<td></td>
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<td></td>
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<td>+</td>
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<td>23 (n = 5)</td>
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<td>+</td>
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<td>24 (n = 4)</td>
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<td>+</td>
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<td>25 (n = 5)</td>
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<td>+</td>
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<tr>
<td>26 (n = 4)</td>
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</table>

*Note. Each injection of virus was made at somite stage I (HH stage 13–15), and the embryos were fixed at day 7–10 (HH stage 31–36). Cr corresponds to labeling in the cranial region of the rib, and Ca corresponds to the caudal part of the rib.*
stage of each embryo, a small tear was made in the vitelline membrane overlying the proposed injection site by using a tungsten needle. Previously pulled injection quality glass micropipettes (Clark Instruments) were backfilled with retroviral supernatant and connected to a PV820 pneumatic picopump (World Precision Instruments) set at approximately 10 psi. The micropipette was lowered into a specific region of the somite of study, and a volume of retroviral supernatant was delivered. For each batch of injections, several embryos received sham injections, which contained only DMEM. Detailed records were made of each injection, before sealing each egg with adhesive tape and returning it to the incubator.

**Location of retroviral injections**

Overall, injections of lacZ-encoding retrovirus were made into specific regions of somites 19–26 at embryonic stages 13–18 and somite stages I–X (Fig. 1). Somite staging was based on the nomenclature of Christ and Ordahl (1995). Precise injection strategies are given in the Results.

**Histochemical detection of β-galactosidase**

All control and injected embryos were incubated until stages 31–36 and subsequently stained for β-galactosidase activity. Each embryo was quickly decapitated, staged, and subsequently fixed in 2% paraformaldehyde. After an extensive period of washing in PBS (0.01 M phosphate-buffered saline, pH 7.2), embryos were transferred to a minimal volume of X-gal staining solution. The solution contained 20 mM K$_3$Fe(CN)$_6$, 20 mM K$_4$Fe(CN)$_6$·3H$_2$O, 2 mM MgCl$_2$, and 1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl β-d-galactopyranoside; Molecular Probes) in PBS. Embryos were incubated in the dark at 37°C and left for 16–24 h to allow development of the blue precipitate. Following refixing in 2% paraformaldehyde, embryos were washed in PBS before transferring to 70% ethanol. Embryos were analyzed as whole mounts for the obvious appearance of the blue precipitate before carefully dissecting to reveal any deeper labeling. All labeling, whether superficial or deep, was carefully recorded, and photographic images were made at each stage of the dissection process by using Kodak 64T film. Images were scanned with a CanoScan 2700F (Canon) and formatted by using Adobe Photoshop (version 5.5).

**Histological processing**

For skeletal preparations, embryos were stained with Alcian blue and, in some cases, also Alizarin red. Briefly, embryos were deskinned and eviscerated, followed by fixation in 100% ethanol for 24 h. Staining was performed overnight at room temperature with embryos incubated in a solution containing 0.05 mg/ml Alcian blue (BDH) in 80% ethanol/20% glacial acetic acid. Specimens were all washed in 1% potassium hydroxide, and some were subsequently incubated in a solution containing 0.1 mg/ml Alizarin red (BDH) in 1% potassium hydroxide. All embryos were cleared in 20% glycerol with 1% potassium hydroxide overnight before transferring into 50% and 80% glycerol. Photographic images were made on Kodak 64T film and prepared as above.

**Results**

Specific regions of individual somites (numbers 19–26) were injected with a lacZ-encoding retrovirus at embryonic stages 13–18, in order to determine the cellular contribution that somites and their various compartments make to the ribs. Due to the naturally high titer of the harvested virus (10$^6$ CFU/ml), further concentration of the virus was not necessary, and results from the injections demonstrated that sufficiently large numbers of somitic cells had been infected with the virus. The exact volume of viral supernatant injected into each somite could not be accurately recorded, although it was clear that each injection contained less than 0.5 μl, with the relative volume of virus controlled by the picopump (this allowed either “small” or “large” volumes of supernatant to be administered). The embryos were allowed to reach stage 31–36 before fixing and staining for β-galactosidase. Embryos were dissected and analyzed as whole mounts for the appearance of the blue reaction precipitate, and histology was performed where appropriate. In embryos where sham-injections had been carried out, no endogenous β-galactosidase activity was found in the regions of study. A small proportion of injected embryos presented gross abnormalities, including small limbs or incomplete closure of the body cavity, and these embryos were not included in the analysis. The abnormalities were probably not caused by the virus itself, as sham-injections also resulted in similar proportions and types of deformities (data not shown). LacZ-encoding viruses have been used extensively in cell marking and cell lineage studies over the last two decades, and there have been no reports of either gross or cellular abnormalities resulting from viral infection (Leber et al., 1996).

**Somitic origin of the ribs**

The first set of injections was designed to map the overall contributions made by individual somites to the developing rib cage. It was necessary to label as many progenitor cells as possible, therefore lacZ-encoding retrovirus was injected into the center of the chosen somite (19–26), at somite stage I (Fig. 2A), thus labeling the cells of the somitocoele as well as the surrounding epithelialized cells of the somite wall (the relative volume of virus for these injections was increased to label large numbers of cells). As expected, these injections gave rise to labeling within all somitic tissue lineages, including skeletal muscle, bone/cartilage, dermis
(connective tissue), and endothelial cells, although not all lineages were labeled after every injection (Fig. 2). Labeling was found in a number of different hypaxial muscle groups, including intrinsic and extrinsic muscles of the wing and scapular girdle (from somites 19–20), pectoral muscles (somites 19–22), and the intercostal muscles (described in more detail later). Interestingly, no epaxial muscles were labeled as a result of this series of injections. Overall,
labeled bone cells/chondrocytes were found in all seven thoracic vertebrae as well as one cervical vertebra and one lumbar vertebrae (C14 and L1, respectively). Label was detected in both the proximal and distal parts of all the ribs (see below), but no labeled cells were ever detected in the scapula or sternum. Dermal labeling was often apparent both within the skin overlying the dorsal region as well as within the developing feather buds, while labeled endothelial cells were found in a number of cases throughout the thoracic region.

A total of seven pairs of ribs make up the thoracic cage in the chick, with each rib articulating with its corresponding thoracic region. The labeling of these ribs was investigated with the retrovirus encoding the lacZ gene (Wigmore and Evans, 2002), but also postulated by Kato and Aoyama (1998) to give rise to the ribs. The final injections focused on the region of the somite around the interface of the lateral sclerotome and the ventrolateral dermomyotome. Fig. 4A illustrates the overall experimental schedule.

As expected, these injections did not give rise to all somitic tissue lineages, and instead each compartment/loci contributed to specific derivatives (Table 2). Injections made into the medial sclerotome resulted, in most cases, in the labeling of vertebral bodies and also the proximal parts of the ribs, including the head, neck, and tubercles (Fig. 4B). Injections into the lateral sclerotome always resulted in labeling within the distal bodies of the ribs (Fig. 4C), although occasional labeled cells were also localized to the proximal rib and the vertebral arch. In some cases, only the sternal elements of the distal ribs were labeled (Fig. 4D). In contrast, when the ventrolateral dermomyotome was injected with virus, no rib labeling was detected in either proximal or distal elements, and instead only intercostal muscles were ever labeled (Fig. 4E), except when somites 19–22 were targeted and labeling of wing, scapular, and pectoral muscles also resulted. Finally, viral injections were aimed at the interface between the ventrolateral dermomyotome and lateral sclerotome. Distal ribs were always labeled after these injections and were usually accompanied by a labeled intercostal muscle (Fig. 4F). All the results from this set of injections were repeatable.

Resegmentation of thoracic ribs

The patterns of labeling resulting from injections into somites 19–26 at somite stage I (with labeled cells restricted

<table>
<thead>
<tr>
<th>Anatomical component</th>
<th>Somite compartments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Region 1</td>
</tr>
<tr>
<td>Vertebrae</td>
<td>2</td>
</tr>
<tr>
<td>Proximal rib</td>
<td>4</td>
</tr>
<tr>
<td>Distal rib</td>
<td>1</td>
</tr>
<tr>
<td>Intercostal muscle</td>
<td>0</td>
</tr>
<tr>
<td>Other hypaxial muscles</td>
<td>0</td>
</tr>
</tbody>
</table>

Note: Each injection of virus was made at somite stage V-X (HH stage 15–18), and the embryos were fixed at day 7–10 (HH stage 31–36). Region 1, injections into medial sclerotome; region 2, injections into lateral sclerotome; region 3, injections into ventrolateral dermomyotome; and region 4, injections at the interface between lateral sclerotome and ventrolateral dermomyotome.
to cranial or caudal parts of the ribs) suggested that the process of resegmentation had occurred during the morphogenesis of the ribs. In order to investigate this in more depth, further sets of injections were carried out and these included injections into specific regions of a chosen somite and also into adjacent somites. In some cases, these injections were made into somites at somite stage I, while others were made at stages IV–VII, when the dermomyotome and sclerotomes had formed. Results were consistent in all cases, irrespective of somite stage. Some injections of virus were made deep into either the rostral or caudal half of somites 20–25, targeting the sclerotome in each case. The relative volume of virus injected in these experiments compared with the initial injections was reduced to ensure only minimal spread of the virus. Results revealed that the caudal part of each somite gave rise to all of the proximal region of the rib, including the head, neck, and tubercle, and also the cranial rim of the distal rib (costal shaft), excepting the sternal extremity, where labeled cells were visible throughout the tissue (Fig. 5A). In contrast, injections into the rostral part of each somite resulted in a lack of labeled cells within the proximal rib and cranial distal rim, and instead gave rise to labeled cells in the caudal rim of the distal rib. Labeled cells were again dispersed throughout the tissue within the sternal extremities of the costal body (Fig. 5B).

Clarification of whether resegmentation appeared to affect distal rib morphogenesis in the chick was made using injections directed into the caudal half of somite 24 and the rostral half of somite 25 in the same embryo at either somite stage I or IV (Fig. 5C). Data from these investigations showed, in all cases examined, that labeled cells were distributed throughout the distal part of the rib (Fig. 5D) and not restricted to either the cranial or caudal rims, demonstrating that resegmentation had indeed occurred in the distal rib.

Discussion

The traditional view that all parts of the ribs originate from the sclerotome of the thoracic somites in avian embryos (Christ and Wilting, 1992; Huang et al., 1994, 1996, 2000b) has recently been challenged by an alternative view suggesting that only the proximal rib derives from the sclerotome while the distal rib arises from regions of the dermomyotome (Kato and Aoyama, 1998). In this study, I have used a lacZ-encoding replication-deficient retrovirus to reveal the precise contributions made by somitic cells to the thoracic ribs and associated structures. The stable introduction of a marker gene into the genome of the host somitic cells allows all their subsequent progeny to be tagged with the marker gene, while removal of the specific structural genes ensures that no new infective virions are subsequently produced by the host cell. Results from these studies indicate that the sclerotome compartment of all thoracic somites contributes cells to both the proximal and distal elements of the ribs and that the distal ribs are largely subject to resegmentation during their morphogenesis. Fig. 6 illustrates an overview of some of the main conclusions.

Specific regions of individual somites (19–26) were injected with the lacZ-encoding retrovirus at embryonic stages 13–18. At no stage could I estimate the exact number of somitic cells labeled, as it was impossible to know the exact volume of virus injected, the spread of the virus, or the number of mitotic cells able to incorporate the virus. However, in spite of this, the consistency and overall clarity of the patterns of labeling generated, following the injections, indicate that the virus was largely confined to the site of injection. Application of small amounts of Indian ink to the viral injection medium acted as a reliable indicator during the manipulation that the virus had not spread to adjacent somites/tissues. Indeed, at no stage were β-gal-expressing cells found in more than two adjacent ribs, indicating that the virus had only infected cells within a single somite (based on the assumption that resegmentation had occurred during rib morphogenesis). Introduction of the retrovirus into avian embryos did not appear to cause any cellular or morphological abnormalities, as sham-injections also resulted in similar proportions and types of deformities. LacZ-encoding retroviruses have been used extensively in cell marking and cell lineage studies and have so far been found to be nonpathogenic (Leber et al., 1996).

Somitic origin of the ribs

The first set of injections was designed to map the contributions of individual somites to the developing rib cage and associated structures of the chick. The overall results clearly demonstrate that the thoracic somites (somites 19–26) contribute cells to all parts of the ribs as well as the associated intercostal muscles. Some injections were also made into the lateral plate mesoderm adjacent to somites 19–26, but these did not result in labeling of any somitic lineages and more importantly never gave rise to labeling in the ribs (data not shown), thereby confirming previous studies that the ribs are indeed derived from the somites (Seno, 1961; Pinto, 1969; Sweeney and Watterson, 1969; Christ et al., 1974; Chevallier, 1975).

Injections made into somites 20–25 resulted in the labeling of two adjacent ribs, with each somite having given rise to labeled cells within the caudal region of one rib and the cranial rim of the subsequent rib, while somites 19 and 26 gave rise to cells of just one rib. It is not surprising that somite 19 only gave rise to cells of just one rib, the cranial part of the first rib, as no rib lies superiorly to the first rib for somite 19 to contribute to. Equally, it makes sense that somite 26 only gave rise to cells of the caudal seventh rib as an eighth rib does not exist in the chick. The overall results, however, do indicate that there are probably differences in the mechanisms that recruit cells to a particular rib during
morphogenesis or in the way in which cell proliferation is controlled along the rostrocaudal axis of the thoracic region. The rationalization of the ribs along this axis appears to already be determined in the segmental plate. When prospective thoracic segmental plate is grafted into cervical or lumbrosacral regions, ectopic ribs form, whereas grafting of cervical somites into the thoracic region results in the failure of ribs to form (Kieny et al., 1972).

In addition to the labeling of the thoracic ribs, these injections gave rise to labeling within other somitic tissue lineages, including skeletal muscles, bone/cartilage, dermis, and endothelial cells. Not all lineages were labeled in every case, but this was not surprising, as it was unlikely that all
cells within the injected somite had been infected. The results did indicate, however, that cells of both the future dermomyotome and sclerotome had been infected by the virus. Precursors from all hypaxial muscles groups, including the intercostal muscles, were labeled at some stage during the investigation. Interestingly, no injections gave rise to the labeling of epaxial muscle precursors, and this must be due to the injections never infecting cells within the prospective dorsomedial dermomyotome, the site of epaxial muscle precursors (reviewed in Ordahl et al., 2000). We are currently investigating this issue. Labeled bone cells were found in the thoracic vertebrae, with the pattern of labeling similar to that of the ribs, i.e., one somite contributing to the caudal half of one vertebra and the cranial half of the subsequent vertebrae, indicating that resegmentation had occurred. No labeled cells were ever detected in the sternum, and it is thought that structure is derived exclusively from lateral plate mesoderm (Chevallier, 1975). The scapula was also never labeled following a viral injection and this is more surprising as at least part of the scapula derives from somitic mesoderm (Chevallier, 1975). It is possible that the precursor cells in this case are already subcompartmentalized within the somite and were not labeled by the virus.

Sclerotomal origin of the ribs

Although the results from the first set of injections confirmed the conclusions of other studies that the somites are the source of cells for the developing ribs, and extended our overall appreciation of the contribution of particular somites to the entire rib cage, they did not address the question of which somitic compartment(s) the ribs are derived from. The second set of injections was designed to look directly at this issue. Injections were made into somites at stages V–X, so that distinct somitic compartments could be targeted. The sclerotome and dermomyotome compartments of the thoracic somites are morphologically evident from around somite stage III–IV (Christ and Ordahl, 1995; Ordahl et al., 2000). Injections were made as precisely as possible into each loci by using histological material of embryos, fixed at similar stages, as a guide. In order that only small numbers of progenitor cells were labeled and to minimize viral spread, the relative volume of virus injected was reduced as much as possible. The virus was largely confined to the site of injection, based on the consistency of the results obtained. If the virus had spread to also label cells within neighboring compartments, we would have expected to see infection of the tissue lineages normally restricted to those other compartments. This did not occur in any of the cases examined.

The sclerotome as a whole has been hypothesized by some workers to give rise to the cells that contribute to all parts of the ribs (Christ and Wilting, 1992; Huang et al., 1994, 1996, 2000b); while others suggest that at least the distal ribs derive from the dermomyotome (Kato and Aoyama, 1998). The current investigation appears to concur with the traditional view that it is the sclerotome that contributes cells to all parts of developing ribs. First, the data revealed that the medial sclerotome gave rise to labeling within the proximal rib (and also vertebral bodies). This is unsurprising, as it has previously been demonstrated that the proximal rib originates from the medial somite compartment (Olivera-Martinez et al., 2000). The results of my study, which looks at slightly later stages, suggest that it is in fact the medial sclerotome that is the precise site of origin of the proximal ribs. The factors regulating proximal rib formation are not known at present; however, results of several targeted mutations where proximal ribs are either reduced or totally missing suggest that Pax-1 and Uncx4.1 play major roles (Leitges et al., 2000; Mansouri et al., 2000; Wallin et al., 1994).

Injections directed at the lateral sclerotome gave rise, in all cases, to labeled cells within the distal rib, both sternal and vertebral components, strongly suggesting that this region of the somite is the origin for the distal ribs and confirming results of other studies (Huang et al., 1994). In a number of cases, only the sternal parts of the distal ribs were labeled with the lacZ gene. A population of Pax-1-negative cells has been previously detected in the ventrolateral angle of the sclerotome at an early stage (Ebensperger et al., 1995), and as yet, it is unclear what the fate of these cells might be. It is possible that these cells were infected with the virus during this set of injections and that this “subcompartment” of the sclerotome contributes cells mainly to the sternal elements of the distal ribs. In one other case, labeling was also detected in the proximal rib as well as the distal rib, suggesting that any boundaries between the lateral and medial sclerotome compartments are either not completely fixed during development or maybe slightly varied between embryos.

There were no cases of labeled cells within the proximal or distal rib elements when injections of virus were directed into the ventrolateral dermomyotome. In these cases, only hypaxial muscle labeling resulted, indicating that the dermomyotome contributes, as expected, to the musculature of the rib cage, but not to the ribs themselves, results which are in conflict with those of Kato and Aoyama (1998). However, when the interface of the lateral sclerotome and the ventrolateral dermomyotome were infected with virus, labeled cells were found in both distal ribs and intercostal muscles in most cases. This could suggest that the cells of lateral sclerotome and the ventrolateral dermomyotome are intimately associated during this period. Such a close relationship between these cells supports the hypothesis that the dermomyotome is required for proper rib formation, illustrated in both Myf-5 and Pax-3 (involved in the regulation of dermomyotome differentiation)-deficient mice, for example, where severe distal rib defects, including truncations, fusions, and bifurcations, are evident (Braun et al., 1992; Dickman et al., 1999; Henderson et al., 1999; Tremblay et al., 1998). Pax-3-expressing dermomyotomal cells, for example, may be involved in providing inductive signals re-
required for cartilage rib formation (Sudo et al., 2001) and are required to act at a very local level. The close relationship between sclerotomal and dermomyotomal cells appears to persist as morphogenesis of the thorax continues. As the somitic mesoderm penetrates into the somatopleuric mesoderm within the interlimb regions, the cartilage precursors of the sternal ribs appear to use the penetrating dermatome and myotome as a route to the site of distal rib formation (Sudo et al., 2001). Even though the results of my study clearly show that the sclerotome contributes to all parts of the ribs, both proximal and distal elements, the data do not completely rule out that a small population of dermomyotomal cells located close to the lateral sclerotome also makes a contribution to the distal ribs, and these cells may then dictate the behavior of the cells within the lateral sclerotome that will form the majority of the distal rib.

Resegmentation in the dorsal ribs

The resegmentation theory of the vertebrae and the ribs has existed since the mid-Nineteenth century, when Remak (1855) observed that the boundaries of the somites and the subsequent vertebral bodies do not correspond. The assumption that boundaries shift in order that muscles bridge skeletal elements has often been criticized (reviewed by Brand-Saberi and Christ, 2000; Verbout, 1985); however, a series of cell-lineage studies using chick-quail chimeras, the fluorescent dye Dil, and also retroviral-mediated gene transfer clearly demonstrate that resegmentation does occur during the formation of the vertebral motion segment (Bagnall, 1992; Bagnall et al., 1988, 1989; Ewan and Everett, 1992; Huang et al., 1994, 1996, 2000a). Two groups of investigators have also shown in detail that the process of resegmentation occurs in the distal elements of the ribs (Aoyama and Asamoto, 2000; Huang et al., 1996, 2000a, 2000b). These studies, using chick-quail chimeras, reveal that the cells of the caudal half of one somite form the proximal element and the cranial rim of the distal element of one rib, while the rostral half of the somite contributes to the caudal part of the preceding distal rib. The subtleties of the exact distribution of cells during resegmentation of the ribs as interpreted by these two groups differs slightly based on their alternate views on the origins of the distal ribs (Huang et al., 2000b; Kato and Aoyama, 1998).

The results of the current investigation clearly agree with those of previous studies and show that distal elements of the ribs are subject to resegmentation. Injections into the rostral sclerotome of one somite gave rise to labeled cells in the caudal rim of one distal rib, while injections into the caudal sclerotome contributed cells to the cranial rim of the succeeding distal rib. These data strengthens the idea that sclerotomal cells are restricted to contributing to only two vertebral segments and are not freely able to distribute along the rostral–caudal axis. In all cases, resegmentation was not evident in the most sternal aspects of the distal ribs, where labeled cells were found throughout the tissue. It is likely that substantial cell mixing occurs in these regions. In line with previous reports (Aoyama and Asamoto, 2000; Huang et al., 1996, 2000a), the proximal elements of the ribs were never found to be subject to the process of resegmentation. Results of the current experiments showed that cells within the proximal ribs were all derived from the caudal part of each somite.

Origins and interactions of intercostal muscle precursors

Intercostal muscles were found to derive from somites 19–26, in agreement with Chevallier (1975), with each somite contributing to a single intercostal muscle (both intrinsic and extrinsic bellies). Each intercostal muscle spans two adjacent ribs, inserting at the caudal border of one rib and at the rostral border of the succeeding rib. The data from these experiments revealed that each labeled intercostal muscle was located between two labeled ribs (that were also derived from the same somite), with labeling in the caudal half of one distal rib and the cranial rim of the subsequent rib. This pattern of labeling strongly resembles the subsequent anatomical relationship of the intercostal muscles with the ribs and suggests that the progenitor cells of the distal rib develop and maintain a close relationship with the precursors of the associated intercostal muscle as they invade into the somatopleuric region of the body wall. It has been shown that the precursors of both of these tissues are derived from the lateral compartment of the early somite, suggesting a common origin (Olivera-Martinez et al., 2000); however, my data strongly suggest that they become restricted to different compartments in the later somite, although these two precursor populations may continue to be somewhat associated at this stage. Development of intercostal muscles appears to be under the control of myf5 (Kablar et al., 1997), and it has been suggested that morphogenesis of the distal ribs may be under similar control (Kato and Aoyama, 1998). The close anatomical relationship between both the precursor cell populations and also the subsequent adult tissues would strongly corroborate this hypothesis. Alternatively, the cells present within the interfacing region between the sclerotome and dermomyotome may represent common precursors of muscle and cartilage that subsequently become instructed to follow different fates based on the expression of various muscle- and cartilage-inducing genes. The extensive colocalization of myotomal markers and cMlfh-1, a marker demarcating prospective cartilaginous tissues, within this region (Sudo et al., 2001) would support this hypothesis.

Rib morphogenesis

Overall, this study strongly indicates that the sclerotome compartment of the thoracic somites gives rise to all parts of the ribs, while the intercostal muscles, as expected, arise from the ventrolateral dermomyotome. The data also suggest that the cells that contribute to the distal ribs are
intimately associated with cells of the dermomyotome (possibly intercostal muscle precursors), and that this relationship may be essential for proper rib morphogenesis. Finally, the results show that the distal ribs are largely subject to resegmentation during their morphogenesis. We are currently investigating the spatial relationship between the progenitors of the distal ribs and the intercostal muscles during development from the initial movement of cells into the somatopleuric region of the body wall, through subsequent elongation of precursor cells to the eventual morphogenesis of the tissues.

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