INTRODUCTION

The embryonic precursors of three diverse cellular lineages are contained within the somite; skeletal muscle, dermis of the back, and cartilage of the vertebrae and ribs. Somites are paired, segmented structures, which form at regular intervals in a cranial-to-caudal manner off of mesodermal strips (paraxial mesoderm) lying on both sides of the neural tube. The newly formed somite is an epithelial sphere, but complex morphological changes occur as the somite matures. First, cells of the ventral somite delaminate from the epithelium to form the mesenchymal sclerotome, which gives rise to the cartilage lineage (Christ and Wilting, 1992). The remaining epithelium, the dermomyotome, gives rise to the dermal lineage, to the skeletal muscle cells of the hypaxial domain and to a structure called the myotome, which is formed between the dermomyotome and the sclerotome, and gives rise to the epaxial skeletal muscle (Christ et al., 1974; Ordahl and Le Douarin, 1992; Denetclaw et al., 1997).

The cells of the most newly formed somite (designated as the stage I somite, Ordahl, 1993) are believed to be developmentally equivalent. Rotation of the dorsoventral axis of a stage I somite (or a stage II somite: the next oldest somite located caudally) does not affect the relative positions of myotome and sclerotomal tissue (Aoyama and Asamoto, 1988). Furthermore, when the ventral portions of a stage I somite (prospective sclerotome) are transplanted dorsally, they remained as morphologically unintegrated mesenchyme when grafted into an embryonic day 2 host, but formed only cartilage when placed into an identically aged host. Vertebral body cartilage from embryonic day 7 and embryonic day 8 embryos formed exclusively ectopic cartilage in an embryonic day 2 host. We conclude that cells determined to the cartilage fate do not appear until somite stage XII, but that not all sclerotome cells are determined at this time. The effect of host age on the differentiation and morphogenetic behavior of sclerotome fragment grafts in this assay indicate the existence of developmental eras within the embryo.

When the somite first forms the cells appear to be equivalent in potential. In order to understand the lineage diversification of the somite, the determination of sclerotome cells to the cartilage fate was tested using an in vivo challenge assay in which quail sclerotome fragments were grafted into a dorsal position in a chick host. Grafts containing undetermined cells were expected to differentiate into other tissues while grafts containing determined chondrocyte precursors were expected to consistently give rise to cartilage. We found that grafted sclerotome fragments from somite stages V-XX were capable of giving rise to integrated muscle and dermis and that it was not until fragments from stage XII somites were grafted that cartilage was consistently produced in the assay. Sclerotomal tissue from embryonic day 4-6 embryos remained as morphologically unintegrated mesenchyme when grafted into an embryonic day 2 host, but formed only cartilage when placed into an identically aged host. Vertebral body cartilage from embryonic day 7 and embryonic day 8 embryos formed exclusively ectopic cartilage in an embryonic day 2 host. We conclude that cells determined to the cartilage fate do not appear until somite stage XII, but that not all sclerotome cells are determined at this time. The effect of host age on the differentiation and morphogenetic behavior of sclerotome fragment grafts in this assay indicate the existence of developmental eras within the embryo.

Key words: Sclerotome, Chick, Determination, Pax-1, Somite, Cartilage

SUMMARY

Determination of sclerotome to the cartilage fate

Jennifer L. Dockter and Charles P. Ordahl*
Department of Anatomy and the Cardiovascular Research Institute, Box 0452, University of California San Francisco, San Francisco, CA 94143-0452, USA
*Author for correspondence (e-mail: ordahl@itsa.ucsf.edu)

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shown to support (or induce) the test fragment to differentiate into an alternative cellular/tissue phenotype. The acquisition of resistance by the embryonic tissue to alteration of its differentiation pathway, and its tendency to continue differentiating according to its original fate, are indices of its determination. A notochord challenge assay was recently used to assess the determination of somitic precursors to the myotome (Williams and Ordahl, 1997). Here we describe a dorsal challenge assay that was developed to test the determination state of sclerotome in vivo by transplanting fragments of this ventral, cartilage-fated somite tissue into a dorsal, muscle- and dermis-forming environment. In the dorsal challenge assay, undetermined cells are expected to respond to the new environment by contributing to muscle and/or dermis, while determined cells are expected to be resistant to the new environment and go on to form cartilage. The results of these experiments indicate that determination is a relatively late event in sclerotome development, both in terms of cellular phenotype and tissue morphogenesis.

MATERIALS AND METHODS

Somite and embryo staging

Somites were staged according to Ordahl (1993). The most newly formed caudal somite is at stage I; the older somites are progressively designated by increasing roman numeral values. Embryos were staged according to Hamburger-Hamilton (HH) (1951).

Sclerotome grafts

White leghorn chicken (Gallus gallus domesticus) (Western Scientific Product, Sacramento, CA and Petaluma Farms, Petaluma, CA) and Japanese quail (Coturnix coturnix japonica) (Strickland Quail Farm, Pooler, GA) eggs were incubated at 37.6°C in a forced-draft incubator. Quail donor embryos were poured from the egg into a bowl containing Tyrode's salts (Sigma). The embryo was cut from the yolk with iridectomy scissors (Fine Science Tools) and pinned ventral side up in a glass dish coated with black Sylgard. Under a dissecting microscope, endoderm, and in some cases, neural tube and notochord, were removed with electrochemically sharpened tungsten microscalpels. The sclerotome was then dissected out using tungsten microscalpels and placed in a drop of Tyrode's salts containing 5% fetal calf serum until grafting (15 minutes-1 hour). In the case of embryonic day (ED) 4 or ED5 sclerome fragments to be placed into age-matched hosts, a Nile-blue-sulfate-permeated agarose chip was placed on the sclerotome tissue for a few seconds to lightly stain the tissue before removal (Hamburger, 1966).

Vertebral body cartilage from ED7 and ED8 quail was obtained by dissecting out the vertebral column. Individual vertebrae were dissected apart and the neural arches, neural tube, notochord, adhering muscle and nerve tissue were removed with forceps and/or tungsten microscalpels. The vertebrae were then incubated in a solution of collagenase type II (Sigma)/pancreatin (BRL) in Tyrode's salts at room temperature for 15-20 minutes and triturated to remove any remaining soft tissue. The remaining vertebral body was dissected into small fragments for grafting using forceps and microscalpels.

ED2-ED3 chick hosts were opened by cutting out a small circle of eggshell. Ink (Pelikan black no. 17) was injected under the blastoderm for visualization of the embryo using a drawn-out microcapillary tube and mouth pipetting. After cutting open the vitelline membrane, a slit was made in the ectoderm with a tungsten microscalpel and the quail sclerotome placed underneath the ectoderm, between somite I and the neural tube (Fig. 1). Eggs were sealed and put back into the incubator until harvest.

In the case of ED4 or ED5 chick hosts, the host was opened, but no ink was injected underneath it; the vitelline and chorionic membranes were incised in the thoracic region with a tungsten microscalpel and a small slit was made in the dorsal ectoderm in the thoracic region of the embryo. The graft was held at the end of a micropipette by gentle mouth suction and the pipette was inserted through the ectoderm slit and along the neural tube until the graft was in the brachial region of the embryo. The suction was then released and the pipette withdrawn, leaving the blue-stained graft visible underneath the ectoderm of the host. Eggs were sealed with tape and returned to the incubator.

In general, early and intermediate stage (stages V-XI and stages XII-XX) sclerotome fragments were taken from cervical and brachial levels, while late stage sclerotome fragments and vertebral body grafts (ED4-ED8) were from thoracic levels. The fragments were taken from medial sclerotome and contained both cranial and caudal sclerotome cells.

Histology

Chimeric embryos were harvested at either 4, 8 or 10 days postsurgery, placed into Carnoy’s fix and embedded in paraffin. 7 μm sections were cut on a rotary microtome and stained with the Feulgen reaction to visualize nucleic nuclei (Le Douarin, 1973). For embryos evaluated with antibody staining, adjacent sections were placed on slides and analyzed with the Feulgen stain, with anti-myosin (MF20, Bader et al., 1982; Developmental Studies Hybridoma Bank) and/or with anti-collagen type II (II-I6B3, Linsenmayer and Hendrix, 1980; Developmental Studies Hybridoma Bank). Sections stained with anti-collagen type II were treated with 4000 u/ml of hyaluronidase (Sigma) for 20 minutes at 37°C prior to incubation with the primary antibody. A biotinylated anti-mouse secondary antibody (Vector Laboratories), a streptavidin/alkaline phosphatase-conjugated tertiary molecule (Vector Laboratories), and an NBT/BCIP color reaction were used to visualize the staining pattern. Some Feulgen-stained sections were subsequently stained with the 13F4 muscle-specific monoclonal antibody (Rong et al., 1987; Developmental Studies Hybridoma Bank) or with the anti-collagen type II antibody. A biotinylated anti-mouse secondary antibody (Vector Laboratories), a streptavidin/fluorescein-conjugated tertiary molecule (Amersham) and fluorescence microscopy were used to visualize the staining pattern.

In situ hybridization

Chimeric embryos were harvested at either 24, 48 or 72 hours postgrafting and adjacent sections were cut. One set of sections was stained by the Feulgen reaction to visualize the grafted quail cells. The corresponding adjacent sections were processed for in situ hybridization as previously described (Frohman et al., 1990) and hybridized with 35S-labeled cRNA probes: chick Pax-3, a 336 bp EcoRV/BamHI fragment (Goulding et al., 1991); chick MyoD, a 622 bp PvuI/EcoRI fragment (Lin et al., 1989) and mouse Pax-1, a 313 bp HincII/SacI fragment (Deutsch et al., 1988). Sections were exposed for 7-14 days and counter-stained with hematoxylin-eosin. Hybridization patterns were analyzed using dark-field microscopy.

Quality of donor tissue removal

To ensure that only sclerotome tissue was removed for transplant, some quail embryos were fixed and embedded immediately after sclerotome dissection. Alternate sections of these embryos were stained with the Feulgen stain and the MF-20 anti-myosin antibody. In the embryos examined (n=2), the myotome on the operated side was completely intact, indicating that myotome or dermomyotome tissues were not being transplanted along with sclerotome tissue (data not shown).

Microscopy

All microscopy (bright-field, dark-field and fluorescence) was performed on a Zeiss Axiopt microscope. Images were acquired using a DEI 470 Optronics CCD camera system and a RasterOps
RESULTS

Experimental design

The determination state of sclerotome at various developmental time points was analyzed by transplanting sclerotome fragments from quail embryos beneath the skin ectoderm of chick host embryos between the neural tube and the stage I somite (Fig. 1) an environment where muscle and dermis normally develop. After surgery, chimeric embryos were killed after incubation for various periods of time and quail cells were identified in cross-sections by the Feulgen reaction, which yields a distinctive magenta-colored nucleolus in quail cells (see Fig. 2C,D,E for example) The presence of quail nucleoli in muscle, dermis or cartilage cells or their somitic primordia (myotome, dermotomal mesenchyme and sclerotome) were scored as shown in Table 1. The differentiation capacity of donor fragments was found to vary according to the developmental stage of the somite from which they were derived as detailed below.

<table>
<thead>
<tr>
<th>Graft age*</th>
<th>n</th>
<th>Muscle†</th>
<th>Dermis†</th>
<th>Cartilage†</th>
<th>Mesenchyme†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early grafts (stage V-XI)</td>
<td>5</td>
<td>+‡</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Intermediate grafts (stage XII-XX, ED3.5)</td>
<td>14 (XII-XX)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1 (ED3.5)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>2 (ED3.5)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Late grafts (ED4-ED6)</td>
<td>18</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Vertebral cartilage (ED7-ED8)</td>
<td>6</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*Graft age indicates the somite stage or embryonic day of development from which the quail sclerotome fragment was excised. Grafts were placed dorsally into an ED2 chick host, underneath the ectoderm and between the neural tube and stage I somite.

†The categories muscle, dermis and cartilage include the somitic primordia from which these differentiated tissues derive: myotome, dermotomal mesenchyme and sclerotome respectively. Mesenchyme describes cells which were unidentifiable as belonging to one of the previous three categories.

‡Within each grafting age group, different outcomes were seen; the plus signs indicate that quail nucleoli were found within that tissue.

Early stage sclerotomes (somite stages V-XI)

35 chimeric embryos containing sclerotome fragments from quail embryos were analyzed after either 4, 8 or 10 days of postsurgical incubation. The later time points were taken to ensure that cells found in somite-derived tissues after 4 days would continue to be present in the corresponding older, differentiated tissues. The results are pooled in Table 1 and were not dependent upon the time of harvest. Quail cells contributed to all three tissue types (17 cases), muscle and dermis (11 cases), dermis alone (5 cases) or cartilage and dermis (2 cases). Fig. 2 shows a typical result in which quail nucleoli were found to be present in cells of the dermotomal mesenchyme, myotome and the sclerotome (Fig. 2C-E). In approximately one half of the cases, quail cells were absent in cartilage despite their origin in the sclerotomal compartment of early staged somites (Table 1).

Cells containing quail nucleoli on the operated side were simply mingled with host chick cells and there was no apparent disruption of host structures on the operated as compared to the unoperated sides (Fig. 2B). Although initially transplanted in the dorsal region, cells containing quail nuclei were also found in more ventral positions and, in some cases, were in close proximity to the notochord (data not shown). There was no correlation between the final dorsoventral position of the graft and the tissues contributed to by the graft. Quail nucleoli were never found in cells on the unoperated side of these or any of the chimeric embryos of this study.
Intermediate stage sclerotomes (XII-XX, ED3.5)

Sclerotome grafts into ED2 hosts from somite stages XII-XX always contributed to muscle, cartilage and dermis (Table 1, n=14). Grafts of sclerotome fragments from ED3.5 embryos (Hamburger-Hamilton stage 21) contributed to all three tissues in one case and to muscle and dermis only in two cases. Chimeras were harvested after either 4, 8 or 10 days of postsurgical incubation and the results were independent of the harvest time. Fig. 3 shows the result of a sclerotome fragment graft from a stage XX somite that was allowed to develop 10 days postgrafting (to ED12). Quail nucleoli can be distinguished in differentiated, somite-derived tissues: dermal papillae of feather germs, muscle fibers and in the neural arch portion of the cartilaginous vertebra (Fig. 3C-E). As in early stage sclerotome grafts, cells bearing quail nucleoli were intermingled with host chick cells in anatomical structures that were morphologically identical to those on the unoperated side.

Sclerotome fragments from stage XV-XX somites were taken from HH stage 18 embryos (ED3). Chimeric embryos harvested at ED10 or ED12 that contained fragments from ED3 donors frequently had patches of black feathers on the skin overlying the operated area (the host chicken embryo strain ordinarily produces white feathers), consistent with the presence of quail-derived melanocytes. Due to the pigment contained in these cells, the presence of quail nucleoli in the melanocytes could not be definitively ascertained. Quail nucleoli could also be found in other neural-crest-derived tissues such as dorsal root ganglia, sympathetic chain ganglia and spinal nerves (data not shown). No quail nucleoli were...
detectable in neural crest derivatives of chimeras containing sclerotome fragments from any other age donor.

Neural crest cells are known to be migrating through the sclerotome at ED2 and at HH stage 18 (Bronner-Fraser, 1986; Serbedzija et al., 1989). Chimeras containing ED2 donor fragments did not have detectable quail nucleoli in neural crest derivatives, indicating that these cells possibly either died, converted phenotype or that there were so few cells in those structures that they could not be detected by the Feulgen method. (A,B) The grafted cells have formed a large mesenchymal structure which disrupts the host anatomy and are not found integrated into any host structures. The dashed line outlines the approximate domain where quail nucleoli are contained. The same domain is outlined in C and D, it does not appear exactly the same because the sections are adjacent, not identical. (C,D) The grafted quail cells do not appear to have differentiated as muscle or cartilage. The grafted quail nucleoli do not appear to express either myosin (C) or collagen type II (D). Areas of positive staining are dark brown-black in color and are indicated by the black arrows.

Late stage sclerotomes (ED4-ED6)

19 grafts of sclerotomal tissue fragments from ED4, ED5 and ED6 embryos into ED2 hosts were performed (Table 1); these chimeras were harvested after 4 days postsurgical incubation. In only 1 of 19 chimeric embryos were quail nucleoli found within morphologically distinct muscle and dermis tissues. In the other 18 embryos of this series, quail nucleoli were present in a mass of unorganized, loose mesenchyme that was separate from surrounding host tissues (Fig. 4A,B). Host tissues were always disrupted in the vicinity of the graft region. Individual quail cells were morphologically indistinguishable from surrounding chick mesenchymal fibroblasts and expressed neither myosin nor type II collagen (Fig. 4C,D).

A hypothesis to explain these results is that the environment of an ED2 embryo is different from that of an ED4-ED6 embryo and is incapable of supporting the development of grafts from these ages. To test this hypothesis, grafts of ED4 or ED5 quail sclerotomal fragments were grafted into a dorsal position, underneath the ectoderm and between the neural tube and the myotome of a brachial level somite, in age-matched chick hosts and killed after 4 days of postsurgical incubation. One ED5 and three ED4 graft experiments were performed. In all experiments, quail nucleoli were found only in cartilaginous structures and not in any mesenchymal populations (Fig. 5). In one experiment the cartilage was ectopic; the other three grafts integrated into normal host cartilage. The morphology of normal host structures was not disturbed.

As a control to ensure that the dorsal environment of an ED4 chick is capable of supporting muscle differentiation, identical grafts into ED4 chick hosts were performed; however, somite stage X-XV dermomyotome fragments from wing-level quail somites were grafted instead of sclerotome fragments. In three out of three chimeric embryos harvested 4 days postsurgery, quail nucleoli were found only in dermis and muscle tissue and not in cartilage. The morphology of host structures was normal (data not shown).

Vertebral cartilage grafts (ED7-ED8)

By ED7, the sclerotome cells have laid down extensive extracellular matrix material and have formed the basic cartilaginous model of the vertebrae with the vertebral body cartilage in a more advanced state of differentiation than the neural arch cartilage (Shapiro, 1992). Grafts of vertebral body cartilage fragments from ED7 and ED8 quail embryos into ED2 chick hosts (harvested at ED6) remained as ectopic

Fig. 4. (A-D) Adjacent cross-sections of a chimeric chick embryo harvested at ED6, 4 days postgrafting of sclerotomal tissue from an ED5 quail and stained with Feulgen stain (A,B), MF20 anti-myosin antibody (C) and anti-collagen type II antibody (D). A is shown using a 10x objective; B-D are shown using a 20x objective. (A,B) The grafted cells have formed a large mesenchymal structure which disrupts the host anatomy and are not found integrated into any host structures. The dashed line outlines the approximate domain where quail nucleoli are contained. The same domain is outlined in C and D, it does not appear exactly the same because the sections are adjacent, not identical. (C,D) The grafted quail cells do not appear to have differentiated as muscle or cartilage. The grafted quail nucleoli do not appear to express either myosin (C) or collagen type II (D). Areas of positive staining are dark brown-black in color and are indicated by the black arrows.

Fig. 5. (A) 5x objective view of a cross-section of a Feulgen-stained chimeric embryo harvested at ED10, 6 days postgrafting of sclerotomal tissue from an ED4 quail just underneath the dorsal ectoderm in the brachial region of an ED4 chick host. The operated side is to the left. The box delineates the area magnified in B. (B) 40x objective view of a portion of the lamina of the vertebra. The darkly staining quail nucleoli, indicated by the arrows, are detected within this portion of the vertebra and its perichondrium.
cartilage in all cases (Fig. 6A). The ectopic cartilage nodules formed by ED7 fragments were similar in appearance to vertebral cartilage in an unoperated ED11 quail embryo in terms of (i) the spacing of the cells, (ii) the appearance of matrix and (iii) the thickness of the perichondrium, the latter being a boundary layer between the quail cells and surrounding chick cells (Fig. 6B). Expression of type II collagen in the nodule was confirmed using antibody staining (Fig. 6C). No chick nuclei were found within the quail-derived cartilage nodule and no quail nucleoli were found outside the nodule. Nodules formed by ED8 vertebral body grafts contained discernible matrix, but very few cells and no perichondrium (data not shown).

**Gene expression after grafting**

To examine changes in gene expression, hosts (which were at ED2 at the time of grafting) bearing early or intermediate stage sclerotome fragments were harvested at 24 and 48 hours postgrafting and probed for the expression of Pax-1, Pax-3 and MyoD mRNAs by in situ hybridization (Fig. 7; Table 2). Pax-1 is a sclerotome marker initially expressed throughout the sclerotome, with its later expression restricted to perichordal cells and the intervertebral discs (Deutsch et al., 1988; Ebensperger et al., 1995; Borycki et al., 1997). Based upon these studies all of the grafts should have been expressing Pax-1 at the time of transplantation. Pax-3 is a dermomyotome marker initially expressed in the segmental plate, becoming restricted to the entire dermomyotome and then to the lateral half of the dermomyotome and the migratory muscle precursors of the limb (Williams and Ordahl, 1994). MyoD is the first of the group of helix-loop-helix transcription factors called the myogenic determination factors (MDFs) to be expressed in the quail and can be seen in the medial portion of a stage II somite (Pownall and Emerson, 1992). As the somite matures, MyoD expression becomes restricted to the myotome and the dorsal medial lip of the dermomyotome (Williams and Ordahl, 1994).

As summarized in Table 2, gene expression in grafted fragments did not depend upon whether the sclerotome graft was from an early or an intermediate stage somite. None of the grafts detectably expressed Pax-1, Pax-3 or MyoD mRNAs at either 24 or 48 hours postgrafting (Fig. 7A-D and E-H). There was one exception: in one case, the grafted cells in one 24 hour embryo clearly expressed Pax-1 (data not shown). In all embryos harvested at 24 or 48 hours postgrafting, expression of these genes in host tissues was not altered (Fig. 7B-D,F-H).

Four embryos containing early or intermediate stage sclerotome fragments grafted into an ED2 host were evaluated at 72 hours postgrafting for expression of MyoD mRNA by in situ hybridization and for expression of myosin heavy chain protein by immunohistochemistry (Fig. 7I-M). The results obtained fell into two categories. In two embryos, the majority of quail nucleoli were found in dermal mesenchyme that was negative for MyoD mRNA and myosin protein (data not shown). The anatomy of host structures was not disturbed and the expression of MyoD and myosin in postoperated tissues was not altered. The few quail nucleoli that could be distinguished in the host myotome appeared to express MyoD mRNA and myosin protein although single-cell resolution was not possible.

Quail nucleoli in the two remaining embryos were found in large, loose mesenchymal structures that spanned the upper two-thirds of the dorsal-ventral length of the normal somite domain (Fig. 7I). Host anatomy and gene expression were severely disrupted on the operated, but not the unoperated side (Fig. 7L,M). As the sections advanced through the grafted region, quail nucleoli were initially detected only in dorsal mesenchyme and then more ventral, with host myotome steadily replaced by quail cells. In sections containing the greatest number of quail nucleoli, little or no host myotome could be detected, either morphologically or by marker expression. The grafted cells formed a subtle arc of tissue, which was similar in position and length to a myotome, but never formed an anatomically distinguishable myotomal structure nor did they ever express MyoD mRNA or myosin protein (Fig. 7J,K).

Since expression of a muscle marker by grafted quail sclerotome fragments could not be detected in time points up to 72 hours after grafting, but grafted nucleoli could be detected morphologically in myotome or muscle tissue in later harvest time points, we wished to confirm expression of muscle proteins by grafted cells at these later time points. Three embryos, two harvested 4 days and one harvested 8 days postgrafting of early or intermediate stage sclerotome fragments into an ED2 host, which had been initially analyzed with the Feulgen stain, were subsequently analyzed by fluorescent immunohistochemistry with 13F4, a monoclonal antibody that marks all types of muscle at both early and differentiated stages and which was known to be capable of

**Table 2. Gene expression postgrafting**

<table>
<thead>
<tr>
<th>Hours post graft*</th>
<th>n</th>
<th>Pax-1†</th>
<th>Pax-3</th>
<th>MyoD</th>
<th>Myosin</th>
<th>13F4</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>7</td>
<td>(4)‡</td>
<td>(7)</td>
<td>(2)</td>
<td>nd§</td>
<td>nd</td>
</tr>
<tr>
<td>48</td>
<td>3</td>
<td>(3)‡</td>
<td>(3)</td>
<td>(2)</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>72</td>
<td>4</td>
<td>nd</td>
<td>nd</td>
<td>(4)</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>96</td>
<td>2</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>+ (2)</td>
<td></td>
</tr>
<tr>
<td>192</td>
<td>1</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>+ (1)</td>
<td></td>
</tr>
</tbody>
</table>

*Chimeric embryos harvested at various times postgrafting of an early or intermediate stage quail sclerotome fragment underneath the ectoderm, between the stage I somite and the neural tube of an ED2 chick host.

†Pax-1, Pax-3 and MyoD mRNA expression was analyzed by radioactive in situ hybridization. Myosin and 13F4 expression was analyzed by immunohistochemistry.

‡The parenthesized numbers indicate the number of embryos analyzed for the marker out of the total number of embryos analyzed at each stage (n). A minus sign indicates no expression of that marker by the grafted cells and a plus sign indicates expression of the marker by the grafted cells.

§nd = not done.
reacting with Feulgen-stained tissue. In all three embryos, quail nucleoli observed within the myotome or muscle fibers of the host bound the 13F4 antibody, indicating that these cells were differentiated muscle, presumably skeletal muscle. Fig. 8 shows a typical result of these experiments in which a somite stage VI sclerotome fragment was grafted into a dorsal position in an ED2 host and was harvested 4 days postgrafting. The bright fluorescent staining was confined to the myotome area (Fig. 8B); quail nucleoli present in the myotome were stained (Fig. 8C,D), while quail nucleoli present in the dermatomal mesenchyme were not.

**DISCUSSION**

**Determination in early and intermediate stage sclerotome**

Fate mapping experiments show that the sclerotome will give rise to the cartilage models of the vertebras and ribs (Christ and Wilting, 1992). The experiments reported here show that despite this ultimate fate, a significant fraction of the cells within the sclerotome retain multipotentiality (i.e. potential for dermis and muscle) until relatively late stages of sclerotome development. For the purposes of this assay, we define determined sclerotome cells as those that would form cartilage despite being transplanted into a dorsal, muscle- and dermis-promoting environment. Sclerotome fragments from early somite stages (V-XI) gave rise to cartilage in only approximately 50% of embryos examined, while muscle and/or dermis tissue resulted in 100% of these cases. Only sclerotome fragments from stage XII to stage XX somites consistently gave rise to cartilage, consistent with the presence of determined chondrogenic precursor cells. Nevertheless, stage XII to XX sclerotome fragments also frequently gave rise to muscle and dermis indicating that undetermined, multipotent precursor cells are retained in the sclerotome even at these relatively late stages of somite development. We cannot rule out the possibility that determined chondrogenic precursors exist in the sclerotome earlier than stage XII but, if present, such precursors do not give rise to cartilage in 100% of transplanted cases. However, by somite stage XII determined chondrogenic precursor cells begin to appear within the somite sclerotome.

The diversity of progenitor cells within the sclerotome cannot be deduced from these experiments. The sclerotome could be composed of either multipotent precursors or a mixture of determined chondrocyte, muscle and dermis precursors, or both. Previous work indicates that committed muscle and cartilage precursors may be present in the chick embryo as early as the epiblast stage (George-Weinstein et al., 1996). Multipotential precursors would give rise to all three phenotypes in challenge grafts from young somite stages, with cartilage becoming the predominant phenotype as the cells' potential became restricted over time. Muscle- and dermis-determined precursors contained within the sclerotome would survive in the challenge environment, but would presumably fail to thrive in the sclerotome in situ due to lack of a survival factor or by active killing of these cells by an environmental factor. Very young chondrocyte precursors may not survive in the challenge environment, possibly explaining the ability of early somite stage sclerotome grafts to give rise to only muscle and dermis.

**Determination in early whole somites versus early sclerotome fragments**

These results are consistent with earlier work demonstrating the ability of either the ventral half, or a piece of the ventral half, of a stage I somite to contribute to dermomyotome and myotome when placed dorsally (Christ et al., 1992; Aoyama, 1993) and with experiments grafting the ventral halves of somites older than stage I into the limb bud (Wachtler et al., 1982). They are in contrast, however, with prior experiments involving inversion of whole somites on the dorsal-ventral axis, which indicated that sclerotomal fate was fixed by somite stage III (Aoyama and Asamoto, 1988) (and see Introduction). According to this interpretation, sclerotome cells have lost multipotency before they have delaminated from the epithelial somite, a conclusion not supported by the results presented here and outlined above.

There are at least two important differences between the previous, somite rotation experiments, and those reported here. The first is that by transplanting somite fragments, as opposed to rotating whole somites, the experiments reported here disrupted the integrity of the somite. The importance of somite integrity is unknown but could act to help maintain the fates of the sclerotome and dermomyotome. Freeing the sclerotome from its normal contacts with the other regions of the somite, and/or with surrounding tissues (notochord and aorta, for examples) may allow it to show a wider range of potential in the dorsal challenge environment.

A second major difference between the present and the previous studies is that, in the latter, the dorsal mesenchyme observed from rotation of stage III somites was not allowed to develop beyond 24 hours postsurgery, which is not long enough for the chondrogenic phenotype to differentiate. In the present study, when examined at 24 hours postgraft, sclerotome fragments from early or intermediate stage somites grafted dorsally had formed a loose mesenchyme that superficially resembled ‘sclerotome’. Nevertheless, that mesenchyme was negative for expression of Pax-1, an established sclerotomal marker gene, even though at the time of transplant the sclerotome fragments were Pax-1 positive. The dorsally located mesenchyme cells were also negative for the early muscle markers Pax-3 and MyoD at 24 hours. By 4 days postsurgery, however, cells from these grafted sclerotome fragments could be detected in organized cartilage, muscle and dermal tissues both by morphological and marker analysis. Thus, the ultimate differentiated phenotype of the mesenchymal cells formed by the graft could not be accurately ascertained at 24 hours postgraft, and this may also be true for the dorsolateral mesenchyme formed by dorsoventral inversion of a whole stage III somite.

**Determination in older sclerotome and vertebral cartilage**

Based upon the results with early and intermediate aged sclerotome, it was expected that grafts of sclerotome from older somites would consistently give rise to cartilage. Surprisingly, however, grafts of sclerotome fragments from ED4-ED6 embryos into ED2 hosts formed a separate mesenchyme that never differentiated into any recognizable tissue type. By contrast, sclerotome grafts from ED4-ED6 grafted into an identically aged host gave rise to only cartilage, and never muscle or dermis. Dermomyotome fragments grafted into ED4 hosts gave rise only to muscle and dermis,
indicating that the dorsal environment of an ED4 chick host is capable of supporting differentiation of these tissues. Because the ED4 dorsal environment can support muscle development, the exclusive formation of cartilage by ED4 sclerotome indicates that this tissue is fully determined and no longer contains pluripotent cells.

Grafts of cartilage fragments from ED7-ED8 vertebral bodies (which have previously been shown to be derived from the ventral sclerotome, Christ and Wilting, 1992) remained as cartilage after transplantation into ED2 hosts. This result is also consistent with the conclusion that determined chondrogenic precursor cells are established by ED4 as stated above. In contrast to grafts of earlier sclerotomal fragments, which integrated into the host tissues whether cartilage, muscle or...
dermis, the cartilage nodules formed by ED7-ED8 grafts were ectopic and were not integrated into the host cartilaginous structures.

**Morphogenetic integration**

Conclusions about the morphogenetic abilities of sclerotome can also be made from the results of the dorsal challenge assay. First, sclerotome appears to remain morphogenetically plastic even after cell type determination for sclerotome fragments has occurred (Table 3). ED2-ED3 sclerotome fragment grafts were found to be undetermined with regard to cell type (see above) and are interpreted to be plastic with regard to morphogenesis, as demonstrated by the ability of these grafts to integrate completely into the normal morphology of ED2 host tissues. However, ED4 sclerotome, which is cell type determined by the criterion of the dorsal challenge assay, is morphogenetically plastic, as shown by its ability to integrate when grafted homochronically. By ED7, the grafts have lost morphogenetic plasticity, as evidenced by their formation of ectopic nodules in an ED2 host.

Second, there appears to be a link between the ability of grafts to differentiate and their ability to morphogenetically integrate. Grafts that differentiated into a recognizable cell type in the dorsal challenge assay (ED2-ED3 sclerotome into ED2 hosts and ED4-ED5 sclerotome into ED4 hosts) also morphogenetically integrated. However, ED4 sclerotome grafted into an ED2 host neither differentiated nor morphogenetically integrated. Grafts of tissue already undergoing differentiation (ED7-ED8 grafts) have presumably already received these cues before transplantation and simply continue in their development.

**Molecular expression and signaling**

Formation of cartilage tissue by sclerotome appears to be a result of their position within the embryo and not of cell autonomous processes (see also above discussion of progenitor cell diversity). If the sclerotome was already determined to form cartilage by the time the cells had delaminated from the epithelial somite, then changing its location within the embryo (thus exposing it to non-cartilage tissue-forming signals) should not have changed the cell types that the graft formed. The results presented here however, demonstrate that some sclerotome cells retain the ability to respond to muscle- and dermis-forming signals long after delamination. Therefore sclerotome cells, which do not form muscle and dermis in their normal embryonic position, probably form cartilage because they are in a position in the embryo that initially prevents them from receiving or responding to signals to do otherwise. They do not appear to require this protection from outside influences once they have produced a large amount of matrix, since vertebral body grafts from ED7 and ED8 were capable of remaining true to fate in the challenge environment.

The role of the notochord as an inducer of sclerotome formation from somites and an inducer of cartilage formation by sclerotome has been supported by many experiments (Watterson et al., 1954; Lash et al., 1957; Cooper, 1965; Brand-Saberi et al., 1993; Pourquie et al., 1993; Fan and Tessier-Lavigne, 1994). The notochord-produced factor most implicated in sclerotome development is *sonic hedgehog*, although its exact role is unclear. Exposure to *sonic hedgehog* has been demonstrated to be sufficient to cause segmental plate mesoderm in vitro to express Pax-1 (Fan and Tessier-Lavigne, 1994) and to cause ectopic Pax-1 expression in vivo (Johnson et al., 1994), although the experiments presented in this paper demonstrate that Pax-1 expression is not sufficient to commit sclerotome to cartilage differentiation (see below). The *sonic hedgehog* gene has been knocked out in mice; homozygote null embryos briefly express Pax-1 in the sclerotomal region, suggesting that *sonic hedgehog* expression is not necessary to initiate sclerotome formation (Chiang et al., 1996). Prolonged expression of *sonic hedgehog* appears to be necessary however for subsequent sclerotome development, as these embryos lack a vertebral column, indicating a failure of the sclerotome to form cartilaginous structures, other than 5 or 6 ribs. Whether this is a proliferative effect (Fan et al., 1995), a survival effect or an effect on determination is not known. It is interesting to note that Sonic hedgehog protein levels and function appear to drop dramatically in the chick notochord at around stage 25 (4.5

### Table 3. Cell type determination versus morphogenetic plasticity

<table>
<thead>
<tr>
<th>Day of Sclerotome Development</th>
<th>Cell Type Undetermined?</th>
<th>Morphogenetically Plastic?</th>
</tr>
</thead>
<tbody>
<tr>
<td>ED2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ED4</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>ED7</td>
<td>–</td>
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days) (Marti et al., 1995), the approximate time at which the sclerotome becomes determined.

The results presented here afford no clear correlation between changes in gene expression in the sclerotome and the first appearance of determined cells by somite stage XII. Early sclerotome markers such as Pax-1 (Deutsch et al., 1988), Pax-9 (Muller et al., 1996), twist (Fan and Tessier-Lavigne, 1994; Gitelman, 1997) and scleraxis (Cserjesi et al., 1995) are already being expressed by this stage and the results of the dorsal challenge assay suggest that this expression is not sufficient to determine sclerotome cells to the cartilage fate nor can expression of these genes be considered definitive markers of sclerotome cells determined to the chondrocyte lineage. Based upon previous expression studies, early and intermediate stage grafts transplanted in the dorsal challenge assay were expressing mRNA for these genes, yet the cells were capable of switching fate. Grafted sclerotome fragments stopped expressing Pax-1 within 24 hours postgrafting and it is possible that, had they maintained expression, the grafts would have resisted the challenge. However, extended expression of these genes by sclerotome cells prior to grafting is not sufficient to allow resistance to the challenge since sclerotome grafts from ED3.5 can still switch fate and various subsets of cells within the sclerotome have been expressing one or a combination of these genes for over 24 hours.

Developmental ‘eras’ in sclerotome determination

Age differences between host and donor had an unusual effect upon experimental outcome in the studies reported here. For example, grafts that were closely matched in age (i.e. ED2-ED3 sclerotome transplanted into ED2 hosts and ED4 sclerotome into ED4 hosts) gave rise to tissues that were well-differentiated with respect to cell type and morphogenesis. In contrast, grafts of ED4 sclerotome transplanted into ED2 hosts never gave rise to differentiated tissues but rather resulted in undifferentiated mesenchyme (see Table 1). We interpret such findings as evidence for developmental ‘eras’; embryonic and fetal periods during which developmental progression of progenitor tissues depends upon a contemporaneous environment.

Fig. 9 summarizes the observations regarding sclerotome development that led to the era hypothesis. First, transplanted sclerotome tissue that remains contemporaneous with its original era can return to a differentiation pathway, even if that pathway is different from the one for which it was originally fated. Second, sclerotome tissue cannot differentiate if it is returned to an era through which it has already passed. Third, sclerotome that is already well differentiated can maintain its phenotype when grafted into an earlier era.

We attempted to test whether sclerotome can develop when advanced into a future era by grafting early and intermediate stage sclerotome fragments dorsally into an ED4 host. Only 4 out of approximately 20 surviving chimeras (20%) contained detectable quail cells, the lowest percentage of any of the surgical experiments. Two chimeric embryos contained quail nucleoli within indeterminate mesenchyme tissue and two embryos contained quail nucleoli integrated into cartilage and/or muscle. The grafts in the remaining 80% of surviving embryos either did not stay in place or did not survive. Because of the low percentage of chimeras containing quail nucleoli and the appearance of indeterminate mesenchyme in 50% of embryos with detectable quail cells, it appears likely that sclerotome cannot develop when transplanted into a future era just as it cannot develop when transplanted into a previous era.

Examination of gene expression and morphology in ED2-ED3 sclerotome fragments at 24, 48 and 72 hours postgrafting into an ED2 host demonstrated an interesting feature of eras (Fig. 9, dashed line). These experiments showed that, at these time points, the transplanted pieces did not integrate into normal host structures, had not formed structures resembling dermomyotome or myotome and were not expressing sclerotome, myotome or dermomyotome markers. Yet, by 4 days (96 hours) postgrafting, the grafts were fully integrated into host structures, displayed the proper morphology for these tissues and, in the case of muscle, expressed an appropriate marker. Thus, ED2 sclerotome fragments transplanted contemporaneously wait to respond to morphogenetic and fate cues in this ectopic spatial environment. This is in contrast to the results from grafting sclerotome fragments from ED4-ED6 into an ED2 host, which places the cells in an era through which they have already passed. These grafts do not differentiate, indicating that they cannot wait for the host to reach the next era for development. Therefore, contemporaneous grafts wait to respond to environmental cues, while un-contemporaneous grafts never respond. Grafts of ED7-ED8 vertebral body cartilage into ED2 hosts did not integrate either; this could be an era effect, these grafts may have already received morphological specification, or this could be due to the fact that the cells were firmly encased in matrix at the time of transplant and were not physically capable of responding to morphological signals.

It is not known if such era restrictions exist for other somite-derived tissues. Experiments studying the expression of desmin, an early muscle marker, in developing somites may delineate eras in early muscle development (Borman and Yorde, 1994). The period between a somite’s formation from
the segmental plate and its subsequent expression of desmin is not identical for all somites. The cranialmost somites do not begin to express desmin until 18-20 hours postformation, while the caudal most somites (somite 20 and subsequent somites) express desmin after 6-7 hours. Whether these changes in rate of desmin expression correlate with eras in early muscle development has not been tested by grafting experiments.

No reported gene expression in the sclerotome strongly correlates with eras in sclerotome development. Only the expression of the matrix protein collagen type II, which in the developing chick vertebral column is first seen in the sclerotome at ED4, corresponds to the timing of a change in eras (Von der Mark et al., 1976). The exact timing of expression in developing chick vertebrae of many cartilage matrix genes such as cartilage link protein has not been reported.

The nature of the mechanisms underlying eras is not known. A switch in eras could be due to an event as global as a change in blood-borne circulating factors or an event as local as a change in extracellular molecules. It seems likely that more data on the properties of eras will have to be accumulated before the mechanism(s) will be elucidated.

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