Emergence of determined myotome precursor cells in the somite

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SUMMARY

Myotome and sclerotome precursor cells are derived, respectively, from cells in the dorsomedial and ventromedial regions of the somite. To assay changes in the specification of myotomal precursor cells during somite maturation, we implanted dorsomedial quadrant fragments, from staged quail somites, next to the notochords of host chick embryos, and superimposed two additional notochords on these implants. In this notochord signalling environment, dorsomedial quadrant cells that are developmentally plastic are expected to differentiate as cartilage, while cells determined to a myogenic fate are expected to differentiate as skeletal muscle. Large numbers of differentiated chondrocytes developed from dorsomedial quadrant grafts of all stages of paraxial mesoderm development tested, indicating that persistent chondrogenic potential in cells fated to form muscle and dermis can be elicited by notochord signals. Differentiated myocytes, however, appeared in two somite-stage-dependent phases. In the first phase, dorsomedial quadrants from segmental plate and early stage somites (II and IV) form small, disorganized clusters of individual myocytes. The frequency of first-phase myocluster formation increases as myogenic factor expression begins in the dorsomedial quadrant, indicating that myogenic determination assayed by this method is closely linked to the expression of myogenic factors in the dorsomedial quadrant. In the second phase, dorsomedial quadrants from somite stages XI-XIII consistently form morphologically organized muscle tissue containing large numbers of parallel-oriented, multinucleated myotubes. Mitotic labelling demonstrated that muscle precursors were determined to the muscle phenotype prior to withdrawal from the cell cycle. Thus, myogenic determination in cells of the dorsomedial quadrant is acquired at earlier stages of somite maturation than the ability to proliferate and form muscle tissue. These results are consistent with the hypothesis that successive lineages of myotome precursor cells with different mitotic and morphogenetic properties arise in the dorsomedial quadrant during somite maturation.

Key words: quail-chick chimera, somite, myogenesis, determination, myoD, myf-5, myotome, notochord signals

INTRODUCTION

Specification of cell types in vertebrate embryos is a conditional process requiring inductive interactions between signalling and responding tissues (Gurdon, 1987). Instructive mechanisms, in which information-rich signalling molecules act on unspecified embryonic cells to impart cell fate, have been suggested for the specification of cartilage precursor cells in the somites of mouse embryos (Fan and Tessier-Lavigne, 1994). Permissive or de-repressive mechanisms, in which selective influences from signalling tissues allow the expansion of pre-specified founder cells (Holtzer, 1978), have been proposed for the appearance of neural precursor cells in the *Xenopus* embryo (Hemmati-Brivanlou and Melton, 1997), and may be responsible for the appearance of skeletal muscle precursor cells in the chick embryo (Choi et al., 1989; George-Weinstein et al., 1996).

Cell-intrinsic components of the specification process have been sought in the skeletal muscle lineage. The myoD family (MDF) of muscle-specific transcriptional regulatory genes, discovered through the manipulation of cell lines, governs contractile protein gene expression (Davis et al., 1987; Braun et al., 1989; Wright et al., 1989; Miner and Wold, 1990). Heterodimers formed between members of the ubiquitously expressed E-protein family and MDF gene products bind to regulatory elements of contractile protein gene promoters to activate transcription (Lassar et al., 1991).

In the vertebrate embryo, myogenic precursor cells of the body (exclusive of certain craniofacial muscles) are derived from somites (Christ et al., 1974), which are compact epithelial spheres that also contain precursor cells for cartilage, dermis and endothelium (Remak, 1855; Rabl, 1888; Christ, 1969; Swalla and Solursh, 1984; von Kirschhofer et al., 1994). MDF members are first expressed in the dorsomedial regions of the stage II somite (Pownall and Emerson, 1992; Ordahl, 1993), and at stage 24 (Hamburger and Hamilton) in the dorsal and ventral regions of the limb mesenchyme (de la Brousse and Emerson, 1990), just prior to the appearance of differentiated myocytes. This pattern of MDF expression, and the apparent absence of differentiated muscle in transgenic mice with targeted disruption of MDF genes (Hasty et al., 1993; Nabeshima et al., 1993; Rudnicki et al., 1993), implicates the MDF members in the differentiation of skeletal muscle in vivo.

It is not clear whether MDF members have additional functions during myogenic specification that precede their role in contractile protein gene expression. When expression of an
MDF member is forced by transfection in primary cultures of non-muscle cells, or in cell lines, a heritable ability to differentiate as skeletal muscle is conferred on the daughters of the original transfectants (Davis et al., 1987; Weintraub et al., 1989; Choi et al., 1990). The maintenance of fidelity to the myogenic differentiation program over several generations in such transfected cells indicates that the transfected MDF member provided or elicited a ‘cellular memory’ (also called determination) for the myogenic cell type. Autoregulatory (and cross-regulatory) loops have been proposed as the molecular mechanism for myoblast determination (Thayer et al., 1989), although experiments that disrupt such loops indicate that myogenic determination probably requires additional molecular events (Tapscott et al., 1989).

Although cell determination is a concept that dates from the earliest days of experimental embryology, unequivocal assays for determined cells have been elusive. Grafting experiments designed to identify ‘determined’ cells (Slack, 1983) must take into account both the type of signalling influence to which precursor cells are exposed, and temporal changes in the response of precursor cells to the signal. For example, previous experiments in which presumptive muscle precursor cells were grafted to the limb bud indicate that ‘determined’ muscle precursor cells already exist within the segmental plate, the region of paraxial mesoderm that gives rise to somites (Wachtler et al., 1982; Krenn et al., 1988). However, while segmental plate cells may develop into muscle in such a permissive environment, notochord implantation experiments (described below) demonstrate that they can also be induced to form cartilage. In other words, muscle precursor cells in the segmental plate have acquired ‘competence’ to form muscle in a permissive environment but they may not yet have acquired ‘memory’ that renders them refractory to induction to other cell types.

Axial structures (neural tube and notochord) of the vertebrate embryo can cause cells from all regions of the somite to undergo cartilage differentiation both in vivo and in vitro (Holtzer and Detwiler, 1953; Waterson et al., 1954; Grobstein and Holtzer, 1955; Lash et al., 1957; Gallera, 1966; Lash, 1967; Jacob et al., 1974; Hall, 1977; Cheney and Lash, 1981; Aoyama and Asamoto, 1988; Aoyama, 1993; Pourquie et al., 1993), although this ability appears to be lost as the somites mature. A supernumerary notochord grafted between the neural tube and the segmental plate of the chick embryo completely suppresses the formation of myotomal muscle, enhances the formation of axial cartilage in the dorsal axial domain, and induces the expression of Pax1 mRNA (a marker for sclerotome) in close proximity to the ectopic notochord (Brand-Saberi et al., 1993; Pourquie et al., 1993). The cells of the segmental plate appear to be more susceptible to this ‘ventralizing’ influence of the notochord, because mature somites produce myotomal muscle when juxtaposed with ectopic notochord (Pourquie et al., 1993).

We have developed a novel, alternative assay for myogenic determination that exploits the cartilage-inducing properties of the notochord to assess the acquisition of myogenic cell memory in cells of the dorsomedial somite. In this assay, dorsomedial quail paraxial mesoderm fragments are transplanted into chick host embryos and then surrounded by host and supernumerary notochord signals. We reasoned that myogenic precursor cells that are determined should be unresponsive to these signals and should continue to make muscle while undetermined cells should respond to the notochord challenge by forming cartilage. We restricted the challenge to myotomal precursor cells by analyzing fragments containing only the dorsomedial quadrant (DMQ) of the paraxial mesoderm. In addition, by taking DMQs from the paraxial mesoderm at different stages of maturation, we hoped to correlate the acquisition of determination with cellular changes in somite development and with the expression of key myogenic determination markers, such as the MDF members. Our results demonstrate that myogenic determination is acquired progressively by cells of the dorsomedial paraxial mesoderm during development. Moreover, there are qualitative and quantitative changes in the muscle that differentiates from DMQ grafts that may reveal important clues to the cellular and molecular processes of muscle formation in the embryo.

MATERIALS AND METHODS

Embryo surgery

Fertile quail eggs were obtained from Strickland Quail Farm (Pooler, GA). Fertile chick eggs were obtained from Western Scientific Products (Sacramento, CA). Egg incubation and general embryo surgical procedures were performed as described (Williams and Ordahl, 1996; Ordahl and Christ, 1997). Notochords from stage 15HH chick embryos, taken from the level of somites I-VII, were prepared as previously described (Pourquie et al., 1993) and kept in holding solution (2% fetal calf serum in Tyrode’s solution) until transplantation. All donor tissue fragments were implanted in the right-hand side of the host embryo at the level of the developing limbs (somites 15-20).

Preparation of somite dorsomedial quadrants from quail embryos

Two day quail embryos (stage 12-13HH) were pinned ventral side up in dissection dishes and a longitudinal midline incision was made in the endoderm adjacent to somites stages I-XV (Fig. 1A). Adherent tissues, such as aorta or endoderm were teased away using a microscalpel to expose the notochord and ventral surface of the somites. A longitudinal incision was then made through the somites to separate their medial and lateral halves. Sclerotome tissue of the ventromedial quadrant of the somite was then removed by aspiration with a micropipette after a brief treatment with collagenase (0.5%, Sigma). The remaining dorsomedial quadrant of the somite (Fig. 1B), was then teased away from the underlying ectoderm using the flat side of a microscalpel and transferred to holding solution using a P-20 Pipetman until ready for implantation into the host.

In order to evaluate the quality of the surgery, some donor embryos were fixed after the excavation of the ventromedial quadrant, while the dorsomedial quadrant (DMQ) was still in place in the donor embryo. The embryo was then sectioned and stained with the MF20 antibody (Developmental Studies Hybridoma Bank, University of Iowa), which recognizes striated muscle myosin, to assay the quality of sclerotome removal and the presence of already differentiated muscle cells in the dorsomedial quadrant of the somite.

Cell numbers in the dorsomedial quadrant

A series of donor DMQs from somites II (n=9) and XI (n=8) were prepared as above, and used to estimate the number of quail cells initially grafted to the host embryo. Cells were incubated in a 20 µl droplet of 25 µg/ml Hoechst stain 33342 (Molecular Probes, Eugene, OR) in Tyrode’s solution at 37°C for 5 minutes, on a microscope slide. An additional 20 µl of 0.5% collagenase and 20 µl pancreatin (1X) were added and the DMQ reincubated for an additional 5 minutes. A
coverslip was used to squash the DMQ so that the nuclei were separate and distinct, and an image of the separated nuclei was obtained using a Zeiss Axiophot microscope under epi-illumination. The image was printed and the number of nuclei counted manually.

**Preparation of host embryos**

Chick embryos containing between 15 and 20 somites (stage 12-13HH) were used as hosts in this study (Fig. 1C) and were prepared for surgery in ovo as described by Ordahl and Christ (1997). The paraxial mesoderm at the levels of the segmental plate through somites stage I-V was exposed via an incision in the ectoderm between the neural tube and the paraxial mesoderm (Fig. 1D).

**Implantation of donor DMQs and overlying notochords**

The quail donor DMQ was transferred to the chick donor by micropipette (Fig. 1E) and placed deeply into the medial position of somite I using a microscalpel (Fig. 1F). Once the donor DMQ was securely in place, two chick notochords were placed directly over the graft and remaining host lateral half somite (Fig. 1G), and the ectoderm flap replaced. Preliminary experiments were identical except that only one donor notochord was implanted.

Care was taken in several aspects of this procedure. First, donor notochords were implanted over the donor somite fragment so that they spanned a 6 somite-equivalent distance cranially and caudally relative to the implanted donor DMQ. This was done to ensure that signals from the notochord surround, as completely as possible, the donor DMQ being tested. Second, donor DMQs were transplanted into host embryos within 40 minutes of removal from donor quail embryos. Third, donor notochords were implanted within 90 minutes of being removed from donor chick embryos. Fourth, two donor notochords were implanted to restrain the grafted quail cells to the ventral epaxial domain, to prevent them from coming under the muscle promoting influence of tissues of the dorsal neural tube and skin ectoderm, while at the same time extending the range of effective notochord signalling.

**Analysis of cell division by undifferentiated muscle precursor cells**

In a separate series of experiments, donor DMQs from stage IV or stage XII somites were incubated for 90 minutes at 37°C in 100 mM BrdU (Sigma cat no. B-5002) in Tyrode’s solution. After two rinses, the labelled DMQ was implanted as above and the chimeric embryo was incubated for an additional 48 hours. The number of differentiated muscle cells that incorporated BrdU prior to differentiation was estimated in Feulgen-stained sections by simply counting nucleoli in Feulgen-stained sections that were estimated by counting nucleoli in Feulgen-stained sections that were visualized using a Zeiss Axiophot microscope. Numbers of quail nucleoli present in muscle tissue were estimated by counting nucleoli in Feulgen-stained sections that were present in MF20 staining tissues on the adjacent section. Numbers in cartilage tissue were estimated in Feulgen-stained sections by simply counting nucleoli in cartilage sections.
counting nucleoli present within the cartilage model. Images were collected using the DEI 470 Optronics CCD video camera system (Goleta, CA) and processed using Adobe Photoshop 3.0 software.

RESULTS

Anatomy of the notochord challenge environment

Preliminary experiments were designed to test the ability of cells within the dorsomedial quadrant (DMQ) of the somite to switch fates, from presumptive muscle and/or dermis, to cartilage. Single supernumerary notochords were implanted ectopically in 24 chick embryos as previously described (Pourquie et al., 1993) except that the medial half of the host stage I somite was replaced with a DMQ of quail somites from stages I to X (Ordahl, 1993). The quail nucleolar marker was then used to identify graft-derived cells in cartilage, muscle, paravertebral mesenchyme, mesenchyme surrounding the neural tube, the connective tissue sheath surrounding the spinal nerve, and the endothelial lining of several blood vessels. 21 out of 24 chimeric embryos showed an abundance of graft-derived cells in the differentiated cartilage models that surrounded both the host and chick donor notochords. Gifts from older somites also consistently produced large numbers of differentiated skeletal muscle cells (≥1000 nuclei), while DMQ grafts from stage IV and younger somites varied widely in the number of differentiated skeletal muscle cells produced. In 25% of the cases, graft-derived cells were located in the dorsal epaxial domain of the chimeras, distant from both the host and supernumerary notochords. The presence or absence of dorsal donor muscle in all of these chimeras was unrelated to the somite stage from which the dorsomedial quadrant was isolated. We reasoned that dorsally displaced somite cells might have received muscle-promoting signals arising from the dorsal neural tube and/or skin ectoderm, (Christ et al., 1992; Buffinger and Stockdale, 1994, 1995; Fan and Tessier-Lavigne, 1994; Munsterberg et al., 1995; Munsterberg and Lassar, 1995; Stern et al., 1995; Stern and Hauschka, 1995) thereby confounding the cartilage-inducing influence of the implanted notochords. For these reasons, we did not analyze muscle development in these chimeras further.

A more rigorous challenge to the fate of donor DMQ cells was performed in an additional 46 chimeras. Double supernumerary notochords were deployed to maximize notochord signalling and as an obstacle to prevent the migration of graft-derived cells into the dorsal epaxial domain, where they might receive muscle-promoting influences from the dorsal neural tube or dorsal ectoderm. Donor test fragments were implanted deep within the medial somite compartment close to the host notochord and two chick notochords were overlaid.

In 39 of these chimeras (85%), the donor-derived cells remained ventral to the implanted notochords, the position in which notochord signalling is expected to be maximal (see Fig. 2). The appearance of donor muscle in this ventral epaxial domain was correlated with the stage of somite development from which the donor dorsomedial fragment was derived. The data from these experiments is summarized in Table 1 and accompanying histological analysis described in detail below. Implantation of fragments of dorsal neural tube or intermediate
mesodermal tissue into the same position as notochords induced neither increased amounts of muscle nor the formation of additional ectopic cartilage from subjacent quail DMQ implants (data not shown). Whereas overlaid notochords tended to restrict cells from the subjacent DMQ to ventral regions of the epaxial domain, no such restriction was seen with neural tube or intermediate mesodermal implants and graft-derived cells contributed widely to muscle and cartilage throughout the epaxial domain of the host embryo (data not shown).

### Muscle differentiation in the dorsal and ventral epaxial domains differs in a stage-dependent fashion

The position of graft-derived cells in relation to host structures and donor notochords at 24 hours, 48 hours and 6 days post-surgery in double notochord challenge chimeras is shown in Fig. 2. Panel A shows the arrangement of tissues in the notochord challenge 5 hours after implantation of the dorsomedial quadrant of a stage IV somite and overlaying of two donor notochords. Quail cells are present in mesenchyme and in an 'epithelioid' structure closely apposed to the ventral neural tube. By 24 hours (Fig. 2B), the mesenchyme has greatly expanded and some quail cells are clustered in a contiguous group near the notochord. No recognizable host dermomyotome epithelium is evident on the operated side by 24 hours post-surgery. Antibody staining with BEN, a marker for floorplate and motor neuron cell bodies (Pourquie et al., 1990), indicated an expansion in the number of motor neuron cell bodies in the host neural tube on the operated side, but floor plate formation (assessed morphologically) was not consistently observed (data not shown).

Six days after surgery, adjacent sections from 46 chimeras were analyzed for the presence of quail cells in differentiated skeletal muscle and in the vertebral cartilage model. Fig. 2C,D show an immunohistological analysis of adjacent cross sections through the grafted region of a chimera. Ectopic cartilage has differentiated on the operated side of these chimeras, in the form of distorted vertebral bodies and lamina as seen previously (Pourquie et al., 1993). Extensive incorporation of quail cells into these cartilage structures (arrow) surrounding the neural tube was seen in all but one of the 46 chimeras evaluated (Table 1). Host-derived and donor-derived cartilage cells in chimeric structures were segregated into well-defined domains.

In seven cases (15%) analyzed separately in this study, somite cells escaped confinement in the ventral epaxial domain bounded by three notochords and were displaced dorsally in close proximity to the dorsal neural tube (Table 1). Fig. 2C,D demonstrates the response seen in this minority of cases. Muscle tissue (panel D) in such specimens was composed of thousands of quail cells (panel C) incorporated into large, elongated myofibrils that appear to run transverse to the body axis. The myonuclei are roughly equal in number to the quail cells found in the cartilage model of the neural arch. Such dorsally located donor muscle tissue appeared in some cases to lie partially inside the vertebral lamina surrounding the neural tube. Dorsomedial quadrant cells from each of the different maturational stages tested were capable of this behavior in the dorsal epaxial domain. Donor muscle cells were never found laterally in the host hypaxial domain.

### Chondrogenic potential within the somite dorsomedial quadrant

Feulgen-stained cross sections of 6-day old chimeras were examined for the presence of quail cells in differentiated cartilage models. Quail-derived cartilage cells were detected in all but one of the double supernumerary notochord chimeras (38 out of 39 specimens; Table 1; Fig. 2C), and from all stages of donor DMQs tested. In all but 2 of these cases, cartilage cells were present in large numbers (Table 1). To evaluate the possibility that sclerotome precursor cells may have been carried along with the DMQ grafts, we examined cross sections to investigate the cellular complexity of DMQs prepared in the same way as for graft implantation. Fig. 3 shows adjacent cross sections through donor somites immediately after excavation of the underlying sclerotome cells in the ventromedial quadrant of the somite. Fig. 3C is a cross section through a stage XI somite, with most of the underlying medial sclerotome cells removed. A few mesenchymal cells of unknown origin remain between the dorsomedial lip of the dermomyotome and the neural tube. Fig. 3D is a cross section adjacent to that shown in Fig. 3C, stained with the MF20 antibody, showing that already differentiated skeletal muscle cells (arrowhead) are contained within the

### Table 1. Contributions to ventral muscle and cartilage from the dorsomedial quadrant of brachial somites at different stages of maturation

<table>
<thead>
<tr>
<th>Donor somite</th>
<th>n</th>
<th>None</th>
<th>Small</th>
<th>Medium</th>
<th>Large</th>
<th>None</th>
<th>Small</th>
<th>Medium</th>
<th>Large</th>
<th>Dorsal escape† n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seg. Plate</td>
<td>10</td>
<td>6</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>Somite II</td>
<td>15</td>
<td>7</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>14</td>
<td>1</td>
</tr>
<tr>
<td>Somite IV</td>
<td>11</td>
<td>1</td>
<td>5</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>Somites X-XIII</td>
<td>10</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>7</td>
<td>2</td>
</tr>
</tbody>
</table>

Numbers are the number of embryos showing the particular feature

*Small colonies are fewer than 20 cells, medium sized colonies are 100-150 cells, and large colonies are greater than 200 cells (typically thousands).

†Dorsal Escape breakdown:

<table>
<thead>
<tr>
<th>Graft</th>
<th>Muscle colonies</th>
<th>Cartilage colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seg. Plate</td>
<td>1-none, 1-small</td>
<td>2-large</td>
</tr>
<tr>
<td>Somite II</td>
<td>1-none</td>
<td>1-large</td>
</tr>
<tr>
<td>Somite IV</td>
<td>1-none, 1-small</td>
<td>2-large</td>
</tr>
<tr>
<td>Somite XI-XIII</td>
<td>2-large</td>
<td>1-small, 1-large</td>
</tr>
</tbody>
</table>
DMQ that will be transferred to the host embryo. Thus, while the mesenchyme cells present in DMQs of mature somites (stages XI-XIII) may represent sclerotome cells, at least in part, it is unlikely that sclerotome was included with the DMQs of early somites (stages II & IV). These results are in agreement with previous findings (Aoyama and Asamoto, 1988; Pourquie et al., 1993), and are consistent with the notion that cells with chondrogenic potential persist within the somite dorsomedial quadrant, and that the cartilage fate is elicited by exposure to the powerful inductive influence of the notochord.

Small myoclusters from the dorsomedial quadrant of the segmental plate

Two segmental plate DMQ chimeras (25%) formed small disorganized clusters of differentiated myocytes. We refer to these small clusters of differentiated myocytes as 'myoclusters', rather than 'colonies', to avoid misunderstandings about the (unknown) number of founder cells present in the donor segmental plate fragment. A histological analysis of small myoclusters is presented below.

Myoclusters are characteristic of dorsomedial fragments from stage II and stage IV somites

The frequency and size of myoclusters formed in the notochord challenge environment increased in a somite-stage-dependent fashion. Small myoclusters were detected in seven (50%) of the chimeras resulting from transplantation of stage II somite DMQs (Table 1). In the remaining seven chimeras, no muscle was formed. Fig. 5 shows the analysis of a chimera containing a single small myocluster. Morphologically identifiable donor
muscle tissue was not grossly evident, but the very small, disorganized cluster of differentiated muscle cells surrounded by donor mesenchyme cells could be detected by anti-myosin staining (panel B). Colocalization of the quail nucleolar marker (panel C) and myosin staining (panel D) demonstrate that many, and possibly all, of the myonuclei in this cluster are quail-derived. Three features of these myoclusters distinguish them from bona fide muscle tissue. First, small myoclusters contain fewer than 20 individual differentiated quail myocytes, each with a single nucleus. Second, myoclusters are disorganized. Individual myocytes are neither bipolar nor aligned with neighboring myocytes. Cross striations are not evident. Quail myocytes are interspersed with, and morphologically indistinguishable from surrounding mesenchyme cells (see also below). Third, myoclusters are always found in proximity to peripheral nervous tissue. This is evident in Fig. 5A,B where the donor myocluster is located near a nascent vertebral foramen through which the spinal nerve passes. All 15 cases of small myoclusters were found in comparable proximity to peripheral nerve tissue.

Approximately half of the chimeras resulting from transplantation of stage IV somite DMQs gave rise to small myoclusters with the same characteristics as those described above for stage II somite dorsomedial fragments (Table 1). Three stage IV somite DMQ chimeras, however, gave rise to larger ‘medium-sized’ myoclusters estimated to contain between 100 and 150 donor myonuclei. Otherwise, these myoclusters had the same characteristic disorganization and proximity to spinal nerves as noted above for small myoclusters.

**Muscle tissue from the DMQ of somites stages XI-XIII**

All DMQ grafts of stage XI-XIII somites formed muscle (Table 1), and the majority (75%) formed large, anatomically distinguishable muscle tissue in a position ventral to the implanted notochords (Fig. 6B). Such muscle tissue consisted predominantly of quail nuclei arranged in bipolar, multinucleate myotubes (Fig. 6C,D). In peripheral regions, chick cells also were seen to contribute to donor-derived muscle tissue in some cases. Cross striations (not shown) and centrally located nuclei are clearly evident. These muscle masses showed well-organized fiber bundles (Fig. 6D) containing thousands of nuclei, organized separately from other surrounding tissues. In the example shown, as in most other cases, the direction of donor fiber alignment was perpendicular to the host body axis. There was no correlation between the formation of these ectopic muscle tissue masses and the proximity of a spinal nerve. Adjacent sections from stage XI DMQ chimeras stained with the QCPN and HNK antibodies 24 hours post-surgery indicated that neural crest cells were not included in the grafts (data not shown).

**Characterization of mesenchyme cells surrounding myoclusters**

As indicated above, donor myoclusters reside within mesenchyme tissue that contains a mixture of both host and donor cells. We wanted to know if donor mesenchyme cells might be arrested at some earlier stage of muscle specification. Therefore, in situ hybridization was used to determine if surrounding, donor-derived mesenchyme cells express early muscle specification markers. Fig. 7 shows adjacent cross sections of an 8-day old chimeric embryo containing a graft of a quail stage IV somite DMQ. Fig. 7B shows an enlargement of the area marked in panel A where mesenchyme is populated with donor cells. This region is negative for myosin protein (Fig. 7C), and for the messenger RNAs for myoD (Fig. 7D), myf5 (Fig. 7E), and Pax-3 (Fig. 7F). We conclude that the surrounding mesenchyme does not represent muscle precursor cells arrested at an early stage of myogenic specification definable by these markers.

**Early response to the notochord challenge**

It is unknown whether the unusual microenvironment of the notochord challenge is conducive to the survival of muscle precursor cells. To determine if muscle precursor cells in the dorsomedial somite quadrant can differentiate in close
proximity to notochord, we analyzed chimeras for the presence of nascent muscle tissue 24 hours after grafting stage XI DMQs into the notochord challenge. Fig. 8A shows that cells derived from the grafted quail somite DMQ remain ventral to the implanted notochords. Those cells derived from the graft that are clustered in a group express sarcomeric myosin protein (Fig. 8B); myf5 and myoD mRNAs (Fig. 8C and D, respectively) but not Pax-3 or Pax-1 mRNAs (Fig. 8E and F, respectively). Graft-derived cells that are in the mesenchymal state do express Pax-1 (Fig. 8F, white arrow), but do not express any of the other muscle lineage markers (Fig. 8B-E). Analysis of chimeras produced with stage IV somite fragments showed a similar rapid appearance of differentiated muscle, but with many more graft-derived cells present in loose mesenchyme (data not shown). These results demonstrate that robust muscle differentiation can occur in close proximity to donor and/or host notochords.

Mitotic skeletal muscle precursors in the dorsomedial quadrant of the somite
A mitotic labelling experiment was used to determine whether the differentiated muscle cells in grafts from older somites were derived from mitotically active precursors or from postmitotic cells. DMQs from stage XII somites were incubated with the thymidine analog BrdU for 90 minutes prior to implantation, and collected 48 hours after placement into the notochord challenge (Fig. 9). Post-mitotic myocyte precursor cells in the graft would not be expected to incorporate this label, while mitotically active myotome precursor cells would be expected to take up the label and eventually form differentiated muscle with BrdU labelled nuclei.

MF20-stained skeletal muscle tissue (Fig. 9A,B) contains quail cells (Fig. 9C) that are labelled with BrdU (Fig. 9D). The appearance and number of cells present in BrdU-treated grafts
from the stage XI somite was indistinguishable from untreated grafts collected at this age. Therefore, while some already differentiated skeletal muscle cells are initially present in the stage XI donor DMQ (as shown in Fig. 3D), undifferentiated muscle precursor cells that have not yet withdrawn from the cell cycle progress through at least one S-phase prior to differentiation after 48 hours in the notochord challenge environment.

To estimate the number of quail donor somite cells that were initially placed in the challenge, dorsomedial quadrants of somites II (n=9) and XII (n=8) were labelled with Hoechst stain and nuclei counted on images taken with a fluorescence microscope (data not shown). Dorsomedial quadrants from somite II contained approximately 280 nuclei (±40 s.e.m.), whereas dorsomedial quadrants from somite XII contained 700 nuclei (± 100 s.e.m.).

**DISCUSSION**

**An in vivo assay to score cell fate decisions between cartilage and skeletal muscle**

We have developed a cell determination assay that challenges prospective skeletal muscle precursor cells with a powerful chondrogenic inducer, the notochord. Previous work has established the notochord as an important source of the signal(s) that induce ventral somite cells to form sclerotome, the precursors of vertebral cartilage (Holtzer and Detwiler, 1953; Watson et al., 1954; Grobstein and Holtzer, 1955; Lash et al., 1957; Lash, 1967; Brand-Saberi et al., 1993; Pourquie et al., 1993; Fan and Tessier-Lavigne, 1994). This assay is a refinement of earlier determination assays (Slack, 1983), in that it tests temporal changes in the response of muscle precursor cells to signals that are known to influence them at an earlier stage of development in the embryo. Undetermined somite cells in this assay should respond to notochord signals by differentiating into chondrocytes, while determined myogenic precursor cells that have acquired phenotype memory should no longer respond to the cartilage-inducing signals of the notochord and continue to differentiate as muscle. By challenging prospective muscle precursor cells from somites at precise developmental stages, the temporal acquisition of muscle lineage memory can be assessed.

This in vivo assay has several advantageous features. First, it employs transplantation of lineage-marked prospective skeletal muscle precursor cells from quail embryos (Le Douarin, 1973). Second, microsurgical procedures (Williams and Ordahl, 1996; Ordahl and Christ, 1997) are employed to restrict analysis to myogenic precursor cells from a well-defined region of the somite (the DMQ), permitting the developmental potential of the myotome lineage to be tested independently of myogenic precursor cells located in the lateral half of the somite, which behave very differently (Ordahl and LeDouarin, 1992; Rong et al., 1992; Gamel et al., 1995). Third, by scoring the differentiation of both muscle and cartilage, the coexistence of both chondrogenic and myogenic potential in the same cell population can be described. Fourth, the in vivo environment allows 3-dimensional relationships between tissues to influence development, as for example in the case of myocusters forming in close proximity to nerve (see also below). Fifth, the incorporation of BrdU into graft-derived nuclei prior to differentiation as skeletal muscle in the notochord challenge clearly demonstrates that determination of muscle precursor cells in vivo precedes withdrawal from the cell cycle. Finally, tissue morphogenesis is fully elaborated in vivo.

Spinal nerve may supply muscle-specific trophic factors, or a spatially favorable environment for myocuster differentiation. The presence of donor-derived mesenchymal cells that do not express muscle markers in close proximity to nerve would appear to rule out a muscle-inducing property of the spinal nerve. Nerve tissue is known to promote the differen-

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**Fig. 8.** Rapid differentiation of muscle precursor cells can occur in close proximity to grafted notochords. (A) A Feulgen-stained cross section of a chimeric embryo harvested 24 hours after surgery (56x). The host received a graft of the DMQ from a stage XI brachial somite overlaid with two notochords. The quail cells are surrounded by the dashed line. Host and donor notochords are marked with asterisks. A large mass of contiguous quail cells is present immediately ventral to the two donor notochords; a few loose quail mesenchymal cells are seen ventral to and surrounding this mass. (B) An adjacent section stained with the MF20 antibody (56x). Note that the contiguous mass is differentiated muscle cells, and that the myotome on the unoperated side of the chimera is also MF20-positive. (C) An adjacent section hybridized to the quail myf5 probe, indicating hybridization in the differentiated muscle cell regions seen in B (50x). (D) An adjacent section hybridized to a myoD probe, again showing strong expression in the regions of muscle differentiation (56x). (E) An adjacent section hybridized to a Pax3 probe, showing that the implantation of donor chick notochords has effectively abolished Pax3 expression in cells of the operated paraxial mesoderm, and has extinguished some expression in the dorsal neural tube on this side of the embryo as well (56x). (F) An adjacent section hybridized to a Pax1 probe, showing expression in the mesenchymal cells on both sides of the chimera, with some enhancement of expression (arrow) seen immediately adjacent to the muscle domains on the operated side of the embryo (56x).
tiation of muscle from somites (Vivarelli and Cossu, 1986; Kenny-Mobbs and Thorogood, 1987; Rong et al., 1992; Buffinger and Stockdale, 1994; Stern and Haushka, 1995), and a lineage of nerve-dependent myoblasts is known to arise in the developing limb (Bonner, 1978). While we favor the hypothesis that most of the muscle-fated precursor cells in early somites and segmental plate are converted to the cartilage fate by notochord signals, an alternative possibility is that they die due to their isolation from muscle-specific trophic factors. Thus, determined myogenic precursor cells could be present even in the segmental plate, but only acquire the ability to thrive independent of nerve-associated trophic factors during maturation within the somites. The infrequent appearance of myofibrils from segmental plate grafts suggests that repressive mechanisms are evident in the notochord challenge assay that are not apparent in dispersion cultures (George-Weinstein et al., 1994). Myogenic repressors such as twist (Hebrok et al., 1994; Fuchtbauer, 1995; Spicer et al., 1996) and notch (Reaume et al., 1992; Kopan et al., 1994) that are expressed in the rostral tip of the segmental plate could be inactivated by the presence of adjacent neural tissue.

**Cellular organization and cell fate specification in the somite**

Dorsal half-somite transplant experiments indicate that cells in the dorsal half of the somite are fate to form skeletal muscle but not cartilage (Christ et al., 1978, 1992; Aoyama, 1993). Medial half-somite transplant experiments indicate that the medial portion of the dorsal half will give rise to the muscle and dermis of the epaxial domain (Ordahl and LeDouarin, 1992). Despite these fate assignments under normal conditions in vivo, we have demonstrated that chondrogenic potential remains in the dorso-medial quadrants of mature somites from which well-organized muscle tissue will develop. Such chondrogenic potential in the mature dorsal somite is not evident in somite-rotation experiments, which indicate that cells of the dermomyotome are resistant to chondrogenic influences by somite stage III (Aoyama and Asamoto, 1988). In addition, dorsally grafted notochord induces ectopic cartilage only from segmental plate but not from formed somites (Pourquie et al., 1993). Such observations, together with the findings reported here raise the possibility that the organization of intact somites may help maintain the fate of unspecified dermal and/or skeletal muscle precursor cells within the dorso-medial quadrant and that surgical disruption of the somite epithelium may reduce the resistance of somite dermomyotome cells to cartilage inducing signals from the notochord.

There may be two steps in the response of the somite to excess notochord signals; (i) cells of the somite epithelium are first induced to scatter as mesenchyme and, subsequently (ii), become susceptible to cartilage inducing signals from the notochord. Unclustered, mesenchymal quail cells in both early and late somite grafts extinguish the expression of Pax3 mRNA under notochord influence within 24 hours and begin to express the sclerotomal marker Pax1 (Fig. 8), as seen previously (Pourquie et al., 1994). However, mitotically active, graft-derived quail cells that remain clustered differentiate rapidly as skeletal muscle within the same time frame, even when immediately adjacent to supernumerary notochords (Fig. 8). Pax3 expression is repressed below background levels in these cells, consistent with the previously observed repression of Pax3 expression in somites (data not shown), suggesting that the epithelium of the mature somite is more resistant to the mesenchyme-inducing influence of the notochord.

**Tissue morphogenesis and mitotic expansion are linked to somite maturation**

Both the incidence of differentiated muscle cells appearing in double notochord challenge chimeras, and the number of myocytes that differentiated in each chimera, increased with increasing developmental stage of the donor somite (Fig. 10, 8). Determined muscle precursor cells are mitotically active. (A) shows a cross section of a 3 day chimeric embryo 24 hours after grafting a stage XI dorso-medial quadranl that was labelled with BrdU for 90 minutes prior to grafting into the notochord challenge. This section (56×) is stained with the MF20 antibody, revealing differentiation of skeletal muscle tissue; the boxed area is magnified in B-D. (B) The region containing differentiated skeletal muscle tissue, as identified by the MF20 antibody (350×). (C) An adjacent section showing the location of quail cells in the grafted region by identification with the QCPN anti-quail antibody (350×). D is another adjacent section showing that many of the nuclei in the grafted tissue that differentiated as muscle are labelled with BrdU, as detected by the IU-4 anti-BrdU antibody (350×).
and Table 1). In addition, a somite-stage-related increase in the ability to organize differentiated muscle cells into tissue is acquired during somite maturation. Myocellular clusters were interspersed with non-muscle mesenchyme cells and were similar in appearance to mononucleate and multipolar differentiated muscle cells seen in culture (Rong et al., 1992; Buffinger and Stockdale, 1994; Gamel et al., 1995; Stern and Hauschka, 1995). In terms of cell numbers, myoclusters represented a small fraction of the cellular component of a normally sized myotome in the dorsal epaxial domain which has been estimated to contain approximately 500 myonuclei in the mouse (Nicolas et al., 1996). DMQs from stage XI-XIII somites, in contrast, formed highly organized muscle tissue comparable in size and myonuclei numbers to the host myotome. Such muscle tissue appeared to organize independently of surrounding host structures and donor myofibers were typically oriented perpendicular to the host axis. These differences are reminiscent of changes in Xenopus muscle precursor cells, which require interaction with neighboring cells in order to differentiate during early development (Gurdon, 1988), and lose this requirement as they mature in the paraxial mesoderm (Kato and Gurdon, 1993).

It is unlikely that the increased number of skeletal muscle cells seen in older somite grafts is a result of the implantation of already differentiated skeletal muscle cells, which are known to be mitotically inactive (Langman and Nelson, 1968; Sechrist and Marcelle, 1996). Incorporation of BrdU into the nuclei of muscle tissue formed from older somite grafts confirmed the presence of mitotically active determined muscle precursors at the time of grafting. Although there was an approximately two-fold difference in the number of cells in stage II versus stage XII DMQ fragments prior to transplantation, the more than 5-fold difference in the number of muscle cells derived from these grafts suggests that either (i) a fixed number of determined myogenic precursors acquires increased mitogenic and morphogenetic capacity, or (ii) increasing numbers of determined myogenic precursor cells are induced from previously unspecified cells, or (iii) both. The timing of appearance of highly proliferative determined myotomal precursors corresponds to the initiation of expression of the muscle specific FGF receptor, FREK (Marcelle et al., 1995) that may render determined myotome precursor cells responsive to ligands for FREK that are expressed in the somite (i.e. FGFs 4, 6 and 8) (Niswander and Martin, 1992; deLapeyriere et al., 1993; Crossley and Martin, 1995; Crossley et al., 1996; Grass et al., 1996).

The different mitotic and morphogenetic properties seen in this assay could reflect the presence of distinct lineages of muscle cell precursors within the somite. Whether these two populations arise from equivalent, multipotent precursor cells responding to changes in the type or duration of signals from the axial structures, or from pre-specified, unequivalent precursor cells in the dorsomedial quadrant of the somite is unknown. Precedent for the idea of separate and distinct muscle precursor populations that arise early in somite development has been established by molecular biological methods (Papatoutian et al., 1995; Braun and Arnold, 1996; Cosso et al., 1996) and experimental embryological methods (Ordahl and LeDouarin, 1992). Finally, the possibility that a population of non-muscle cells arises later within the somite DMQ to direct muscle development should not be overlooked. Connective tissue cells, for example, are believed to control skeletal muscle morphogenesis in the limb (Chevallier and Kienny, 1982).

Fig. 10. Summary of muscle precursor cell properties. Myogenic precursor cells from the segmental plate. Dorso-medial segmental plate rarely forms differentiated myoclusters of fewer than 20 cells, and is converted almost exclusively to cartilage. The paired-box transcription factor, Pax3, is expressed throughout this tissue, at levels detectable by in situ hybridization. Myf5 expression can only be detected by PCR amplification, and myoD is not expressed in this tissue. Myogenic precursor cells from the stage II somite. DMQs of the stage II somite form myoclusters only 50% of the time, and are completely converted to cartilage in the remaining cases. In situ detectable myoD expression begins in the dorsomedial epithelium of the stage II somite; at this time, Pax3 expression is extinguished in these cells. Myogenic precursor cells from the stage IV somite. DMQs of the stage IV somite often form small myoclusters and occasionally form large numbers of differentiated, disorganized muscle cells. Cartilage is always elicited from these grafts. MyoD expression is still detectable, and the onset of in situ detectable myf5 expression is seen. Myogenic precursor cells from older somites (XI-XIII). DMQs from older somites have acquired an enhanced mitotic potential that results in the consistent formation of large numbers of skeletal muscle cells organized into recognizable muscle tissue. In situ detectable expression of myoD, myf5 and myogenin is seen, as well as the presence of fully differentiated myotomal fibers expressing myosin heavy chain. The expression of Pax3 is restricted primarily to the lateral border of the somite.
Correlation of transcription factor expression and the appearance of determined skeletal muscle precursor cells

The paired-box transcription factor Pax3, a marker of the skeletal muscle lineage expressed prior to myoD and myf5 (Bober et al., 1994b; Goulding et al., 1994; Williams and Ordahl, 1994), is expressed in the DMQ of the segmental plate during somitogenesis (see Fig. 10). Consistent conversion of DMQ cells of the segmental plate and somite dermomyotome to the cartilage fate in the notochord challenge indicates that Pax3 expression in paraxial mesoderm does not irreversibly commit them to the muscle fate (Fig. 10). RT-PCR experiments have demonstrated low-level (covert) expression of at least one MDF member in the segmental plate (Kopan et al., 1994; Maroto et al., 1997). Overt expression of myoD transcripts is first detectable by in situ hybridization at somite stage II (Pownall and Emerson, 1992), in correlation with an increase in the incidence of determined cells detected by this assay (Fig. 10). Myf5 expression begins in the dorsal-medial stage IV somite (Pownall and Emerson, 1992), coincident with an enhanced mitotic potential demonstrated in the notochord challenge. If myoclusters are the same cells that earlier expressed MDF members in the paraxial mesoderm (Pownall and Emerson, 1992; Kopan et al., 1994; Maroto et al., 1997), additional neural influence(s) may be required for their persistence as muscle when challenged with the powerful cartilage inducing influence of the notochord.

The effect of notochord signalling on muscle cell specification

Notochord signals, in combination with signals from the neural tube, result in muscle differentiation or the expression of myoD in chick segmental plate cells cultured in vitro (Buffinger and Stockdale, 1994, 1995; Munsterberg et al., 1995; Munsterberg and Lassar, 1995; Stern et al., 1995; Stern and Hauschka, 1995). A two signal model for muscle specification has been proposed, in which notochord signals induce the first steps of specification in the paraxial mesoderm in preparation for the reception of maintenance and growth signals from the neural tube (Munsterberg and Lassar, 1995). Notochord alone produces a weak muscle differentiation response from early epithelial somites, but is insufficient to cause muscle differentiation in the segmental plate in vitro (Buffinger and Stockdale, 1994; Stern and Hauschka, 1995). Epithelial organization may thus be necessary to carry cells in the first stages of specification forward into differentiation in vitro.

The two-signal model has also been supported by experiments in vivo, showing that ablation of the neural tube prevents differentiation of muscle, but weak myoD expression is initiated if the notochord is left intact (Bober et al., 1994a). Likewise, supernumerary notochord grafts which act to suppress later differentiation of muscle precursor cells result in weak up-regulation of myoD expression nearby (Bober et al., 1994a), however this effect is seen even in the absence of neural tube (Pownall et al., 1996). These latter results have led to the hypothesis that notochord signals alone are sufficient to cause MDF member expression in the segmental plate in vivo (Pownall et al., 1996; Borycki et al., 1997).

A trio of BMP antagonists, chordin, noggin and follistatin, are expressed in the notochord and/or organizer of Xenopus embryos and have been implicated in directing the cell fate decision between epidermis and neural tissue in Xenopus (Hemmati-Brivanlou and Melton, 1997). Localized mis-expression of BMP4 in avian embryos influences the expression of genes involved in patterning and cell fate specification in the somite (Pourquie et al., 1996). Expression of follistatin in the avian notochord and somite (Patel et al., 1996; Amthor et al., 1996) suggests that BMP antagonists may also influence the differentiation of avian somite cells.

Identification of sonic hedgehog as a candidate morphogen secreted by the notochord has led to the hypothesis that it is involved in directing cell fate decisions in the paraxial mesoderm. Explant cultures of paraxial mesoderm respond to sonic hedgehog by expression of the sclerotal marker Pax1 (Fan et al., 1995; Munsterberg et al., 1995). sonic hedgehog in combination with Wnt family members has been shown to up-regulate the expression of myoD and myosin heavy chain mRNA in explants of paraxial mesoderm (Munsterberg et al., 1995). In the zebrafish, correct timing and position of myoD expression requires an intact notochord (Weinberg et al., 1996), which presumably acts to provide signalling by sonic hedgehog. Interference with negative regulation of sonic hedgehog signalling enhances the onset of myoD expression in the zebrafish (Hammerschmidt et al., 1996), and unregulated, ectopic expression of sonic hedgehog in chick embryos expands myoD expression domains in the paraxial mesoderm (Johnston et al., 1994).

A putative receptor for sonic hedgehog, patched, has recently been identified (Stone et al., 1996). patched expression, which is upregulated in response to sonic hedgehog signalling (Goodrich et al., 1996), is localized to the sclerotome of developing somites, but is not seen in the myotome or dermomyotome. Expression of two zinc finger transcription factors, Gli and Gli3, is differentially responsive to active sonic hedgehog signalling, providing a sensitive bioassay for regions of the paraxial mesoderm that receive the sonic hedgehog signal (Marigo et al., 1996). Gli expression is up-regulated in response to sonic hedgehog, and is localized to the sclerotome. Gli3 expression, which is down-regulated in response to sonic hedgehog signalling, is seen exclusively in the myotome, and is excluded from the sclerotome of developing somites (Marigo et al., 1996). These expression patterns suggest that active sonic hedgehog signalling affects the formation of the sclerotome, but not the myotome, in vivo.

Targeted disruption of the sonic hedgehog gene in the mouse embryo affects the axial and limb skeleton, but does not prevent the formation of differentiated skeletal muscle (Chiang et al., 1996). In sonic hedgehog null mice, the expression domain of Pax3 is expanded ventrally into the region of the somite that normally forms sclerotome (Chiang et al., 1996). MyoD and myf5 expression, which are believed to mark two separate muscle lineages in the mouse embryo (Braun et al., 1994; Braun and Arnold, 1996), are differentially affected by the absence of sonic hedgehog. The appearance of myf5 expression in the medial somite is severely reduced, but myoD expression in the lateral half of the somite appears to be unaffected (Chiang et al., 1996). These results argue that while the formation of a myf5-dependent medial somite lineage may be directly or indirectly dependent on the presence of sonic hedgehog, the myoD-dependent lineage, which fully rescues skeletal muscle in adult mice lacking the myf5 lineage (Rudnicki et al., 1992), is independent of sonic hedgehog sig-
nalling from the notochord. Thus, the overall consequences of sonic hedgehog signalling for skeletal muscle appear to be minimal.

Nevertheless, these considerations necessitate caution when interpreting the results of an assay which uses notochords to impose a cartilage fate on skeletal muscle precursor cells. The notochord has long been postulated to be a source of trophic factors that affect the development of surrounding tissues, in particular axial cartilage (Gordon and Lash, 1974; Teillet and Le Douarin, 1983). sonic hedgehog stimulates mitogenesis in somite explant cultures (Fan et al., 1995), and may function as a mitogen for precursor cells of the developing retina (Jensen and Wallace, 1997), providing further support for this idea. Whether the notochord acts to instruct multipotent paraxial mesoderm cells to adopt cartilage and skeletal muscle fates, or simply provides a mitogenic support for expansion of previously specified cells, it is clear that muscle precursor cells in the dorsomedial quadrant of the somite differentiate as skeletal muscle in a somite-stage-dependent fashion when challenged with notochord signals. The identification of these somite-stage-dependent differences in muscle precursor cell potential provides an embryological starting point for the characterisation of the molecular causes of formation of skeletal muscle tissues in vertebrate embryos.

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