Location and growth of epaxial myotome precursor cells

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SUMMARY

The skeletal muscle progenitor cells of the vertebrate body originate in the dermomyotome epithelium of the embryonic somites. To precisely locate myotome precursor cells, fluorescent vital dyes were iontophoretically injected at specific sites in the dermomyotome in ovo and the fates of dye-labeled cells monitored by confocal microscopy. Dyelabeled myotome myofibers were generated from cells injected along the entire medial boundary and the medial portion of the cranial boundary of the dermomyotome, regions in close proximity to the dorsal region of the neural tube where myogenic-inducing factors are thought to be produced. Other injected regions of the dermomyotome did not give rise to myotome fibers. Analysis of nascent myotome fibers showed that they elongate along the embryonic axis in cranial and caudal directions, or in both directions simultaneously, until they reach the margins of the dermomyotome. Finally, deposition of myotome fibers and expansion of the dermomyotome epithelium occurs in a lateral-to-medial direction. This new model for early myotome formation has implications for myogenic specification and for growth of the epaxial domain during early embryonic development.

Key words: myotome, somite, myogenesis, muscle, determination factor, chick, fate mapping, DiI, DiO, confocal microscopy

INTRODUCTION

The cellular mechanisms that generate skeletal muscle have been a subject of controversy for over a century. Fischel (1895) was the first to identify the dermomyotome epithelium of the somite as the source of myogenic progenitor cells. However, only recently have lineage tracing and embryo surgery experiments fully confirmed his hypotheses regarding the origin of epaxial (back) and hypaxial (limb and body wall) muscle (Reviewed in Christ and Ordahl, 1995). Briefly, somites are epithelial spheres that form on either side of the developing neural tube by budding off from the cranial end of the segmental plate, a region of the paraxial mesoderm in the posterior regions of the embryo. Epithelial somites are then patterned in response to signals received from a variety of axial and lateral tissue sources. Ventral somite cells lose their epithelial organization to form the scleromere, a mesenchyme that gives rise to the vertebrae and ribs. The remaining dorsal epithelial cap is known as the dermomyotome because it gives rise to dermis and skeletal muscle.

The cellular movements and translocations by which differentiated skeletal muscle cells emerge from progenitor cells within the dermomyotome epithelium are varied and complex. Appendicular muscle precursor cells migrate over considerable distances and times prior to differentiating (Christ et al., 1977, 1978; Williams and Ordahl, 1994; Hayashi and Ozawa, 1991). Epaxial (dorsal) muscle initially forms as a layer of mononucleate (unfused), postmitotic, differentiated myocytes immediately subjacent to the dermomyotome epithelium and is referred to as the myotome.

Fate mapping using chick-quail surgery identifies the medial half of the dermomyotome as the location of precursor cells for all epaxial muscle (Christ et al., 1978; Ordahl and Le Douarin, 1992). The precise location of myogenic precursor cells within that epithelium, however, remains controversial. Williams (1910) first proposed that myotome cells are formed along the medial lip (edge) of the dermomyotome. More recently, Kaehn et al. (1988) proposed that myotome cells emerge only from the mediocranial corner of the dermomyotome. A third hypothesis suggests that myotome cells arise from all regions of the dermomyotome epithelium simultaneously (recently reviewed in Ede and El-Gadi, 1986; Christ and Ordahl, 1995; Cossu et al., 1996). No experimental determination of the location of myogenic precursor cells within the somite dermomyotome has been performed.

We used cell fate mapping to identify the source of myogenic precursor cells within the somite dermomyotome. The dermomyotome epithelium is too small (100 μm²) for detailed fate mapping using quail-chick embryo surgery (Le Douarin, 1973). Fluorescent vital dyes, however, are well established as lineage tracers in the chicken embryo and have been shown to produce no aberrant developmental effects (Serbedzija et al., 1989; Hayashi and Ozawa, 1991; Bagnall, 1992; Hatada and Stern, 1994). We iontophoretically injected fluorescent carbocyanine dyes at specific sites in the dermomyotome and monitored these labeled cells and their progeny as they differentiated into myotome cells in ovo. In addition to identifying the precise location of early myogenic precursor cells, these results yield information about the directions of nascent myofiber growth.
and the relative age of myotome fibers. This information is potentially useful for understanding the cellular and molecular changes accompanying both myogenic specification, and growth in the embryonic epaxial domain.

MATERIALS AND METHODS

Chick embryo preparation for dye labeling

Fertilized White Leghorn chicken (Gallus domesticus) eggs were obtained from a commercial supplier and grown at 39°C, for 2 days, in a humidified incubator. Chick embryos at stages HH 13-16 (Hamburger and Hamilton, 1951) were windowed by sawing a small opening in the top of the egg shell. The embryo was then floated above shell level with Tyrode’s saline solution (Sigma) and Pelican India ink, diluted 1:6 in Tyrode’s, was injected below the endoderm to provide visual contrast for observing somites and other tissue structures. For vital dye injections, electrolytically sharpened tungsten needles were used to open the vitelline membrane. A small longitudinal incision was made through the overlying ectoderm along the groove marking the separation of the neural tube and somite. Pancreatin (Sigma), diluted 1:10 in Tyrode’s, was used to assist in the removal of the ectoderm layer, to directly expose the dermomyotome for microelectrode dye labeling. Enzyme activity was stopped with 10% fetal calf serum and tissue hydration was maintained by periodic addition of Tyrode’s saline solution over the embryo. The columnar epithelial cells of the dermomyotome were labeled with a 0.2% (weight/volume) concentration of 1,1′, di-octadecyl-3,3,3′,3′¢-tetramethylindo-carbocyanine perchlorate, DiI, or with 3, 3′-dioctadecyloxacarbocyanine perchlorate, DiO, (Molecular Probe, Inc.) solution dissolved in 100% glycofurol (Sigma). DiO crystals were initially not directly soluble in glycofurol and required prior solubilization in 100% ethanol, before additional dilution with glycofurol (1:1) to the 0.2% working stock.

Dermomyotome dye-injection sites

The process of somitogenesis is correlated with progressive morphogenetic changes in the somite as newer somites are formed caudally resulting in a maturation gradient where younger somites are located caudally and older ones are more cranial in position. A classification scheme that accounts for somite developmental age has been proposed where the newly budded somite is referred to as stage I, the next older somite is stage II, and so forth (Ordahl, 1993). Thus, according to this classification scheme, a somite can be identified and correlated with specific developmental events. The somite staging references used in this study are based upon this staging system.

Nine sites were labeled in the dermomyotome of stage VI to IX forelimb level somites of 2-day-old chick embryos, a stage of development when myotome fibers are first detected (Kaehn et al., 1988) (see Fig. 1a). A critical aspect of the procedure was labeling of small numbers of cells at precise locations. Fig. 1b-e shows several labeling sites and the size of the typical dye injection spots as visualized through the stereomicroscope used for surgery. Good injection spots were estimated to contain approximately 25 labeled cells by confocal microscopy (data not shown). Another critical aspect is the ability to deposit label within the medial lip of the dermomyotome. In order to verify the accuracy of injection site localization, transverse sections of five such injected somites were analyzed by confocal microscopy as shown in Fig. 1f.g. In each case, dye localization was restricted to the dermomyotome sites indicated.

Dye injections were performed under a dissecting microscope (Zeiss, model SV-11) using a micromanipulator (Leica) to position the microelectrode. Glass needles with tip resistances of 3 MΩ in 3 M KCl were made using a horizontal micropipette puller (Sutter, Model P-87) and glass tubing (OD = 1.2 mm; ID = 0.9 mm) with filament (Fisher). For injections, the glass needles were backfilled with the DiI or DiO working solution until the tip was filled. Microelectrodes were then made by backfilling with 3M KCl immediately before somite labeling. Electrical current was applied through a Ag/AgCl wire placed in the KCl solution and a 5 second pulse, with 90 nA of current, was used to drive the dye solution out of the microelectrode. Dye delivery by iontophoresis into the myotome of older somites completely labeled elongated myotome fibers by 1.5 hours postinjection (data not shown). However, dye labeling of younger somites from 2-
day-old chick embryos, at the stages reported above, did not result in labeled myotome fibers over this same time period, indicating that elongated myotome fibers were not yet established in these somites.

**Fluorescence imaging of dye-labeled somites**

A Zeiss SV-11 stereo dissecting microscope was modified for fluorescence or rhodamine fluorochrome detection, which allowed in vivo visualization of DiO and DiI labels, respectively. The microscope was also fitted with a thermoelectrically cooled, color video-rate CCD camera (ZVS-47 DEC, Zeiss-Optronics). A halogen light source with dual fiber optic cables was used to illuminate the embryo, and placement of interference filters in the light path yielded blue or green fluorescence excitation. In addition, the SV-11 was fitted with a beam splitter to direct 100% of light to an emission filter positioned in front of the camera for fluorescence detection. The camera had a 1/2" interline transfer HyperHAD™ CCD chip with 768 horizontal and 494 vertical active pixels and produced a resolution of 450 horizontal and 350 vertical TV lines. The camera was powered by an external power supply housed in a processor unit which controls on-chip integration, automatic or manual gain, image freeze-framing, edge sharpening, and red and blue color adjustments. Dye-labeled sites in the dermomyotome were detected by 8 seconds of signal integration under maximum camera gain.

The analog signal was exported from the camera processor unit in RGB mode and images were displayed on a Sony Trinitron color television monitor (PVM-1343MD). The images were then sent to a frame grabber board (Scion, model LG3) housed in a Macintosh IICi computer. Each image frame is 640 horizontal by 480 vertical pixel array captured at 8-bits/pixel and is stored in a temporary 4 MB memory buffer in the LG3 board until transferred to the computer. Image display and capture on the computer were controlled using the NIH-Image version 1.59 public domain imaging software (developed at the US National Institutes of Health and is available from the Internet by anonymous FTP from zippy.nimh.nih.gov or on floppy disk from the National Technical Information Service, Springfield, VA, part number PB95-500195GE1).

Embryos labeled with dye were fixed with 4% formaldehyde, either immediately or after overnight incubation. Prior to fixation, embryos were removed from the egg and cleaned of extraembryonic membrane material. After fixation, embryos were washed once in PBS and temporarily stored at room temperature until viewed by confocal microscopy. Embryos were prepared for confocal viewing by cutting the embryo in half along the neural tube and removing a segment of the embryo in half along the neural tube and removing a segment of the tissue that included the injected somite. The tissue section was then placed in a custom-made chamber well produced by layering several strips of electrical tape on a glass slide and cutting a small square through the tape. The chamber minimized tissue distortion when a coverslip was laid over the specimen. The embryo fragment was bathed in PBS and sealed in the chamber with nail polish.

DiI and DiO labeling of myotome in somites was analyzed using a Zeiss LSM410 laser scanning confocal microscope with its packaged operating system software. The dye-labeled cells were examined through a 20×/0.5 NA Plan-Neofluor objective (Zeiss). The green line (568 nm) or blue line (488 nm) of an argon-krypton ion laser was used to excite DiI- or DiO-labeled cells, respectively, and the fluorescence emission occurring at ≥610 nm (DiI) or at 515-540 nm (DiO) was collected by a photomultiplier tube. Confocal image files were 512×512 pixels and were captured in RGB channels. Confocal images were single images of cumulative optical serial sections at various depths and are described in the figure legends. NIH-Image (version 1.59), and Adobe Photoshop (version 3.0) were used for image analysis and image processing.

**RESULTS**

Somites formed at the wing level of 2-day-old chick embryos (HH-14) were chosen as targets for the present study because the most information regarding myotome formation is available for somites at this axial level (Christ and Ordahl, 1995). The earliest differentiated myotome cells (Kaehn et al., 1988) can be detected at somite stage VII (Fig. 2a). During the next 24 hours, myotome cells form that span the cranio-caudal width of each somite (Fig. 2b). Several features of the myotome at this stage of development are of interest. First, myotome cells are postmitotic, mononucleate, myofibers whose axes are aligned with the embryonic axis (Kaehn et al., 1988). As differentiated muscle fibers, myotome cells express muscle-specific proteins, such as desmin (Fig. 2b). Second, differentiated myotome fibers can be divided into two classes according to their length: (a) unit length differentiated fibers that reach from the cranial to caudal margins of the somite, and (b) intermediate length differentiated fibers that are progressively shorter at their caudal extents giving a right-angled triangle appearance to the lateral portion of the myotome (Fig. 2e). Third, myotome fibers form a third layer of tissue in the somite interposed between the dermomyotome and sclerotome (Fig. 2f).

Nine dye-labeled sites within the dermomyotome epithelium formed a grid pattern shown in Fig. 2c. Labeling at site B is diagrammatically represented in transverse section in Fig. 2d, and such site-specific labeling was experimentally confirmed by confocal microscopy (see Materials and Methods). Fig. 2e,f show diagrammatically the position of myotome and dermomyotome cells after overnight incubation as determined by confocal microscopy. Fig. 2g-i shows three images from a 20-series (2.3 μm interval) z-scan through a somite that had been labeled with DiI at position (B) in the dermomyotome the previous day. The first image (Fig. 2g) shows a punctate labeling pattern that reflects labeling of columnar epithelial cells of the dermomyotome whose long axes are perpendicular to the confocal section plane (see Fig. 2d,f). The second and third images (Fig. 2h,i) show two scans through different portions of the myotome, where labeled cells are thin and highly elongated in the caudal-caudal axis, and in the same plane as that of the confocal scan. The final image (Fig. 2j), is a composite of all 20 images in this z-series, and is the manner in which the dermomyotome fate map results will be presented. Note that the punctate pattern is superimposed over the elongate labeling. Punctate labeling was never found lateral to elongate labeling, although punctate cells often trailed medial to the elongate labeling (see Fig. 2j). Finally, dye-labeled cells were never found in the ventral somite compartment, the sclerotome, nor were dye-labeled cells from the injected somite ever found to translocate to neighboring somites in the course of these experiments.

**The fates of dermomyotome cells labeled at the nine dye injection sites**

To ascertain where myotome precursor cells are located in the dermomyotome and to monitor myotome growth, somites from 2-day-old chicken embryos were systematically injected in ovo with fluorescent vital dyes (Fig. 3; Table 1). Dye-labeled myotome cells resulted when injections were made at sites along the entire medial lip region of the dermomyotome. A myotome labeling incidence of 77%, 80% and 64% was observed for site C, site B and site A, respectively. One other position, site F, of the dermomyotome, also gave rise to myotome cells in 59% of the cases.

The other dye labeling sites (D, E, G, H and I) in the dermomyotome result in label remaining within the dermo-
myotome but never gave rise to labeled myotome cells. Injections at sites A, B, C and F also resulted in labeled dermomyotome cells whose position overlapped the correspondingly labeled myotome and often trailed medially. Labeled dermomyotome cells were never found lateral to labeled myotome cells. Finally, injection site (I) gave rise to small numbers of labeled migratory cells enroute to the adjacent limb bud consistent with previous studies (Christ et al., 1977, 1978).

**Simultaneous injection at different sites**

The above results indicated that myotome precursor cells arise along the entire medial lip and the medial portion of the cranial lip of the somite dermomyotome, and not solely at the mediocranial corner (site C) as predicted by a recent model for myotome formation (Kaehn et al., 1988). To determine if myotome precursor cells translocate from other sites to site C before initiating myotome formation, we performed simultaneous injections of different colored dyes.

Myotome fibers resulting from simultaneous injection at sites C and F maintain the same relative and non-overlapping position (Fig. 4a-c). Labeled dermomyotome cells from both injection sites were also positionally conserved relative to their initial injection sites. A trailing pattern of a few dermomyotome cells labeled at site F overlapped site C-labeled myotome fibers, but never vice versa.

Simultaneous injection at sites A and C, on the other hand, yielded a partially over-lapping population of unit-length fibers laterally, with shorter fibers medially that were non-overlapping because they were restricted to the caudal or cranial halves of the somite, respectively (Fig. 4d-f). The restriction of the shorter fibers to the same region of the somite as the original injection site indicates that myotome fiber elongation and differentiation was initiated at the injection site and proceeded in a cranial (for site A) or caudal (for site C) direction (see below). The false color image of the overlap region (Fig. 4f) shows no dye mixing (yellow color) that would be expected if dye was transferred from one cell to another, or if myotome cells were fusing. As above, labeled dermomyotome cells were positionally conserved relative to the initial injection sites. Simul-

![Fig. 2](image)

**Fig. 2.** A 2-day-old chick embryo, somite desmin expression, illustrations of dermomyotome vital dye labeling and confocal microscopy analysis of dye-labeled myotome fibers. (a) An image showing a 27-somite embryo (HH 15) and a stage VIII somite injected with vital dyes. Forelimb level somites are indicated by the white bar. Somite size increases in the medial-lateral axis as newly formed epithelial somites (stage I) become compartmentalized into sclerotome and dermomyotome layers (stage VIII), and later, express elongated myotome fibers (stage XV). (b) Thoracic level somites (stages XI to XV) in a 35-somite embryo labeled with desmin monoclonal antibody. A right-angle myotome growth pattern is visible in somites where myotome completely spans the somite medially, but are progressively shorter as the myotome fibers extend laterally along the cranial edge of the dermomyotome. Arrowheads mark the most caudal extent of myotome fibers contributing to their right-angled triangle appearance in the somite. (c-f) Illustrations of dermomyotome vital dye labeling. (c) A dorsal view of a stage V somite and site B labeling in the dermomyotome. (d) A transverse section view of the somite shows that labeled cells from site B labeling were in the medial lip area of the dermomyotome. (e) Site B injections resulted in labeled myotome (filled rods) and dermomyotome cells (filled circles). (f) A transverse section showing the labeled myotome and dermomyotome cells, migrating myogenic precursor cells (mm) exiting the lateral dermomyotome, and the direction of confocal optical scans, in z-series, through the dorsal half of the somite (large arrow). (g-j) DiI injections at site (B) in two adjacent forelimb level somites result in labeled myotome fibers after overnight embryo growth. One somite is shown in its entirety and a partial view of the other somite is visible. (g) A confocal scan showing only punctate dermomyotome cell labeling with some labeling of the ectoderm over the neural tube region. Confocal scans progressively deeper into the somite show initially dermomyotome and myotome labeling (h), and later, only labeled myotome (i). No neural tube labeling was seen in these deeper confocal sections. (j) An image resulting from the compilation of 20 confocal scans at 2.3 μm intervals. In each panel, and in subsequent figures, the cranial direction is to the right and dorsal is at the top of the displayed image. The asterisk indicates the somitocoel cavity; nt, neural tube; nc, notochord; mt, myotome; sc, sclerotome; dm, dermomyotome.
taneous injection at sites A, B and C with two differently colored dyes (Fig. 4g-i) resulted in three partially overlapping populations of mature fibers, whose overlapping lateral-to-medial relationship was C>B>A.

We conclude that myotome precursor cells reside along the entire medial lip and the medial half of the cranial lip of the dermomyotome. Precursor cells do not translocate prior to initiating myotome cell elongation and differentiation. Moreover, myotome cells can initiate elongation and differentiation at all active sites simultaneously.

**Age dependence in myotome fiber deposition**

In each of the experiments described above, unit length myotome fibers were always located lateral to shorter myotome fibers. Since nascent myotome fibers must elongate to span the entire somite, we assume that shorter fibers represent myotome fibers in the process of elongation. For example, labeled myotome from injection at site A describes a right triangle with one short side represented by the caudal border, a second short side by unit length myotome fibers that span the somite, and a hypotenuse represented by the tips of the progressively shorter myotome cells (Fig. 3a). This triangular medial myotome labeling pattern can also be seen for labeling at site C (see Fig. 3c). We interpret this right-triangular pattern as reflecting the relative maturity of nascent myotome fibers in which the shorter (younger) fibers always reside medial to the longer (older) fibers. Taken together, these observations lead to the conclusion that myotome fiber ages increase in a medial-to-lateral direction.

To directly assess the timing of myotome fiber arrival in the myotome, we labeled the mediocranial corner of the somite (site C) twice; first with DiO and then, 7 hours later, with a second injection of DiI (Fig. 5). After incubating the embryo overnight, the first injection with DiO produced labeled myotome fibers completely spanning the lateral region of the somite (Fig. 5b). The second injection with DiI produced labeled myotome fibers that were located more medially (Fig. 5c). The myotome fibers resulting from each injection were non-overlapping (Fig. 5d). In contrast, trailing dermomyotome (punctate) labeling with DiO partially overlapped the DiI-labeled region, but not vice versa. These results indicate that the lateral-medial position of myotome fibers reflect their relative age.

**DISCUSSION**

The experiments reported here provide the first detailed fate map for the dermomyotomes of forelimb-level somites at early stages of chick development. That fate map shows that myotome cells arise from cells along the cranial and medial edges, or lips of the dermomyotome (see Fig. 6). This restricted location of early myotome precursor cells eliminates earlier hypotheses that myotome precursor cells are located: (1) over the entire area of the dermomyotome (see also below), (2) in all of the lips of the dermomyotome or (3) within the subjacent sclerotome. Our results are consistent with previous suggestions that the medial lip of the dermomyotome is the site of intense myogenic activity (Williams, 1910; Holtzer and Detwiler, 1953; Cossu et al., 1996). Our results are also in accord with those of previous surgical experiments. First, dye-labeled sclerotome cells were never observed after dermomyotome injection, consistent with previous conclusions that dermomyotome fates include muscle and dermis but not cartilage, a sclerotome derivative (Christ et al., 1978). Second, after labeling in the craniolateral lip region (site I) of the dermomyotome, cells were observed that had

**Fig. 3.** Representative examples of resulting myotome and dermomyotome cell labeling from dye-injections at the nine dermomyotome sites. Myotome fiber labeling resulted from injections at sites A-C and F, which were in the dermomyotome medial lip area and at the cranial midline (see Table 1). In contrast, no myotome fiber labeling occurred when injections were made at sites D,E,G-I. (a-c) Dye-labeled myotome fibers resulting from injections along the entire medial lip were always lateral to labeled dermomyotome cells. (h) Double labeling of the somite resulted in myotome labeling only at site B, but not at site H (see arrow). (i) Dye injections at site I resulted in labeled dermomyotome and migratory myogenic precursor cells shown here moving into the developing forelimb (see arrow). In a and f, fluorescent cells in neighboring somites resulted from separate dye-injections in these somites.
translocated towards the adjacent limb bud, consistent with previous observations about the migration of lateral-half somite cells to the limb (Christ et al., 1977, 1978; Ordahl and LeDouarin, 1992; Williams and Ordahl, 1994). Finally, cells labeled by injections in other dermomyotome sites (D, E, G and H) remained within the dermomyotome epithelial layer and did not contribute to either the myotome or any other somitic structures during the course of the experiment. Such cells would be expected to contribute to muscle and/or dermis at later stages of somite development (Christ and Ordahl, 1995).

Translocations of myotome precursor cells

Myotome precursors within the epithelial layer of the dermomyotome must translocate into the subjacent myotome layer (Fig. 6). The mechanism of this translocation is unknown but the location of precursor cells within the medial and mediocranial lips suggests that translocation may occur over a short distance since the lip regions of the dermomyotome are recurved ventrally, towards the myotome layer.

The possibility that myogenic precursor cells might first translocate to a single site within the dermomyotome epithelium before translocation to the myotome layer was analyzed carefully because an earlier study of myotome formation showed that the first myotome cells emerge only at the corner of the dermomyotome where the medial and cranial lips meet (Kaehn et al., 1988). We wanted to know, therefore, if myotome precursor cells labeled for example at either site A or F would translocate to the mediocranial corner (site C) before initiating elongation into mature myotome fibers. In several experiments to test this hypothesis (Fig. 4), we failed to identify such translocations. We conclude that, after the initiation of myotome cell generation at the mediocranial corner, the entire medial and mediocranial portions of the dermomyotome lips actively generates myotome cells.

Our results do suggest, however, that the cranial half of the medial lip continues to be the region of most intense myogenic activity for two reasons. First, labeling at these sites (B,C) gave the highest incidence of myotome fiber labeling (Table 1) and,

![Fig. 4. Labeled myotome fibers result from multiple dye injections along the entire medial lip region and the cranial midline of the dermomyotome, and myotome fiber growth proceeds simultaneously in cranial-ward and caudal-ward directions. (a-c) Confocal image of labeled myotome and dermomyotome cells following 13 hours of embryo growth after injecting sites C and F, in two adjacent somites (stages XI and XII), in a 23-somite embryo. (a) DiI- and (b) DiO-labeled cells are shown in a single monochrome image resulting from the compilation of 8 parasagittal optical sections taken at 2.5 μm intervals. (c) A false-color image of combined DiI and DiO monochrome images shows that the resulting labeled myotome fibers maintain their relative positions to each other in the somite (n=7). (d-f) Confocal images of labeled myotome and dermomyotome cells following 17 hours of embryo growth after injecting somite stage VIII, at sites A and C, from a 26-somite embryo. (d) DiI- and (e) DiO-labeled cells are shown in a single monochrome image resulting from the compilation of 19 and 15 parasagittal optical sections, respectively, taken at 2.5 μm intervals. (f) A false-color image of combined DiI and DiO monochrome images shows labeled myotome that completely span the somite located laterally. Also, myotome fibers, located medially in the somite, appeared to become...
Fig. 5. Myotome formation in the somite is medial in direction. Confocal images of a DiO- and DiI-injected stage VIII somite from a 25-somite embryo labeled at the same relative mediocranial position (site C) in the dermomyotome, but separated by 7 hours between the first and second dye injections. (a) An illustration showing the first injection with DiO (solid asterisk) and the second injection, 7 hours later, with DiI (open asterisks). (b) DiO- and (c) DiI-labeled cells are shown in a single monochrome image resulting from the compilation of 7 and 12 parasagittal optical sections, respectively, taken at 2.5 μm intervals. (d) A false-color image of combined monochrome images for DiO, DiI and the transmitted light image of the specimen (image not shown). Dye-labeled myotome fibers from the first (DiO) and second (DiI) injections were laterally and medially located in the somite (n=5), respectively.

Fig. 6. A model for myotome formation and epaxial growth. (a-d) A diagrammatic representation of the results of dye injection fate mapping of the somite dermomyotome. (a) A dorsal view of a stage VII somite and the grid of nine injection sites employed in these experiments. For purposes of illustration, sites A and C are colored blue and red, respectively. (c) A transverse section through the center of the same somite (see dashed line, a) that intersects positions B, E and H. (b,d) Equivalent views of the same somite at stage XV, after approximately 12 hours incubation. Myotome fibers in a layer immediately subjacent to the dermomyotome and are colored blue or red, respectively, corresponding to the labeling of their precursor cells at positions A and C in the medial lip of the dermomyotome at somite stage VII (a). Black lines in b and black circles in d represent myotome fibers that began to form prior to the time of injection. In b, the central portion of the dermomyotome epithelium is not shown to reveal the underlying myotome. In c and d, both the myotome and dermomyotome layers are shown. In a-d, the line x indicates the length of the mediolateral span of the dermomyotome while the arrow i shows the incremental growth of the dermomyotome after 12 hours of additional embryo development (b,d). (e) A 3-dimensionalized view of the myotomal layer towards its ventral surface and illustrates three stages in the translocation of myotome precursor cells from site B within the medial lip of the dermomyotome into the myotome layer and their subsequent maturation. In the first stage (arrow 1), dermomyotome cells within the medial lip translocate to the myotome layer concomitant with medial-directed extension of the dermomyotome (arrow labeled ‘growth’). In the second stage, myotome precursor cells elongate simultaneously in a cranial and caudal direction (short black arrows). In the third stage, unit length myotome fibers span the somite connecting the cranial and caudal lips of the dermomyotome. During stage 1, myotome precursor cells express myoD but not pax-3 (Williams and Ordahl, 1994). Elongating (stage 2) and unit-length myotome fibers express differentiated muscle markers such as desmin (Kaeah et al., 1988) and myosin (not shown). Myotome precursor cells and fibers remain mononucleate during all three stages. (e) Color coding: green, lip regions of the dermomyotome where myogenic precursor cells are located; grey, dermomyotome and caudal lip areas that do not contain immediate precursors of the myotome; orange, myotome precursor cells in the medial lip of the dermomyotome and elongating and unit-length myotome fiber cells derived thereof; light blue, myotome layer of the somite.
second, myotome fibers labeled at site C were always located more laterally than those simultaneously labeled at more caudal sites (A, B) (see Fig. 4g-i) indicating that the birthrate of myotome fibers is highest at site C (see also below). Thus, the mediocranial corner of the dermomyotome may be a focal site for the activity of extrinsic or intrinsic factors governing myogenic specification.

Myotome fiber growth

Nascent myotome cells elongate under the dermomyotome and are parallel to the axis of the embryo until they span the caudocranial width of the somite (Fig. 6). In all the dye labeling experiments reported here, unit-length (presumptive mature) myotome fibers were found lateral to shorter-than-unit length (presumptive growing) myotome cells. Similarly, the shortest (youngest) labeled myotome cells were always positionally conserved, relative to the original site of injection. In addition, the increasing length of myotome fibers lateralward described a triangular shape that we interpret as reflecting the progressive elongation of myotome fibers. Based upon this interpretation, we conclude that myotome cell extension can occur in cranial, or caudal or both directions simultaneously (see Fig. 6). The mechanism(s) of myotome fiber elongation is unknown.

Relative age and position of myotome fibers

The lateral and medial location of the longest and shortest labeled myotome cells, respectively, suggested that differentiated muscle cells appear within the myotome in a lateral-to-medial sequence. Two color dye injections separated by 7 hours at the equivalent site unequivocally established the relative myotome fiber age proceeds from medial to lateral. That conclusion is inconsistent with the results presented here and we conclude that the triangular pattern of the lateral-most myotome cells reflects phenomena unrelated to relative fiber age. A possible explanation for the triangular pattern of the lateral-most (oldest) myotome fibers could be related to the position of the somitocoel. Initially, the somitocoel lies under the entire dermomyotome (see Fig. 2d), but later becomes caudolaterally restricted in a shape complementary to the lateral fibers of the early myotome (see Fig. 6). Differentiated myotome fibers are always located medial to the somitocoel in transverse section (see Williams and Ordahl, 1994, for example), suggesting that myotome cells can neither penetrate the somitocoel nor bypass it ventrally or dorsally. We hypothesize that the relatively static triangular pattern of the earliest fibers at the lateral boundary of the primary myotome reflects restricted elongation in this region, possibly due to impedance by the somitocoel.

The dermomyotome as an epaxial growth center

Somite dermomyotomes elongate along their mediolateral axes in concert with the regional growth of the epaxial domain in the early embryo (see, for example, Fig. 2a). The dermomyotome injection experiments reported here indicate that such growth is medial-directed. First, dermomyotome cells resulting from labeling at any of the nine sites were always

Table 1. Somite dermomyotome injection grid and myotome labeling

<table>
<thead>
<tr>
<th>Somite site labeled</th>
<th>Embryo age in somite</th>
<th>Somites stage (labeled)</th>
<th>Myotome labeled</th>
<th>Myotome not labeled</th>
<th>Incidence of labeling</th>
<th>Total number</th>
</tr>
</thead>
<tbody>
<tr>
<td>A 24±3</td>
<td>VII±I</td>
<td>64% (n=9)</td>
<td>36% (n=5)</td>
<td>1.8</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>B 24±3</td>
<td>VII±I</td>
<td>80% (n=32)</td>
<td>20% (n=8)</td>
<td>4</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>C 24±2</td>
<td>VII±I</td>
<td>77% (n=44)</td>
<td>23% (n=13)</td>
<td>3.4</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>D 21±2</td>
<td>VII±I</td>
<td>0% (n=0)</td>
<td>100% (n=8)</td>
<td>0</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>E 21±2</td>
<td>VII±I</td>
<td>0% (n=0)</td>
<td>100% (n=6)</td>
<td>0</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>F 25±3</td>
<td>VIII±I</td>
<td>59% (n=19)</td>
<td>41% (n=13)</td>
<td>1.5</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>G 22±1</td>
<td>VIII±I</td>
<td>0% (n=0)</td>
<td>100% (n=3)</td>
<td>0</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>H 21±1</td>
<td>VII±I</td>
<td>0% (n=0)</td>
<td>100% (n=4)</td>
<td>0</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>I 22±1</td>
<td>VII±I</td>
<td>0% (n=0)</td>
<td>100% (n=4)</td>
<td>0</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

*Somite dermomyotome dye labeling sites correspond to the letter positions shown in the diagram below; embryo age and somite stage are shown as mean ± s.d.; incidence of labeling refers to the number of examples of dye labeled myotome/unlabeled myotome; dye injected embryos were incubated 28 hours before being analysed.

Somite dye injection sites:
displaced lateral-ward but were never displaced medial-ward. Second, dermomyotome cells labeled from injection sites along either the caudal or cranial lip were never appreciably displaced cranially or caudally, respectively. Third, cells injected along the lateral edge either remained in situ or underwent lateral migration. Fourth, large numbers of medial lip cells are leaving the dermomyotome to be deposited in the myotome layer and the dermomyotome cells resulting from medial lip injections were always superimposed over or medial to the corresponding myotome fibers. Fifth, unlabeled myotome fibers and dermomyotome cells always appeared medial to their labeled counterparts after long incubation times.

Taken together, these results indicate that net growth of the somite dermomyotome epithelium occurs principally along its medial lip and, as a result, dermomyotome expansion occurs at this site and in a medial direction (Fig. 6). That this region is also simultaneously involved in myotome cell deposition suggests that the medial expansion of the dermomyotome and the medial deposition of new myotome fibers may be linked processes. Such expansion at this site is in accordance with earlier proposals regarding the process of myogenesis in the somite (Williams, 1910; Holtzer and Detwiler, 1953). We hypothesize that this medial-directed expansion of the dermomyotome, in concert with the already established directed expansion pattern of the neural tube dorsally (Fowler and Watterson, 1953; Gasser, 1979), is responsible for the growth of the epaxial domain of the early embryonic body. The control of that expansion would therefore be expected to affect, and possibly determine, the early growth pattern in the epaxial domain of the embryo.

The results of these experiments cannot distinguish whether such medial growth is a consequence of (1) relatively intense mitotic activity within a confined region, such as the medial lip of the dermomyotome, or (2) general mitotic activity in the dermomyotome with medial directed growth resulting from escape of myotome precursor cells medially and/or the site-restricted breakdown of cell-cell adhesion along the medial border. In the latter case, myogenic precursor cells would be recruited from the expanding dermomyotome population because of their position. The former case, on the contrary, predicts that the medial lip contains an expanding population of myogenic stem cells that grow medial-ward as they deposit nascent myotome cells. Further experimentation will be required to distinguish between these possibilities.

**Gene expression patterns in the paraxial mesoderm and the somite dermomyotome**

The analysis of myogenic specification in flies implicates specific transcription factors and cell signaling molecules in the generation of small cellular clusters that intercommunicate giving rise to myogenic stem cells (Jan and Jan, 1993). Vertebrate counterparts of many of the same molecules are implicated in vertebrate myogenic specification, particularly transcription factor members of the MyoD family of genes (MDFs) (Reviews: Weintraub et al., 1991; Olson, 1992; Buckingham, 1993). For example, mRNA encoding the mouse MDF, *myf-5*, is expressed in the medial lip of the early mouse dermomyotome (Ott et al., 1991; Smith et al., 1994). Similarly, *gmyf-1*, the quail equivalent of mammalian *myoD1* is expressed in the medial lip of the quail dermomyotome (Pownall and Emerson, 1992). This localized medial lip expression of MDFs is consistent with the hypothesis that cells within the medial lip of the dermomyotome are the direct precursors of myotome muscle (Williams, 1910; Holtzer and Detwiler, 1953; Cosso et al., 1996). Interestingly, homozygous knockout of the *myf-5* locus in mice yields a phenotype that includes absence of early myotome development and reduced muscle mass in neonates (Braun et al., 1992) consistent with ablation of the early myotome lineage in these mutants.

Dermomyotome cells immediately adjacent (lateral) to the medial lip are negative for MDF gene expression but are positive for expression of Pax-3 (Williams and Ordalh, 1994), an unrelated transcription factor whose expression marks early somite lineages (Bober et al., 1994; Goulding et al., 1994). It will be interesting to determine how the interface between MDFs and Pax-3 gene expression might be related to the medial-directed growth of the dermomyotome and/or the specification of myogenic precursor cells.

The early myotome also shows regionalized differences in gene expression (Smith et al., 1994). Based upon the results presented here, we interpret those regionalized differences in gene expression to be related to myotome fiber age. Similarly, the recent suggestion that *MyoD* and *MRF4* expressing myotome fibers represent different waves of myogenesis (Patapoutian et al., 1995) is also consistent with the results presented here. Such regionalized expression of contractile protein isoforms in the early somite myotome may yield information about the early steps in myogenic differentiation that were previously addressable only in vitro (Lin et al., 1994).

**General applicability of this model**

These fate mapping experiments permit conclusions to be made about the source, direction of elongation and relative age of the early primary myotome fibers in forelimb-level somites of the 2-day-old chick embryo. These myotome fibers give rise to the muscles of the back, specifically muscles of the epaxial domain of the body (Ordalh and Le Douarin, 1992; Ordalh, 1993). Epaxial muscles are formed by most, if not all, somites in the embryo. The primary myotome of somites at other axial levels (i.e. cervical, thoracic, abdominal, leg and tail levels) of the chick embryo are similar in morphological appearance to that of the forelimb level somites and show similar patterns of expression of key genes in myogenic specification, such as *MyoD* and *Pax-3*. We predict, therefore, that myotome formation in somites at other axial levels will be essentially similar (or identical) to that described here.

The muscles of the limbs and body wall (hypaxial muscles), on the contrary, form from cells located in the lateral region of the somite (Ordalh and LeDouarin, 1992; and C. P. O, unpublished observations). The lateral dermomyotome cells of forelimb- and hindlimb-level somites migrate to the developing limb buds and form muscle as a mesenchyme (outlined in Introduction), by mechanisms that are incompletely understood but which may be fundamentally different from the epithelial-myocyte transitions described here. The body wall muscle at thoracic and abdominal levels, however, begins to form under the lateral lip of the somites in a manner essentially identical, but mediolaterally inverted from, that described here (Christ et al., 1983; Tosney et al., 1994; Grothe et al., 1996).

We hypothesize that the process of body wall (hypaxial) muscle formation at these axial levels is essentially identical to that described here for epaxial muscles. One important difference, however, is the opposite orientation of the growth pattern.
Thus, for body wall muscle, relative ages would increase from medial to lateral. Moreover, if this prediction is correct, the later growth in the hypaxial domain of the embryo may be driven by lateral-ward expansion of the dermomyotome and myotome in a manner essentially similar (or identical) to that proposed above for epaxial growth.

Finally, somitogenesis in mammalian embryos shows many similarities to that of avian embryos, with some differences in the sequence of expression of MDFs (Buckingham, 1993). Moreover, mouse somites have been shown to survive and differentiate well after transplantation into chick embryos (Fontaine-Perus et al., 1995). We predict, therefore, that myotome formation as described here for avian embryos will be essentially similar in mammalian embryos. Such may not be the case for amphibian (Kielbowna, 1981) and fish (Weinberg et al., 1996) embryos, however, where the cellular movements of early muscle progenitors are substantially different.

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