The roles of cell migration and myofiber intercalation in patterning formation of the postmitotic myotome

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SUMMARY

We have previously found that the postmitotic myotome is formed by two successive waves of myoblasts. A first wave of pioneer cells is generated from the dorsomedial region of epithelial somites. A second wave originates from all four edges of the dermomyotome but cells enter the myotome only from the rostral and caudal lips. We provide new evidence for the existence of these distinctive waves. We show for the first time that when the somite dissociates, pioneer myotomal progenitors migrate as mesenchymal cells from the medial side towards the rostral edge of the segment. Subsequently, they generate myofibers that elongate caudally. Pioneer myofiber differentiation then progresses in a medial-to-lateral direction with fibers reaching the lateralmost region of each segment. At later stages, pioneers participate in the formation of multinucleated fibers during secondary myogenesis by fusing with younger cells. We also demonstrate that subsequent to primary myotome formation by pioneers,

growth occurs by uniform cell addition along the dorsoventral myotome. At this stage, the contributing cells arise from multiple sources as the myotome keeps growing even in the absence of the dorsomedial lip. Moreover, as opposed to suggestions that myotome growth is driven primarily and directly by the medial and lateral edges, we demonstrate that there is no direct fiber generation from the dorsomedial lip. Instead, we find that added fibers elongate from the extreme edges. Altogether, the integration between both myogenic waves results in an even pattern of dorsoventral growth of the myotome which is accounted for by progressive cell intercalation of second wave cells between preexisting pioneer fibers.

Key words: Avian embryo, Cell delamination, Desmin, Dermomyotome, Epithelial-mesenchymal conversion, Myoblast migration, Pioneer myoblasts, Somite

INTRODUCTION

In the early embryo, muscle progenitor cells arising in the somites differentiate into myofibers that give rise to the transient embryonic myotome. The myotome then develops into skeletal muscles that include epaxial, intercostal and abdominal components. Thus, the anatomy of skeletal muscles in vertebrates results from complex patterning and morphogenetic processes that can be already evidenced during embryogenesis. The mechanisms that underlie initial myotome formation and subsequent growth have been investigated intensively during recent years; these encompass the elucidation of tissue interactions and signal transduction pathways that initiate myogenesis (for a review, see Borycki and Emerson, 2000), the identity and function of myogenic regulatory factors (MRFs) (reviewed by Tajbakhsh and Buckingham, 2000), and the actual origin and cellular mechanisms that govern myotome ontogeny (Brand-Saberi and Christ, 1999; Cossu et al., 1996b; Currie and Ingham, 1998; Kalcheim et al., 1999). The latter aspect is particularly intriguing, as in addition to providing an excellent experimental system for the study of cell migration,

differentiation and morphogenesis, precise knowledge of the mechanisms of myotome formation is essential for correct interpretation of the function played by MRFs and by other associated genes.

The somite-derived dermomyotome has been identified as the major source of myotomal fibers and of limb muscle (Christ et al., 1978). However, the precise timing, origin and mode of colonization of the myotome remained unclear and were the subject of numerous studies. Based on observation of sectioned material, it was proposed that myotome cells arose from the medial edge of the dermomyotome (Williams, 1910; Ede and El-Gadi, 1986; Boyd, 1960; Hamilton, 1965). Langman and Nelson (Langman and Nelson, 1968) suggested instead that the entire dermomyotomal sheath gave rise to myocytes by delamination, and Christ et al. (Christ et al., 1978) proposed that the myotome was formed from cells that originated in all four inwardly curved lips of the dermomyotome. In a later study based on desmin and acetylcholinesterase localization, Christ and colleagues (Kaehn et al., 1988) identified the rostromedial corner of the dermomyotome as the initiation site of myogenesis. Myofibers were then shown to arise from along the entire rostral edge and elongate caudally. Thus, a model

involving progressive fiber differentiation in both rostrocaudal and mediolateral directions was proposed.

In our previous studies, we have distinguished two separate waves in the formation of the postmitotic myotome, based on spatial and temporal considerations. A first wave originates along the entire dorsomedial region of the somite while it is still epithelial, in a group of progenitors that express MyoD and Myf5 and consequently become the first postmitotic cells in the somite (Kahane et al., 1998a). Upon somite dissociation, they first bend underneath the dermomyotome, delaminate and relocate from their source towards the rostral somitic domain. From this region, myofibers are generated in a rostral-to-caudal direction leading to formation of a primary myotome that spans the entire dorsomedial to ventrolateral extent of each segment (Kahane et al., 1998a). Because they are the first myoblasts to form a unit-length myotomal structure, we called them pioneer cells.

A second wave of myoblasts follows and even slightly overlaps in time the formation of the primary myotome by the pioneer cells. This wave accounts for growth of the pre-existing structure and emanates from all four dermomyotome lips (Kahane et al., 1998b; Cinnamon et al., 1999). Recent quail-chick grafting experiments fully confirmed our data by showing that the myotome is furnished by progenitors residing in all four edges of the dermomyotome (Huang and Christ, 2000). Further evidence from our laboratory has distinguished between the mechanisms by which cells from the different lips enter the myotome. We have reported that cells from the rostral (R) and caudal (C) lips directly generate myofibers in a direction that is parallel to the pre-existing pioneers (Kahane et al., 1998b). By contrast, muscle precursors from along the dorsomedial lip (DML) and ventrolateral lip (VLL) first delaminate into an intermediate zone between these epithelia and the myotome, that we termed the sub-lip domain (SLD), and migrate longitudinally through this pathway as mesenchymal cells towards the R and C lips. Only when reaching the latter edges, they begin differentiating into myofibers and colonize the myotome by intercalating among pre-existing fibers, as part of the second wave (Cinnamon et al., 2001). These experimental findings support a model by which the myotome expands as a whole in the mediodorsal to ventrolateral extent containing both old and younger fibers all along.

At variance with our views, Denetclaw et al. and Denetclaw and Ordahl have suggested that the DML drives medial growth of the myotome and that the VLL is responsible for myotome expansion in a ventral direction (Denetclaw and Ordahl, 2000; Ordahl et al., 2001). This model predicts an incremental mechanism of myotome growth by which the oldest part of the myotome remains centrally located and the youngest fibers are added towards both extreme dorsomedial and ventrolateral regions. Moreover, based on ablation and back-grafting experiments, it was inferred that the DML is both necessary and sufficient to drive epaxial growth of the myotome (Ordahl et al., 2001). Further DiI tracing experiments also suggested that the mechanism by which the DML provides cells to the myotome is by direct cell translocation from any point along the epithelium into the myotome layer followed by differentiation with no prior longitudinal translation (Denetclaw et al., 1997; Denetclaw et al., 2001). Hence, these data predict that myofibers elongate bi-directionally in situ rendering at any time a group of partial-length fibers arranged in a staggered fashion and localized in an intermediate region between DML and dorsalmost unit-length fibers of the myotome (Denetclaw et al., 2001).

Therefore, several basic issues concerning the mechanisms of myotome formation still remain controversial. These comprise the nature of the contribution of the medial edge of the segment (medial epithelial somite versus later DML), the role of cell migration in myotome morphogenesis and, finally, whether overall myotome growth is incremental or, alternatively, of an intercalatory nature. The present study was carried out to further resolve these discrepancies. We demonstrate, first, that pioneer myotomal progenitors migrate as mesenchymal cells from the medial side of the somite to its rostral edge and then generate myofibers that elongate caudally; thus, we further substantiate that migratory movements are an integral part of the formation of the primary myotome (Kahane et al., 1998b; Cinnamon et al., 1999). Second, differentiation of the earliest myofibers, the pioneers, progresses in a dorsomedial-to-ventrolateral direction generally consistent with the proposal by Kaehn et al. (Kaehn et al., 1988) but not with Ordahl and colleagues (Ordahl et al., 2001). Third, differentiated pioneer myofibers attain the ventralmost region of each segment which later elongates into the somatopleure and gives rise to intercostal/abdominal muscles. Hence, medial somite-derived pioneers also participate in the formation of hypaxial body wall muscles (Cinnamon et al., 1999), suggesting that there is no strict separation of epaxial and hypaxial lineages as far as the first wave is concerned. Fourth, pioneers participate in the formation of multinucleated fibers during secondary myogenesis by fusing with younger cells. Fifth, we directly demonstrate that addition of new cells to the growing myotome occurs uniformly along its entire dorsoventral aspect, even in the absence of the DML. In addition, we show that there is no direct addition of myofibers from the DML, as no partial-length fibers are found adjacent to this lip. Alternatively, they elongate from the extreme R and C edges. Therefore, as the DML is not the primary source of cells contributing to myotome growth, incremental epaxial growth driven by the DML, as proposed by Ordahl et al. (Ordahl et al., 2001), cannot solely account for myotome expansion. Instead, a model of intercalatory growth driven by the four dermomyotome lips accounts for all experimental findings.

MATERIALS AND METHODS

Embryos
Fertile quail (*Coturnix coturnix Japonica*) eggs from commercial sources were used in this study.

Whole-mount desmin immunostaining
Twenty five- to 32-somite quail embryos were removed from the eggshell, washed in phosphate-buffered saline (PBS, pH 7.4) and fixed in 4% formaldehyde in PBS for 3 hours at room temperature. Blocking of nonspecific staining and tissue permeabilization were performed for 3 hours in PBS containing 10% fetal calf serum and 0.5% Triton X-100. This solution also served for diluting antibodies and subsequent washes. Monoclonal anti-desmin antibody (1:20) and a secondary antibody (FITC-conjugated goat anti-mouse, 1:100) were

from Sigma (St Louis, MO). All incubations and washes were performed overnight at 4°C on a vertical shaker, yielding very specific immunolabeling patterns.

CM-DiI labeling of pioneer myofibers

The dorsomedial quarter of epithelial somites was labeled with CM-DiI as previously described (Kahane et al., 1998a; Cinnamon et al., 2001). Experiments were performed in interlimb-level segments (22-27) of embryos aged 25- to 28-somite pairs. In the first experimental series, a discrete spot of dye was applied iontophoretically to the caudalmost part of the medial somite (see Fig. 2A). In the second experimental paradigm, a relatively large focus of cells resulting from repetitive dye applications was labeled in the center of the medial somitic aspect midway between adjacent intersomitic clefts (see Fig. 3A). Embryos were reincubated for periods ranging between 8 and 36 hours prior to fixation. Whole-mount analysis and histological processing were performed as previously described (Cinnamon et al., 2001).

DiI labeling of fixed tissue

To examine the length of the medialmost fibers of the myotome, a combination of DiI labeling of fixed tissues with whole-mount desmin immunostaining was preformed. Thirty two- to 34-somite quail embryos were fixed in 4% formaldehyde in PBS for 3 hours at room temperature. After fixation, a unilateral row of paraxial mesoderm was microdissected free of sclerotome, notochord and neural tube. The tissue was pinned medial side up in a silicon-containing dish filled with PBS and placed under a stereoscope with epifluorescent attachment. Borosilicate tubes with filament (OD=1.0 mm, ID=0.5 mm) were pulled using a vertical puller (Sutter model P-30). The tip was broken to an estimated outer diameter of 2 μm. Micropipettes were backfilled with 1,1′, di-octadecyl-3, 3,3′,3′-tetramethylindo-carbocyanine perchlorate (DiI, Molecular Probes, 0.25% in ethanol). DiI injections were performed using air pressure, directed to the mediorostral or mediocaudal edges of the myotomes. Alternating myotomes were labeled at somitic levels 8 to 25. Residues of DiI solution were thoroughly washed out and tissue fragments incubated overnight at 38°C. During this incubation period, the lipophilic dye diffuses through the plasma membrane of the myofiber from the labeled edge to the other extreme. Tissues were then immunostained with desmin antibodies as described above.

Laser-scanning confocal microscopy

Laser-scanning confocal microscopy and digital imaging were preformed as already described (Cinnamon et al., 2001).

Detection of post-mitotic pioneers by [³H]thymidine labeling

Two pulses of [³H]thymidine (10 μCi), 5 hours apart, were delivered to quail embryos aged 22 somites. Embryos were then reincubated for additional 6 days until secondary myogenesis was well under way. After fixation in 4% formaldehyde, embryos were embedded in paraffin wax, sectioned frontally at 8 μm, immunostained for desmin and processed for autoradiography as described (Kahane et al., 1998a). Cells that continued dividing actively during the reincubation period efficiently diluted the radioactive metabolite and became unlabeled. By contrast, the early postmitotic cells corresponding to pioneer myoblasts, retained the label over their nuclei. At the time of labeling or shortly thereafter, pioneer myoblasts are born at interlimb levels (somites 22-27). Therefore, labeled nuclei within multinucleated muscle fibers belong to pioneer cells.

Quantification of the distribution of myofibers added to the growing myotome

Quail embryos aged 35-somite pairs received two pulses of [³H]thymidine (10 μCi), 3 hours apart, followed by a chase with cold thymidine, as described (Kahane et al., 1998a). Embryos were then reincubated for 3 additional days. Only those progenitors that exited the cell cycle immediately after labeling and generated myofibers, retained the label. Embryos were fixed and sectioned as described in the previous section, and then processed for desmin immunolabeling. The dorsoventral extent of the myotomes in segments 22-27 was subdivided into six equivalent regions. Labeled myotomal nuclei were counted in serial frontal sections and the total number per region was determined. The total number of labeled cells per myotome was normalized to 100%. Results represent the mean±s.d. of the proportion of labeled nuclei per region averaged from six flank-level segments.

DML ablation

The DMLs of segments 10-16 in embryos aged 28-somite pairs, were ablated in ovo. Immediately after surgery, one pulse of radiolabeled thymidine was delivered and embryos were further incubated overnight and fixed as described above. Serial transverse sections (8 μm) were immunostained for desmin protein using a peroxydase-conjugated secondary antibody, and processed for autoradiography to visualize the distribution of new myocytes being added to the myotome.

RESULTS

Formation of the pioneer myotome involves cell delamination, migration in a caudal-to-rostral direction and a mediolateral order of myofiber generation

Whole-mount desmin immunolabeling

In a previous study based on desmin immunostaining of sections, Kaehn et al. (Kaehn et al., 1988) assumed that the rostromedial corner of the dermomyotome is the initiation site of myogenesis. From this site, myofiber differentiation was observed to progress both caudally and laterally until a unit-length myotome formed. To increase the sensitivity of the technique, whole embryos aged 31-somite pairs were immunolabeled with desmin antibodies and subjected to confocal microscopy. Desmin signal first appeared in somite 29 in epithelial cells along the entire medial half of the somite (rather than only in the rostromedial corner), where it was predominant at their basal aspect (Fig. 1A). Further rostral (somite 27, Fig. 1B), mesenchymal cells expressing the protein were observed within the rostral half of segments (Fig. 1C,D, open arrowheads) and an overall triangular pattern of desmin immunoreactivity was evident. The first myofibers appeared at somitic level 25. These abutted the medial epithelium of the somite; furthermore, they were attached to the rostral edge of each dermomyotome but their opposite end was free within the segment (Fig. 1C,D, between arrows and filled arrowheads), suggesting they were in the process of elongating in a caudal direction. The number of fibers progressively increased in a mediolateral direction and more fibers reached the caudal edge of the segments (Fig. 1D-F). A lateral front of mesenchymal myoblasts located close to the rostral edge of the dermomyotome was present until cell differentiation was completed, resulting in formation of unit-length myofibers attached to both R and C edges. Notably, the newly formed DML epithelium retained desmin reactivity throughout formation of the primary myotome (Fig. 1A-E) but then the signal mostly disappeared and remained in only a few epithelial cells (Fig. 1F).

Cells from the caudal-most part of the medial wall of epithelial somites migrate to the rostral edge prior to generating myofibers

Observation of desmin-stained embryos suggested that pioneer myoblasts originate all along the medial half of epithelial somites. The presence of desmin-positive myoblasts in only the rostral half of dissociated segments suggests that once converted into mesenchyme, these progenitors migrate rostrally. This is further substantiated by the rostrocaudal direction of initial fiber formation. In addition, a previous study documented the caudal-to-rostral relocation of pioneer myoblasts by following the movement of postmitotic (thymidine negative) nuclei in embryos constantly pulsed with [^{3}H] thymidine (Kahane et al., 1998a). To provide additional, independent proof for rostralward movement, the medial side of epithelial somites was focally labeled with DiI. Cells located near the caudal corner of the segment (Fig. 2A,A′) were labeled to optimally demonstrate the polarity of cell movement. This enabled us to directly trace the migration of dye-labeled cell bodies by performing timecourse confocal analysis of whole segments.

Between 6 and 12 hours, post-injection-labeled cells were apparent lateral and rostral to the original injection site with an increasing number of mesenchymal progenitors reaching already the inner aspect of the rostral edge of the corresponding dermomyotomes (arrowheads in Fig. 2B,B′,C,C′). From 12 hours onwards, partial length myofibers appeared that clearly elongated from the rostral edges in a caudal direction (Fig. 2D,D′, delimited by arrows and data not shown) far from the original caudal spot of dye. Hence, these results demonstrate that during formation of the pioneer myotome, cells from along the dorsomedial epithelial somite convert into mesenchyme, migrate from their origins to the rostral margin and only then generate myofibers towards the caudal edge until they form a full rectangular scaffold (see also Fig. 3B). This polarized migration pattern is unique to the pioneer wave and was not observed during later waves of myotome formation (Kahane et al., 1998b; Kahane et al., 2001; Cinnamon et al., 1999; Cinnamon et al., 2001).

The fate of pioneer myofibers

Pioneer myofibers extend along the whole epaxial-hypaxial extent of the myotome

We employed CM-DiI to label the medial aspect of epithelial somites at interlimb levels of the axis (Fig. 3A). The distribution of dye-labeled fibers was examined 36 hours later in wholemounts (Fig. 3B) and transverse sections (Fig. 3C). The earliest myogenic progenitors derived from a focal spot in the center of the medial somitic aspect, differentiated into unit-length myofibers that spanned the entire mediolateral extents of the segment (Fig. 3B,C) where they were interspersed among

desmin-positive/DiI-negative fibers (Fig. 3C). Furthermore, transverse section analysis revealed that these myofibers were superficially localized, adjacent to the dermomyotomal side of the desmin-positive myotome (arrows in Fig. 3C). These results are fully consistent with data stemming from previous birthdating experiments (Kahane et al., 1998a; Kahane et al., 1998b). Taken together, our results further substantiate the notion that the myotome grows in thickness from the dermal to sclerotomal sides and dorsoventrally by progressive cell intercalation among pioneers. Our data, however, strongly contrast with the proposal that the myotome grows incrementally in both medial and lateral directions leaving the oldest myofibers concentrated at the center (Denetclaw et al., 1997; Denetclaw and Ordahl, 2000; Denetclaw et al., 2001; Ordahl et al., 2001).

Pioneer myofibers participate in the formation of multinucleated muscles

We next examined the fate of pioneer fibers during secondary myogenesis. Somites were labeled with radioactive thymidine
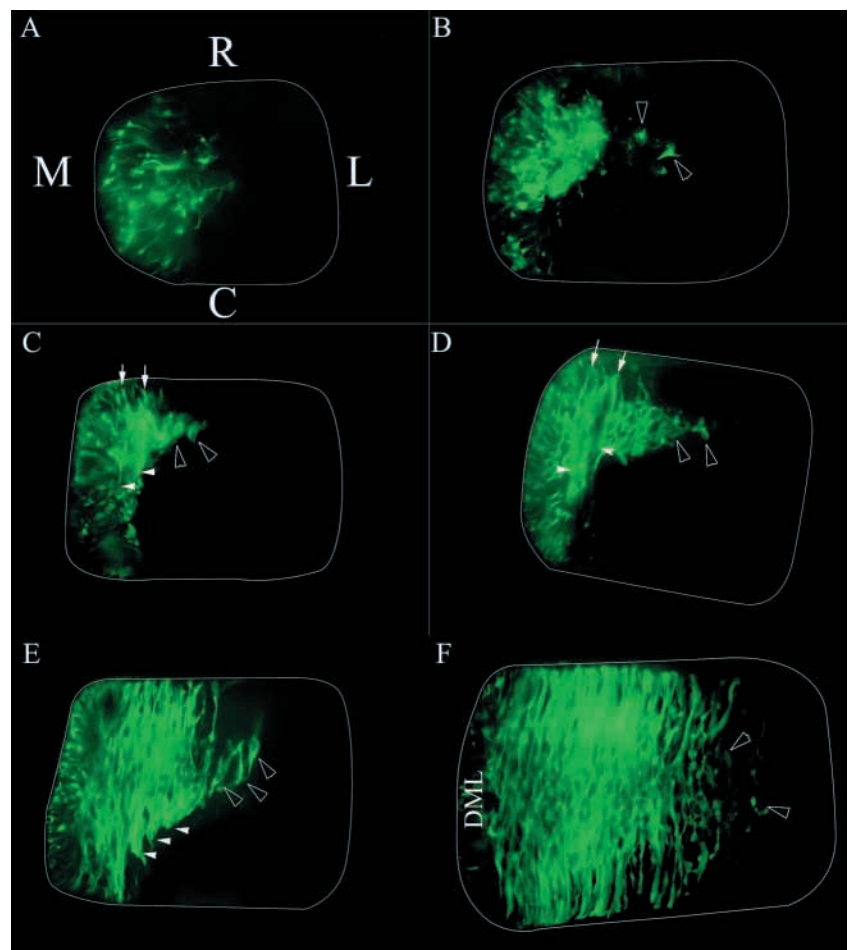

Fig. 1. Dynamic expression of desmin immunoreactivity during development of the pioneer myotome. Confocal analysis of whole somites of an embryo aged 31-somite pairs. Consecutive panels illustrate somitic levels A, 29; B, 27; C, 25; D, 23; E, 21; and F, 18. Open arrowheads indicate mesenchymal cells that delaminate from the epithelium. Growing fibers are delimited between arrow and white arrowheads. DML, dorsomedial lip; C, caudal; L, lateral; M, medial; R, rostral.
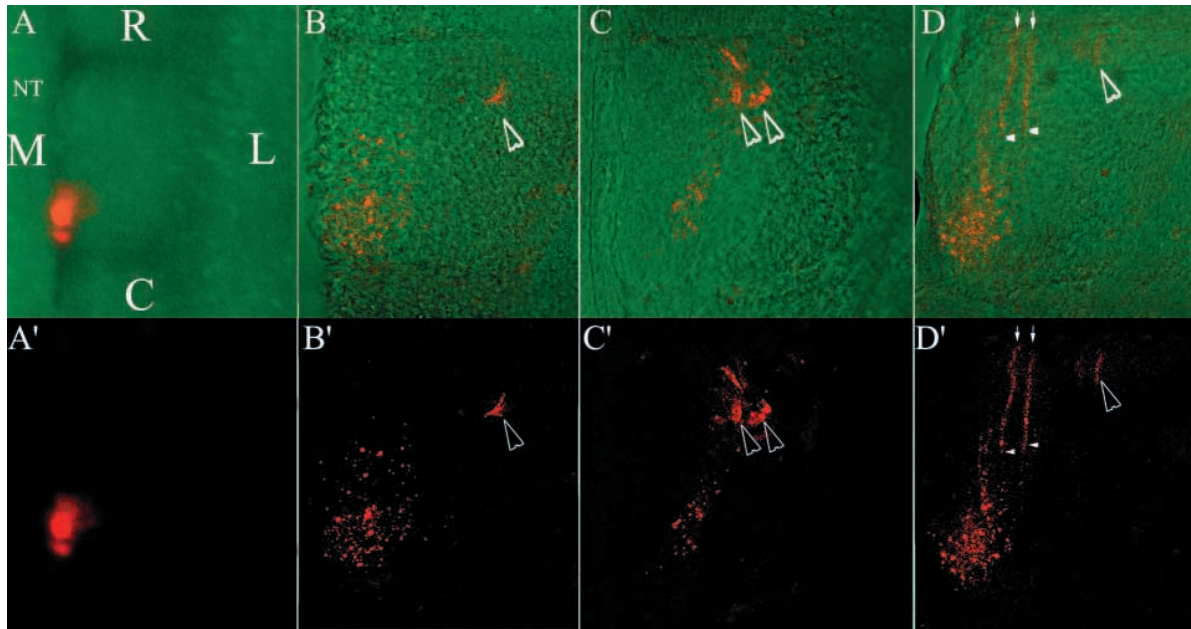
Fig. 2. Pioneer myoblasts migrate from caudal regions of the medial epithelial somite to the rostral edge prior to generating myofibers. The medial wall of epithelial somites at interlimb levels of the axis was focally labeled with CM-DiI approaching the caudal tip of the segment (A,A′ time 0). Six (B,B′) and 12 (C,C′) hours after injection, mesenchymal myoblasts (open arrowheads) have relocated towards the rostral half of the somite. (D,D′) Eighteen hours after labeling, differentiating fibers (between arrows and white arrowheads) are detected that are attached to the rostral pole and elongate caudally. Mesenchymal myoblasts located laterally always begun differentiating later than medial ones (see also Fig. 1). (A-D) Dye labeling on top of the phase contrast image. (A′-D′) Dye-labeled fibers only. C, caudal; L, lateral; M, medial; R, rostral; NT, neural tube.

Fig. 3. The primary myotome derived from the medial somite extends throughout the whole segment and pioneer fibers localize superficially.
(A) Microinjection of CM-DiI to the center of the DMQ of an epithelial flank-level somite.
(B) Formation of dye-labeled fibers that span the entire rostrocaudal and mediolateral extents of a segment.
(C) Transverse section counterstained for desmin reveals the dorsoventral distribution of pioneer fibers (arrows pointing to red cells), which localize from the DML to the VLL and occupy a superficial position (towards the DM epithelium) within the desmin-positive myotome (green). C, caudal; L, lateral; M, medial; R, rostral; DM, dermomyotome; DRG, dorsal root ganglion; EC, ectoderm; Myo, myotome; NT, neural tube; Scl, sclerotome. Scale bar: 10 μm.
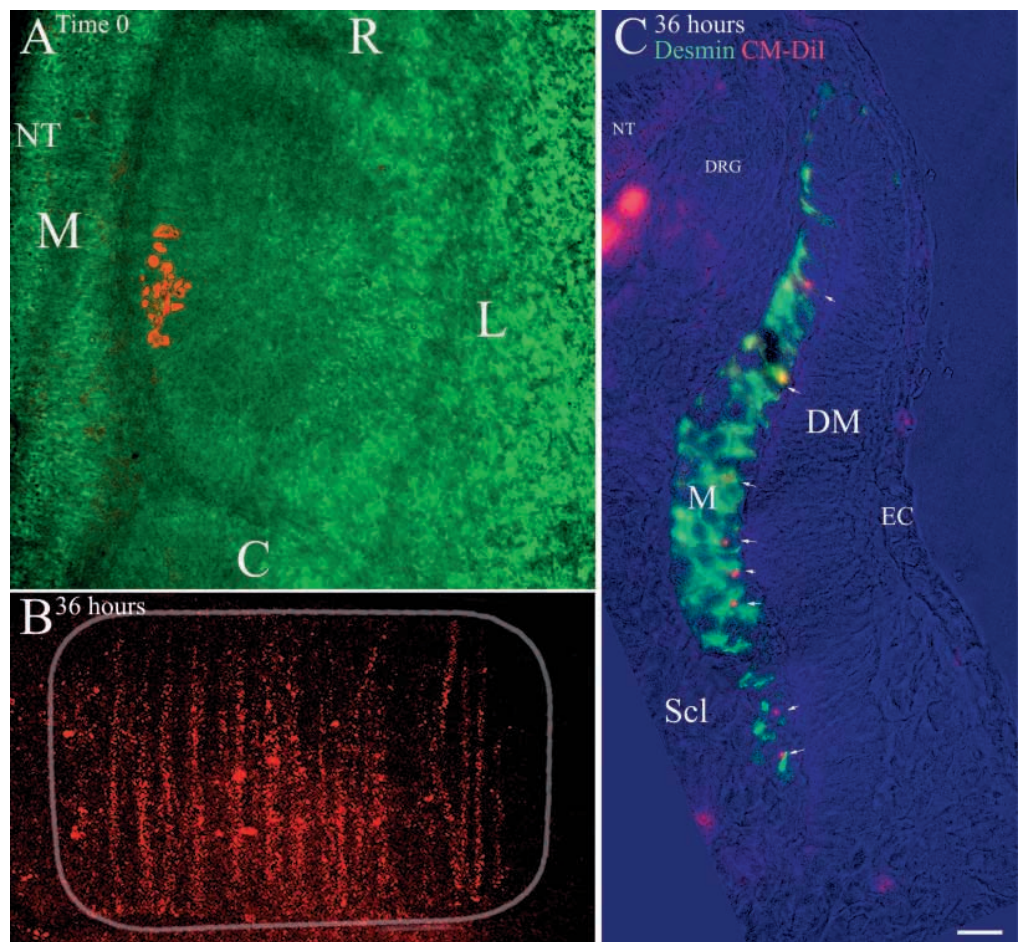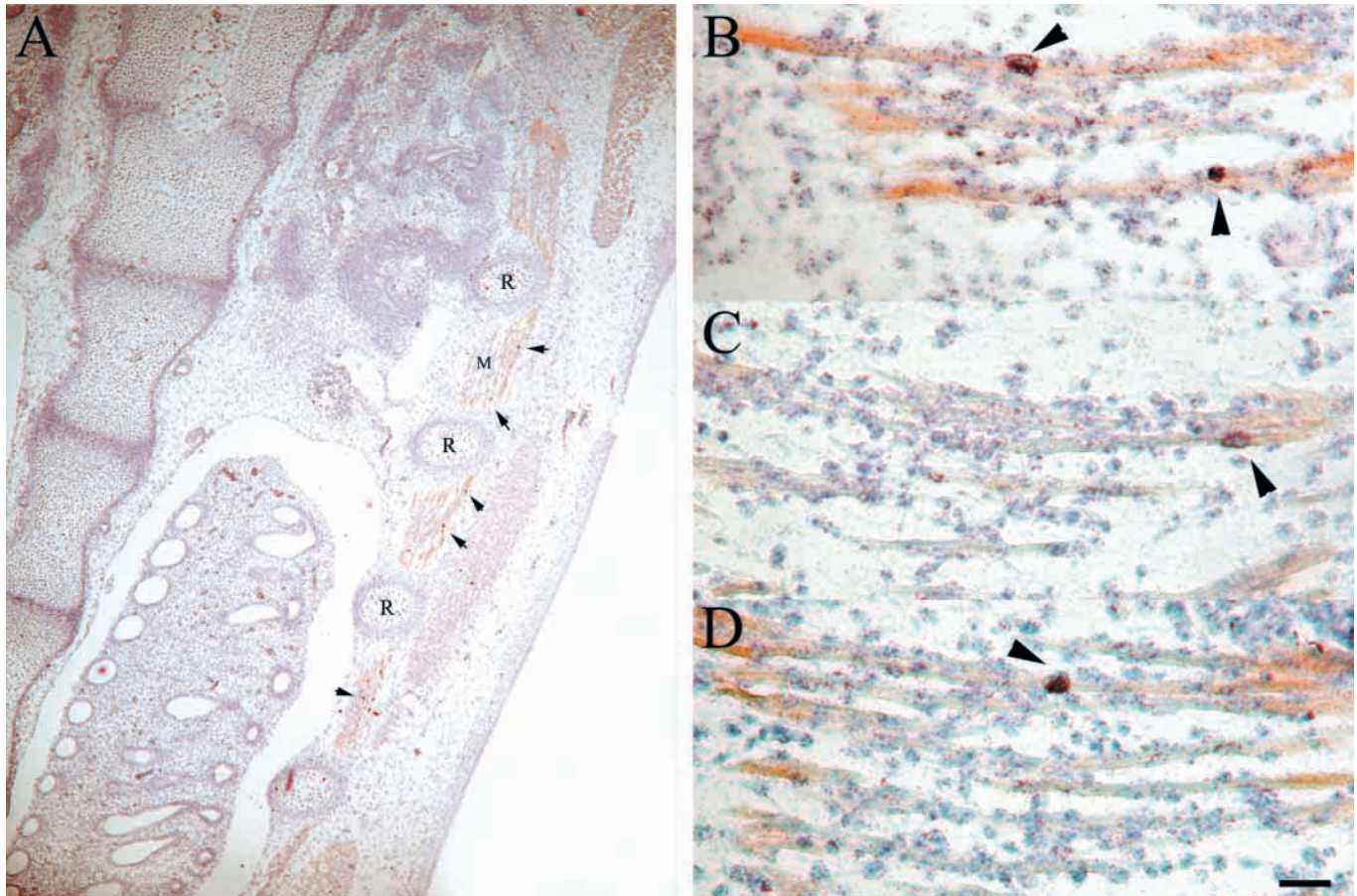
Fig. 4. Pioneer myofibers fuse with younger cells during development of multinucleated intercostal muscles. Frontal sections through a E8 quail embryo pulse-chased with thymidine and counterstained for desmin (brown reaction product) as described in the Materials and Methods. (A) Low magnification to show localization of intercostal muscles (M) between successive ribs (R). Arrows indicate clearly distinguishable fibers containing nuclei with thymidine grains. (B-D) Higher magnifications illustrating several parallel fibers each containing multiple nuclei stained with Hematoxylin (light blue). Arrowheads indicate thymidine-positive pioneer nuclei which are an integral part of the multinucleated fiber. Scale bar: 100 μm in A; 22 μm in B-D.

at the epithelial stage. Embryos were further incubated for 6 days until secondary myogenesis was well under way. During this incubation period, all mitotically active cells diluted the label, whereas the early post-mitotic pioneer cells remained positively labeled (Fig. 4). These myotomal pioneer fibers were found in the hypaxial region of E8 embryos, including intercostal (Fig. 4) and abdominal muscles (not shown), consistent with previous findings on the extended epaxial-hypaxial distribution of these primary fibers (Cinnamon et al., 1999). At this stage, pioneer nuclei were included into individual multinucleated, desmin-immunoreactive fibers (arrowheads in Fig. 4B-D) along with many other unlabeled nuclei (Hematoxylin positive/thymidine negative). Thus, we show for the first time, that the earliest postmitotic nuclei corresponding to pioneer fibers fuse with younger myogenic cells to become integrated into mature intercostal muscles. This observation indicates that in addition to providing a framework for the growth of the early myotome, pioneer myofibers might serve a function during later stages of muscle development.

Taken together, our results show that myotome expansion occurs both in the dermal to sclerotomal direction and also homogeneously along the dorsoventral axis by progressive intercalation. An incremental mode of myotome growth, which assumes that the oldest fibers remain grouped in the center (Ordahl et al., 2001), cannot explain the observations of pioneer myofibers being distributed evenly and later colonizing the hypaxial intercostal domain.

Addition of new cells along the dorsoventral extent of the myotome is uniform

To further address whether the myotome grows by incremental as opposed to even dorsomedial to ventrolateral cell intercalation, we examined the pattern of cell addition to the myotome in a direct way. Thirty-somite embryos were pulsed with radioactive thymidine followed by fixation 1 day later, as described in the Materials and Methods. At cervical levels of the axis, pre-existing myotomal fibers are postmitotic at the beginning of the pulse; therefore, the presence of labeled cells within the myotome must reflect newly added cells; i.e. progenitors that at the time of the pulse were still in the dermomyotome lips where they incorporated the label, then exited the cell cycle and entered the myotome as myofibers. Fig. 5A (control side) reveals that labeled nuclei are evenly
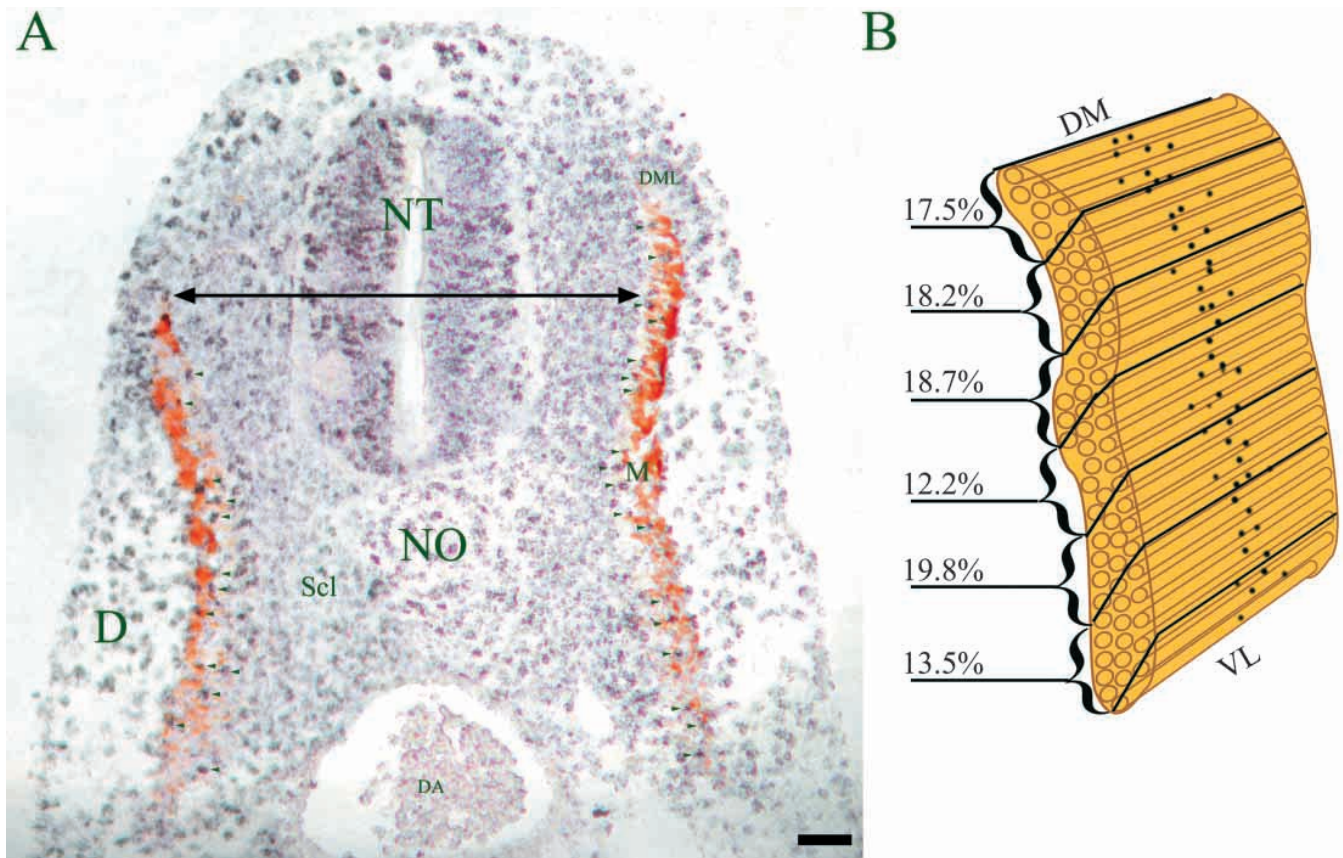
Fig. 5. Growth of the myotome occurs by even cell intercalation all along its dorsoventral extent and newly added fibers localize medially abutting the sclerotome. (A) Transverse section showing the homogeneous entry of labeled progenitors (newly added postmitotic fibers) along the entire dorsoventral extent of the desmin-positive myotome. Note the localization of labeled nuclei to the medial (sclerotomal) side of the myotome (arrowheads). The left side of the section shows the result of a DML ablation performed 1 day earlier. Note that both the DML and the dorsalmost part of the myotome are missing (dorsal to the long arrow). Nevertheless, the remaining myotome is an active structure growing by a similar, homogeneous addition of new cells all along the dorsoventral extent. (B) Quantification of the distribution of second-wave fibers that entered the myotome during an approximate period of 6 hours (two pulses of radiolabeled thymidine, each available to the embryo for 3 hours) (Kahane et al., 1998a; Kahane et al., 1998b). The proportion of newly added cells was counted in serial frontal sections, as described in the Materials and Methods. Calculated values (mean±s.d., n=6 somites) for six contiguous regions per segment are: 17.5±3.17; 18.2±3.8; 18.7±8.6; 12.2±1.63; 19.8±6.7; and 13.5±4.0. D, dermis; DA, dorsal aorta; DML, dorsomedial lip; M, myotome; NT, neural tube; NO, notochord; Scl, sclerotome. Scale bar: 30 μm.

spread along the entire dorsoventral extent of the myotome (arrowheads), as expected from a simultaneous and even intercalation mode of myotome expansion. However, if the incremental model was correct, we would expect to find these newly added fibers concentrated in the dorsomedial and ventrolateral areas exclusively, due to the respective contributions by DML and VLL. Notably, these second wave fibers mainly localize towards the sclerotomal portion of the myotome, consistent with the superficial to deep order of myotome growth already documented (see Fig. 3C) (Kahane et al., 1998b).

We next quantified the precise distribution of the newly added myofibers. To this end, 35-somite embryos were pulsed for 6 hours with radioactive thymidine followed by a prolonged chase (3 days) with cold nucleotide to minimize the background labeling and thus optimize the counting procedure. The precise distribution of second wave myofibers being added to the myotome throughout this 6 hour period was quantified at flank levels of the axis. As shown in Fig. 5B, and in

agreement with the qualitative impression (Fig. 5A), the distribution of newly added fibers that entered the myotome following establishment of the pioneer structure was equivalent along the entire dorsomedial-to-ventrolateral extent, demonstrating unequivocally a homogeneous pattern of myotome colonization.

As the DML of the dermomyotome was proposed by Ordahl and colleagues to be a necessary and sufficient engine that drives epaxial myotome growth (Ordahl et al., 2001), we ablated the DML along several segments and analyzed the pattern of cell addition to the growing myotome. If this view was correct, the ablated myotome would be expected to remain small, as at the time of operation, and not demonstrate addition of new cells. In striking contrast, DML ablation resulted in absence of only the dorsalmost region of the myotome (Fig. 5A, operated side). Furthermore, the remaining myotome continued growing similar to the contralateral intact one by intercalatory addition of postmitotic nuclei (Fig. 5A, arrowheads) all along its dorsoventral aspect despite the

absence of the DML. These results demonstrate that the DML is only one among several sources (the four dermomyotome lips) that contribute to overall expansion of the myotome.

Second-wave myofibers elongate directly from the extreme rostral and caudal lips and not from the DML

We proposed that DML- and VLL-derived cells first migrate longitudinally as mesenchymal cells through the SLD until reaching the R and C lips from which they begin elongation in the opposite direction (Cinnamon et al., 2001). Ordahl and colleagues, however, have suggested that epaxial growth of the myotome, driven by the DML, occurs in a medialward direction, leaving the oldest fibers laterally and the youngest close to the DML (Denetclaw et al., 1997; Denetclaw et al., 2001). Based on dye-labeling studies, these authors defined an area between DML and full-length fibers of the myotome, that contains partial-length fibers in the process of bi-directional elongation. These were suggested to differentiate once separating from the epithelium without prior longitudinal movements. A mirror image was proposed for VLL-derived muscle (Denetclaw and Ordahl, 2000). If this view was correct, we would expect to be able to detect a region containing a significant amount of those partial length fibers with whole-mount desmin immunolabeling, as we did for elongating pioneer myofibers (Fig. 1). By contrast, we find that the dorsomedial-most desmin-positive fibers detected in the myotome appeared to be anchored to both extremes of the segments (with the central nucleus-containing area less stained) and no partial fibers could be visualized approaching the DML (Fig. 6A). To further examine whether these are indeed unit-length fibers, DiI was applied to the rostromedial extreme of fixed segments, left overnight to let the dye diffuse across the fibers and subjected the tissue fragments to desmin immunolabeling. Under these conditions, only full-length fibers would allow the dye to spread towards their caudal extremity. Consistently, DiI applied to the rostral extreme (asterisk) diffused along the fibers until reaching their caudal end (Fig. 6, arrows in B,C). In addition, Fig. 6C reveals no partial-length desmin-positive fibers extending medial to the full-length fibers stained with DiI. Such partial fibers, if present, would be expected to express desmin protein as shown in Fig. 1 and Fig. 6D. We therefore conclude that no partial-length fibers exist in the sub-DML region. Instead, elongating fibers could be visualized emerging from either the R or C lips by whole-mount desmin immunostaining (Fig. 6D, between arrow and arrowhead). Taken together, these results directly show that there is no intervening region with partial-length fibers, as proposed by Ordahl and colleagues, and therefore no in situ differentiation of DML-derived myofibers occurs in this area. Instead, our results are consistent with previous findings by Cinnamon et al. (Cinnamon et al., 2001), showing that DML-derived cells delaminate and migrate as mesenchymal progenitors through the SLD towards the R and C lips from which fiber differentiation takes place.

DISCUSSION

In a series of studies, we showed that the myotome of avian embryos develops in at least three successive and
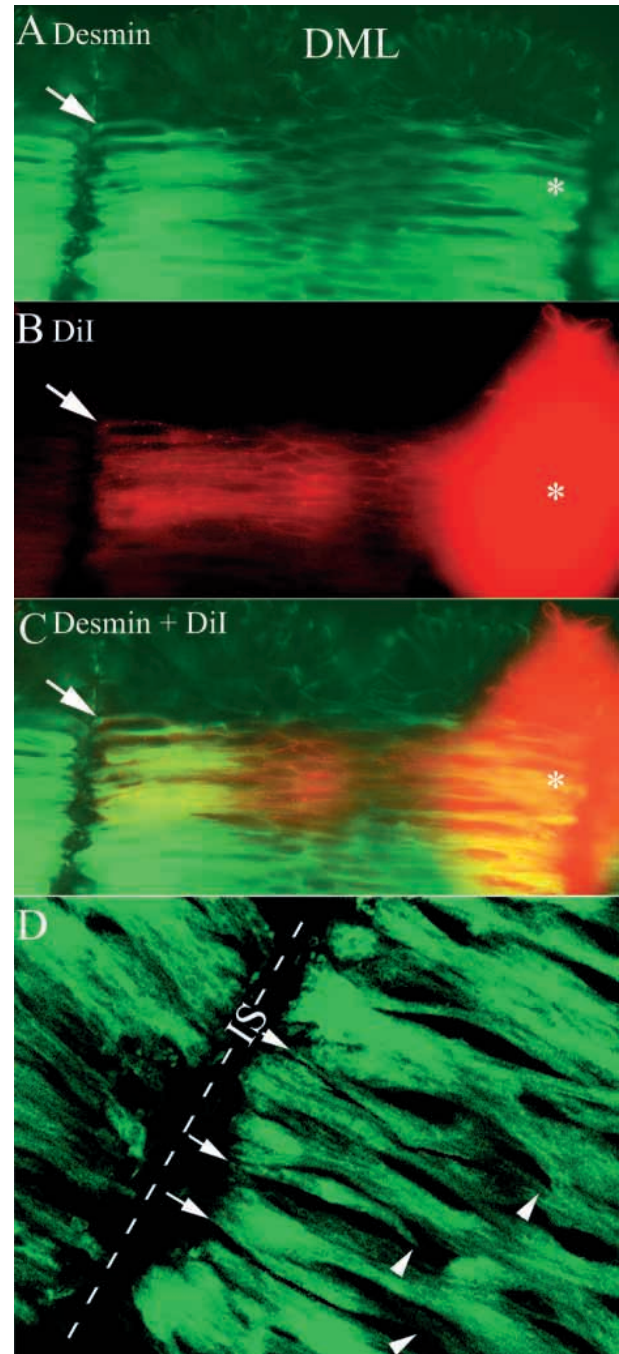

Fig. 6. The dorsalmost region of the desmin-positive myotome contains unit-length myofibers, with myofiber generation primarily beginning from the extreme edges of the dermomyotome. (A-C) The dorsomedial-most desmin-positive fibers detected in the myotome are anchored to both extremes of the segment. The central nucleus-containing area is less stained and no partial fibers can be visualized approaching the DML. DiI applied to the rostral extreme of a segment (asterisk) diffused along the fibers until reaching their caudal end (arrows in B,C). Co-localization of DiI and desmin staining define that dorsalmost fibers are of full length and highlight the absence of partial-length fibers. (D) Elongating fibers (between arrows and arrowheads) emerge from an extreme lip adjacent to the intersomitic region (IS, and broken line separating adjacent segments). Notably, unit length fibers had broad end-feet attached to the edges, whereas growing fibers demonstrated tapered attachments.

distinguishable phases. The first two waves create a post-mitotic myotome and a third wave of precursors which remains temporarily proliferative contributes to dramatic growth of the structure prior to secondary myogenesis (Kahane et al., 2001). Hence, the essence of our model assumes a complex pattern of myotome development. In this study, further evidence is provided that different cellular mechanisms govern establishment of the two initial waves.

Pioneer myoblasts migrate towards the rostral edge before differentiating

Using DiI to label epithelial cells of the medial somitic wall, we directly traced the movement of the labeled progenitors after somite dissociation. A clear rostralward migration of labeled mesenchymal progenitors was observed that was maximized by marking the cells close to the caudal margin of the segment. Importantly, myofiber differentiation followed arrival of these cells to the rostral pole of the segment and proceeded in a caudal direction. These results are fully consistent with our previous findings on a rostralward relocation of postmitotic pioneer nuclei prior to fiber generation (Kahane et al., 1998a). Notably, on their way to the rostral edge, these progenitors give rise to a transient triangular pattern that was now also evidenced by wholemount desmin immunostaining. This triangular shape is formed primarily by mesenchymal cells and slightly later by a combination of growing fibers and of mesenchymal progenitors. Hence, several independent lines of evidence demonstrate now that migration of myoblast progenitors of the first wave is an essential event during formation of the primary myotome (Fig. 7).

Such migrating cells could not be detected by Kaehn et al. (Kaehn et al., 1988) who observed the earliest expression of desmin at the dorsomedial corner of the dermomyotome. While these authors interpreted this site as the origin of the myotome, our staining method allowed the detection of desmin immunoreactivity at earlier stages and at higher resolution, thus evidencing the pioneer wave throughout its formation. This includes the transition between three consecutive expression patterns, a linear pattern corresponding to the pioneer cells still localized along the entire medial somite, a triangular pattern reflecting migration and early differentiation and finally, a rectangular shape corresponding to the formation of unit-length fibers spanning the entire segment. These results are also consistent with previously observed patterns of MRF expression (Borycki et al., 1997; Pownall and Emerson, 1992; Pownall et al., 1996; Ott et al., 1991) and altogether, provide now a comprehensive picture of the cellular mechanisms underlying primary myotome formation (Fig. 7).

Pioneer fibers elongate in a general dorsomedial to ventrolateral direction

Using desmin immunolabeling, the first growing myofibers were detected close to the forming DML while lateral to them, progenitors were still mesenchymal. Generation of pioneer myofibers then proceeds simultaneously in medial to lateral and in rostral to caudal directions, leading at intermediate stages to the triangular shape with a laterally directed apex (Fig. 7) (reviewed by Kalcheim et al., 1999). This general growth pattern was already noticed by Kaehn et al. (Kaehn et al., 1988) using desmin immunostaining and our results are in
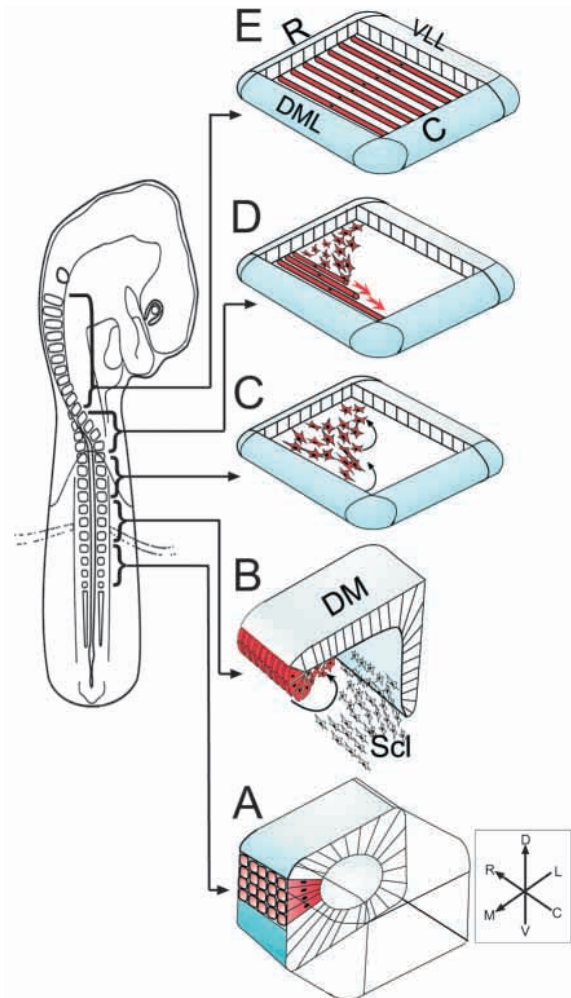

Fig. 7. The formation of the primary myotome by pioneer myoblasts. Successive stages in the formation of the early myotome (first wave, red color) represented as a function of somitic level in a 28-somite embryo. (A) Pioneer myoblasts originate along the entire R-C extent of the dorsomedial wall of epithelial somites. (B) During somite dissociation, pioneer myoblasts bend underneath the forming DML, delaminate and begin migrating to rostral portions of the somite (curved arrow). White mesenchymal cells represent sclerotome (Scl). (C) Migrating mesenchymal myoblasts give rise to a transient triangular pattern. (D) The first growing myofibers differentiate apposed to the DML. The direction of myofiber formation proceeds from R to C (red arrows) and from M to L. Mesenchymal cells are located lateral to the differentiating fibers. The overall pattern of the forming myotome is still triangular. (E) All fibers have reached the caudal and lateral edges, giving rise to the early myotome (rectangular pattern), which is attached to the R and C lips of the dermomyotome. These are mononucleated fibers whose nuclei concentrate at the center (black dots). The second wave of myotome colonization (not shown) begins at segmental levels D/E. The drawings represented in the figure depict characteristic phases, yet the processes of myoblast delamination, migration and fiber generation are continuous and overlapping over discrete segmental levels. Hence, segments are found that reveal simultaneously delaminating and migrating cells, or migrating and differentiating cells. In C-E, only the four dermomyotome (DM) lips (DML, VLL, R and C) were left. The dorsal layer of the DM has been removed to appreciate the underlying myoblasts. Intersecting arrows represent coordinates for orientation: R, rostral; C, caudal; M, medial; L, lateral; D, dorsal; V, ventral.
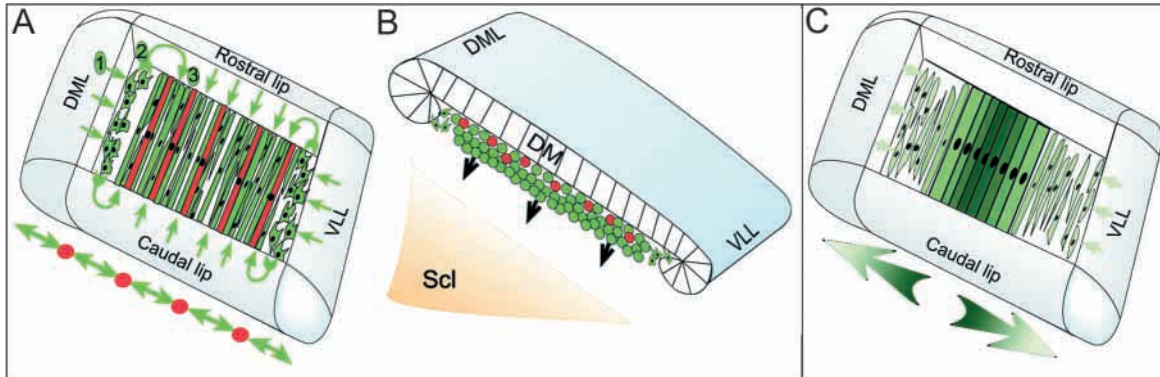
Fig. 8. Even growth of the myotome by progressive cell intercalation as opposed to incremental cell addition. (A,B) Simultaneous expansion of the myotome in the dorsoventral and transverse planes is accounted for by two successive myoblast waves first wave of pioneers in red and second wave in green). (A) Even pattern of myotome growth in the DM to VL orientation by progressive cell intercalation. Green arrows illustrate the contribution of the second wave stemming from all four lips of the DM. Fibers are directly generated from all along the rostral and caudal lips. By contrast, cells from along the DML and VLL first delaminate into the sub-lip domain (1), mesenchymal myoblasts then migrate longitudinally to one of the extreme edges (2) and generate myofibers (3) upon joining their rostral and caudal counterparts. Myofiber elongation occurs in a direction that is parallel to pre-existing pioneer fibers (red) and newly added cells intercalate among adjacent pioneers. This results in an even pattern of dorsoventral growth (arrows below the scheme) with young and old fibers evenly spread. (B) Growth in the transverse plane occurs in a superficial (DM) to deep (Scl) direction. Black arrows indicate the direction of myotome growth, leaving the pioneer fibers (red dots) superficially localized apposed to the DM. (C) Incremental growth of the myotome driven by the DML and VLL (small arrows) as proposed by Ordahl et al. (Ordahl et al., 2000). The oldest fibers are of unit length (dark green) and localize to the center of the myotome. Further growth occurs both medially and laterally (large arrows in which the green gradient represents relative myofiber age). Accordingly, cells from the DML and VLL delaminate into an intermediate domain where they begin differentiating bi-directionally in situ with no prior longitudinal relocation. This growth domain is therefore assumed to contain partial-length fibers in a staggered configuration subjacent to both medial and lateral edges, respectively.

agreement. By contrast, these results clearly illustrate an opposite direction of myofiber differentiation to that suggested by Ordahl and colleagues (Denetclaw et al., 1997; Ordahl et al., 2001), who maintain that medial expansion by DML-derived cells yields older (longer) myofibers in the ventrolateral region of the segment and younger ones (shorter) towards the medial growing zone (Fig. 8C).

The mechanism by which the dorsomedial region of the somite contributes to the myotome changes with development

As discussed in the preceding sections, the first wave of pioneer cells originates along the medial aspect of the still epithelial somites (for example somites 20-25 in a 25-somite embryo) and formation of the pioneer myotome is completed about 16-20 hours later (Kahane et al., 1998a). Thus, the ongoing stages of pioneer myotome formation can be observed throughout the recently formed 15-20 somites of embryos aged 30-somite pairs. During this phase, the myotome is primarily driven by medial somite-derived myoblasts differentiating in a medial to lateral direction (see above and Fig. 7). Consistent with the hypothesis that the medial somitic quarter provides pioneer cells, ablation of this region of epithelial somites indeed prevented myotome development (Ordahl et al., 2001).

During formation of the pioneer myofibers, the medial aspect of the somite gives rise to the DML of the dermoyotome. This structure can be defined as such only rostral to the 10th recently formed somite pair in embryos aged 30 somites, and at more rostral levels in younger embryos. Formation of the DML and the additional lips of the dermomyotome characterize the onset of the second wave, which begins with some temporal overlap with respect to the pioneer wave, peaks

at E3 and progressively decreases until E4 (Kahane et al., 1998b; Kahane et al., 2001; Cinnamon et al., 1999; Cinnamon et al., 2001). When DML ablation was performed following establishment of the pioneer scaffold, only the dorsalmost part of the myotome was missing (10-15% of total myotomal nuclei) (Kahane et al., 1998b). In DML ablations performed at similar stages, Ordahl et al. (Ordahl et al., 2001) reported on dorsally truncated myotomes. From whole-mount preparation analysis of these truncations, they concluded that growth was arrested because of the lack of the DML. By contrast, direct examination of sectioned embryos comprising the addition of new cells clearly revealed that the myotomes kept growing by an even pattern of dorsoventral cell addition even in the absence of a DML (see Fig. 5). Hence, the dorsomedial region at the epithelial somite stage is the earliest source of all pioneers, but the later DML is only one among several sources of progenitors that furnish the growing myotome.

Along this line, when attempting to perform the complementary experiment, i.e. ablating the entire DM epithelium except for the DML, we found it difficult to leave intact the DML epithelium lacking the flanking R and C lips without severely affecting the structure of the somite. Therefore, we, instead, ablated a significant proportion of the DM epithelium lateral to the DML (about two-thirds), leaving about one-third of the medial DM that comprises the DML and adjacent R and C lips. After overnight incubation, and as expected, a significant truncation of both the lateral DM and the desmin-positive myotome were obtained (N. K. and C. K, unpublished). Notably, the remaining DM epithelium was thinner than the contralateral one. In addition, the entire segment was shorter in the rostrocaudal extent, suggesting that a structural reorganization of the cells occurred to

accommodate or compensate for the ablation. This result further suggests that normal myotome development requires the integrity of the DM and all its component lips. These results differ from the normal pattern of myotome development obtained after similar ablations performed by Ordahl et al. (Ordahl et al., 2001). The differences may stem from the relative amounts of ablated DM and/or from the axial levels that concerned the surgery. The latter authors mostly focused on limb levels of the axis, where most of the lateral part of the DM contributes to limb muscles rather than to the myotome, thereby underestimating the extent of truncation. Taken together, the DML can be neither necessary nor sufficient to drive myotome expansion, as suggested by Ordahl et al. (Ordahl et al., 2001).

Our studies also distinguish two different mechanisms whereby the medial somite generates myofibers. As discussed in the preceding sections, pioneer myoblasts arising in the medial epithelial somite migrate rostralwards and generate fibers in a caudal direction (see also Kahane et al., 1998a). At variance, the DML at later stages provides myogenic progenitors that delaminate into the SLD, migrate longitudinally as mesenchymal cells to the R or C edges of the dermomyotome, and only then generate myofibers through both extreme edges (Cinnamon et al., 2001) (Fig. 8A). An additional difference between the early medial somite and the subsequent DML is that the former gives rise to myofibers expanding throughout the entire mediolateral extent of the segment, contributing also to hypaxial muscles of the body wall, whereas the latter only contributes fibers that localize to the dorsomedial region of the growing epaxial myotome (Kahane et al., 1998b; Cinnamon et al., 1999). Thus, the early medial somitic region that drives formation of the pioneer scaffold significantly differs from the later DML that locally contributes to its growth. Altogether, our results point to two successive and distinct modes of myotome formation that are inconsistent with the view proposed by Ordahl et al. (Ordahl et al., 2001), who envisage a single mode of myotome ontogeny throughout both formation and growth phases, driven by medialward expansion of the DML (Fig. 8C).

As part of their model, Ordahl et al. (Ordahl et al., 2001) also suggested that the mechanism by which the DML provides fibers to the myotome is by cell translocation into an intermediate, sub-DML zone followed by in situ bi-directional fiber differentiation, a process requiring no prior longitudinal cell movement (Denetclaw et al., 2001) (Fig. 8C). In striking contrast, using direct desmin/DiI visualization we could not evidence such a zone containing growing fibers between the DML and the myotome. Instead, we clearly document the existence of cell migrations both at the pioneer phase of myotome formation as well as during growth in both sub-DML and sub-VLL regions, processes that clearly precede generation of myofibers and unidirectional elongation.

Growth of the myotome by progressive intercalation of precursors from all four dermoyotome lips

Recent studies suggested that the myotome expands in a medial direction by incremental ingression of DML cells (Denetclaw et al., 1997; Denetclaw et al., 2001; Ordahl et al., 2001) and in a lateral direction by addition of VLL-derived progenitors (Denetclaw and Ordahl, 2000). We also found that following establishment of the pioneer structure, the DML of the

dermomyotome contributes to its medial expansion (Kahane et al., 1998b) and the VLL to lateral growth (Cinnamon et al., 1999). Thus, consensus has been reached that these lips give rise to muscle (Huang and Christ, 2000; Venters et al., 1999). Yet, while the group of Ordahl and colleagues claim that these two lips are the main engines for growth in opposite directions (Fig. 8C), our own data, now confirmed by the results of Huang and Christ (Huang and Christ, 2000) and Venters et al. (Venters et al., 1999), already agree that all four edges of the epithelial dermomyotome provide cells that account for myotome growth (Fig. 8A) in both avians and mice. This is further substantiated by the finding that in homozygous mice with an *nlacZ* reporter gene targeted into the Myf5 locus, muscle progenitor cells delaminate but remain developmentally arrested and accumulate along all four edges of the dermomyotome (Tajbakhsh and Buckingham, 2000).

This raises the question of the overall mechanism of myotome growth. If cells of the DML and VLL were the only source of the myotome, then a model of incremental growth in the medial and lateral directions would be correct. However, we presently report that addition of new cells in intact embryos occurs in a homogeneous pattern along the entire dorsoventral extent of the growing myotome, rather than exclusively at the medial and lateral extremes. This is consistent with our previous data directly documenting myofiber elongation from along the entire rostral and caudal dermomyotome lips (Cinnamon et al., 1999; Kahane et al., 1998b). Even more significantly, ablation of the DML does not prevent ongoing cell addition to the myotome and only results in truncation of its dorsalmost portion. Thus, the DML on its own is not sufficient to drive myotome growth and coherent addition from all four edges is required.

Furthermore, we document in the present study that the newly added cells intercalate among preexisting myofibers. Conversely, we show that pioneer myofibers become interspersed among younger fibers as well (this study) (Kahane et al., 1998b; Cinnamon et al., 1999). Thus, even at mature stages, pioneer myofibers can be found throughout the entire dorsoventral myotome, rather than concentrated in the center of this structure, as would be expected if growth was incremental. These data are fully consistent with results of previous studies in which we showed that progenitors of all four lips progressively integrate by intercalation among a pre-existing scaffold formed by the pioneer myofibers (Kahane et al., 1998a; Cinnamon et al., 1999) (Fig. 8A,B). Thus, based on all the data presented, we propose that overall myotome growth occurs in a uniform manner along the dorsomedial to ventrolateral extent rather than by local increments (Fig. 8).

Notably, using timely expression of myogenic markers, Venters et al. (Venters et al., 1999) also reported that in the mouse, Myf5-positive progenitors enter the myotome from the R and C lips by an intercalatory mechanism. Recently, Eloy-Trinquet and Nicolas (Eloy-Trinquet and Nicolas, 2001), used clonal analysis to follow myotome formation in the mouse. These authors reported that there is a direct relationship between myotome precursors in the dermomyotome and their daughter cells in the myotome, therefore refuting the model based on two opposite stem cell systems located in the DML and VLL, and supporting our view of direct translocation from the dermomyotome into the corresponding region of the myotome. In addition, in all the clones analyzed in their study,

Eloy-Trinquet and Nicolas (Eloy-Trinquet and Nicolas, 2001) have noticed that unlabeled myocytes intercalated with genealogically related, labeled fibers. This finding fully supports our model of intercalation of new among older cells, a process that results in even expansion of the structure.

Fate of the pioneer myofibers

Once completed, the primary myotome composed of pioneer fibers spans the entire mediolateral extent of each segment attaining both the DML and VLL, respectively (Kahane et al., 1998a) (Fig. 3). As these progenitors are the first to exit the cell cycle, their number is limited and they are rapidly overgrown by progenitors of subsequent waves. Yet, their fate at relatively late stages of muscle development could be traced by positive labeling of their postmitotic nuclei in timely pulse-chase experiments. Indeed, we find that pioneer fibers become incorporated into multinucleated fibers of both epaxial (N. K. and C. K., unpublished) as well as hypaxial flank muscles (this paper). In *Drosophila* embryos, fusion-competent myoblasts are unable to fuse in the absence of founder cells (Bate, 1990; Ruiz-Gomez et al., 2000; San Martin and Bate, 2001; Baylies et al., 1998). In addition, Stockdale and Holtzer (Stockdale and Holtzer, 1961) showed that mononucleate myocytes isolated from chick somites were unable to fuse alone but were incorporated into multinucleated myotubes when cultured with cells taken from older muscle. This would suggest that the primary myotomal fibers of vertebrates may act as founder cells during secondary myogenesis. Our analysis of intercostal muscles reveals the presence of about one pioneer nucleus per fiber. Nevertheless, there were many fibers devoid of pioneer nuclei, as the number of secondary fibers greatly exceeds that of the pioneers.. As the initial postmitotic mononucleated fibers of the myotome are composed of cells from both the first as well as the second waves, the possibility exists that progenitors of the two waves altogether act as founder cells during fusion into mature myofibers.

Epaxial-hypaxial segregation of myogenic lineages

The notion that epaxial and hypaxial muscles are composed of two separate lineages was initially proposed based on quail-chick chimerism at the limb levels of the axis (Ordahl and Le Douarin, 1992). Yet, at these levels, the hypaxial muscles derive exclusively from the VLL of the dermomyotome and do not contain a myotomal contribution. Further analysis at flank levels also suggested distinct and opposite stem cell systems located in the DML and VLL that account for the formation of epaxial and hypaxial domains of the myotome, respectively (Denetclaw et al., 1997; Denetclaw and Ordahl, 2000; Olivera-Martinez et al., 2000) with a quiescent zone in the center between both domains composed of the oldest fibers (Denetclaw and Ordahl, 2000). In this and previous studies (Kahane et al., 1998a; Kahane et al., 1998b; Cinnamon et al., 1999) we have shown that the primary wave of pioneers, which originates along the medial epithelial somite and is specified by medial signals, develops to span the entire mediolateral extent of the segment. At later stages, pioneer fibers even incorporate into abdominal and intercostal muscles. Thus, pioneer myofibers clearly participate in the formation of both epaxial and hypaxial muscles. Therefore, our findings oppose this view and instead favor the notion that as far as the pioneer myotome is concerned, no such lineage segregation exists.

A more restricted pattern of myotome colonization is observed during the second wave. This consists of a medial domain (formed by cells of the medial part of the extreme R and C edges of the dermomyotome and the DML) and a lateral domain (formed by cells of the lateral part of the extreme R and C edges and the VLL) (Kahane et al., 1998a; Cinnamon et al., 1999). Yet, there is a clear mediolateral continuity of myotome colonization, reflected by an equivalent proportion of second wave fibers entering along the dorsal to ventral extent of the structure (Fig. 5). These experimental observations also lead to the notion that epaxial as opposed to hypaxial muscles do not derive from lineally distinct progenitors.

Furthermore, it has been noted that the expression patterns of some genes crucial for the myogenic process define two and even three distinct domains in dermomyotome and myotome (medial, central and lateral, by Myf5, En1 and MyoD, respectively) (Sporle, 2001; Tajbakhsh and Buckingham, 2000), and that Myf5 expression is independently regulated in different mediolateral domains of the myotome by distinct enhancers (Hadchouel et al., 2000). This only indicates that gene expression is related and influenced by cellular regionalization and local interactions (Cossu et al., 1996a; Dietrich et al., 1998; Epstein et al., 1996; Hirsinger et al., 1997; Marcelle et al., 1997; Pourquie et al., 1995; Reshef et al., 1998; Tajbakhsh et al., 1998). This argument does not, however, necessarily reflect a clonal separation between the ascendance of the corresponding progenitors.

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