Erratum


Part A of Fig. 4 is missing in the printed version. The correct figure is given below and in the online version.

A

\[ \Sigma C = \sum C(i) \]

B

\[ \Sigma C \text{ (number of clones)} \]

C

\[ \Sigma C \text{ (number of clones)} \]
INTRODUCTION

Nearly all skeletal muscles of vertebrates are derived from somites, which form the segmented paraxial mesoderm. Only the anterior head muscles develop from the prechordal and paraxial head mesoderm (Christ and Ordahl, 1995; Wachtler and Christ, 1992). Somites bud off from the cranial end of the presomitic mesoderm, which represents the caudal, unsegmented region of the paraxial mesoderm (Christ and Ordahl, 1995). The entire paraxial mesoderm derives from the primitive streak. Somites then progressively differentiate into different tissues under intrinsic and extrinsic influences. First, the ventral half of the somite gives rise to the sclerotome, a mesenchymal tissue that will develop into the vertebrae and ribs. The dorsal half of the somite, referred to as the dermomyotome, remains epithelial and then expands mediolaterally (Denetclaw et al., 1997; Denetclaw and Ordahl, 2000). During this expansion, cells from the dermomyotome translocate ventrally to form the myotome, an embryonic muscle. The remnant of the dermomyotome later undergoes an epithelio-mesenchymal conversion to form the dermis of the back. It remains unclear if the dermomyotome also contributes to the formation of the distal ribs (Huang et al., 2000; Kato and Aoyama, 1998).

Two types of muscles are derived from the somites: dorsomedially the epaxial myotome and epaxial muscles (muscles of the back), and ventrolaterally the hypaxial myotome and hypaxial muscles (muscles of the bodywall at trunk level, and of the limbs at limb level). The formation and differentiation of these two muscle types appears to result from different extrinsic signals [extensively reviewed by Hirsinger et al. (Hirsinger et al., 2000)]. For example, formation of the epaxial myotome in the dorsomedial quadrant of the somite is controlled by a number of secreted factors. BMP4, WNT1 and WNT3A are produced by the dorsal neural tube (Hirsinger et al., 1997; Ikeya and Takada, 1998; Marcelle et al., 1997; Tajbakhsh and Sporle, 1998), SHH by the notochord and floor plate (Borycki et al., 1998) and other members of the WNT family are also secreted from the dorsal ectoderm (Cossu et al., 1996; Tajbakhsh and Sporle, 1998). The spatial and temporal coordination of these signals induces expression of the myogenic regulatory factors (MRFs) Myf-5 and MyoD (Ikeya and Takada, 1998; Maroto et al., 1997; Tajbakhsh and Sporle, 1998). The dorsolateral quadrant, on the other hand, responds to signals from the dorsal ectoderm (including members of the WNT family) and from the lateral plate (BMP4) (Dietrich et al., 1998; Hirsinger et al., 1997; Pourquie et al., 1996) by expressing hypaxial markers, including Pax-3, Sim1 and the MRFs (Dietrich et al., 1998; Hirsinger et al., 1997; Pourquie et al., 1996; Tajbakhsh and Sporle, 1998). The situation is somewhat different at the limb level. Cells of the lateral part of the dermomyotome migrate away from the somite to form the limb musculature. These hypaxial cells also receive signals from the lateral plate (Dietrich et al., 1998) but express
different hypaxial markers, such as Lbx1 (Dietrich et al., 1998; Mennerich et al., 1998).

Although the differentiation of somite derivatives has been intensively studied at the genetic level, a complete comprehension of muscle development requires that these molecular signals be linked to the successive formation of the involved structures and to the underlying cellular processes that result in their formation. However, cell behaviour during muscle development remains controversial, particularly with respect to the formation of the myotome from the dermomyotome (Cinnamon et al., 2001; Cinnamon et al., 1999; Denetclaw et al., 2001; Denetclaw et al., 1997; Denetclaw and Ordahl, 2000; Kahane et al., 1998a; Kahane et al., 1998b). It has been shown that epaxial and hypaxial myotomes derive from the medial and lateral parts of the dermomyotome, respectively (Denetclaw and Ordahl, 2000; Huang and Christ, 2000; Ordahl and Le Douarin, 1992). However, it is not known how strict this separation is, whether it is progressively established, or if it implicates one or several factors. The exact location of myotomal precursors in the dermomyotome also remains unclear. Some studies in the avian embryo indicate that myocytes of the primary myotome (the initial myocytes that form the most dorsal cellular layers of the myotome) translocate directly from the dorsomedial (DML) and ventrolateral (VLL) lips of the dermomyotome (Denetclaw et al., 2001; Denetclaw et al., 1997; Denetclaw and Ordahl, 2000; Ordahl et al., 2001), whereas other experiments indicate that the myocytes essentially derive from the rostral and caudal lips, and that the myocyte precursors deriving from the DML and VLL first delaminate into a sub-lip domain and then migrate to the rostral and caudal lips before elongating as myocytes (Cinnamon et al., 2001; Cinnamon et al., 1999; Kahane et al., 1998b). To further complicate this issue, it is possible that the spatial origin of myotomal cells changes with maturation of the dermomyotome (Hirsinger et al., 2000).

To investigate the origin of the separation of epaxial and hypaxial myotomes, we used the LaaZ method (Bonnerot and Nicolas, 1993; Eloy-Trinquet et al., 2000; Mathis and Nicolas, 1997; Mathis and Nicolas, 1998) to perform a clonal analysis of myotome formation at the thoracic level of the embryo. This powerful method previously allowed us to propose a model for formation of the myotome from a self-renewing pool of cells in the primitive streak (Eloy-Trinquet et al., 2000; Nicolas et al., 1996). In this model, self-renewing cells (S) in the primitive streak give rise to daughter S cells and to precursor cells (P) that will leave the primitive streak to contribute to a few myotomal segments in the paraxial mesoderm. These P cells subsequently divide into post-mitotic myoblasts. This stem cell system is also at the origin of limb muscles, and persists in the tail bud to form the sacral and caudal segments (Eloy-Trinquet and Nicolas, 2001). Although this model explains the anteroposterior formation of the myotome and probably of all the paraxial mesoderm, it does not address its mediolateral organisation. In the present study, we have analysed the mediolateral contribution of LaaZ/LacZ chimeras in the mouse myotome. A systematic description of the mediolateral position of β-gal+ cells in the resultant clones led to the following conclusions: (i) the precursors of the myotome are mediolaterally regionalised before somite segmentation, (ii) superimposed on this regionalisation is a clonal separation of epaxial and hypaxial precursors, at the time of, or shortly after, allocation of precursor cells to one segment, and (iii) there is a direct relationship between myotome precursors in the dermomyotome and their daughter cells in the myotome.

**MATERIALS AND METHODS**

**Transgenic animals**

The mouse transgenic lines used in this study have been previously described (Klarefeld et al., 1991; Bonnerot and Nicolas, 1993; Nicolas et al., 1996). Briefly, they express the nlsLacZ (RzNLZ2 line) or the nlsLacZ (α-2 line) reporter gene under control of the chick promoter of the α2 subunit of the acetylcholine receptor, which drives expression of the transgene in skeletal muscle cells and the myotome. The embryos that were analysed resulted from crosses between homozygous transgenic males and (C57Bl/6xDBA/A F1) females. The morning after copulation is taken as 0.3 days post coitum (p.c.) (E0.3). For in toto X-gal analysis, the embryos were fixed, histochemically stained and then cleared as previously described (Mathis and Nicolas, 1998).

**Description of the myotome in RzNLZ2 embryos**

RzNLZ2 embryos were observed under a stereomicroscope equipped with a camera (3-CCD, JVC). The length of the myotomal segments and the epaxial and hypaxial domains of the myotome were measured with the LIDA and its accompanying Calibration Server softwares (Leica).

**Histological analysis and in situ hybridisation**

Histochimically stained RzNLZ2 E11.5 embryos were transferred into 1× PBS containing 30% (w/v) sucrose for 48 hours. They were next embedded in Tissue Freezing Medium (Jung). 50-60 μm cryostat transversal sections were obtained at the trunk level. Sections were fixed in 1% paraformaldehyde (PFA) and rinsed twice in 1× PBS, before mounting and observation under microscope. To visualize the pattern of expression of the LaaZ transgene in the α-2 line, in situ hybridization with a LaaZ probe was performed on whole-mount embryos, as described (Mathis and Nicolas, 1998). In addition, an embryo with a long bilateral clone, SC 346, was cut transversally with a razor blade to reveal a segment containing a large number of labelled cells.

**Description of mediolateral position of β-gal+ cells in the LaaZ/LacZ embryos**

153 of the clones used in this study were previously described (Nicolas et al., 1996). 162 additional clones were generated for this study and were analysed as previously described (Nicolas et al., 1996). The analysis includes all of the unilateral monosegmented and bisegmented clones that exhibit β-gal+ cells only in thoracic somites (segments 12-24). The mediolateral position of the β-gal+ cells for each clone was assessed in each labelled segment using a stereomicroscope equipped with a camera (3-CCD, JVC). The position of each β-gal+ cell was expressed as a percentage of the length of the myotomal segment, using LIDA and its accompanying Calibration Server softwares (Leica). Position 0% represents the medial end of the segment and position 100%, its lateral end. Some α-2 embryos with unilateral monosegmented clones were cut transversally with a razor blade to better visualize the position of the labelled cells in relation to the morphological indentation of the bodywall. The number and position of the labelled cells detected in these sections were identical to measurements obtained in intact embryos.

**RESULTS**

To understand the mediolateral cellular organisation of the myotome, we analysed LacZ clones obtained at the thoracic
Regionalisation of myotomal precursors

We first verified that the $\alpha$-2 transgenic line expresses the $\beta$acZ transgene in the myotome and limb buds, by performing an in situ hybridisation with a $\beta$acZ probe (Fig. 1B). We complemented this study by a detailed analysis of the labelled cells in a number of LacZ clones. The $\beta$-gal$^+$ cells are all included in myotomal structures and none were observed in either the dermomyotome or the sclerotome (Fig. 1D,E). To define the myotomal segments in E11.5 embryos more completely, we made use of the control transgenic line $\alpha$2NLZ2, which recapitulates the expression pattern of the $\alpha$-2 line used to produce the clones. At E11.5, an average of 40 myotomal segments are visible (Fig. 1A). At the thoracic level (segments 12-24), the labelling exhibits a brushing aspect in the medial part of each segment. In the lateral part of the segment, the labelling is more linear (Fig. 1A). The limit between the two parts of the myotome corresponds to the morphological indentation of the body wall shown on a cryostat transversal section (Fig. 1).

Table 1. Length of thoracolumbar segments of the E11.5 embryo

<table>
<thead>
<tr>
<th>Domain of the myotome</th>
<th>Length (µm)</th>
<th>Proportion of the myotome (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epaxial</td>
<td>469±25</td>
<td>43.1±2.3</td>
</tr>
<tr>
<td>Hypaxial</td>
<td>621±39</td>
<td>56.9±2.3</td>
</tr>
<tr>
<td>Total</td>
<td>1090±38</td>
<td>100±0</td>
</tr>
</tbody>
</table>

Measurements are means of segments 12-24 of 3 $\alpha$2NLZ2 E11.5 embryos.
MEDIOLATERAL CONTRIBUTION OF MYOTOME PRECURSORS

We first analysed the 45 unilateral monosegmented clones (Fig. 2). For each clone, the mediolateral position of each β-gal + myocyte was determined and represented on a relative scale from 0 (medial) to 100 (lateral) (Fig. 2A). A comparison of the 45 clones reveals two important properties. Firstly, β-gal + myocytes are almost systematically intercalated by β-gal − myocytes (Fig. 2A, Fig. 1F-K), which shows that cell intermingling occurs during the formation of the myotome. Secondly, none of the monosegmented clones spread along the entire mediolateral (ML) axis of the myotome (Table 2), the largest clone comprising a maximum 43% of the total segment (Fig. 2A, clone SC349). In other words, the contribution of monosegmented clones is regionalised, since they do not disperse freely throughout the entire myotome.

To learn whether regionalisation of the myotome precursors occurs before their allocation to one segment, we compared the distribution of labelled cells within the mediolateral axis of each clone. The mediolateral contribution of each myotome clone was determined and represented on a relative scale from 0 (medial) to 100 (lateral) (Fig. 2A). For each clone, the mediolateral position of each labelled myocyte was determined and represented on a relative scale from 0 (medial) to 100 (lateral) (Fig. 2A). A comparison of the 45 clones reveals two important properties.

Table 2. Mediolateral extension of monosegmented and bisegmented clones

<table>
<thead>
<tr>
<th>Clones</th>
<th>x≤25%</th>
<th>25%≤x≤50%</th>
<th>50%≤x≤75%</th>
<th>x&gt;75%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monosegmented (n=45)</td>
<td>39 (86.7)</td>
<td>6 (13.3)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Bisegmented (n=16)</td>
<td>8 (50.0)</td>
<td>6 (37.5)</td>
<td>1 (6.3)</td>
<td>1 (6.3)</td>
</tr>
</tbody>
</table>

Mediolateral extension of bisegmented clones was measured on pooled thoracolumbar labelled segments.
occurs before their allocation to one segment, we analysed the total potentiality of each labelled precursor for the 16 unilateral bisegmented clones (Fig. 3). To accomplish this, we determined the mediolateral position of each labelled cell. Each white rectangle represents a segment and the closed circles are labelled cells. MB, medial border; LB, lateral border of the clone. (B-D) Mediolateral position of the labelled cells in the thoracic unilateral bisegmented clones. Each white rectangle represents a clone with its two labelled segments. The code of colours is the same as in Fig. 2. The clones are classified by their property of not crossing (B–C) or of crossing (D) the clonal separation at the middle of the segment. m, medial; l, lateral.

**A clonal separation of two myotomal domains**

Since the clones appear to overlap (Fig. 2A, Fig. 3B-D), there is no evidence of obvious clonal separations that would divide the ML axis into several clonal domains. To investigate with greater accuracy the possibility of a discrete clonal separation somewhere in the myotome, we next analysed the ability of clones to cross-over at any mediolateral level, by examining clones at 5% intervals along the axis of the myotome. If a clonal border occurs at any of these intervals, there will be a corresponding lower frequency of clones crossing-over at this level. For this type of analysis, we used a parameter defined as the ‘crossing index’ or $C(i)$ (Mathis and Nicolas, 2000) (Fig. 4A). The $C(i)$ of the monosegmented family of clones has a marked minimum at a value of 40-50% along the length of the ML axis (Fig. 4B). This result suggests that, in addition to a regionalised mode of myotome production, there is a strict
clonal separation that subdivides the myotome into two clonal domains: a medial domain for the first 40-50% of the ML axis (Fig. 2B), and a lateral domain for the last 50-60% (Fig. 2C). It should follow, therefore, that this clonal border revealed within the myotome is also a property of the pool of precursors at the origin of single segments. Moreover, this clonal separation between medial and lateral domains at 40-50% of the ML axis of the myotome correlates with the morphological limit between epaxial and hypaxial myotomes (Fig. 1A,C and Table 1), suggesting that the two clonal domains correspond to the epaxial and hypaxial myotomes.

The clonal separation is not yet established before allocation of myotomal precursors to a single segment

In order to evaluate whether the clonal separation between medial and lateral myotomes is already established before the allocation of precursor cells to a single segment, we next analysed the family of clones that contribute to two adjacent thoracic segments (Fig. 3B-D). Most of their labelled precursor cells probably trace back to before the budding-off of the somite from the segmental plate. The crossing index of the 16 bisegmented clones exhibits no minimum at the middle of the ML axis (Fig. 4C). On the contrary, it is maximum at the 40-50% level, corresponding to the clonal separation, indicating that there must be many clones that are unrestricted to either the epaxial or hypaxial myotome. Therefore the clonal separation does not effectively separate the medial and lateral precursors when they still possess the potentiality to contribute to two somites. Furthermore, there is no minimum value of the crossing index at any other mediolateral level (Fig. 4C), suggesting that there is no clonal separation at any level of the myotome prior to allocation of the precursor cells to a segment. The same analysis was made on longer clones contributing to 3-6 adjacent segments (data not shown), and the absence of minimum values in their crossing index reinforce the conclusion that clonal separation is not established in the segmental plate. The bisegmented clones were further classified with respect to the clonal separation (Fig. 3B-D).

Eight bisegmented clones (out of 16) are restricted to either the medial (Fig. 1L, Fig. 3B) or lateral (Fig. 1N, Fig. 3C) myotomes, and thus do not cross the clonal border. Interestingly, these eight restricted bisegmented clones demonstrate that there is a significant proportion of precursors that, probably due to their position in their pool, are already assigned to either the medial or the lateral domain, before their allocation to a single segment.

The myotome is not produced from two permanent stem cell systems

In order to understand how the separation between epaxial and hypaxial precursors occurs, we then wanted to describe some aspects of their formation. An attractive model for the production of myocytes from the dermomyotome is one in which the epaxial and hypaxial domains of the myotome are formed from two stem cell systems located in the dorsomedial and lateromedial lips of the dermomyotome, respectively (Denetclaw and Ordahl, 2000). This model would result in myocytes being deposited in a lateral-to-medial direction for the epaxial domain and in the opposite orientation for the hypaxial domain (Fig. 5A). Such a polarised mode of myocyte production from two stem cell systems could result in a clonal separation of the epaxial and hypaxial myotomes. In another version of this model, the stem cell systems remain in the central part of the dermomyotome (Fig. 5B). In this case, stem cell systems deposit cells in the center of the myotome, thus displacing the formerly produced myocytes in a lateral-to-medial direction for the epaxial domain, and in a medial-to-lateral direction for the hypaxial domain.
Regionalisation of myotomal precursors

117

To test the hypothesis that two permanent stem cell systems could produce myocytes during the entire process of myotome building, we analysed different characteristics of the monosegmented clones. Two stem cell systems located at the edges of the dermomyotome (Fig. 5A) should produce epaxial restricted clones that always contribute to the medial extremity of the epaxial domain, and hypaxial restricted clones that always contribute to the lateral extremity of the hypaxial domain. Therefore, an increase in the number of clones (clonal complexity) contributing to the two most distal parts of the myotome is expected, compared to the number of clones contributing to the middle part of the segment (Fig. 5A,C,D, insets a). However, representation of the clones classified by their medial border for the epaxial restricted clones and their lateral border for the hypaxial ones, does not reveal a systematic contribution of the longest clones to the medial or lateral extremities of the myotome, respectively (Fig. 5C).

Furthermore, the clonal complexity of the clones with more than 1 labelled cell (pluricellular clones) has no marked minimum in the middle of the segment (Fig. 5D). These results thus refute the model that myocytes are produced from two permanent stem cell systems located at the edges of the myotome. Similarly, the alternative model with the two stem cell systems remaining located in the central part of the dermomyotome (Fig. 5B) can be rejected, since the prediction of this model about clonal complexity (Fig. 5D, inset b) is not verified: there is no maximum clonal complexity value. On the contrary, this value is constant between 20% and 80% of the ML axis (Fig. 5D). Furthermore, the longest clones do not extend up to the extremities of the myotome (Fig. 5C). In addition, a major prediction of both models of permanent stem cell systems, located either in the edges or in the central part of the dermomyotome, is that clones contributing to two segments will always contribute to the whole ML length of the

Fig. 6. Models for the spatial relationship between myocytes and their precursors in the pool for two adjacent segments. The pool of precursors before the formation of the clonal boundary is shown above two consecutive myotomal segments. The colour gradient symbolises the regionalisation of the precursors. In the inverted model (A), cells in the extreme parts of the pool of precursors (closed circles) give rise to myocytes in the middle of the myotome, and cells in the middle part of the pool of precursors (open circles) to myocytes in the extreme parts of the myotome, resulting in an inversion of the gradient. Consequently, labelling of a precursor cell in the middle of the pool (open circle) before the establishment of the clonal separation will give rise to cells on both sides of the boundary, and will result in a clone contributing only to the two medial and lateral extremities of the myotome. In the direct model (B), precursors in the pool for two segments give rise to myocytes, keeping the orientation of the gradient. Labelling of a cell in the middle of the pool of precursors (open circle) will result in a clone that is contiguous across the boundary. 

Fig. 7. Models for the production of the myotome from the dermomyotome. (A-F) Represented above are the two pools of precursors in the dermomyotome (d), in the middle the myotome (m), and below (horizontal thin lines), the clones resulting from labelling of a precursor in the dermomyotome. The clonal boundary in the dermomyotome is represented as a black vertical line. Arrows indicate the genealogical relations between precursors and the myocytes. (A) Non-regionalised model based on extensive cell mixing of the precursors and of the myocytes. (B) Non-regionalised model based on two stem cell systems located in the edges of the dermomyotome. (C) Non-regionalised model based on two stem cell systems located in the central part of the dermomyotome. (D) Regionalised model with a direct relationship between the precursors and the myocytes. (E) Regionalised model with an inverted relationship between the precursors and the myocytes. (F) Regionalised and temporal model based on two stem cell systems with rapid recruitment and loss of new cells. The pool is first composed of black cells that contribute to the myotome (vertical arrows) and self-renew in the dermomyotome (horizontal arrows). These black cells are progressively replaced by the gray cells and then by the white cells.
myotome or of one of the medial and lateral domains, because they will correspond to labelling of precursors of the stem cells before their allocation to one of the two domains. However, 30 of the 32 segmental contributions of the bisegmented clones contribute to less than 50% of the ML axis (Fig. 3B-D).

Thus our results refute models of myotome production based on permanent stem cell systems in the dermomyotome. However, they do not exclude more complex situations involving transient stem cells which, after some rounds of asymmetric divisions, become postmitotic and enter the myotome (see Discussion).

A direct relationship between myocytes and their precursors

A striking property of the bisegmented clones represented in Fig. 3D is that they always contribute to regions nearest to the clonal border, such that their participation seems contiguous across the clonal border. More generally, we have never observed a clone that crosses the clonal border, which participates in both extremities of the myotome without also participating in the middle region.

This property of the clones that cross the clonal border allows us to distinguish between two possible relationships between myocytes and their precursors (Fig. 6). With respect to the production of the myotome from a regionalised pool of precursors, this observation suggests a direct topographic relationship between the precursors of these clones and their descendants in the myotome (Fig. 6B). Indeed, in an inverted relationship model (Fig. 6A), clones that cross the clonal border are expected to contribute discontinuously to the two most distal regions of the myotome. The direct topographic relationship between the precursors of the clones which cross the clonal border and their descendants indicates both regional and coherent modes of growth in the central region of the paraxial mesoderm and during the subsequent translocation of the myocytes.

Comparison of the potentialities of the medial and lateral precursors

To compare the mode of production and growth of the medial and lateral parts of the myotome, we analysed several parameters of the clones restricted to these two parts of the myotome (Table 3). Firstly, the mean numbers of cells per clone are similar in the medial and lateral domains, indicating that their precursors have identical potentialities to produce myocytes. Secondly, however, the mean ML extension of the clones is smaller in the medial than in the lateral domains, and this results in a higher density of cells in the medial part of the myotome (Table 3). These data indicate that significantly more intermingling of myocytes occurs in the hypaxial, compared to the epaxial domain. These results indicate that, despite the identical potentiality of medial and lateral precursors to produce myocytes, medial and hypaxial domains exhibit differences in their cellular organisation.

DISCUSSION

Previous analyses at the cellular level have described various features of the mediolateral organisation of the paraxial mesoderm and myotome. However, these analyses are limited in their scope, particularly in the mouse due to the inaccessibility of the post-implantation embryo after E4.5. The LacZ method of clonal analysis used here to study the formation of the myotome is based on a spontaneous genetic labelling event in any cell of the targeted tissue. It allowed us to reveal many points concerning the mediolateral organisation of the myotome, including (i) an early regionalisation of the myotome precursors before their allocation to one segment, (ii) a late clonal separation between medial and lateral myotomes in the somite, (iii) that these clonal domains probably correspond to epaxial and hypaxial myotomes, (iv) that these domains are not produced from two permanent stem cell systems, and (v) a direct spatial relationship between the myocytes and their precursors in the dermomyotome. These findings have important implications for the understanding of the formation of the myotome.

Formation of the epaxial and the hypaxial myotomes from the dermomyotome

Several models that accomodate the existence of separate precursor pools for the epaxial and hypaxial myotomes could theoretically explain the relationship between myotomal cells at E11.5 and their precursors in the dermomyotome (Fig. 7). There are non-regionalised models based on extensive cell mixing (Fig. 7A), resulting in a high proportion of clones contributing to the whole epaxial or hypaxial domain, or based on two permanent stem cell systems, one in which the stem cell systems are located at the edges of the dermomyotome (Fig. 7B), and another in which the stem cell systems remain in the central part of the dermomyotome (Fig. 7C), resulting in temporally inverted orientations of myocyte production. There are also regionalised models, in which myocyte precursors give rise to descendants in only a fraction of the ML axis of the myotome. In these models, myocyte precursors in the dermomyotome are organised in relation to the future position of their descendants in the myotome, either with a direct topographic relationship (Fig. 7D) or with an inverted one (Fig. 7E). Models based on transient stem cell systems in which the precursors, after some rounds of divisions, become postmitotic and enter the myotome, can be included in this category (Fig. 7F).

Our results clearly refute non-regionalised models based on two permanent opposite stem cell systems located in the dorsomedial and ventrolateral lips of the dermomyotome (Fig. 7B, Fig. 5A,C,D). Moreover, as the participation of all the monosegmented and bisegmented clones along only a fraction of the mediolateral axis of the myotome excludes the non-regionalised models (Fig. 7A-C, Fig. 2, Fig. 3B-D), we favour models involving a regionalisation of the myotome precursors (Fig. 7D-F). Finally, the existence of plurisegmented clones, which are contiguous across the line that separates the two domains, excludes an inverted relationship between cells in the dermomyotome and myotome (Fig. 7E, Fig. 3C, Fig. 6A). Therefore our results are consistent only with the direct relationship between myocytes and their precursors.
regionalised models of formation of the myotome from the
dermomyotome (Fig. 7D,F).

It is interesting to draw a parallel between this regionalisation
of the myotome and recent findings in birds, which indicate that
the central part of the dermomyotome contributes to the
formation of the medial part of the hypaxial myotome, and the
lateralmost dermomyotome contributes to the lateral part of the
hypaxial myotome (Olivera-Martinez et al., 2000). Moreover,
the expression patterns of some genes, like enl or siml in the
E10.5 mouse embryo, suggest the existence of three domains in
the dermomyotome and myotome (Spörle et al., 2001; Tajbakhsh
and Buckingham, 2000), in which the third, central domain
could produce the subjacent myotome. Furthermore, Myf-5
expression has been shown to be regulated independently in
different mediolateral subdomains of the myotome by distinct
enhancers (Hachouel et al., 2000). These data indicate that
regionalised gene expression is superimposed on cellular
regionalisation within the somite.

The direct relationship between myocytes and their precursors
in the dermomyotome distinguishes this translocation event from
the indirect one that occurs between epiblast and the mesoderm
during gastrulation (Keller and Danilchik, 1988; Lawson et al.,
1991). We propose that this regionalisation could allow the early
establishment of differential signals in relation to the final
position of myocytes in the myotome. The direct relationship
between precursors in the dermomyotome and myocytes could
then allow the latter to remain in the same mediolateral signaling
environment after their translocation. For instance, daughters of
a precursor cell located near the neural tube will stay nearby, and
dughters of a precursor cell close to the lateral plate will
maintain this localization.

**A clonal separation in the myotome and between its
immediate precursors**

Regionalisation of myotomal precursors

Circumstantial evidence in birds first suggested a separation
between medial and lateral precursors of the paraxial mesoderm
(Selleck and Stern, 1991), which was later proposed for the precursors of the epaxial and hypaxial musculature in somites at
the limb bud level (Ordahl and Le Douarin, 1992) and at the
thoracic level. In contrast, substantial cell mixing occurs
between epaxial and hypaxial muscles derived from grafted
halves of thoracic somites (Ordahl et al., 2000). More recently,
other studies in the dermomyotome have shown distinct
localisations of the precursors of the epaxial and hypaxial
domains of the myotome, at both limb and trunk levels
(Denetclaw et al., 1997; Denetclaw and Ordahl, 2000; Olivera-
Martinez et al., 2000; Huang, 2000).

In this study, we have shown that from the time of their
formation, the medial and lateral domains of the myotome are
clonally distinct and that their immediate precursors in the
dermomyotome (and probably in the somites as well) are
organised into two strictly distinct pools. This clonal separation
is apparently established by the time of budding of the somites
from the paraxial mesoderm, because we did not observe any
monosegmented clones contributing to both medial and lateral
regions of the myotome. Furthermore, four of the eight
restricted, bisegmented clones have only one cell in the posterior
segment. As weak mixing may occur between cells of adjacent
somites, these four clones may have been generated after the
time of segmentation. This hypothesis would then significantly
strengthen this demonstration of the existence of a clonal
separation in the somite. This clonal separation does not,
however, occur before the allocation of precursors to a segment,
because the precursors of half of the bisegmented clones do not
respect this separation. The 8 remaining bisegmented clones
restricted to one or the other clonal domains could result either
from a possible start of the establishment of the clonal separation
in the presomitic mesoderm, or, more probably, from the general
regionalisation of the precursors of the myotome established
earlier.

We showed that the boundary between the two clonal domains
correlates with morphological indentation of the body wall,
which marks the limit between epaxial and hypaxial myotomes
in E11.5 mouse embryo (Hachouel et al., 2000; Tajbakhsh
and Buckingham, 2000) (S. Tajbakhsh, personal communication).
This suggests that epaxial and hypaxial myotomes are clonally
distinct and thus represent two cellular compartments in the
thoracic segments. This boundary could, in fact, provide a strict
separation between two types of muscle formation that require
different, and maybe incompatible, regulatory pathways: the
epaxial muscles that form in situ, and the hypaxial muscles that
are generated from migrating populations of cells. It is not
possible, at the present time, to extrapolate our data to somites
anterior to the forelimbs and posterior to the hindlimbs.
However, if the comparison between mouse and avian embryos
is extended further, it can be hypothesised that this clonal
boundary also exists at the limb bud level (Denetclaw et al.,

If clonal separation is established just after segmentation, at a
time when the dermomyotome and sclerotome have not
differentiated, it will be of interest to determine whether other
somitic structures are also involved. Analyses done in HH15-17
chick embryos show a separate origin of the medial and lateral
precursors of the dermomyotome (Denetclaw et al., 1997;
Denetclaw and Ordahl, 2000). It has also been suggested that the
proximal and distal parts of the skeleton derive from the medial
and lateral parts of the sclerotome, respectively (Christ and
Wilting, 1992), but this has only been demonstrated for the
whole somite and not at the sclerotome level (Olivera-Martinez
et al., 2000; Ordahl et al., 2000). The existence of clonal
compartments has also been proposed for the dorsal and ventral
surface ectoderm at the limb and flank levels, with the separation
residing along a line drawn between the bases of the wing and
leg buds (Altabef et al., 1997). We suggest that this line may
correspond to the morphological indentation that marks the
boundary between the epaxial and hypaxial body domains.
Although the timing of these latter restrictions is not known, it
suggests that the clonal separation observed here for the
myotome and its precursors may reflect a more general
separation between dorsal and ventral domains, which may
involve the whole somite and surface ectoderm. Furthermore, a
single mechanism may be involved to establish the clonal
separation in these different tissues.

**Establishment and maintenance of the clonal
boundary**

Several elements may be involved in the establishment of this
clonal separation. For example, coherent cell growth and
behaviour in the epithelial somite and epithelial dermomyotome
may be a crucial component of this process. However, if such
coherent growth is not oriented differently in the two domains,
such a mechanism alone would not be sufficient to prevent cells
from crossing the clonal boundary. Thus, the existence of an active frontier seems necessary between the medial and lateral halves of the structures. This frontier could be based on precise cell-cell and/or cell-matrix interactions that would prevent mixing or promote cell-sorting between the different clonal domains, as in the rhombencephalon (Mellitzer et al., 1999; Wizenmann and Lumsden, 1997; Xu and Wilkinson, 1997). Although many adhesion molecules are expressed in the somite, most are not expressed preferentially in one or the other of the clonal domains. A more systematic analysis of gene expression or adhesive properties in the medial and lateral cells of the somite, dermomyotome and ectoderm may reveal important differences and should contribute to our comprehension of the formation of this boundary. At a later stage, during the growth of the dermomyotome, the formation of a quiescent zone between the epaxial and hypaxial precursors (observed in the chick) (Denetclaw and Ordahl, 2000) may serve to maintain and reinforce the initial separation of the two precursor pools. The maintenance of the clonal boundary during formation of the myotome may also result from passive or active mechanisms. Our observation of intercalating myocytes along the whole mediolateral axis of the myotome, and especially near the clonal boundary, excludes the hypothesis that the clonal boundary in the myotome is maintained simply due to coherent cell behaviour. Thus, other characteristics of the system must be involved. The lag between the start of the epaxial and the hypaxial myotome formation (Denetclaw and Ordahl, 2000) could intervene in the persistence of the clonal boundary. Another attractive possibility is the temporal production of the epaxial and hypaxial myotomes in opposite orientation, from two ‘stem cell’ pools residing at the edges of the dermomyotome (Denetclaw and Ordahl, 2000). But to be in agreement with a regionalised model of production of the myotome from the dermomyotome, such pools should be dynamic (rapid recruitment and loss of new cells, Fig. 7F). This last model, which is compatible with our results, is both regionalised (Cinnamon et al., 1999; Denetclaw et al., 2001; Kahane et al., 1998b), and temporal (Denetclaw and Ordahl, 2000). Alternatively, the acquisition of different adhesive properties between the epaxial and hypaxial cells that will translocate to the myotome, could also function by preventing cell mixing among the myocytes of the two clonal domains, or by allowing a separation of the epaxial and hypaxial cells through cell sorting. Finally, it is interesting to note that En-1, homologue of engrailed, which is involved in maintenance of the rostrocaudal boundary in the drosophila wing (Blair, 1992), is also expressed at the level of the boundary in the dermomyotome of the mouse embryo (Davis et al., 1991; Spörle et al., 2001), and in the dermomyotome, myotome and surface ectoderm of the chicken embryo at the limb and flank levels (Gardner and Barald, 1992), which suggests that this gene may play a role in the maintenance of the dorsoventral boundary in these tissues.

**Formation of the medial and lateral myotomes**

We observed more extensive intercalation among myocytes of the monosegmented clones in the lateral domain, compared to the medial domain of the myotome. This may be due to differences in the degree of coherence during the growth of the precursors in the dermomyotome, the degree of intercalation during the translocation of the myocytes, or the subsequent migration of lateral cells to form the bodywall muscles. Moreover, the more rapid growth of the hypaxial part of the dermomyotome and myotome (Denetclaw and Ordahl, 2000), could by itself explain elevated intercalation in the lateral domain.

Despite this difference, the other properties of myotome precursors (such as the number of cells produced, their regionalisation along the ML axis and the existence of intercalation) are remarkably similar in the medial and the lateral domains. This finding suggests that the modes of myocyte production in the two domains are similar, which is also the case in avian embryos (Cinnamon et al., 1999; Denetclaw and Ordahl, 2000). Although this similarity at first seems surprising, because of the known differences between the extrinsic and intrinsic signals in the medial and lateral domains of the dermomyotome [reviewed by Hirsinger et al. (Hirsinger et al., 2000)], it could be that the characteristics involved in production of the medial and lateral myocytes are established independently of these signals. Alternatively, completely different signalling pathways could converge on similar modes of production of differentiated cells.

**Intercalation of the myocytes**

The existence of a clonal separation during the formation of the myotome and, before this, the regionalisation formation of the myotome from the dermomyotome, could not be explained without a certain degree of cell coherence during the formation of the myotome and between myocytes, in addition to that observed in the precursor pool. However, in all the clones analysed, either monosegmented, bisegmented or longer, we observed unlabelled myocytes intercalated with the genealogically related labelled myocytes, indicating that intercalation nevertheless occurs during these processes. Such intercalation has also been suggested to occur in avian embryos, between the myocytes of the primary myotome (Cinnamon et al., 1999; Denetclaw et al., 1997). Because both the primary and the secondary myotomes (already formed at E11.5) can be labelled in our clones, intercalation must also involve myocytes of the secondary myotome. Our observation of intercalation between myocytes in E12.5 mouse embryos (data not shown) is also consistent with this idea. Kalcheim and coworkers have proposed that the myocytes of the primary myotome in quail embryos, when translocating from the dermomyotome, intercalate with older, already translocated, ‘pioneer’ fibers, suggesting that intercalation occurs between the myocytes produced in successive waves (Cinnamon et al., 1999; Kahane et al., 1998a; Kahane et al., 1998b).

This intercalation of clonally related myocytes could result from different mechanisms. Before the segmentation occurs, the myotome precursors could undergo a ‘coherent intercalation’ that would respect their mediolateral regionalisation. However, such a mechanism is unlikely for the precursors located in the somite (monosegmented clones), because intercalation does not normally occur in epithelia (Gardner and Lawrence, 1985). Another possibility is that the mediolateral growth of the dermomyotome, together with the formation of the myotome (Denetclaw et al., 1997; Denetclaw and Ordahl, 2000), simply results in a mechanical intercalation of the myocytes, due to a shift in the relative positions of the precursors located in the dermomyotome. Finally, intercalation could occur during translocation of the myocytes into the myotome (Cinnamon et al., 1999). Indeed, intercalation could result from the necessary convergence and extension of myoblasts that translocate from an epithelium (formed by many rostrocaudal layers of cells) to
produce fewer layers of unit-length cells, as described during gastrulation in the Xenopus embryo (Keller and Daniilchik, 1988; Keller and Tibbetts, 1989).

Whatever the mechanism(s) involved, this intercalation results in the physical separation of myocytes and their precursor cells, and of myocyte daughter cells. Intercalation may serve to disrupt interactions between genealogically related cells and, thereby, permit novel interactions with other types of cells. It is possible that these new interactions are necessary and instrumental in controlling the coordinated growths of the dermomyotome and myotome and/or in further patterning of these structures.

We thank Luc Mathis for careful reading of the manuscript and for experimental advice, and Shahraghim Tajbakhsh for helpful discussions. We also thank Robert Kelly for careful reading of this version of the manuscript. This work has been financially supported by grants from the Pasteur Institute, the CNRS (Centre national pour la Recherche scientifique), the ARC (Association pour la Recherche contre le Cancer) and the AFM, (Association française contre les Myopathies). S.E.-T. was a recipient of a fellowship from the MENRT (Ministère de l'Education nationale, de la Recherche et de la Technologie). J.-F.N. is from the INSERM (Institut national de la Santé et de la Recherche médicale).

REFERENCES


Cinnamon, Y., Kahane, N., Bachelet, I. and Kalcheim, C. (1999). Characterization of the myotome and/or in further patterning of these structures. controlling the coordinated growths of the dermomyotome and that these new interactions are necessary and instrumental in interactions between genealogically related cells and, thereby, and of myocyte daughter cells. Intercalation may serve to disrupt


