Cell coherence during production of the presomitic mesoderm and somitogenesis in the mouse embryo

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SUMMARY

In this study, we investigated (in the early mouse embryo) the clonal properties of precursor cells which contribute to the segmented myotome, a structure derived from the somites. We used the lacZ method of single cell-labelling to visualise clones born before segmentation and bilateralisation. We found that clones which contribute to several segments both unilateral and bilateral were regionalised along the mediolateral axis and that their mediolateral position was maintained in successive adjacent segments. Furthermore, clones contributed to all segments, from their most anterior to their most posterior borders. Therefore, it appears that mediolateral regionalisation of myotomal precursor cells is a property established before bilateralisation of the presomitic mesoderm and that coherent clonal growth accompanies cell dispersion along both the mediolateral and anteroposterior axes. These findings in the mouse correlate well with what is known in the chick, suggesting conservation of the mode of production and distribution of the cells of the presomitic mesoderm. However, in addition, we also found that the mediolateral contribution of a clone is already determined in the pool of self-renewing cells that produces the myotomal precursor cells and thus that this pool is itself regionalised. Finally, we found that bilateral clones exhibit symmetry in right and left sides in the embryo at all levels of the mediolateral axis of the myotome. All these properties indicate synchrony and symmetry of formation of the presomitic mesoderm on both sides of the embryo leading to formation of a static embryonic structure with few cell movements. We suggest that sequential production of groups of cells with an identical clonal origin for both sides of the embryo from a single pool of self-renewing cells, coupled with acquisition of static cell behaviour, could play a role in colinearity of expression of Hox genes and in the segmentation system of higher vertebrates.

Key words: Clonal analysis, lacZ, Self-renewing cells, Stem cell, Primitive streak, Presomitic mesoderm, Segmentation, Mouse embryo, Clock, Patterning

INTRODUCTION

A central aspect of vertebrate embryo development is the segmentation and specification of its longitudinal axis (Keynes and Stern, 1988). This segmentation is visible in different structures, such as the rhombencephalon (which is organised into metameric units called rhombomeres) and paraxial mesoderm posterior to the otoc vesicle (which is organised into somites). Somites initially formed as paired epithelial balls arranged rostrocaudally on both sides of the notochord and neural tube. They bud off from the cranial end of an unsegmented region of the paraxial mesoderm, the segmental plate or presomitic mesoderm (Christ and Ordahl, 1995). Subsequently, somites differentiate into several cell types under intrinsic and extrinsic influences (reviewed by Hirsinger et al., 2000). In particular, they produce the segmented myotome.

Recently, there has been increased insight as to how the presomitic mesoderm is patterned and its link with segmentation (Vasiliauskas and Stern, 2001). It has been suggested that the timing of segmentation of the paraxial mesoderm is regulated by a molecular oscillator, the segmentation clock, and a determination front level in the presomitic mesoderm (Forsberg et al., 1998; Jouve et al., 2000; McGrew et al., 1998; Palmeirim et al., 1997; Dubrulle et al., 2001) (reviewed by Maroto and Pourquié, 2001). Furthermore, Zakany et al. (Zakany et al., 2001) proposed that the segmentation clock could provide a counting mechanism for Hox gene transcription, which might coordinate the production of novel segments with the specification of anteroposterior (AP) identity.

These complex molecular processes require precise production and organisation of cells in the presomitic mesoderm. It is therefore crucial to examine cell production, movement and distribution in relation to cell specification in the early embryo. Information essentially stems from studies in the chick and to a lesser extent (owing to technical difficulties) in the mouse. Pioneer works (Psychoyos and Stern, 1996; Selleck and Stern, 1991; Selleck and Stern, 1992; Stern et al., 1988) suggest restricted movements in the chick
precursor cells are organised at a given time during the formation of somite precursors (Psychyos and Stern, 1996). It was shown that: (1) when somite precursor cells are labelled at rostral positions, cells become confined to the medial aspect of the paraxial mesoderm and as the injection is made more posteriorly, the pathway taken by the cells becomes progressively more lateral; and (2) that the emerging cells that contribute to the somite form a stream from the injection site with some labelled cells still remaining in the area of the regressing node, suggesting that some cells remain in the primitive streak for at least 24 hours. This is consistent with the hypothesis of the existence of stem cells in the node (Selleck and Stern, 1991). In other experiments (Selleck and Stern, 1991), it was shown that injection into a cell in the midline of the node often provided labelled progeny to both left and right sides of the embryo and injection into more lateral cells gave rise to progeny that were confined to the ipsilateral side of the latter. Finally, injection of a single cell within the rostral or caudal part of the segmental plate indicates that 2 days later clones were still discrete, being confined at most to one segment long region (Stern et al., 1988).

In the mouse, with respect to the organisation of the primitive streak, more caudal regions of the latter give rise to progressively more lateral mesoderm (Lawson et al., 1991; Smith et al., 1994; Tam and Beddington, 1987), and injections of fluorescent dye at the most rostral level of the primitive streak principally label cells of the paraxial mesoderm (Smith et al., 1994). Furthermore, groups of prospective mesodermal cells ingress bilaterally from the primitive streak. As in chick, it has been hypothesised that cells remaining in the streak or tail bud at the termination of prospective labelling studies represent a minority in the population composed of stem cells (Beddington, 1994; Tam and Beddington, 1987; Wilson and Beddington, 1996). Direct evidence for stem cell precursors of the myotome (Nicolas, 1996; Eloy-Trinquet and Nicolas, 2000; Eloy-Trinquet and Nicolas, 2002) strengthens this hypothesis.

Despite this large amount of information, there are major questions that remain incompletely or totally unresolved. This information is crucial for complementing what is known in chick and providing the missing data in mouse and for comparing embryonic operations in a phylogenetic perspective. For example, the degree of overlapping at the clonal level of the precursor cells of one somite and the amplitude of cell regionalisation of the early precursors of subregions of one somite are not known. This is because the available observations concern labelling of groups of cells rather than injection into a single cell. Similarly, the extent of mediolateral (ML) dispersion of genealogically related cells in one somite and in somites at different rostrocaudal levels populated by related cells has never been documented. Finally, apart from observation of the bilateral contribution of cells of the midline in the node (Selleck and Stern, 1991), nothing is known in chick or mouse about the contribution of single cells to the left and right sides of the structure, and their degree of symmetry along the AP or ML axis. Nevertheless, this kind of information is indispensable for determining if and how the precursor cells are organised at a given time during the production of these structures and for establishing how precursor cells can generate two distinct pools of cells for each side of the embryo. As discussed above, this missing information is necessary for confronting the cellular aspects of somitogenesis with the genetic basis of segmentation and of specification.

To address these issues, we used the lacZ method to clonally analyse the formation of the myotome. This method is based on the generation of a functional lacZ reporter gene during normal development of transgenic mice carrying an inactivated lacZ transgene through a random intragenic recombination event that re-establishes the open reading frame of the reporter gene (Nicolas et al., 1996; Eloy-Trinquet and Nicolas, 2000). This method previously led us to propose a model for formation of the myotome from a stem cell system located in the primitive streak (Nicolas et al., 1996; Stern et al., 1988; Selleck and Stern, 1991; Psychoyos and Stern, 1996). However, although this model accounts for the AP production of the myotome, and probably of the entire paraxial mesoderm, it does not address the issues listed above. In this study, we present for the first time a complete clonal analysis of the mediolateral contribution of lacZ/lacZ chimeras in the mouse myotome. The description of the position of each β-gal+ cell in these clones leads us to the conclusion that ML regionalisation of the precursors of the myotome can be traced back to a single pool of self-renewing cells before bilateralisation, and that it is accompanied by coherent cell behaviour up to the time of formation of the myotome. We also considered how the cells of a single pool of precursors could generate two pools of precursors for each side of the embryo, an issue never addressed previously. These new data facilitate discussion of the clonal patterning of the precursors of the myotome in relation to concomitant segmentation by the segmentation clock and genetic specification by Hox genes.

MATERIALS AND METHODS

Transgenic animals

The α-2 mouse transgenic line used in this study is the same as that described by Nicolas et al. (Nicolas et al., 1996). It harbours a nlslaacZ gene under the control of the chick promoter of the α2-subunit of the acetylcholine receptor that drives the expression of the transgene in the myotome. Consequently, only the myotome descendants of the labelled cells are detected. All embryos resulted from crosses between homozygous transgenic males and C57BL/6·DBA/2 F1) females. The morning of copulation is taken as 0.3 days post coitus (E0.3). The embryos where fixed, stained histochemically and cleared as previously described (Mathis and Nicolas, 1998).

Description of position of β-gal+ cells in the lacZ/lacZ embryos

Clones used in this study are those described by Nicolas et al. (Nicolas et al., 1996) and 162 new clones. The analysis concerns all the long clones (anteroposterior extension beyond seven segments) and those short clones which contribute only to thoracic somites (segments 12 to 24). ML position of the β-gal+ cells of the clones was described in each labelled segment using a stereomicroscope equipped with a camera connected to a computer. The position of each β-gal+ cell was determined as a percentage of the length of the myotomal segment, using the ‘LIDA’ and ‘LIDA Calibration server’ software (Leica). Position 0% is at the medial end of the segment and position 100%...
Cell organisation during somitogenesis is at its lateral end. Some α2 embryos with clones and an embryo of the control transgenic line were cut transversely to visualise the position of the β-gal+ cells in the myotome.

**Analysis of β-gal+ clones at the thoracic level**

To obtain clonal information on the regionalisation of the myotome, the α-2 lacZ/laacZ genetic mosaics with clones contributing to the thoracic level of the embryo were analysed. As the myotome in segments 12 to 24 exhibit similar characteristics in both relative arrangement and cell number (Eloy-Trinquet and Nicolas, 2000; Eloy-Trinquet and Nicolas, 2002), all thoracic clones were pooled together.

**RESULTS**

To investigate regionalisation of myotomal precursor cells, we analysed lacZ clones obtained from laacZ E11.5 embryos produced from crosses between α-2 transgenic males and (C57Bl/6xDBA/2 F1) females (Nicolas et al., 1996). α-2 transgenic line expresses the transgene in the myotome of all 40 myotomal segments present at E11.5 and in the forming muscle masses of the limb buds (Nicolas et al., 1996). Analysis of the labelled cells in several lacZ clones confirmed that all β-gal+ cells are included in myotomal structures (Fig. 1O,P).

Among 6232 α-2 E11.5 embryos that express nlslaacZ in the myotome, 315 lacZ clones were obtained. They correspond to random labelling events of the various myotomal precursor cells present during development. The geometrical characteristics of the clones suggest a classification into four categories: monosegmented clones; short plurisegmented clones (up to 6 segments long), either unilateral (Fig. 1F-H) or bilateral (Fig. 1I-P); and long clones (more than six segments long, Fig. 1C-E), mainly bilateral. In a previous study (Eloy-Trinquet and Nicolas, 2002), analysis of unilateral monosegmented and bisegmented clones at the thoracic level showed that there is a ML regionalisation within the myotome, across the entire ML axis, which is established prior to the allocation of precursor cells to one segment. This ML regionalisation is followed, within a segment, by clonal separation of precursors of the medial and lateral parts of the myotome. In this study, we investigate the important issue of the stage at which myotomal precursor cells become mediolaterally regionalised and whether further organisation can be detected at early stages of production of the paraxial mesoderm. To investigate this, we have analysed the mediolateral contributions of plurisegmented clones, both unilateral and bilateral. This analysis was restricted to the thoracic level as thoracic segments exhibit similar geometrical characteristics and cell numbers (Fig. 1A) (Eloy-Trinquet and Nicolas, 2000).

**Mediolateral contribution of short unilateral plurisegmented clones**

We first investigated whether the ML regionalisation observed...
for the precursors of unilateral monosegmented and bisegmented clones (Eloy-Trinquet and Nicolas, 2002) holds true for short unilateral plurisegmented clones. We measured the total ML extension for each of the 23 thoracic short unilateral plurisegmented clones, by superposition of the labelling in successive segments for each clone (Fig. 2A). β-gal+ cells are dispersed over less than 50% of the ML axis in 18 clones out of 23 (Table 1), indicating that their pooled contribution to several segments is restricted to a portion of the ML axis. These regionalised contributions are observed in medial, lateral and intermediate regions of the ML axis (Fig. 1F-H, Fig. 2B, see clones LM38, SC176 and LM23 for examples). These results indicate that ML regionalisation is already established in the pool of precursors of short unilateral clones, and that it is not restricted to one region of the myotome, but is a characteristic of the whole ML axis.

<table>
<thead>
<tr>
<th>Clones</th>
<th>Number of clones of mediolateral extension (%)*</th>
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</thead>
<tbody>
<tr>
<td>Short unilateral plurisegmented clones (n=23)</td>
<td>9 (39.1%) 9 (39.1%) 4 (17.4%) 1 (4.3%)</td>
</tr>
<tr>
<td>Unilateral contributions of short bilateral clones (n=48)†</td>
<td>13 (27.1%) 19 (39.6%) 11 (22.9%) 5 (10.4%)</td>
</tr>
<tr>
<td>Short bilateral clones (n=24)</td>
<td>2 (8.3%) 8 (33.3%) 7 (29.2%) 7 (29.2%)</td>
</tr>
<tr>
<td>Long clones (n=31)</td>
<td>2 (6.5%) 2 (6.5%) 13 (41.9%) 14 (45.2%)</td>
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</table>

*Mediolateral extension was measured on pooled thoracolumbar labelled segments.
†Unilateral contributions of short bilateral clones correspond to the left and to the right contributions of the short bilateral clones, treated separately.

**Table 1. Mediolateral extension of plurisegmented clones**

**Fig. 2.** (A) Definition of the mediolateral extension (MLE) of the labelling in pooled segments of a clone. (B,C) ML contribution of the short thoracic unilateral plurisegmented clones (B) and of the short bilateral clones (C). Each clone is represented by one (B) or by two white rectangles for the left and right sides of the embryo (C). The medial and lateral borders of the myotome are indicated. The height of the rectangles is proportional to the longitudinal extension of the clone (number of segments). Each segment is divided into a hundred parts of equal length. Each part with labelled cells is represented by a coloured bar. The number of labelled cells is colour coded: blue, one cell; green, two cells; yellow, three or four cells; red, five cells or more. m, medial; l, lateral; MB, medial border; LB, lateral border; MLE, mediolateral extension.
Cell organisation during somitogenesis

Cell behaviour of precursors of short plurisegmented clones

How is this ML regionalisation maintained during the divisions that separate the precursor cells of these short plurisegmented clones and differentiated myocytes, in particular during the production of individual somites? A simple hypothesis is that both coherent growth and coherent precursor cell movements are maintained during these processes. According to this hypothesis, the ML position of the contribution of the clones in successive adjacent segments should be maintained.

To investigate this possibility, we calculated the middle position (MidP) of each β-gal⁺ group of cells in successive segments. MidP = (MB + LB)/2, where MB is the medial border of the labelling and LB the lateral border of the labelling (Fig. 3A). This parameter takes into account both the ML position and extension of the labelling in each segment. Note that by doing this, the most extreme values have the same weight than the other. Therefore, this parameter tests stringently the hypothesis of coherent growth. The correlation of the MidP values for adjacent segments has been evaluated graphically (Fig. 3B). Each clone with two labelled segments is represented by a single black point, and clones with more than two labelled segments are represented by several points of different colours. The nearer to the y=x axis the point is, the nearer the MidP values are. To evaluate whether the MidP of successive segments is correlated, a limit of 20% has been chosen, which corresponds to a distance of two cell diameters in a newly segmented somite (the size of the segment is 100 μm, the size of a cell is 10 μm) (Tam, 1981). The majority of points (26 out of 30) are in the |y-x|≤20% area of the graph. The ML contributions of the clones in successive segments are therefore strongly correlated. This correlation is observed at all positions along the ML axis, as indicated by the dispersion of MidP values along the x axis from 18% to 90%.

These characteristics of unilateral clones are also observed for the left and right contributions, treated separately, of short bilateral clones (Table 1 and Fig. 11-P, Fig. 2C and Fig. 3C), suggesting that the pool of myotomal precursor cells has limited coherent clonal growth along the ML axis before the formation of somites, and that the relative positions of the precursors are conserved during segmentation, the tenfold ML expansion of the dermomyotome and myotome, and the translocation of cells from the dermomyotome to the myotome.

Coherent cell behaviour along the anteroposterior axis

Does this limited coherent clonal growth also apply to cell dispersion along the AP axis? If precursor cells were widely dispersed along the AP axis [over more than two segments (150 μm)], they would be expected to frequently participate in non-adjacent segments. However this is rarely the case. For example, majority of the short unilateral plurisegmented clones [18 clones out of 23 (78%)] contribute to all segments from their most anterior to their most posterior borders (Fig. 2B). This characteristic is also found for each side of the short bilateral clones: 42 out of 48 (87.5%) of the plurisegmented labelling contribute to all segments between their anterior and posterior borders (Fig. 2C). This suggests that there is a relatively high level of coherent clonal growth of the pool of myotomal precursors along the AP axis before their allocation to a segment. Together these data suggest a global coherence of myotomal precursor cells along both the ML and AP axes.

Fig. 3. Correlation between the positions of the labelling in consecutive segments of short unilateral plurisegmented clones (B), of short bilateral clones (C) and between the positions of the labelling in the two sides of short bilateral clones (E). (A,D) Definition of the middle position of the labelling (MidP) in one segment (A) and in the segments of one side of the myotome (D). x and y axis represent MidP in, respectively, the most anterior and the most posterior of the two compared segments (B,C), or in the left and the right sides of the clone, respectively (E). In B, when the clone has only two labelled segments, the comparison between these two segments is represented in black, and when the clone has more than two labelled segments, the comparisons for the different couples of segments are represented with the same colour. In C, the comparisons for the different couples of segments of each unilateral contribution of a bilateral clone are represented by the same symbol and colour. [y-x]|≤20% area of the graphs is shaded. m, medial; l, lateral; MB, medial border; LB, lateral border.
Mediolateral contribution of short bilateral clones
We subsequently investigated whether ML regionalisation of myotomal precursor cells precedes bilateralisation of the paraxial mesoderm. We calculated the total ML extension of labelling in pooled left and right labelled segments for 24 thoracic short bilateral clones (Fig. 2C, Table 1), derived from precursor cells that have resided in a non-bilateralised embryonic structure. Again the β-gal<sup>+</sup> cells of short bilateral clones are not fully dispersed along the ML axis of the myotome. Labelled cells are dispersed over less than 50% of the ML axis in 10 clones out of 24 and over more than 75% of the ML axis in only seven clones (Table 1). It appears therefore that the precursors of short bilateral clones are already mediolaterally regionalised. The observation that both unilateral and bilateral clones have a restricted ML distribution suggests that ML regionalisation of myotomal precursors is established before bilateralisation of the presomitic mesoderm, and is maintained throughout segmentation and somite development.

Mediolateral cell behaviour of precursors of short bilateral clones
To investigate whether mediolateral regionalisation of the precursors of short bilateral clones is also associated with coherent cell behaviour, we analysed the correlation of the middle position (MidP values) of labelling in the left and right sides of the embryo (Fig. 3D,E). Seventeen out of 24 clones are in the |y-x|≤20% area of the graph (shaded), for either low or high MidP values, which correspond to medial or lateral clones. This symmetry can be observed in transverse sections of the clones (Fig. 1O-P, compare the left and the right contribution of the bilateral clones). These results indicate that short bilateral clones exhibit symmetry, and that this symmetry concerns the whole ML axis of the myotome. This strongly suggests that the pool of myotomal precursors cells is already coherent in the non-bilateralised structures of the embryo.

Mediolateral contribution of long clones
So far, this analysis has concerned the pool of cells that originally give rise to short bilateral clones, which are in turn derived from self-renewing cells that give rise to long bilateral clones (Nicolas et al., 1996). We next analysed whether ML regionalisation is also a characteristic of this pool of self-renewing cells. For 31 long clones contributing to thoracic segments between the limbs, we measured their maximum ML extension after superposition of labelling in left and right thoracic segments.

Four long clones that contribute to the thoracic level of the embryo are clearly mediolaterally restricted, as they contribute to less than 50% of the ML axis of the myotome (Table 1). Three of these restricted clones are at various medial positions (their ML extremities are 14% to 49%, 21% to 43% and 18% to 34%), and one is at an intermediate position (28% to 67%). These clones show a correlation of the ML positions of labelled cells in adjacent segments and between the left and right sides of the embryo (Fig. 1C,D). This regionalisation is maintained during the formation of 10 thoracic segments. This can only be explained by cell coherence of the precursor cells in the self-renewing pool, and suggests that the ML contribution of the four long clones is already determined in the self-renewing precursor cell population.

To investigate the possibility that the apparently less restricted long clones may also be symmetrical, we compared the ML position (MidP values, Fig. 4A) of the β-gal<sup>+</sup> cells in the labelled thoracic segments of the left and right sides of the myotome. This analysis was performed for the 23 clones that

**Fig. 4.** Comparison of the middle position (MidP values) of the labelling in pooled labelled thoracic segments of the right and left sides of long clones contributing bilaterally to the thoracic level. (A) x axis and y axis represent MidP values in, respectively, the left and the right sides of the embryo (Fig. 3D,E). Seventeen out of 24 clones are in the |y-x|≤20% area of the graph (shaded), for either low or high MidP values, which correspond to medial or lateral clones. This symmetry can be observed in transverse sections of the clones (Fig. 1O-P, compare the left and the right contribution of the bilateral clones). These results indicate that short bilateral clones exhibit symmetry, and that this symmetry concerns the whole ML axis of the myotome. This strongly suggests that the pool of myotomal precursors cells is already coherent in the non-bilateralised structures of the embryo.

**C**

<table>
<thead>
<tr>
<th>Maximal theoretical difference between the MidP values</th>
<th>0</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
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<tbody>
<tr>
<td>obsAMidP&lt;20%</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>20% of maxAMidP&lt;obsAMidP&lt;20%</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>obsAMidP&lt;20%</td>
<td>0</td>
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<tr>
<td>20% of maxAMidP&lt;obsAMidP&lt;20%</td>
<td>0</td>
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<td>maxAMidP&lt;20%</td>
<td>0</td>
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<td>0</td>
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</table>

Clones outside this area are considered to be asymmetric. The grey area corresponds to observed difference between MidP inferior to 20% of the segment length but superior to the 20% maximal theoretical difference (non-informative clones). The blue area corresponds to the observed difference between MidP inferior to 20% of the segment length and inferior to the 20% maximal theoretical difference (symmetrical clones). m, medial; l, lateral; MB: medial border, LB, lateral border.
contribute to both sides of the embryo at the thoracic level. We found that 19 clones have MidP values that differ by less than 20% (Fig. 4A, shaded area). However, if the ML extension of a clone is large on both sides of the embryo, each MidP value will be close to 50% and the difference between left and right MidP values will be low. In order to overcome this problem and to test more stringently whether the 23 clones exhibit a significant degree of symmetry, we compared the observed difference between their left and right MidP values (Fig. 4C, y axis) with the maximal theoretical difference (Fig. 4C, x axis). The maximum theoretical difference was calculated by displacing the left labelling to the medial extremity and the right labelling to the lateral extremity of the myotome (Fig. 4B). We consider that a clone is significantly symmetrical if the observed difference between the MidP values is inferior to 20% of the maximal theoretical difference (Fig. 4C, blue area). Six clones are non-informative because, although their MidP values differ by less than 20% of the segment length, this corresponds to more than 20% of the maximal theoretical difference (Fig. 4C, grey area). However, the other 13 clones (76.5% of the informative clones) are symmetrical (Fig. 4C, blue area) and only four are not symmetrical (Fig. 4C, white area).

This symmetry of the ML contribution observed for the majority of long clones on the left and right sides of the embryo, together with the existence of four mediolaterally restricted long clones, suggest that such a ML contribution is already determined in the pool of self-renewing cells that produces the myotomal precursor cells, and thus that this pool is regionalised.

**Prefiguration of the mediolateral regionalisation of the myotome in the pool of precursor cells before bilateralisation**

The ML regionalisation of β-gal + cells in short and long bilateral clones suggests ML regionalisation is already prefigured in the pool of precursors that contribute bilaterally to the myotome. The symmetry of the clonal contribution observed on the left and right sides of the embryo suggests that left and right sides of the myotome originate from a single pool of precursor cells. But still several possibilities could explain these observations. The major classes of hypothesis of how the regionalised ML axis of the myotome (Fig. 5, green arrows and colour gradients) is represented in the pool of precursors of bilateral clones (Fig. 5, red arrows and colour gradients) are presented below.

First, left and right sides of the myotome could be partially separated in the precursor pool (Fig. 5A). According to this hypothesis, the ML axis would be already duplicated in the precursor pool (Fig. 5A, red double-arrow). Second, both sides of the myotome could be derived from a common pool of precursor cells before bilateralisation. In this case, three situations could be envisaged: (1) there is no prefiguration of the ML axis in this unique pool and the left and right contributions of the descendants of each precursor are random as far as their original ML position is concerned (Fig. 5B); (2) the ML axis of the pool prefigures the ML axis of both the left and right myotomes (Fig. 5C); (3) an axis orthogonal to the ML axis of the myotome prefigures both left and right myotomes (Fig. 5D). In the latter case, a topographic transformation must occur during bilateralisation of the paraxial mesoderm.

The properties of short and long bilateral clones exclude all proposed models except the last one. In the hypothesis of separate representations of the left and right sides (Fig. 5A), the majority of the clones are expected to be unilateral and bilateral clones are expected to correspond to only one category of left-right symmetry (all medial). However, there are only a few long unilateral clones and the bilateral clones display all possible ML distributions (medial, intermediate and lateral). In the hypothesis of a unique representation of the left and right myotomes and a random distribution of the daughter cells (Fig. 5B), the clones are not expected to be symmetrical, whereas the majority of short and long clones are symmetrical (Fig. 1C-D, I-P, Fig. 2C, Fig. 3E, Fig. 4). In the hypothesis of a prefiguration along the ML axis (Fig. 5C), the clones are expected to be systematically disymmetrical (medial-lateral and lateral-medial), and again this is not observed. To predict
all the categories of observed clones successfully, and in particular the symmetry of the lateral short bilateral clones (Fig. 5D, close circles), the prefiguration of the ML axis must be orthogonal in the pool of precursor cells before bilateralisation.

Therefore, future ML regionalisation of both the left and right sides of the myotome is most probably prefigured orthogonally in the precursor pool prior to bilateralisation of the paraxial mesoderm. A comparison with fate mapping mainly in the chick (Psychoyos and Stern, 1996) and in the mouse (Smith et al., 1994) suggests that this ML regionalisation corresponds to the AP axis of the streak (see Introduction and Discussion) There is an obligatory reorientation associated with coherent cell behaviour during bilateralisation of the paraxial mesoderm.

**Left-right contributions of β-gal+ clones to the myotome**

So far, this analysis has revealed that the pool of precursor cells of short and long bilateral clones are organised along one axis that prefigures the organisation of the ML axis of both the left and right sides of the myotome. But how are two pools of precursor cells for each side of the embryo produced from a unique pool of self-renewing cells? To investigate this point, we analysed the left and right contribution of the 85 long and 58 short bilateral clones (irrespective of their contribution to thoracic segments of the embry).

Strikingly, there are seven long clones (out of 85) that contribute to only one side of the embryo (Fig. 1E), three of which have an AP extension exceeding 19 segments. These clones reveal that the cells of the precursor pool are heterogenous with regard to their participation in myotomes on either side of the embryo, and that this characteristic can be maintained by a precursor cell during the formation of at least 30 segments.

To test whether this unequal left-right contribution characterises a specialised cell type, or is an extreme case of a more general property, we analysed the relative contribution of long clones to the left and right sides of the embryo. We calculated their coefficient of bilaterality defined as \( \frac{nR-nL}{nR+nL} \), where \( nR \) and \( nL \) are the number of labelled segments in respectively the right side and the left side of the embryo. The number of clones (y axis) for each value (x axis) of the coefficient is represented. 0 corresponds to a perfectly equal contribution of clones to the right and left sides and –1 and +1 to unilateral clones.

Together these results show (1) that the left and right myotomes are formed similarly, as the coefficient of bilaterality is continuous across both sides of the embryo (–1 to 0 and 0 to 1); (2) that differences in cell properties of myotomal precursors on the left and right sides of the embryo form a continuum; and (3), that as a consequence of these properties, there is no strict clonal separation between the left and the right sides of the embryo in the pool of precursor cells prior to bilateralisation of the paraxial mesoderm.

**DISCUSSION**

**Regionalisation and coherence of myotome precursor cells**

We report a significant regionalisation of myotomal precursors preceding bilateralisation of the paraxial mesoderm. This regionalisation is maintained during production of the paraxial mesoderm, segmentation of the somites, translocation of myocytes from the dermomyotome and the 10-fold ML growth of the dermomyotome and myotome (Fig. 7). This result is reminiscent of the cellular coherence observed in the segmental plate of the chick, where ML regionalisation was also detected (Selleck and Stern, 1991; Ordahl and Le Douarin, 1992). In a previous article (Eloy-Trinquet and Nicolas, 2002), we showed that there is a direct topographic relationship between myocytes and their precursors in the dermomyotome. This relationship can now be interpreted as a continuation of the ML coherence of more ancestral precursor cells.

The topography of the bilateral clones further suggests that ML regionalisation of the myotome is represented in the pool of self-renewing cells before bilateralisation. The production of new paraxial mesoderm is accompanied by a change of the relative position of cells in relation to the longitudinal axis of the embryo. Previous fate mapping studies in the chick...
posterior cells contribute to a lateral one more posterior to extra-embryonic mesoderm. Psychoyos and Stern (Psychoyos and Stern, 1996) and Wilson and Beddington (Wilson and Beddington, 1996) have shown in chick and mouse embryos, respectively, that precursor cells of the medial and lateral paraxial mesoderm are also arrayed anteroposteriorly in the primitive streak. Together with these findings, our results suggest that the pool of cells in the primitive streak at the origin of myotomal precursor cells is also anteroposteriorly regionalised. It should be noted, however, that although the most straightforward explanation for the pattern of cell allocation is regionalisation of progenitors, another mechanism involving cell sorting or complex but stereotypic tissue movement cannot be excluded. An important issue that remains to be resolved is whether cells at different regions along the AP axis in the primitive streak produce cells that remain associated until their definitive localisation in the paraxial mesoderm or whether the movements associated with the reorientation of the axes disrupt their association. For example, if the movement associated with reorientation corresponds to a 90% rotation, then the relative position of cells is likely to change quite significantly, the most lateral cells being shifted posteriorly (compare the white and the black arrows in Fig. 7).

Regardless of these considerations, ML regionalisation of the paraxial mesoderm in the self-renewing precursor cell pool in the primitive streak, followed by ML coherence in the presomitic mesoderm, could permit early mediolateral patterning of distinct mesodermal cell types. Indeed, many genes are expressed in restricted anteroposterior regions of the primitive streak (reviewed by Tam and Behringer, 1997), although this has not been observed for separate populations of paraxial precursor cells of mesoderm. By contrast, however, heterotopic grafts have shown that mesodermal precursor cells in the primitive streak are not irreversibly determined as to their mediolateral fate (Garcia-Martinez and Schoenwolf, 1992), and that the mediolateral fate of cells is not irreversibly determined even in the somite (Ordahl and Le Douarin, 1992). Progressive and reversible patterning could occur in this system, permitting readjustment of decisions until terminal differentiation. Whatever the case may be, a major consequence of ML coherence is that muscle precursor cells remain in the same signalling environment until translocation from the dermomyotome. Additionally, the ability of cells to signal to their environment may be determined by their position in the pool.

**Organisation of the self-renewing pool and its bilateral contribution to the myotome**

Several long unilateral clones and a continuum in the value of the coefficient of bilaterality of long and short bilateral clones (Fig. 6) was observed. This suggests that the position of self-renewing cells in the pool might determine the probability of their contribution to the left and right sides of the paraxial mesoderm. One possibility is that the decision as to which side of the embryo descendants of the self-renewing precursors will contribute to is determined by their distance from the midline. According to this model, descendants of medial cells before bilateralisation of the paraxial mesoderm will have an equal probability of contributing to the two sides of the myotome (Fig. 7, white and black arrows); descendants of more lateral
cells of the non-bilaterised pool will have an increasingly high probability of contributing to the corresponding side of the embryo, and descendants of the most lateral cells will contribute to only one side of the embryo (Fig. 7, grey arrow). This property concerns all cells of the pool of progenitors and is maintained during elongation of large parts of the AP axis. The major consequence of these results is that it implies ML cell coherence in the pool of self-renewing cells.

Although similar extensive data are missing for the other vertebrate species, it is interesting to note that in the chick node, injections into a cell in the midline often contribute labelled progeny to both left and right sides of the embryo and injections into more lateral cells give rise to progeny that are confined to the ipsilateral side of the embryo (Selleck and Stern, 1991). Therefore, it is possible that in both mouse and chick, the position of the cells in the pool might determine the probability of their contribution to the left and right sides of the paraxial mesoderm.

This model explains how the left and right sides of the myotome originate from a single pool of precursor cells. A major consequence of this organisation is that it maximises the clonal diversity of each side of the embryo, while conferring almost the same polyclonal identity to both sides. Thus, if dynamic regulatory events are important at these early stages (see below) such an organisation will assure a synchrony between left and right sides. Such a synchrony is indeed observed (for example the contribution of bilateral clones to the same segments in Fig. 2C). It would be difficult to obtain this coordination with two independent pools of cells and duplicated signals.

**Clonal coherence of the pool of myotome precursors along the longitudinal axis**

After the results of grafts of presomitic mesoderm cells, it was proposed that extensive cell mixing could occur along the AP axis in the mouse presomitic mesoderm (Tam, 1988; Tam and Beddington, 1987). If such mixing had occurred, we should have observed a majority of unilateral clones contributing to non-adjacent segments, but we did not, suggesting that cells do not disperse extensively along the presomitic mesoderm.

Previous clonal analyses in the chick embryo showed that presence of precursor cells in the segmental plate remained discrete, being confined at most to one segment-long region within the somite mesoderm (Stern et al., 1988). Therefore, clonal coherence is likely to be a property of the longitudinal axis of the paraxial mesoderm shared by mouse and chick embryos.

The longitudinal production of the myotome relies on a self-renewing cell system that produces cells of different segments through a temporal mode (Nicolas et al., 1996). This system is dispersive, as descendants of the self-renewing cells that enter the paraxial mesoderm are produced sequentially from the pool. Subsequently, however, these cells behave coherently in the presomitic mesoderm. Therefore there is a clear transition between a self-renewing cell system located in the caudal part of the embryo, and the coherent behaviour of cells in the structures produced from it. The production of cells from this organised self-renewing pool leads to formation of a static embryonic structure, the presomitic mesoderm, with few cell divisions (data not shown) and few cell movements.

**Cell behaviour and spatiotemporal gene activity during segmentation**

Over the past few years, several studies have shown that the segmentation process is associated with a molecular oscillator, the segmentation clock, and a determination front in presomitic mesoderm. The molecular clock has been visualised by the periodic expression patterns of chick *hairy1* and *hairy2*, *Lnfg* and *HES1* in the chick and mouse segmental plate (Forsberg et al., 1998; Jouve et al., 2000; McGrew et al., 1998; Palmeirim et al., 1997). These genes are expressed as a wave sweeping caudorostrally across the whole presomitic mesoderm. The determination front represents the site at which FGF8 concentration drops below a critical threshold (Dubrulle et al., 2001). More recently, transcriptional activation of some Hox genes has been shown to occur in the presomitic mesoderm in dynamic stripes of expression (Zakany et al., 2001). This dynamic expression pattern appears to depend upon Notch signalling, as does the periodic expression of the oscillating genes (reviewed by Maroto and Pourquié, 2001). This strongly suggests a link between patterning and morphogenesis through the segmentation clock. However, to explain how an oscillating signal is transformed into linear activation of Hox genes, it must be hypothesised that the accessibility of Hox genes within a complex progressively increases within the posterior presomitic mesoderm, which would result in a time-dependent activation of all accessible Hox genes (Zakany et al., 2001).

Such models of somitogenesis generate constraints at the level of cell behaviour. Concerning the process of segmentation: (1) the position of the determination front must be regulated to avoid differences in somite size during the period of production of identical somites and must remain at the same level on both sides of the embryo; (2) cells in the caudal part of the presomitic mesoderm must coordinate their molecular clock along both the ML (same value) and AP (different values) axes. This coordination is probably required during several cycles before passing the determination front; (3) once the front has been passed and future somite boundaries determined, the structure must remain in place until segmentation to ensure the differentiation of a precise boundary. Concerning the regulation of gene expression: (1) cells in the presomitic mesoderm, which express a specific group of Hox genes, must not mix with cells determined for a different group; (2) cells that have been newly integrated into the presomitic mesoderm must receive information about which group of Hox genes they make accessible; and (3) these genes must be made accessible in a coordinated manner on both sides of the embryo.

Our results in mice indicate that cell behaviour during somitogenesis conforms to many of these constraints. There are also indications that these properties are shared by the chick embryo, suggesting conservation of cellular operations between birds and mammals. Myotomal precursor cells are organised before bilateralisation and produce a static structure along both ML and AP axes. This could facilitate both the coordination of the clock between cells and the coherent growth of cells with the same accessible group of Hox genes. ML expansion of the structure and strict clonal separation of medial and lateral myotome precursors occur only after segmentation (Eloy-Trinquet and Nicolas, 2002), suggesting that the structure remains in place beyond the determination front before being relaxed. Finally, cells newly integrated into
presomitic mesoderm are derived from a single pool of cells residing permanently in the most caudal part of the structure from the time of formation of the primitive streak. These new cells are produced sequentially over time from this unique and permanent pool in which it is easy to integrate a time function with the process of activation of the Hox complexes. Furthermore, symmetry in the production of somitic cells on both sides of the embryo assures synchrony and identical polyclonal composition between left and right paraxial mesoderm. Such a permanent structure is also well-adapted to the maintenance of an expression domain of products involved in positioning the determination front. Clearly, other systems of production of the paraxial mesoderm such as from a changing pool, from a regionalised pool, or from two pools, would have to rely on more complex systems of coordination.

In conclusion, sequential production of groups of cells with an identical clonal origin for both sides of the embryo, coupled with the acquisition of static cell behaviour could be a very elegant and simple way to coordinate intrinsic cell properties (at the basis of sequential colinearity) with a system of segmentation mainly based on extrinsic elements (the clock, the determination front).

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