Evidence for medial/lateral specification and positional information within the presomitic mesoderm

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SUMMARY

In the vertebrate embryo, segmentation is built on repetitive structures, named somites, which are formed progressively from the most rostral part of presomitic mesoderm, every 90 minutes in the avian embryo. The discovery of the cyclic expression of several genes, occurring every 90 minutes in each presomitic cell, has shown that there is a molecular clock linked to somitogenesis. We demonstrate that a dynamic expression pattern of the cycling genes is already evident at the level of the prospective presomitic territory. The analysis of this expression pattern, correlated with a quail/chick fate-map, identifies a ‘wave’ of expression travelling along the future medial/lateral presomitic axis. Further analysis also reveals the existence of a medial/lateral asynchrony of expression at the level of presomitic mesoderm. This work suggests that the molecular clock is providing cellular positional information not only along the anterior/posterior but also along the medial/lateral presomitic axis. Finally, by using an in vitro culture system, we show that the information for morphological somite formation and molecular segmentation is segregated within the medial/lateral presomitic axis. Medial presomitic cells are able to form somites and express segmentation markers in the absence of lateral presomitic cells. By contrast, and surprisingly, lateral presomitic cells that are deprived of their medial counterparts are not able to organise themselves into somites and lose the expression of genes known to be important for vertebrate segmentation, such as Delta-1, Notch-1, paraxis, hairy1, hairy2 and lunatic fringe.

Key words: Somite, Segmentation, Molecular clock, Primitive streak, Chick embryo, hairy1, hairy2, lunatic fringe

INTRODUCTION

In the vertebrate embryo, somites constitute the basis of the segmental pattern of the body. They not only give rise to segmented structures such as vertebrae, intervertebral disks, epaxial muscles and ribs, but they also impose a segmental pattern upon a variety of tissues, such as peripheral nerves, ganglia and vascular primordia (Keynes and Stern, 1984; Rickmann et al., 1985; Teillet et al., 1987; Wilting et al., 1997). Somites appear progressively, in a rostral-to-caudal order, as each pair of somites is fixed and characteristic of each species.

In the chick embryo, this budding off occurs every 90 minutes at the thoracic level. Concomitant with epithelial somite formation at the anterior end of PSM, gastrulation continues in the most posterior part of the embryo, causing progressive PSM formation and embryo elongation (Christ and Ordahl, 1995; Gossler and Hrabe de Angelis, 1998). By using the quail/chick chimaera technique, a cell lineage study in six-somite stage embryos demonstrated that somitic cells originate from a region located in the posterior midline of the sinus rhomboidalis (posterior open neural plate), not adjacent to the median pit (a region containing the midline precursor cells) (Catala et al., 1996).

A previous study (Palmeirim et al., 1997) has presented evidence for the existence of a molecular clock that underlies the process of chick somitogenesis by showing that, in 15- to 20-somite stage embryos, PSM cells undergo several cycles of hairy1 gene expression, with a 90-minute periodicity, corresponding to the time required to form one segment. These hairy1 mRNA oscillations of expression occur in each PSM cell until it is incorporated into a somite. As previously described, the expression of hairy1 mRNA appears as a caudal to rostral ‘wave’ that spans across the entire length of PSM once during the formation of each somite. This same type of behaviour has also been described for a gene coding for a closely related transcription factor, hairy2 (Jouve et al., 2000), and for another gene encoding the secreted protein lunatic fringe (McGrew et al., 1998; Aulehla and Johnson, 1999). Cycling genes expressed during somitogenesis have also been recently described in mouse (lunatic fringe (Forsberg et al., 1998; Aulehla and Johnson, 1999); Hes1 (Jouve et al., 2000); Hey2 (Leimeister et al., 2000)) and in zebrafish embryos (her1) (Holley et al., 2000).
For many years, scientists have tried unsuccessfully to disturb the pattern of somite formation. Heterotopic grafts of both anterior to posterior and posterior to anterior part of the PSM of a chick embryo to another chick embryo of the same stage give rise to somite formation that respects the segmentation timing of the donor embryo (Packard, 1978). Similarly, the anterior/posterior inversion of a region of PSM tissue leads to a caudal to rostral somite formation in the grafted tissue (Menkes and Sandor, 1977; Palmeirim et al., 1998). In fact, PSM is able to segment even in complete isolation from environmental tissues (Packard and Jacobson, 1976; Sandor and Fazakas-Todea, 1980). This can be observed by the progressive appearance of Delta-1 stripes in cultured explants of isolated PSM (Palmeirim et al., 1998). In these explants, despite disruption of morphological somite formation, genes known to be involved in the segmentation process are present, exhibiting a normal pattern of expression. Therefore, segmentation and somite formation are distinct processes: the former is independent of environmental tissues and the latter is dependent on the overlying ectoderm (Sosic et al., 1997; Palmeirim et al., 1998).

The aim of this work was to evaluate the behaviour of the molecular clock at the PSM prospective territory, during the formation of one somite. A detailed whole-mount and cross-section analysis of the expression patterns of cycling genes (hairy1, hairy2 and lunatic fringe) show that concomitantly with an anterior/posterior PSM ‘wave’ of expression, a medial/lateral ‘wave’ can also be observed both in PSM and in its prospective territory. This result has driven us to evaluate further the heterogeneity within the medial/lateral PSM axis. We have found that the information for morphological somite formation and PSM genetic segmentation is present in medial but not in lateral PSM cells. We present data to support the idea that medial and lateral halves of PSM originate from distinct prospective territories and are also differently committed in what concerns segmentation and somite formation.

MATERIALS AND METHODS

Eggs and embryos

Fertilised chick (Gallus gallus) and quail (Coturnix coturnix japonica) eggs obtained from commercial sources were incubated at 37°C in a 45% humidified atmosphere. The embryos were staged according to the number of formed somites (Hamburger and Hamilton, 1951). We consider a somite to be completely formed when we observe a definite cleft separating it from the PSM. We refer to the forming somite as somite 0 (caudal cleft not yet completely formed). The newly formed somite is referred to as somite I, as proposed by Christ and Ordahl (Christ and Ordahl, 1995).

RNA probes

The digoxigenin or fluorescein-labelled RNA probes were produced as described previously: hairy1 (Palmeirim et al., 1997), hairy2 (Jouve et al., 2000), lunatic fringe (Sakamoto et al., 1997), Delta-1 and Notch-1 (Henrique et al., 1995), paraxis (Barnes et al., 1997), BMP-4 (Francis et al., 1994), Ch-Tbx6L (Knezovic et al., 1997) and Noggin (Connolly et al., 1997).

Whole-mount in situ hybridisation

Embryos and explants were fixed overnight at 4°C in 4% formaldehyde 2mM EGTA in phosphate-buffered saline (PBS), rinsed in PBT (PBS, 0.1% Tween 20), dehydrated through a methanol series and stored in 100% methanol at –20°C. Whole-mount in situ hybridisation was performed according to the procedure described by Henrique et al. (Henrique et al., 1995). For double in situ hybridisation, two RNA probes were hybridised simultaneously, one labelled with digoxigenin-UTP and the other with fluorescein-UTP. Both types of labelling were detected by alkaline-phosphatase-coupled antibodies (Roche). After the development of the first reaction, and in order to eliminate the alkaline-phosphatase extremitiies coupled to the first antibody, a 30 minute incubation at 70°C with MABT was performed, preventing any cross-reaction. The RNA probes were detected either with NBT-BCIP (blue staining) or INT-BCIP (red staining).

Cross-sections

The hairy1, hairy2 and lunatic fringe-labelled embryos were dehydrated in a series of ethanol, embedded in methacrylate (TECNOVIT 8100) and processed for sectioning at 20 μm using an ultramicrotome (LK-B Ultrotome). The slides were mounted in Neomount (Merek) and photographed using a Leica DC 200 camera coupled to an Olympus BH-2 microscope.

Quail-chick grafts

Chicken and quail eggs were incubated for 30 to 36 hours in order to obtain six-somite stage embryos. A window was performed in the shell of the chicken eggs, Indian ink was injected into the sub-germinal cavity and the vitelline membrane was pulled apart using a tungsten microscalpel. Quail embryos were collected from the egg yolk into resin-coated petri dishes in PBS without Ca²⁺/Mg²⁺. At the caudal part of the embryo we identify the sinus rhomboidalis (open neural plate). At the centre of this structure, a pit can be observed. We consider the median pit to be the region comprising the pit itself and the slope surrounding it. Quail midline fragments, which extended caudally from the posterior limit of median pit over 100-150 μm, were grafted homotopically into stage-matched chick hosts (n=6) (see Fig. 4A). In order to avoid the grafting of prospective neural cells, the graft tissue only comprised the deeper layer under the surface epithelium. The chimerae were incubated overnight, after which they were fixed in modified Carnoy solution (100% ethanol, 37% formaldehyde solution and glacial acetic acid (6:3:1, V:V:V)), embedded in paraffin, serially sectioned (5-7 μm) and processed overnight with the monoclonal antibody (mAB) QCQN (Developmental Studies Hybridoma Bank), followed by a HRP-conjugated anti-mouse IgG antibody (Southern Biotechnology) and DAB revelation to evidence quail nuclei, as described by Charrier et al. (Charrier et al., 1999).

Embryo culture experiment

Fifteen- to 20-somite stage embryos were collected from the egg yolk into resin-coated petri dishes in PBS without Ca²⁺/Mg²⁺. All the microsurgical procedures were performed in the left side of the embryo, taking the right-hand side PSM as a control. Different incisions were made, using a tungsten microscalpel, in order to create two types of ablations.

Lateral PSM ablation

A slit was performed in the ectoderm between the intermediate mesoderm and the entire length of PSM. After a brief treatment with 4x pancreatin (Gibco), a longitudinal incision was made in the PSM, dividing it into a medial (M-PSM) and a lateral (L-PSM) half. The L-PSM was removed by cutting transversally the anterior and posterior extremities of this piece of tissue.

Medial PSM ablation

A slit was made in the ectoderm between the neural tube and the PSM. The PSM was again longitudinally subdivided into two halves and the M-PSM was excised. Foetal calf serum was then used to inactivate pancreatin and the operated embryo was cultured as described by Palmeirim et al.
(Palmeirim et al., 1997). After 6-9 hours of incubation (the time required to form four to six pairs of somites; note that under these conditions somitogenesis proceeds normally), the embryos were fixed in 4% formaldehyde-PBS without Ca\textsuperscript{2+}/Mg\textsuperscript{2+}, 2 mM EGTA pH8, overnight at 4°C. They were then washed in PBT, progressively dehydrated and kept in methanol at –20°C. Finally, we assessed for morphological somite formation and for Delta-1 and Tbx6L gene expression by performing whole-mount in situ hybridisation with the appropriate antisense RNA probes.

**Explant culture experiment**

Embryos with 15 to 20 somites were processed as described above in the embryo culture experiment. Subsequently, two types of explants were precisely delimited, excised and cultured. The entire left-hand side of the PSM was subdivided into a medial and a lateral half by performing a longitudinal incision throughout the three embryonic germ layers. Two transverse cuts were then made: one precisely beneath somite 1 (cells from somite 0 are included in both explants) and another in the most posterior part of PSM. Both explants were incubated separately for 6 hours using the in vitro tissue culture technique already described (Palmeirim et al., 1997). Explants were then fixed and hybridised with hairy1, hairy2, lunatic fringe, paraxis, Notch-1, Delta-1, Tbx6L, BMP-4 and Noggin digoxigenin-labelled antisense RNA probes. All hybridised explants and embryos were photographed as whole-mounts in PBT/0.1% Azide, using a Leica DC 200 camera.

**RESULTS**

**Variable expression patterns of hairy1, hairy2 and lunatic fringe genes at the level of sinus rhomboidalis**

Because in six-somite stage embryos the presumptive somitic territory has been located in the posterior midline region of the sinus rhomboidalis (Catala et al., 1996), we assessed for the pattern of expression of hairy1 (n=37), hairy2 (n=41) and lunatic fringe (n=24) in this domain. At the level of sinus rhomboidalis the embryos presented very different patterns of expression: some embryos present a strong staining in this region (Fig. 1A,E,H) while others are not stained at all (Fig. 1C,F,I). As expected, and already described for 15- to 20-somite stage embryos, very different expression patterns were observed, at the level of PSM, defining a PSM caudal-to-rostral wave of expression for each of these genes (Fig. 1A-I) (Palmeirim et al., 1997; McGrew et al., 1998; Aulehla and Johnson, 1999; Jouve et al., 2000).

In order to compare the expression patterns of hairy1, hairy2 and lunatic fringe genes at the level of sinus rhomboidalis, we performed double whole-mount in situ hybridisation with the following combination of genes: hairy1/hairy2 (n=14), hairy1/lunatic fringe (n=16) and lunatic fringe/hairy2 (n=7), in six-somite stage embryos (Fig. 2A-I). The hairy1 expression domain is located within the borders of the sinus rhomboidalis (Fig. 2A-F). The lunatic fringe gene presents a pattern of expression that is frequently coincident with the one of hairy1 (Fig. 2E,F). Nevertheless, in some embryos, the region of expression of lunatic fringe extends slightly more posteriorly (Fig. 2D). By contrast, the domain of expression of hairy2 is broader, extending beyond the limit established by the expression patterns of both hairy1 and lunatic fringe (Fig. 2A-C,G-I). Analysis of these results allows us to conclude that in the posterior part of the sinus rhomboidalis, the expression patterns of these genes is variable and that this variation is not superimposed. (Fig. 1, Fig. 2). A wave of expression is in progress at the level of sinus rhomboidalis

The whole-mount analysis did not permit a precise localisation of the cells undergoing hairy1, hairy2 and lunatic fringe cycles of expression. We analysed a series of posterior-to-anterior cross-sections, which reveals the way cells are progressively...
organised in the sinus rhomboidalis. Starting at the posterior limit of the sinus rhomboidalis, a cross-section shows a superficial layer, with a slight groove, forming a continuous sheet of cells over a layer of mesenchymal tissue (Fig. 1, cross-sections at level 4). Rostral to this region, the superficial median groove disappears and the layer of mesenchymal cells is progressively enlarged (Fig. 1, cross-sections at levels 2 and 3). Progressing anteriorly, the median pit is reached. This region contains the prospective notochord and floor plate cells (Catala et al., 1996), as well as a region essential for the caudalward regression of Hensen’s node (Charrier et al., 1999).

Highly variable levels of expression can be observed in the mesenchymal tissue underlying the prospective neural plate cells, from the posterior limit of the median pit (Fig. 1, cross-sections at level 1) to a region located slightly caudal to the sinus rhomboidalis (Fig. 1, cross-sections at level 4), defining a dynamic ‘wave’ of expression that moves in the longitudinal axis of this region. The analysis of these results suggests that simultaneous to the caudal-to-rostral ‘wave’ displacement that occurs at the level of PSM, another ‘wave’ of expression is in progress in the most posterior part of the embryo, at the level of sinus rhomboidalis (Fig. 1A-I).

This gradient of expression defines a 100-150 \( \mu \)m sub-region, located just behind the median pit, that very often presents a clear asynchrony with the more posterior regions (compare Fig. 1 cross-sections at levels 2 and 3). This sub-region was not described by Catala et al. (Catala et al., 1996) as being part of the somitic prospective territory.

**The medial part of PSM originates from deep cells located just behind the median pit**

The somitic prospective territory described by Catala et al. (Catala et al., 1996), is located in the posterior part of sinus rhomboidalis but it is not adjacent to the median pit. Their work states that the more superficial layers of the region adjacent to the median pit give rise to the neural tube, but they did not assess for the fate of the deeper mesenchymal layers. We used the quail-chick chimera technique to determine the fate of the cells located just behind the median pit, underneath the presumptive neuroectoderm. The latter was lifted and a 100-150 \( \mu \)m fragment of deep mesenchymal tissue was surgically removed and replaced by the equivalent tissue from a quail embryo of the same stage (Fig. 3A). One day after the graft, the chimeraes were analysed using the QCPN monoclonal antibody, which allows the recognition of quail cells in the chimeraes. The quail donor cells are exclusively located along the entire length of medial PSM (M-PSM) and in medial epithelial somites (Fig. 3B,C). Furthermore, no quail cells are observed in the lateral PSM (L-PSM) (Fig. 3B,C). These results show that medial PSM cells arise from a region located just behind the median pit, evidenced by quail cells only contributing to PSM and somites and being restricted to the most medial part of these tissues. These results
extend the accepted idea that, at the primitive streak, more anterior cells will give rise to more medial mesodermal structures, while more posterior cells will give rise to more lateral mesodermal structures, to the cellular organisation within the PSM prospective territory.

**Somites are not formed in the absence of medial PSM cells**

As medial PSM cells originate from a different region of that of lateral PSM cells, we wondered whether they were equally committed to form somites. To answer this question, we performed ablations of the L-PSM territory corresponding to the length of approximately ten prospective somites on one side of 15 to 20-somite stage embryos (n=20) (Fig. 4A). These operated embryos were then cultured for 6-9 hours, which corresponds to the time required to form 4-6 pairs of somites. The analysis of the results showed that the same number of somites was formed in both operated and control PSM (Fig. 4B). Nevertheless, in the absence of L-PSM tissue, the first three to four developed somites were smaller than the control ones. By contrast, the most posterior formed somites are the expected size, showing the recovery capacity of PSM lateral tissue (Fig. 4B).

In another series of experiments we excised the M-PSM, keeping in place the L-PSM tissue (n=33) (Fig. 4E). After 6-9 hours of culture, the control PSM segmented and gave rise to four to six pairs of somites, as expected. Strikingly, no somite formation can be observed in the operated PSM, suggesting that in the absence of M-PSM cells, the lateral half of this tissue is not able to form somites (Fig. 4F). The results obtained suggest that M-PSM cells have the information for somite formation, in contrast to L-PSM cells.

We performed whole-mount in situ hybridisation in both medial and lateral PSM aborted embryos, using the Delta-1 antisense RNA probe. The results obtained showed that somites formed in the absence of L-PSM cells express Delta-1 in their posterior border, precisely in the same way as the non-operated PSM (Fig. 4C). By contrast, when the M-PSM domain is excised, not only does the L-PSM tissue not form morphological somites, but Delta-1 expression pattern is perturbed: its anterior limit is shifted posteriorly and stripes of Delta-1 expression cannot be observed in the operated PSM (Fig. 4G). Whole-mount in situ hybridisation was also performed using Ch-Tbx6L, a presomitic marker (Knezevic et al., 1997), which confirmed the presence of PSM tissue in both types of ablations (Fig. 4D,H).

**The information for molecular segmentation is also segregated within PSM**

It has already been established that the process of molecular

![Fig. 4](image-url)

Fig. 4. Somites are not formed in the absence of medial PSM cells. (A,E) The type of PSM ablation performed on 15 to 20 somite stage embryos. (B) Dorsal view of an embryo cultured for 9 hours after lateral PSM ablation. The black arrowheads indicate somites formed during the in vitro culture period. The same number of somites is formed both in operated and control sides, although in the former the size of the somites appears smaller. (C) Lateral PSM ablated embryo hybridised with Delta-1. Somites formed in culture express Delta-1 in its normal pattern of expression. (D) Lateral PSM ablated embryo hybridised with Tbx6L present a normal pattern of expression. (F) Dorsal view of an embryo cultured for 9 hours, whose medial PSM has been removed. The brackets indicate the operated side where no somites have been formed in the absence of medial PSM tissue. (G) Medial PSM ablated embryo hybridised with Delta-1 reveals that the remaining lateral PSM does not express the Delta-1 gene and that it is restricted to a more caudal domain, when compared with the control PSM. (H) Medial PSM ablated embryo hybridised with Tbx6L clearly shows that lateral PSM tissue remains in the operated side (arrowhead). Scale bars: 200 μm in B-F; 300 μm in C,D,G,H.
segmentation is independent of the epithelialization phenomenon that leads to somite formation. We therefore assessed for the molecular segmentation of PSM in the absence of either medial or lateral PSM cells, by analysing the expression of the following molecular markers: Delta-1, Notch-1, paraxis, hairy1, hairy2, lunatic fringe and Tbx6L. We performed an in vitro culture of separated lateral and medial PSM explants devoid of the caudal part of the embryo (Fig. 5A), since it is likely to influence the results by constantly adding cells from the prospective PSM territory. As a control for the microsurgical incision, some explants were randomly chosen, and immediately fixed and hybridised with probes for Delta-1 (n=5) or with BMP-4 (n=4) (Fig. 5). These control experiments show that the Delta-1 domain of expression is longitudinally subdivided into two halves. In the BMP-4 hybridised explant, a thin domain negative to this gene is present corresponding to the lateral PSM tissue. Scale bar: 250 μm.

The expression of Delta-1, Notch-1, paraxis, hairy1, hairy2, lunatic fringe and Tbx6L was assessed after 6 hours of culture. The analysis of the results obtained shows that the M-PSM domain, isolated from the neighbouring L-PSM domain, is not only able to form somites, as previously described, but also expresses all the tested molecular markers, in a similar pattern to the non-operated PSM (Fig. 6A-I). By contrast, L-PSM cells, isolated from more medial cells, lose the expression of Delta-1 (n=16), hairy1 (n=11), hairy2 (n=17) and lunatic fringe (n=15) genes (Fig. 6A,G-I). Notch-1 (n=9) and paraxis (n=10) are weakly expressed in cells located in the most anterior part of L-PSM (Fig. 6B,C), corresponding to the cells that were already expressing these genes at the moment of the operation. However, de novo expression is never detected in more posterior cells, in contrast to what happens in the control PSM (Fig. 6B,C). The expression domain of BMP-4 and Noggin is conserved in both explants: the BMP-4 domain is still delimiting the L-PSM half, the same way Noggin remains in the frontier between PSM and lateral plate tissue.
Cycling genes and medial/lateral PSM identity

DISCUSSION

The molecular clock is already operating at the prospective PSM territory

Originally, it was demonstrated that presomitic cells begin expressing pulses of hairy1 mRNA as they enter the paraxial mesoderm. The presence of cycling genes transcripts at the level of the tailbud has been mentioned (Aulehla and Johnson, 1999), although this region gives rise to several embryonic tissues that have not been fate-mapped. A cross-section analysis on the expression patterns of hairy1, hairy2 and lunatic fringe genes, in 6-somite stage embryos, reveals an oscillation of expression of these genes from the posterior limit of the median pit to a region slightly posterior to the sinus rhomboidalis. The results from our quail/chick cell lineage study add new data to the previous fate-map performed by Catala et al. (Catala et al., 1996), allowing us to determine that this region, which exhibits a cyclic behaviour, corresponds exactly to the somitic prospective territory. In our study, we clearly show that the levels of hairy1, hairy2 and lunatic fringe mRNA are already oscillating in prospective PSM cells well before they are incorporated into PSM, and that the cells presenting this cyclic behaviour are restricted to the prospective PSM territory (Fig. 1). It is also evident that within the prospective PSM territory we have a ‘wave’ of expression of cycling genes spreading in its longitudinal axis (Fig. 1).

The double whole-mount in situ hybridisation allowed us to perform a combinatory analysis of the expression patterns of the cycling genes. It is clear that cells located within the frontier defined by the sinus rhomboidalis, undergo cycles of expression of hairy1, hairy2 and lunatic fringe. Nevertheless, the patterns of expression of these genes are very often not overlapped (Fig. 2). This can be explained either by an asynchrony in the transcription of the genes or as a consequence of different lifetimes of their mRNAs. Furthermore, posterior to the sinus rhomboidalis, mesenchymal cells undergo cycles of expression of hairy2 and lunatic fringe but unexpectedly, they never seem to express the hairy1 gene. In an even more posterior region, we can only observe transcription cycles of hairy2, while the expression of hairy1 and lunatic fringe genes is never observed in this region.

The number of mRNA cyclic waves of expression exhibited by PSM cells depends on the time these cells spend until they incorporate a somite. Given that we have shown that prospective PSM cells are already undergoing cyclic waves of mRNA expression in their prospective territories, these cells must undergo more than the 12 cycles of expression they perform in the PSM (Palmeirim et al., 1997). However, as development proceeds, the PSM length is gradually reduced, suggesting that prospective somitic cells spend progressively less time in the PSM tissue. Moreover, the number of cycles undergone before somite formation could be maintained constant if, at later stages of development, prospective PSM cells spent more time in their prospective territory. Indeed, in our quail-chick chimaeras, quail donor cells can still be observed in this prospective PSM region after 24 hours of incubation. These remaining quial cells, or their progeny, are thus undergoing several cycles of expression without exiting the prospective PSM region.

The molecular clock is providing medial/lateral positional information

In 1996, Catala et al. determined the fate of several superficial regions of the sinus rhomboidalis by performing quail/chick orthotopic grafts at the six-somite stage, concluding that somite precursors are located in the posterior part of sinus rhomboidalis, not adjacent to the median pit (Catala et al., 1996). We performed grafts of the deep layer of the region caudal to the median pit (see Materials and Methods) and found that, 1 day after the operation, quail cells were situated in the M-PSM adjacent to notochord and neural tube. Thus, these cells located behind the median pit (which contains the precursors of more axial structures) and under the prospective neural plate will give rise to medial presomitic cells. Hence, we designate this region PM-PSM (presumptive medial – presomitic mesoderm; see Fig 1, Fig. 3). In agreement with our fate map, Charrier et al. (Charrier et al., 1999) showed that the paraxial mesodermal marker Tbx6L expression domain is juxtaposed to the HNF3β axial marker expression domain. Our result redefines, in the chicken embryo, the anterior limit of the prospective PSM territory, which is similar to the one described for the mouse embryo (Wilson and Beddington, 1996; Tam et al., 2000).

Our results show that within the prospective PSM territory, more anterior cells will be located in more medial positions. This suggests that more posterior cells will be located in more lateral positions. A Dil fate map created by Selleck and Stern (Selleck and Stern, 1991) at stage 4 (HH) has already revealed different origins for medial and lateral PSM cells. Therefore, the ‘wave’ of expression of the cycling genes spreading along the longitudinal axis of the PSM prospective territory corresponds to a ‘wave’ spreading along the future medial/lateral PSM axis. In agreement with this, if we reanalyse the stripes of expression of the cycling genes at the level of PSM, we can observe a medial/lateral asynchrony,
which is evidenced by the appearance of cross-stripes. In fact, an asynchrony in the medial/lateral expression could explain the appearance of a cross-stripe as a transition state between two horizontal stripes (Fig. 7). A very interesting conclusion is that the molecular clock is providing cellular positional information for both anteroposterior and mediolateral axes.

Medial and lateral PSM cells are differently committed to form somites

Medial and lateral PSM cells have different prospective territories, as reported in this study for six-somite stage embryos and for stage 4 embryos (HH) (Selleck and Stern, 1991). However, the fact that both medial and lateral parts of PSM have different origins does not imply that these two domains are differently committed as far as somite formation is concerned. We assessed for this issue, by performing both medial and lateral PSM ablations. Our results show that the M-PSM is able to form somites in the absence of the L-PSM, but surprisingly, no epithelial somite formation can be observed when the L-PSM is isolated from its medial counterpart. Selleck and Stern (Selleck and Stern, 1992) suggested that, at stage 4 (HH), cells from the lateral sector of the node (prospective medial PSM cells) are the ones that determine the spacing of the metameric pattern. Our experimental data clearly demonstrate that immediately before somites are formed, the information for morphological somite formation is only present in the medial PSM cells. Therefore, it is likely that medial PSM cells have to recruit lateral ones in order to form somites. Furthermore, PSM and epithelial somites can be subdivided into a medial and a lateral compartment according to their fate (Ordahl and Le Douarin, 1992; Olivera-Martinez et al., 2000). Hence, medial and lateral parts of PSM have different origins, are differently committed to somite formation and give rise to different embryonic structures.

Medial/lateral PSM dissociation disturbs the intrinsic program of PSM molecular segmentation

Several data from the literature support the idea that molecular segmentation occurs independently of epithelial somite formation (Burgess et al., 1996; Sosic et al., 1997; Palmeirim et al., 1998). In mice, targeted inactivation of several genes that are known to be important for vertebrate somitogenesis leads to a disruption of morphological somite formation (Conlon et al., 1995; Oka et al., 1995; Hrabe de Angelis et al., 1997; Kusumi et al., 1998), although somite derivatives such as muscles and skeleton retain a segmented pattern. In addition, morphological somite formation can be impaired by the ablation of the PSM upper-layer ectoderm (Sosic et al., 1997; Palmeirim et al., 1998), whereas genetic segmentation of PSM is not dependent on any signal coming from neighbouring tissues, making it an intrinsic property of this tissue (Palmeirim et al., 1998). For example, in cultured isolated PSM, epithelial somites do not form, although striped expression pattern of Delta-1 is observed, corresponding to the Delta-1 pattern of expression at the level of somites. The dynamic expression pattern of the cycling genes exhibits a remarkable degree of autonomy. The pattern of expression of these genes is completely independent of any signal coming either from the posterior or anterior part of the PSM, as cutting the PSM into as many as ten pieces, does not disturb the expression pattern of these genes (data not shown). In this work, we not only demonstrate that morphological somite formation is prevented in the absence of M-PSM, but also that the process of molecular segmentation is perturbed. The L-PSM isolated from its medial counterpart loses the expression of molecular segmentation markers such as Notch-1, Delta-1, paraxis, hairy1, hairy2 and lunatic fringe (Fig. 6). For the first time we have disturbed the expression pattern of the cycling genes in L-PSM cells by isolating them from the medial ones.

In the chick embryo, the PSM is neighboured by the lateral mesoderm that expresses high levels of BMP-4. The PSM is protected from the action of BMP-4 protein by the product of Noggin expressed in the border between PSM and lateral mesoderm (Tonegawa and Takahashi, 1998). In cultured explants, the domains of expression of these genes are maintained (Fig. 6G,H). These results suggest that, in the absence of M-PSM cells, its lateral counterpart is unlikely to be re-specified into lateral mesoderm.

It is now generally accepted that the Notch and Delta signalling pathway plays a role in the process of somitogenesis. The analysis of mouse mutants for the transmembrane receptor Notch1 (Conlon et al., 1995), for its ligands Delta1 and Delta3 (Hrabe de Angelis et al., 1997; Kusumi et al., 1998) and for the transcription factor RBPJ-kappa (Oka et al., 1995) has demonstrated the importance of these proteins for establishing the somitic boundaries and defining anterior and posterior somitic identities. Furthermore, it has recently been observed that the Delta1 mutant mice lack the dynamic expression of Hes1 (the gene claimed to be the hairy2 homologue in the mouse) (Jouve et al., 2000). Several lines of research are now trying to unveil the relationship between the Notch and Delta signalling pathway and the molecular clock that underlies somitogenesis (Aulehla and Johnson, 1999; Holley et al., 2000; Jiang et al., 2000). Taking this into account, our observation that hairy1, hairy2 and lunatic fringe genes are not expressed in the isolated lateral PSM could be explained by the absence of an operating Notch and Delta signalling pathway in this tissue.

The absence of expression of the segmentation genes, including the cycling genes, in the L-PSM deprived from its medial counterpart, cannot be explained by the lack of a signal provided by axial structures, as PSM is able to undergo normal segmentation isolated from these structures (Palmeirim et al., 1997; Palmeirim et al., 1998; McGrew et al., 1998; Jouve et al., 2000). Therefore, it is more likely that a signal supplied by M-PSM would be responsible for the upregulation of these genes in the L-PSM. However, the nature of this signal remains to be identified.

In summary, we unveil that medial PSM cells are the ones possessing the information for molecular segmentation and somite formation. We propose that in order to control morphological somite formation and molecular segmentation, a signal travels along the mediolateral PSM axis. Furthermore, we demonstrate that the molecular clock that underlies somitogenesis is already operating in PSM precursor cells. At this level, a ‘wave’ of expression of hairy1, hairy2 and lunatic fringe genes spreads along the longitudinal axis of the whole PSM prospective territory, which corresponds to the future mediolateral PSM axis. A very interesting conclusion of this work is that the molecular clock is providing cellular positional information in at least two dimensions: not only in the anteroposterior but also in the mediolateral PSM axis.
REFERENCES


