A clock-work somite

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Summary

Somites are transient structures which represent the most overt segmental feature of the vertebrate embryo. The strict temporal regulation of somitogenesis is of critical developmental importance since many segmental structures adopt a periodicity based on that of the somites. Until recently, the mechanisms underlying the periodicity of somitogenesis were largely unknown. Based on the oscillations of *c-hairy1* and *lunatic fringe* RNA, we now have evidence for an intrinsic segmentation clock in presomitic cells. Translation of this temporal periodicity into a spatial periodicity, through somite formation, requires Notch signaling. While the *Hox* genes are certainly involved, it remains unknown how the metameric vertebrate axis becomes regionalized along the antero–posterior (AP) dimension into the occipital, cervical, thoracic, lumbar, and sacral domains. We discuss the implications of cell division as a clock mechanism underlying the regionalization of somites and their derivatives along the AP axis. Possible links between the segmentation clock and axial regionalization are also discussed. *BioEssays* 22:72–83, 2000.

> "Tout ce qui est simple est faux, tout ce qui est compliqué est inutilisable" ("Everything simple is false, all that is complicated is unusable") Paul Valéry

Introduction

The correct positioning and patterning of organs and tissues within an organism during development is a process that requires exquisite chronological regulation. One striking example of this strict temporal control during vertebrate embryonic development is the process of somitogenesis. This process generates, sequentially along the antero–posterior (AP) axis, the earliest and most overt mesodermal segments of the embryo: the somites. The somites are transient structures whose later derivatives, such as the axial skeleton and the skeletal muscles, also retain a segmental arrangement. A number of models have been proposed to address the mechanism underlying the generation of such a periodic pattern. In this review we focus on the topic of the "segmentation clock" by discussing these models in light of recent molecular data.

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Segmentation strategies

Somitogenesis is the earliest manifestation of segmentation within the cephalochordate and vertebrate subphyla. Segmentation per se is a core feature of development in several invertebrate phyla as well as in the vertebrate phylum and refers to the generation of repeated units along the AP embryonic axis. This process follows one of two general modes.[1–3] One mechanism, exemplified by long germ band insects, such as *Drosophila melanogaster,* proceeds via the stepwise subdivision of a preexisting territory into successively smaller metameric units.[4] In vertebrates, a similar process occurs when the developing hindbrain becomes subdivided into rhombomeres.[5] Such simultaneous segment formation is in sharp contrast to the more widely observed mode of metamerism employed by, for example, annelids, short germ band insects, and other arthropods.[6] Segments in these species are sequentially produced from a terminal growth zone, located posteriorly, which contains stem cells. During vertebrate somitogenesis a variation of this latter mode of segmentation is seen (Ref. 7 and references therein). Thus, the somites are sequentially produced from the anterior end of the unsegmented or presomitic mesoderm (PSM). This tissue exists as two mesenchymal rods on either side of the posterior neural tube, which extend back to the regressing primitive streak and tail bud. The recruitment of new presomitic tissue from the primitive streak into the posterior end of the PSM, together with cell division within it, keeps pace with somite budding anteriorly, permitting the PSM to maintain its longitudinal dimension[1] (Fig. 1).

Since somite number increases at a steady rate during early development, these structures serve as a means of measuring developmental time. Moreover, as morphological landmarks they define a staging system for early development. Thus, somitogenesis is a temporally regulated process, which continues throughout early embryogenesis and is an integral part of body axis generation.

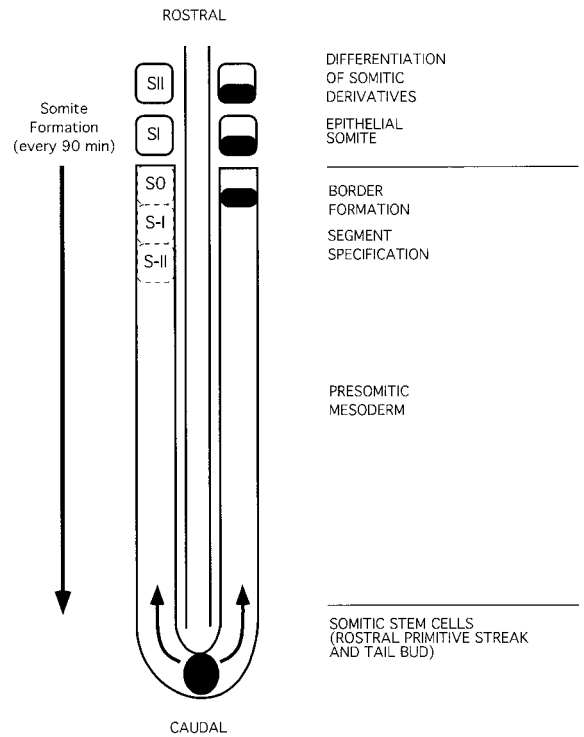

Figure 1. Schematic representation of the sequential stages of somitogenesis. Subsequent to their ingression from the epiblast into the streak, the precursors of the paraxial mesoderm form a resident population of somitic stem cells in the rostral primitive streak and, later, in the tailbud. Asymmetric cell divisions of these cells are thought to generate self-renewing daughter cells, which remain in the streak and PSM progenitor daughters, which are released into the caudal PSM. Concurrent with this event, a new pair of somites buds from the rostral end of the streak every 90 minutes. Thus, as somitogenesis proceeds the relative position of a cell becomes displaced rostrally. In the rostral PSM, during the generation of the next somite, cells fated to segment together increase their adhesive properties and undergo a mesenchymal-epithelial transition and a border forms caudal to the prospective caudal domain of this forming somite. According to Ordahl's[83] nomenclature for newly formed somites, the most recently formed epithelial somite is termed SI, the last but one somite is called SII, and so on. We propose to call the forming somite, whose boundaries are not completely formed, somite 0 (S0). We also propose to call blocks of PSM cells of one somite length, located caudal to S0, somite -I, -II, and so on. Having acquired AP polarity within the rostral PSM, the cells of the epithelial somite, SI, and the last but one somite, SII, etc., only now become competent to respond to inductive signals from the adjacent tissues, which act to pattern somites along the dorsoventral and mediolateral axes. These events are integral to their progressive differentiation along the three somitic lineages.

Somitogenesis is genetically and environmentally controlled

The generation of regularly spaced fissures, which demarcate the somites, proceeds according to regular time intervals that are species-specific, as is the total number of somites produced. Among the diverse range of vertebrate species, there is surprisingly little meristic variation in somite number, which is usually around 50–70.[8] There are exceptions to this rule, however, since this statistic can rise to several hundred in the case of snakes. Within a given species, variability of the somite number is unusual and seldom varies by more than 5%.[9]

While somite formation and somitic derivatives are similar in all vertebrates, the time schedule of this process can vary dramatically between species. In cold-blooded vertebrates, the species-specific pace of somitogenesis is temperature-dependent and, in the case of salmon embryos, has even been shown to be specific to the particular genetic strain.[10] Moreover, hybrid salmon embryos will adopt the somitogenesis rate of the male parent strain, indicating that there is a genetic component to this regulation. These data imply that regulating the somitogenesis pace is imperative for the correct overall development of an embryo. The lability of this parameter appears to be under environmental influence.

Somites derive from a stem cell population

Fate-mapping studies have demonstrated that the presumptive somitic territory derives from bilaterally located regions in the epiblast (mouse and chick) or marginal zone (amphibia).[11–13] The cells in these regions ingress at the blastopore (amphibia) or primitive streak (mouse and chick) during gastrulation. There is evidence that in chick and mouse, after these cells have ingressed, they become a resident population of somitogenic stem cells in the node/primitive streak, and then in the tail bud.[14,15] Once in place they are believed to divide asymmetrically, giving rise to a progenitor and a stem cell daughter. The PSM derives from these progenitors, and thus the stem cells contribute progeny to somites along the entire body axis, (Fig. 1).

The evidence for a cell population with stem cell properties comes from two independent approaches. The first is a cell lineage analysis in chick using injection of single cells in the primitive streak and Hensen's node with a fluorescent tracer.[15,16] The second employs a retrospective clonal analysis in the mouse using the LaacZ system.[14] This latter strategy suggested that the murine streak contains a pool of 100–150 somitic stem cells, which generate the progenitors of all the somites along the body axis.

Until recently, the existence of similar somitic stem cells in other vertebrate groups, such as fish and amphibia, was not widely accepted. It was believed that, due to little cell division taking place after gastrulation, trunk

somitogenesis in frogs and fish resulted from sequential segmentation of a pre-existing territory, which involutes during gastrulation. Tail somites, in this view, are formed by a second mechanism involving tail bud extension. Nevertheless, several lines of evidence argue against this classical view and suggest that the somitic territory might be produced in a way similar to that of chick and mouse. First, evidence suggesting the existence of somitic stem cells has been provided through zebrafish fate-mapping studies[17,18] and through cell lineage analyses of gastrula stage *Xenopus* embryos.[19] In addition, it is now well established that tail bud extension is a continuation of the gastrulation process, arguing against the existence of two different modes of paraxial mesoderm segmentation in amphibia.[20] These data suggest that, at early stages in the development of vertebrates, a population of paraxial mesoderm stem cells become specified. This population will continuously generate progeny that will populate the somites, throughout the duration of body axis extension.

One important implication of this somitic stem cell model is the idea that AP regional identity is in no way preprogrammed in the precursor cells of the streak. The inference is that regional identity is acquired with time during development. Since the stem cells are a proliferating population, a first step in establishing regional identity along the AP body axis may be linked to mitosis, such that a more caudal identity correlates with an increasing number of precursor cell divisions. This concept, though unproven, agrees with a number of experimental observations and theoretical models, as will be discussed in detail in the second part of the review, which deals with segment regionalization.

Models for the generation of a metameric pattern

Segmental prepattern in the presomitic mesoderm

Many of the classical somitogenesis models were based on the observation that it is not possible to modify the intrinsic schedule of PSM segmentation experimentally. For instance, placing PSM fragments in a challenging in vivo environment or culturing isolated PSM explants in vitro does not alter their endogenous segmentation program (Refs. 1, 21, 22 and references therein). These studies, together with the fact that there is very little cell movement in the PSM,[23,24] suggest that cells resident in the PSM are already prepatterned along the AP axis with respect to their subsequent grouping into somites. Thus, an underlying tenet of early models was that the decisive segregation of cells fated to belong to the same somite has to be generated in the caudal-most PSM, at the region of its transition from the primitive streak and later from the tail bud. In other words, the periodicity of segmentation would reflect the ingression of pregrouped somitogenic cells from the streak into the PSM (see Refs. 25, 26). "Prepattern"-type models, however, fail to explain the mechanisms of how periodicity is generated in the first place.

Ticking models for metamerism

A second category of model directly addresses the temporal and spatial aspects of periodicity regulation. These models postulate the existence of an oscillator or clock that operates in the PSM cells with a periodicity corresponding to the time required to form one somite. These mechanistic models place the realization of metamerism at the level of the rostral PSM, immediately prior to somite formation. Consequently, no prior segmentation of the presomitic mesoderm is postulated in these models, although some degree of synchrony is assumed between cells that will populate the same somite.

Clock and Wavefront

Cooke and Zeeman devised the "Clock and Wavefront" model[27,28] in order to explain global regulation of vertebrate somite number as seen in both haploid amphibian embryos[29,30] and in size-reduced amphibian embryos (after experimental cell ablation).[9] This model incorporates elements from another well-established model, namely the Positional Information Theory.[31] Thus, the model postulates that the positional information (p.i.) variable of each presomitic cell is a function of developmental time. At some critical point in development, the value of this variable is such that the cell undergoes a sudden behavioral change, for example in cell adhesivity. Since development proceeds along a smooth AP gradient, the observed behavioral change in response to cells attaining the threshold p.i. value over time would be manifest as a smooth wave. This "wave," however, is proposed to operate in conjunction with an intracellular oscillator continuously operating in presomitic cells. The oscillator would be phase-linked between neighboring cells and would gate the response to the onset of behavioral change, by alternately promoting and then inhibiting the response to the wavefront. In other words, there would be a smooth anterior–posterior developmental wavefront of behavioral change in cells, but the oscillatory nature of the response gives the impression of discrete successive populations assuming the change (Fig. 2). In this way, the model invokes some inherent cyclic property of the cells, which underlies the temporal precision and periodicity of the somitogenesis process.

Experimental support for the model was provided from studies in which amphibian embryos were subjected to a pulse of heat shock. These embryos subsequently developed segmentation defects located in a set number of somites caudal to the somite that was in the process of forming at the time of treatment.[32,33] This result was interpreted as evidence for the progression of a wavefront tra-

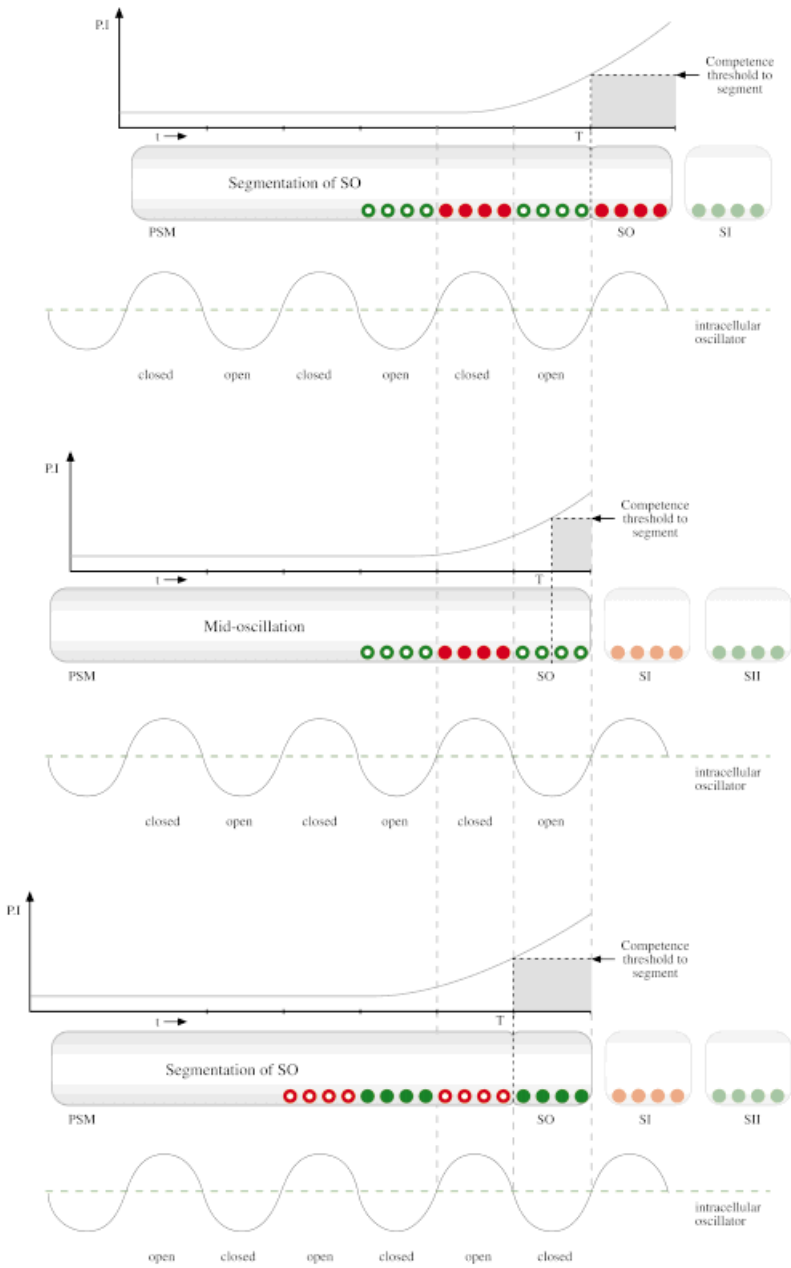

Figure 2. The Clock and Wavefront model. The graph plots the positional information (P.I.) variable of the cells against time (t) spent in the PSM. Time T marks the point at which cells reach a certain P.I. value that indicates their competence to undergo a behavioral change, i.e., to segment. The PSM cells are also subject to a second level of temporal regulation, dictated by their intracellular oscillator, which controls their response to this competence trigger. The oscillations are represented as fluctuations between open and closed states. The red and green circles represent groups of phase-linked PSM cells oscillating between these states. Thus, onset of "competence" is only realized when cells are in a certain point of their oscillation cycle, represented here as "closed circles." Therefore, as is shown in the middle panel, cells having attained the P.I. value that bestows them with competence to segment, but which are in mid-oscillation will not segment until they are in the closed state. Cells within the formed somites are all represented as closed circles. Anterior is to the right. S0, forming somite. SI, newly formed somite. SII, last but one somite.

versing zones of phase-linked, differentially responsive cells in the PSM, but it provided no clues to the nature of the oscillator component of the model.

Meinhardt's model

A second model, which provides an explanation for the periodic regulation of somitogenesis and which is based on reaction-diffusion mechanisms, is that proposed by Meinhardt.[34] This model also addresses the separate issue of intrasomitic AP polarity. While the onset of dorso-ventral and medio-lateral specification occurs only after the somite has formed, the AP polarity of each somitic compartment is already established within the cells of the unsegmented rostral PSM.[35] This AP subdivision of the somites has important consequences, since it underlies the phenomenon of resegmentation, during which each vertebra forms by fusion of the posterior part of a somite with the anterior part of the next caudal somite.[36] Moreover, anterior and posterior sclerotome compartments exhibit differ-

ent properties with respect to neural crest cell and motor axon migration.[1,37–39] This AP compartmentalization in the somite is therefore also responsible for segmental organization of the peripheral nervous system.

Meinhardt's model, like the Clock and Wavefront hypothesis, also incorporates the idea of an oscillatory mechanism operating within the presomitic cells producing an alternation between cell states, such as anterior (A) and posterior (P). He proposed that the periodicity of each complete oscillation would be the time taken for a somite to form and that the cells in opposite "states" would be unable to mix. According to his model this fluctuation between cell states would become stabilized at the anterior limit of the PSM as the somite buds off and, consequently, cells of "like-state" become grouped separately to those in an "unlike-state" (Fig. 3).

In addition, segmentation requires the formation of intersomitic boundaries. The apposition of A and P cells, however, would not suffice as a mechanism, for this would also lead to the formation of intrasomitic borders. Therefore, to explain formation of the somite boundaries Meinhardt postulated a third state representing the intersomitic border cells. This somitogenesis model relies on and defines the establishment of AP polarity within the forming somite.

Thus, these two models succeed in explaining various features underlying somitogenesis, but they account for different aspects. The Clock and Wavefront model naturally explains the global regulation of somite number against embryonic size,[28] whereas Meinhardt's model accounts for AP polarity in the somites.[34]

Molecular evidence, from both chick and mouse studies, has recently provided further support for the existence of a developmental clock that is linked to vertebrate somitogenesis. Facets of both models described above appear to be at play during this process. These data are discussed below.

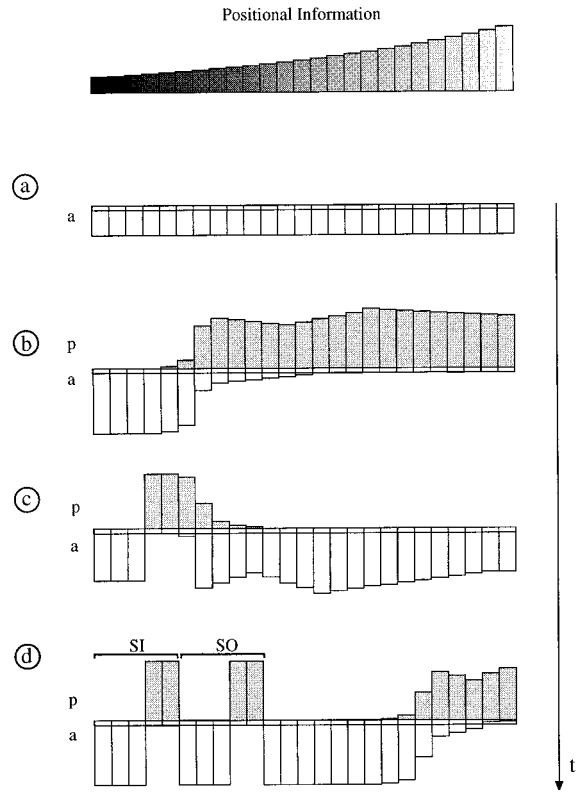

Figure 3. Meinhardt's model (adapted from Ref. 34). Stages in the generation of a periodic and a sequential pattern under the control of a morphogen gradient. The antero-posterior gradient, at the top of the figure, provides positional information. **a**: The initial stable gradient. Cells are initially in one "state," here depicted as anterior. **b**: At a specific morphogen concentration, cells switch to the "posterior state." **c**: Cells on each side of this transition point stabilize each other mutually in the "a" and "p" states. "p" cells at a distance from the border and which are thus too far to receive a stabilizing influence revert to an "a" identity. The somitic border is formed between the posterior cells of one somite and the anterior cells of the caudally adjacent somite. **d**: The next P-A border requires an increased morphogen concentration. The process continues in this way until the entire field is subdivided into the periodic pattern of anterior and posterior. S0, forming somite. SI, newly formed somite. Anterior to left. "t" represents developmental time.

A molecular clock linked to vertebrate segmentation

c-hairy1 *expression identifies a molecular clock linked to somitogenesis*

A recent article[40] provided the first molecular evidence that the cells of the PSM do display "clock-like" or oscillatory behavior prior to segmentation, as had been postulated 20 years earlier.[28] Palmeirim and colleagues describe the PSM expression in avian embryos of *c-hairy1,* a vertebrate homolog of the *Drosophila hairy* gene which is one of the members of the fly segmental patterning pathway[41] (Fig. 4). They demonstrate that presomitic cells oscillate in synchrony with their neighbours in terms of *c-hairy1* RNA expression. These oscillations are bilaterally synchronous and appear as caudal-rostral waves of expression that sweep across the PSM approximately every 90 minutes, which equals the formation time of one somite in chick (Fig. 4a,b). This study revealed that the oscillatory property of the PSM is autonomous and does not result from the propagation of a node-derived signal. The strict correlation between the periodicity of the oscillator and somite formation implies that somitogenesis is temporally

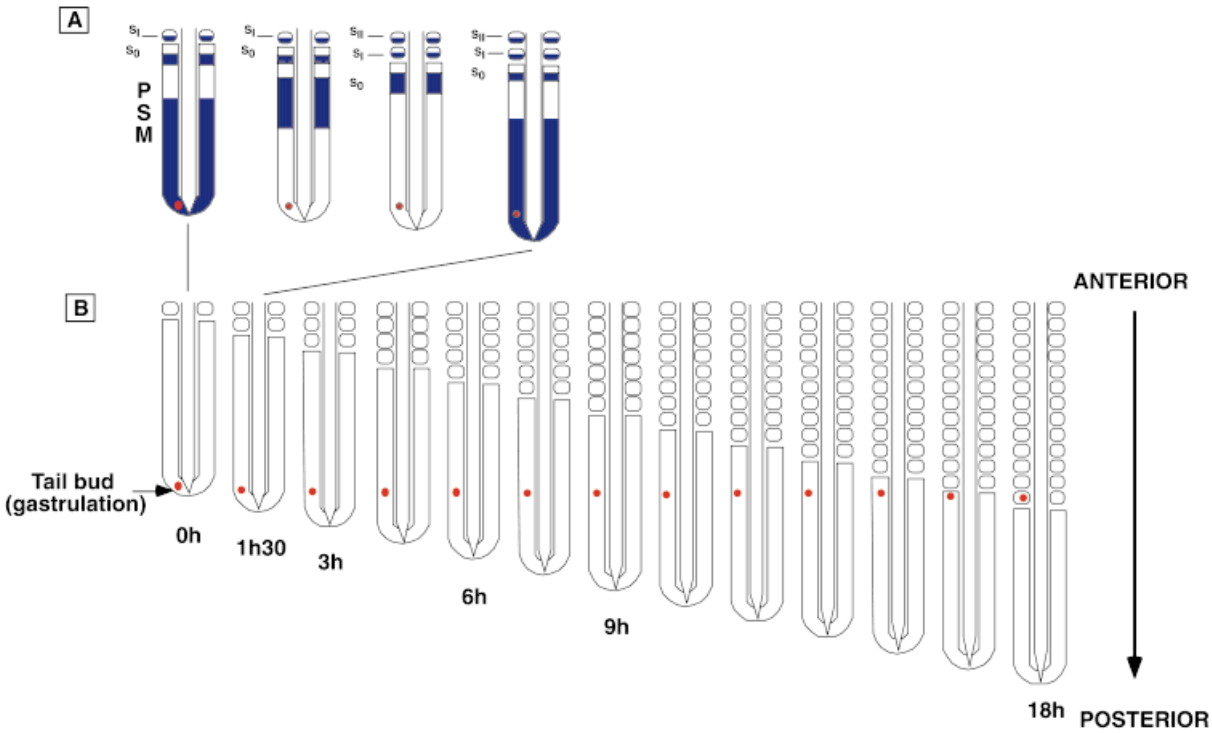

Figure 4. Schematic representation of *c-hairy1* expression in the PSM, with respect to an individual presomitic cell. **A**: The four diagrams of the segmental plate during the formation of the next somite show static intervals of the *c-hairy1* expression wave that sweeps across the PSM caudo-rostrally every 90 minutes. Expression is initiated in a broad caudal domain that narrows as it progresses anteriorly and is finally restricted to the caudal domain of the next somite to be formed. Thus, an individual PSM cell (red dot) will experience a *"c-hairy1* on" phase and a *"c-hairy1* off" phase during each oscillation. S0, forming somite. SI, newly formed somite. SII, last but one somite. **B**: History of a presomitic cell (red dot) in the PSM: from the time it exits the domain of self-renewing stem cells in the streak/tailbud and becomes resident in the PSM (0h), until it is incorporated into a somite (18h). This time interval corresponds to the formation of 12 somites, which is the number of prospective somites in the PSM tissue. Thus, during the time it resides in the PSM each cell will undergo 12 cycles of *c-hairy1* expression. Since the onset of cycling RNA expression is initiated in the stem cells of the streak, somitic cells in the anterior somites will have experienced fewer cycles prior to their entry into the PSM than those cells that will populate more caudal somites. Thus, the total number of expression cycles for cells in distinct somites will be different and will correspond to their location along the AP body axis. These oscillations therefore define a clock linked to both somite segmentation and possibly to regionalization of the somites along the AP body axis.

regulated by the biochemical activity of an intrinsic clock "ticking" in the PSM cells.

c-hairy1 — *Is it a clock component or under clock control?*

The *c-hairy1* homolog in the fly is a primary pair-rule gene which encodes a basic helix-loop-helix transcriptional repressor.[41,43–48] Similarly, the murine *HES1* gene, which is structurally highly related to the *c-hairy1* gene, encodes a transcriptional repressor, and has been shown to bind to its own promoter.[49] Thus, one might imagine that *c-hairy1* may regulate its own transcription in an oscillatory cycle as a crucial clock component.[42] Inhibiting protein synthesis, however, does not arrest cyclic *c-hairy1* expression,[40] which rules out this idea. The data imply, rather, that *c-hairy1* production is an output rather than a key regulator of the oscillator and also that the clock acts at the post-transcriptional level, by an as yet unknown means, to regulate *c-hairy1* transcription (Fig. 5).

Specifying half-segments

During boundary formation and budding of the new somite at the rostral end of the PSM, the expression of *c-hairy1* becomes fixed. Cells in the anterior half of the forming somite no longer express *c-hairy1*, whereas those cells in the future posterior half which are expressing *c-hairy1* at this time maintain expression in the formed somite.[40] Thus, the dynamics of *c-hairy1* expression are

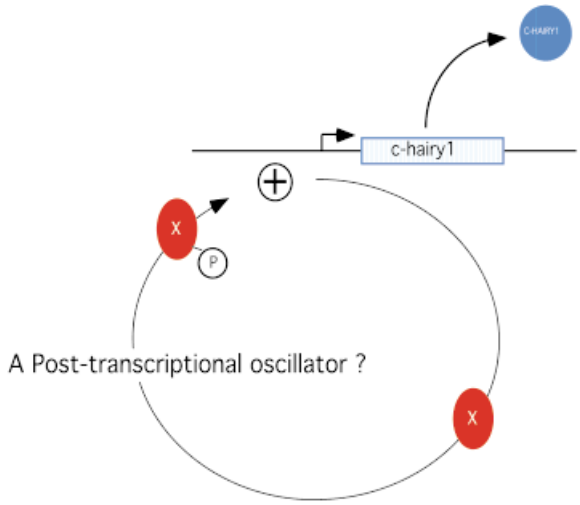

Figure 5. c-hairy1 protein; an output of the clock. Regulation of cyclical *c-hairy1* expression may reside in posttranscriptional modulations to extant proteins in PSM cells. Thus, the factors driving the *c-hairy1* oscillations may be periodically activated through the tight regulation of posttranscriptional events such as phosphorylation and dephosphorylation. In this case, c-hairy1 protein product would be an output of the segmentation clock rather than a key component in clock control.

strikingly reminiscent of those predicted for the oscillator in Meinhardt's model[34] (for review see Ref. 50). In this version of the model, cells in the PSM oscillate between anterior (*c-hairy1* off) and posterior (*c-hairy1* on) states which become stabilized in the rostral PSM. It is tempting to speculate that these oscillations of *c-hairy1* expression are part of the mechanism acting to establish half-somite identity.

Building a border

The oscillating expression profile of *c-hairy1* reflects a clock-like activity within PSM cells that could function to synchronize cell behavior and thus regulate the temporal periodicity of somitogenesis. At a defined time the morphogenetic response to this somitogenesis clock is the epithelialization event of border formation. What mechanisms underlie how and when the somitic borders form?

We now know that the Notch signaling pathway is of critical importance in this process (reviewed in Ref. 7). A requirement for these genes during somitogenesis has been demonstrated in mice carrying mutations in many different components of the pathway. These include *Notch1, Delta1*, *Delta3*, *RPB-JK*, and *Presenilin* mutants.[51–57] The mutant embryos all exhibit segmentation defects related to the size, shape, bilateral synchrony, and AP polarity of somites. These data implicate the pathway in somite boundary formation and establishment of AP compartments. Nevertheless, a basic metameric pattern is maintained when Notch activity is impaired, which suggests that Notch signaling lies downstream of the "clock." In the *Xenopus* and zebrafish systems, overexpression of injected RNA encoding proteins that inhibit or constitutively activate components of the Notch pathway has demonstrated the conserved role of this signaling system in coordinating vertebrate somitogenesis[58,59] (for review, see Ref. 35).

Like border formation, AP specification occurs in the rostral PSM. This has been demonstrated by inverting regions of the PSM in vivo in chick embryo.[60,61] Specification in the PSM is further supported by the expression of several genes, such as *Delta1* and *Delta3*, which are expressed in the PSM and which become anteriorly or posteriorly restricted in the somites, where their role in AP somitic identity specification has been demonstrated in mutational mouse studies.[53,54]

Unlike *c-hairy1*, most of the Notch pathway components display broad, static expression domains within the PSM (see Fig. 6). Superimposed on these domains are restricted regions of upregulated *Notch* and *Delta* expression in the rostral PSM.[22,57,62] This pattern is of significance since, as noted above, this is the region where Notch signaling activity appears to be of critical importance during somitogenesis. The question arises: What is the link between the temporal periodicity which is governed by the somitogenesis clock and revealed by dynamic *c-hairy1* expression and the spatial periodicity, which requires Notch signaling and is revealed by evenly spaced somite-border formation?

Clock control on the Notch signaling pathway

Since the description of *c-hairy1* expression, a second gene, *lunatic fringe,* has been demonstrated as having a cyclical expression in the PSM of both chick and mouse.[63–65] This gene is a vertebrate homolog of the fly *fringe* gene,[66] which encodes a putative secreted protein that has been implicated in Notch signaling.[67–69] During wing morphogenesis *fringe* functions to establish the position of the dorso-ventral boundary by both promoting Notch activation by Delta and inhibiting Notch activation by Serrate. This activity leads to a feedback system in which Notch expression becomes restricted to and subsequently establishes the position of the wing margin.

The spatio-temporal kinetics of *lunatic fringe* expression in the PSM appear to be similar in chick and mouse embryos and are almost identical to those of *c-hairy1* in chick. While the two genes appear to be implicated in the clock phenomenon, there is some evidence to suggest that *lunatic fringe* may act even further downstream than *c-hairy1*, since de

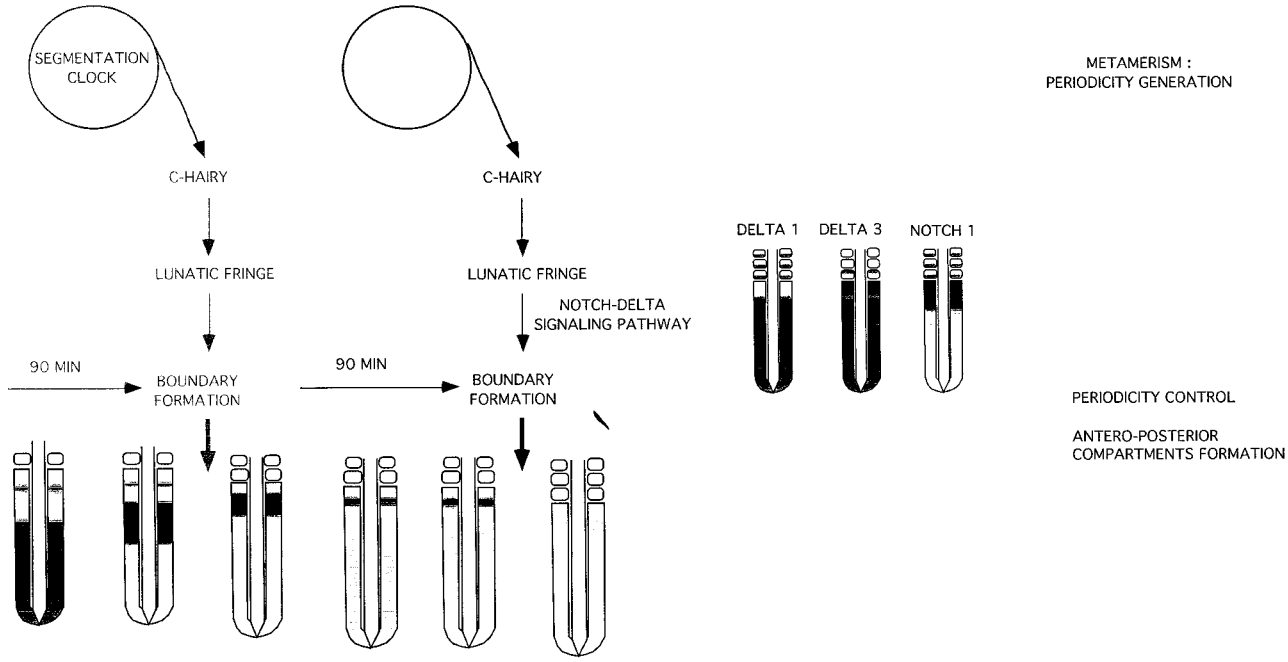

Figure 6. Schematic diagram of how the segmentation clock may modulate border formation during somitogenesis in the PSM. There is one cycle of the clock in each somitic cell every 90 minutes, which is manifest in the expression of *c-hairy1* and *lunatic fringe* RNA. In the bottom part of the diagram, each block of the same color corresponds to expression domains of the same wave as it spreads caudo-rostrally across the PSM. The different shades correspond to consecutive waves of RNA expression. This clock activity may be what generates the periodicity of metamerism and this property is shared by all presomitic cells, from the time they are resident in the streak until they are incorporated into a somite. These regular RNA oscillations then become stabilized in the rostral PSM as the prospective somite border is formed. The top part of the figure specifically demonstrates the result of the clock cycle at the rostral end of the PSM during border formation. Within this region the cycling expression of *lunatic fringe* is believed to modulate the activity of the Notch pathway every 90 minutes and thereby activate the formation and positioning of the intersomitic boundary. The static RNA expression domains of *Notch* and *Delta* are shown to the right.

novo protein synthesis is required for the oscillations of *lunatic fringe* expression but not for *c-hairy1*.[64]

A crucial role for *lunatic fringe* during somitogenesis was demonstrated by loss of function mutations of the gene in mice, which cause segmentation defects similar to those seen in the Notch pathway mutant embryos.[70,71] Thus, the segments that form in these mice are irregular in size, display AP polarity defects, and are no longer bilaterally symmetrical. Since the mutant embryos exhibit diffuse boundaries of *Notch* and *Delta* expression and subsequent disruption of segmental structures, one hypothesis is that *lunatic fringe* plays a role upstream of the Notch pathway to delimit domains of Notch-Delta activity and/or the position of border formation. Thus, in this interpretation the somitogenesis clock acts to temporally regulate Fringe modulation of the Notch pathway, which would lead to the periodic arrangement of evenly spaced boundaries (see Fig. 6). Taken together, these data suggest that *lunatic fringe* may act as a linking mechanism between the temporal periodicity imposed by the clock and the spatial periodicity displayed by somite formation.

Clock control over regional specification of segments along the AP body axis

Once the meristic series has been generated it begins to show morphological signs of regionalization along the AP axis. A number of studies have led to the proposal that this regionalization process may also be subject to a type of "clock" control.

The cell cycle as a clock

The results of heat-shock experiments performed in the chick embryo led Stern and colleagues to propose a model for somitogenesis that involves a clock driven by the cell cycle.[24] After one pulse of heat-shock, they observed segmentation abnormalities, which were occasionally repeated, up to three times, at 5–7 somite intervals.[24,72,73] The periodicity of these repeated anomalies closely correlates with

the duration of a cell cycle in these cells, since in the chick a pair of somite segments from the PSM approximately every 90 minutes and the cell cycle time of these cells is about 10 hours.[73] Moreover, they showed that some cell cycle synchrony exists in the PSM. According to the model, heat-shock treatment generates anomalies in those cells fated to belong to the same somite, which are cell-cycle synchronized and which are in a specific heat-shock sensitive phase of the cycle.

It is noteworthy that a 5–7 somite interval, which corresponds to the duration of one cell cycle, correlates with the length of domains of somite derivatives sharing regional identity at the vertebral level. In other words, in most vertebrate species each level of the axis, occipital, cervical, thoracic, lumbar, and sacral is usually comprised of a multiple of 5–7 vertebrae, specific to each region.[73,74] The fact that cell cycles are partially synchronized in the PSM suggests that cell division could control the transition from the generation of somitic derivatives typical of one regional level to the next.

"Einbahnstrasse" model

In a similar vein, the "Einbahnstrasse" model, proposed by Duboule, directly implicates the cell cycle in the temporal regulation of regional identity specification but with specific reference to the role of Hox genes.[75] This cluster of genes and their role in specifying AP identity have been conserved throughout evolution. In vertebrates, the Hox genes display colinearity in both the temporal and spatial onset of their expression along the AP body axis with respect to their linear position in the complex.[76] Thus, those Hox genes, which lie not more than 3′ along the cluster, are expressed earlier in development and more anteriorly than those located more 5′. Activation of Hox gene expression during proximo-distal outgrowth of the limb follows the same colinearity rules. Studies of this latter system led to the "Einbahnstrasse" model.[75] It holds that progression in the activation of Hox genes along their complexes, from anterior to posterior and proximal to distal in the trunk and limb, respectively, would be a function of cell proliferation. Consequently, only those cells that remain in the proliferative zone will undergo sufficient cell divisions to permit their activation of more 5′ genes, which in turn will ultimately drive these cells to adopt a more caudal (or distal in the case of the limb) fate.

The proposed mechanism of control invokes an accumulation of proteins, which bind specifically and with high affinity to DNA in the 5′ region of the Hox complex. This blocks access to the transcription machinery but with successive cell divisions these transcriptional antagonists become progressively titrated out, thereby allowing access to and transcription of the more 5′ genes of the complex.[77] The model can be equally well adapted to the spatial and temporal control of Hox gene expression in the PSM. Thus, successive stem cell divisions in the primitive streak may regulate the sequential activation of the Hox genes in the cells that will populate the PSM. PSM cells, which are produced early and are positioned anteriorly will thus express 3′ genes, while more 5′ genes will be expressed in PSM cells produced later and which are located more posteriorly. Consequently, the complement of Hox genes expressed by PSM cells specifies the regional fate of their somitic derivatives. The regular oscillations, manifest in the cell cycles, and the unidirectionality of the sequence (opening of the cluster in a 3–5′ direction) are features that would make this mechanism an authentic biological clock.

Somitic regionalization and the segmentation clock?

Meinhardt's model also incorporated an idea that could account for how the oscillatory mechanism used to generate the somites could serve to bestow regional identity to the somites. In this scheme, the number of oscillations undergone by each cell determines the segment-specific fate of its derivatives, be that cervical, thoracic, lumbar, etc. He proposed an analogy with a grandfather clock, in which oscillations of the pendulum correspond to the oscillations in the PSM cells.[34] These would drive the rhythmic movements of the clock hands, which can be related to formation of somite units and the regional domains described above.

To be implicated in somite determination along the AP axis such a mechanism implies that the oscillations have to start very early in the development of somitic cells. Regional determination of the paraxial mesoderm cells along this axis is believed to occur immediately after they leave the primitive streak (or tail bud) and enter the PSM.[35,78,79] The PSM cells in which *c-hairy1* and *lunatic fringe* oscillate are, therefore, already determined with respect to their future AP identity and location. Consequently, for a time-counting mechanism such as those proposed by Duboule and Meinhardt to be operational the segmentation clock has to be functional prior to cells entering the PSM, i.e., in the somitic stem cells of the primitive streak.

To date, the studies of *c-hairy1* and *lunatic fringe* expression have not addressed the status of the clock in the presomitic territory of the streak and the tail bud, i.e., prior to entry of these cells into the PSM.[40,63–66] We have observed that in the chick embryo the first appearance of cycling RNA expression in the prospective paraxial mesoderm correlates with its ingression from the epiblast into the primitive streak (Pourquié and Jouve, in preparation). Oscillations of the cycling genes are then detected in the rostral primitive streak and the forming PSM. Therefore, somite precursors in the streak undergo oscillatory expression of these genes prior to their release into the PSM. This suggests that the segmentation clock is already active in the somitic stem cells, which are located in the rostral streak. Consequently,

somitic cells which derive from these stem cells will not only have experienced 12 oscillations as reported in the initial study of *c-hairy1* expression, but a number of oscillations that directly corresponds to their position along the AP axis (Fig. 4). Thus, cells that leave the stem cell zone early, and which will thus be located anteriorly, will experience fewer cycles of gene expression prior to their oscillation cycles in the PSM than cells that populate more posterior somites and which continue to cycle in the streak before entering the PSM.

These data suggest that the number of oscillations experienced by PSM cells characterizes their specific AP position and therefore may be directly linked with the regionalization of their somitic derivatives. One possible means of linking these events is that the segmentation clock could control the cell cycle in these cells and, thus, either directly or indirectly the activation of Hox gene expression.

Calcium clocks in the PSM?

A recent study in zebrafish demonstrated that during gastrulation and somitogenesis the embryo exhibits a periodic series of intercellular, long-range calcium waves with a 5–10 minute frequency. Moreover, these pulses emanate from distinct loci at different developmental stages, notably from the node and tailbud regions during somitogenesis.[80] Whether these periodic waves are in any way linked to somitogenesis remains to be established. Since somite formation time in the zebrafish does not correspond to the frequency time of the calcium waves described, a direct link with the segmentation clock appears unlikely. Nevertheless, the cells remain phase coordinated over a long time span, which, in the absence of cell communication, appears to demand an improbably precise control mechanism within each cell. The high frequency of calcium waves described in the zebrafish suggests a means of intercellular communication, possibly via gap junctions,[81] which could provide a mechanism of "co-ordinating the spatial and temporal regulation of highly localised processes across large cellular domains."[80,82]

Conclusions

Recent molecular approaches to understanding the regulation of somitogenesis have begun to elucidate aspects of the underlying mechanisms. We now have strong supporting evidence for a molecular clock at play during somitogenesis which generates waves of transcriptional activation and/or degradation that sweep across the PSM at an astonishing rate, as demonstrated through analyses of the kinetics of *c-hairy1* and *lunatic fringe* expression in mouse and chick. Thus, molecular candidates for the output components of the clock (*c-hairy1* and *lunatic fringe*) and downstream effectors of somite formation (Notch pathway components) have now been identified and their role in this process is being further characterized. Many questions remain, however. In particular, it remains a mystery as to what is driving the segmentation clock and what its precise role is during somitogenesis. The elucidation of exactly how the interplay between the clock genes and Notch components is established also remains an important challenge for the future. As yet, we have no idea what regulates the arrest of *c-hairy1* and *lunatic fringe* cycling in the forming somite. Does this reflect an arrest in the "clock" itself in these cells or does the cyclic regulation of these clock markers become unhooked at somite formation and the now hidden clock continue to run? Biochemical and genetic research into the upstream regulators of *c-hairy1* expression are sure to provide some answers to these questions.

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