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Vertebrate somitogenesis: a novel paradigm for animal segmentation?

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ABSTRACT In vertebrates, the primary segmented tissue of the body axis is the paraxial mesoderm, which lies bilaterally to the axial organs, neural tube and notochord. The segmental pattern of the paraxial mesoderm is established during embryogenesis through the production of the somites which are transient embryonic segments giving rise to the vertebrae, the skeletal muscles and the dorsal dermis. Somitogenesis can be subdivided into three major phases (see Fig. 1). First a growth phase during which new paraxial mesoderm cells are produced by a growth zone (epiblast and blastopore margin or primitive streak and later on tail bud) and become organized as two rods of mesenchymal tissue, forming the presomitic mesoderm. Second a patterning phase occuring in the PSM, during which the segmental pattern is established at the molecular level. Third, the somitic boundaries are formed during the morphological segmentation phase. In all vertebrates, all cells of the paraxial mesoderm, during their maturation in the PSM, go successively through these three phases, which are tightly regulated at the spatio-temporal level. The first phase of paraxial mesoderm production falls out of the scope of this review, as it essentially pertains to the gastrulation process. Here, I essentially discuss the segmental patterning phase in vertebrates. Recent data suggest that establishment of the segmental pattern relies on a clock and wavefront mechanism which has been conserved in vertebrates. Furthermore, conservation of this system could extend to invertebrates, suggesting that the clock and wavefront is an ancestral mechanism.

KEY WORDS: *somite, FGF, wavefront, Clock, segmentation*

The paraxial mesoderm can be subdivided into two major domains along the antero-posterior (AP) axis (Fig. 2). From the anterior tip of the embryonic axis to the otic vesicle it is called head or cephalic mesoderm and gives rise to several muscles and bones of the head (Noden, 1991; Couly, *et al.,* 1992). No somites form in this region and the issue of its segmentation remains controversial(Gilland and Baker, 1993; Jouve, *et al.,* 2002). Caudal to this tissue is found the somitic region which extends along the body axis down to the end of the tail. The anterior part of the paraxial mesoderm is sequentially produced through invagination of epiblast territory at the blastopore margin or at the primitive streak, as a result of gastrulation. After completion of blastopore closure or primitive streak regression, the gastrulating region becomes restricted to a small region, called tail bud, which is located at the caudal tip of the axis. Somites of the posterior body and tail are subsequently produced by the tail bud which acts as a terminal growth zone. The transition between the invagination mode and the tail bud mode of paraxial mesoderm production is very progressive. Ingression movements similar to those taking place during early gastrulation have been reported in the tail bud suggesting that gastrulation continues in this structure (Gont, *et al.,* 1993; Kanki and Ho, 1997; Knezevic, *et al.,* 1998). However, it has also been argued that the tail bud functions as a blastema of undifferentiated cells(Davis and Kirschner, 2000).

The somitogenesis process begins soon after internalisation of the head mesoderm and continues during subsequent axis production. The first somite forms immediately caudal to the otic vesicle(Hinsch and Hamilton, 1956; Huang, *et al.,* 1997). Segment formation continues in a sequential fashion such that a new pair of somites is regularly added in a rostro-caudal fashion until a fixed species-specific number of somites is reached. The total number of somites produced is highly invariable within a given species but can vary dramatically between species. The speed of somite production is also specific of the species and can vary depending on the temperature. One pair of somites is produced every 20 minutes at 25°C in zebrafish or every 90 minutes at 37°C in the

Abbreviations used in this paper: PSM, presomitic mesoderm.

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chick embryo while it takes one day in the Salmon at 4°C(Gorodilov, 1992; Wood and Thorogood, 1994; Palmeirim, *et al.,* 1997).

Patterning the caudal PSM: the segmentation clock and the FGF wavefront

The newly formed caudal PSM is a loose mesenchyme which does not exhibit any particular segmental pattern. However, it is endowed with some periodic information as it exhibits rhythmic expression of a particular category of genes called the cycling genes (Fig. 3)(Pourquié, 2003). This rhythmic expression begins during gastrulation in the paraxial mesoderm precursors and their descendants and is maintained throughout somitogenesis (Jouve, *et al.,* 2002). These oscillations were first recognized in the chick embryo as rhythmic waves of expression in PSM cells of the mRNA coding for the basic helix loop helix (b-HLH) transcription factor c-hairy1, a vertebrate homologue of the protein encoded by the fly pair-rule gene *hairy* (Palmeirim, *et al.,* 1997). This periodic expression of *c-hairy1* provided evidence for the existence of an oscillator acting in PSM cells, which was called the segmentation clock. The existence of such an oscillator in PSM cells had been predicted in several theoretical models such as the "Clock and Wavefront"(Cooke and Zeeman, 1976). In this model, PSM cells oscillate between a permissive and a non-permissive state for somite formation. These oscillations are phase-linked and controlled cell-autonomously by the Clock. Somite formation is triggered when cells of the rostral PSM in the permissive phase of the clock cycle are hit by a wave-front of maturation that slowly moves caudally along the axis of the embryo. In this model, the Clock generates a temporal periodicity that is translated spatially into the periodic boundaries of the somites.

Several additional genes exhibiting a dynamic behavior similar to *c-hairy1*, have now been identified in fish, frog, chick and mouse embryos indicating that the segmentation clock is conserved among vertebrates (Forsberg, *et al.,* 1998; Mcgrew, *et al.,* 1998; Holley, *et al.,* 2000; Jiang, *et al.,* 2000; Jouve, *et al.,* 2000; Leimeister, *et al.,* 2000; Sawada, *et al.,* 2000; Bessho, *et al.,* 2001; Li, *et al.,* 2003). Most are involved in Notch signaling suggesting that Notch activation plays a critical role in the oscillator. Such genes encode several transcription factors of the Hairy and Enhancer of Split (HES) family, acting downstream of Notch signaling (Holley, *et al.,* 2000; Jouve, *et al.,* 2000; Leimeister, *et al.,* 2000; Sawada,

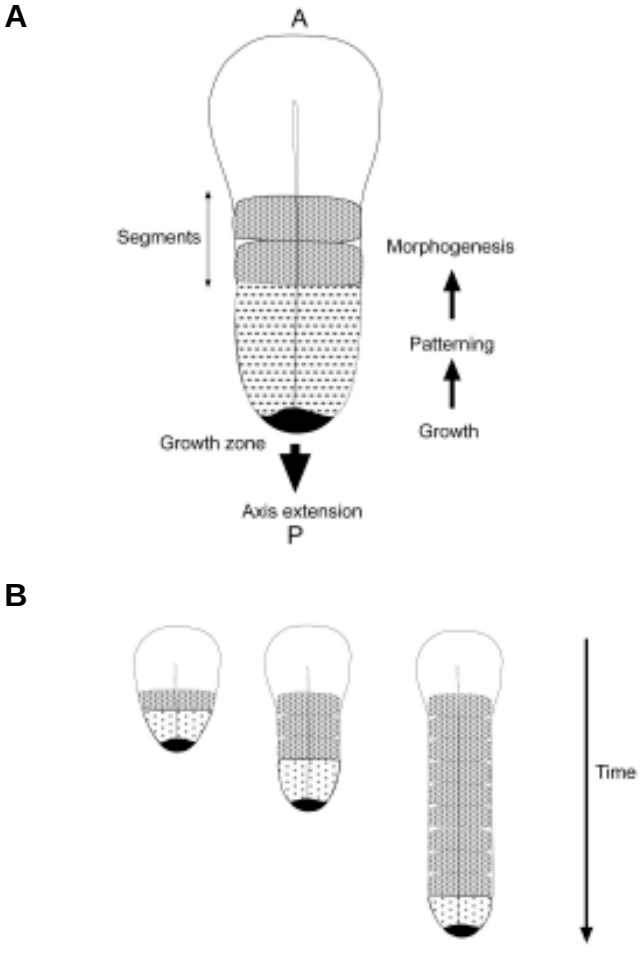


Fig. 1. (A) *Schematized view of the somitogenesis process in vertebrates. Such a scheme also applies for segmentation of many invertebrate species.* **(B)** *Progressive segment formation during embryogenesis. Vertebrates and many invertebrates produce their segments in this way. Abbreviations: A, anterior; P, posterior.*

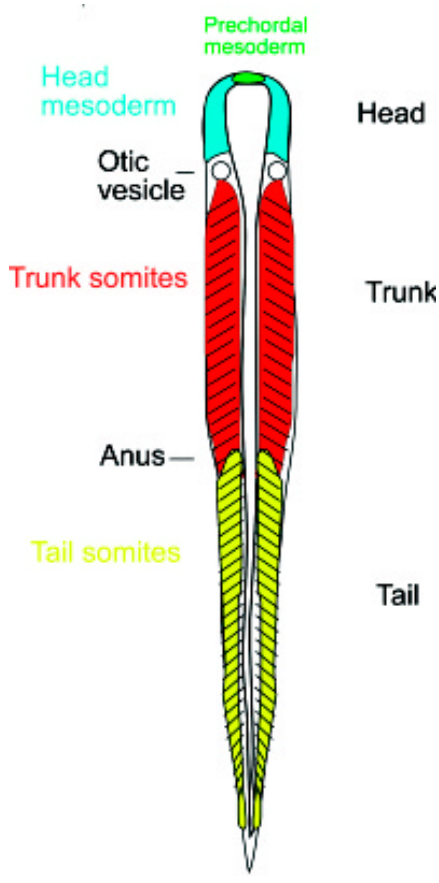
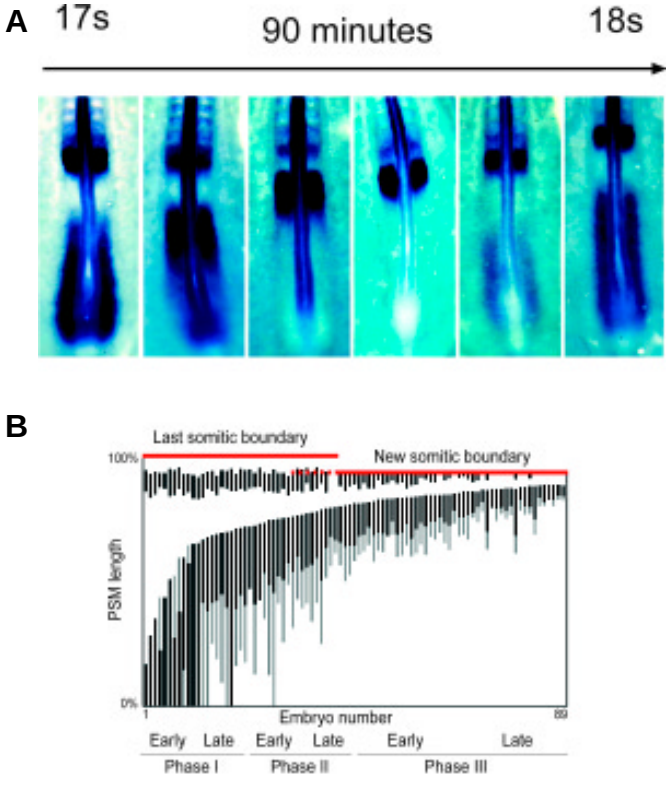


Fig. 2. Major regions of the paraxial mesoderm in vertebrates. *The prechordal mesoderm does not strictly belong to the paraxial mesoderm as it is an axial derivative, but it lies in continuity with it and shares unique characteristics such as the potential to give rise to skeletal muscles. The body region is classically considered to extend from the otic vesicle to the anus and the tail region lies caudal to the anus.*

et al., 2000; Bessho, *et al.,* 2001; Li, *et al.,* 2003), as well as the glycosyl-transferase Lunatic Fringe (Forsberg, *et al.,* 1998; Mcgrew, *et al.,* 1998) and the Notch ligand deltaC (Jiang, *et al.,* 2000). Their cycling behavior in the PSM is regulated at the transcriptional level (Cole, *et al.,* 2002; Morales, *et al.,* 2002). All these genes oscillate largely in synchrony in the PSM, suggesting that they are downstream of a common cycling activator.

In mouse, the inhibitor of Wnt signaling *axin2* is also expressed in a dynamic sequence similar to, but out of phase with that of the Notch-related cyclic genes(Aulehla, *et al.,* 2003). Wnt signaling acts upstream of the Notch-regulated cyclic genes (Aulehla, *et al.,* 2003)indicating that in mouse the segmentation clock is composed of a Wnt-based regulatory loop entraining a series of Notch-based loops. However, the role of Wnt signaling in the mechanism of the clock has not been established in other species than the mouse and the conservation of the Wnt-based loop is currently unknown. The role of the Segmentation clock in the somitogenesis process remains unclear. The clock might serve to coordinate periodic activation of Notch signaling in the anterior-most PSM, which ultimately would result in the rhythmic specification of somite boundaries and subcompartments (Jen, *et al.,* 1999; Takahashi, *et al.,* 2000). However, in mouse and fish mutants in which cyclic gene oscillations are disrupted, like in Notch pathway mutants, paraxial mesoderm derivatives retain some segmental organisation, suggesting that these genes are in fact not critical for establishing the metameric pattern (Conlon, *et al.,* 1995; Hrabe De Angelis, *et al.,* 1997; Jiang, *et al.,* 2000). The bilateral desynchronization of somitic boundaries seen in Notch pathway mutant embryos suggest that the clock plays a role in coordinating the precise timing of boundary production during development. Whether the clock plays a role in the initial establishment of the segmental pattern remains to be demonstrated.

Whereas the segmentation clock is thought to set the pace of vertebrate segmentation, it remains to explain how this pulsation is converted into the reiterated arrangement of segment boundaries along the AP axis. By performing surgical inversion of small PSM fragments in the chick embryo, it was established that the presomitic mesoderm can be subdivided into two broad regions along the AP axis (Fig. 4)(Dubrulle, *et al.,* 2001). First a caudal domain which encompasses almost the caudal two–thirds of the PSM in chick, in



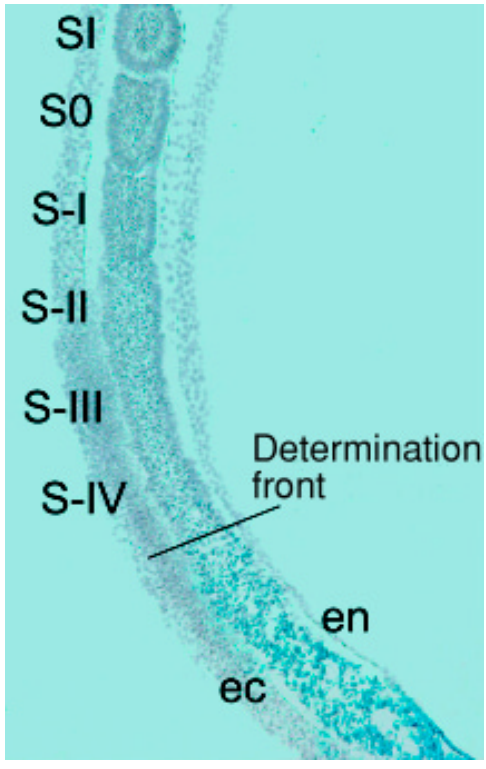


Fig. 3 (Left). The segmentation clock. (A) *Sequence of* lunatic fringe *mRNA expression during formation of the 18th somite in the chick embryo. Rostral to the top. Adapted from Mcgrew,* et al., *(1998).* **(B)** *Wave-like expression of lunatic fringe during one oscillation cycle. Each bar represents the extension along the AP axis of the* lunatic fringe *expression domain in 14 to 18-somite old embryos (x axis). Embryos have been ordered by rostral progression of their anterior expression boundary. The expression domains along the AP axis of the PSM are represented as bars plotted along the y axis, anterior to the top. Darker bars represent stronger expression. After Dale* et al., *(2003).*

Fig. 4 (Right). The wavefront. *Sagittal section of a 15-20 somite chick embryo showing the cellular organisation and* fgf8 *expression (in blue), rostral to the top. The PSM can be subdivided into a caudal mesenchymal domain expressing* fgf8 *mRNA and a rostral region exhibiting a progressive epithelialisation as seen by the alignment of the nuclei facing the ectoderm (ec) and the endoderm (en). The two domains are separated by the determination front. The position of the rostral presumptive somites named according to Pourquié and Tam, (2001) is indicated. Photograph courtesy of Julien Dubrulle.*

which somitic boundaries are not yet determined. This domain is characterized by a mesenchymal organization and by the expression of a set of genes conserved throughout vertebrates, which includes *fgf8, caudal, evx, brachyury*, and *mesogenin/mespo* (Joly, *et al.,* 1993; Duband, *et al.,* 1987; Dush and Martin, 1992; Buchberger, *et al.,* 2000; Yoon, *et al.,* 2000; Dubrulle, *et al.,* 2001; Sawada, *et al.,* 2001). Cells activate their segmentation program when they enter the second domain which corresponds in the chick to the rostral third of the PSM (Dubrulle, *et al.,* 2001). The limit between these two domains which marks a critical maturation step in the PSM was termed the determination front (Fig. 4). In chick and fish embryos, the position of the front was shown to be defined by the concentration of the secreted factor FGF8 whose mRNA is expressed in a graded fashion in the caudal PSM. Overexpressing FGF8 results in an inhibition of somitogenesis and a rostral extension of genes normally restricted to the caudal domain, indicating that FGF8 actively maintains the caudal identity of PSM cells, and therefore controls activation of the segmentation program (Dubrulle, *et al.,* 2001).

The segmentation process is intimately linked to the formation of the antero-posterior axis, and the pace of somite formation is tightly correlated to that of axis elongation (Fig. 1B). Due to the constant posterior elongation of the body axis during early development, the *fgf8* gradient is continuously displaced posteriorly, and thus the absolute position of the determination front along the AP axis undergoes a posterior-ward movement. The determination front could mark the place (or the maturation stage) when PSM cells become competent to respond to the clock signal and initiate the process of boundary formation. Therefore, the constant posterior regression of the determination front ensures that boundaries will be separated by a distance corresponding to the posterior displacement of the determination front during one period of the oscillation. These observations are consistent with the determination front acting as the wavefront of the clock and wavefront model. Whereas the role of FGF8 in the control of the segmentation process was only established in chick and fish embryos, in other vertebrates, FGF8 is similarly expressed in the caudal part of the embryo suggesting that the role of FGF8 in implementing the wavefront has been conserved in vertebrates(Crossley and Martin, 1995; Christen and Slack, 1997).

Moving along the presomitic mesoderm: from the determination front to boundary formation

During their maturation in the PSM, cells begin to exhibit an epithelial organisation, characterized by an increase in cadherin levels and cytoarchitectural changes such as basally aligned nuclei, and formation of a basal lamina(Duband, *et al.,* 1987; Dubrulle and Pourquié, unpublished observations). This transition has been well described in the chick where it begins at the determination front level (Fig. 4). Accordingly, overexpression of FGF8 in the PSM blocks somite epithelialisation suggesting that activation of the epithelialisation process in the PSM is negatively regulated by FGF8 (Dubrulle, *et al.,* 2001). This change in cellular organisation correlates with the activation of genes known to be required for the epithelialisation such as *paraxis* in chick or mouse (Burgess, *et al.,* 1996; Sosic, *et al.,* 1997)or *Tbx24* in fish (Nikaido, *et al.,* 2002). In zebrafish, the *fused somite* mutant in which the T-box gene *Tbx24* is disrupted, completely lacks epithelial somites (Nikaido, *et al.,* 2002). In mouse, the null mutation of the b-HLH gene *paraxis* which is expressed rostral to the determination front also results in a loss of epithelial somites(Burgess, *et al.,* 1996). Interestingly, the paraxial mesoderm derivatives of the two mutants described above exhibit a normal segmental arrangement. This suggests that segmental patterning is independent of the epithelialisation process. Such a conclusion is corroborated by *in vitro* experiments demonstrating that cultured PSM explant can form a striped pattern of gene expression without showing any signs of epithelialisation(Palmeirim, *et al.,* 1998).

In fish, chick and mouse, the first genes exhibiting a striped expression are activated immediately rostral to the determination front, in the epithelialized region. The transcription factor *Mesp2/c-meso1* becomes periodically activated in a segmental domain at this level and marks the earliest manifestation of segmental organisation in the PSM (Saga, *et al.,* 1997; Sawada, *et al.,* 2000; Takahashi, *et al.,* 2000). This gene is initially expressed in a domain wider than a somite which subsequently narrows as cells become more anterior in the PSM to finally end in the future rostral half-somite. Therefore, in vertebrates, like in fly, segmentation proceeds by refining an initial pattern of coarse stripes. In zebrafish, frog, chick and mouse the transcription factors of the Mesp family appear to play a very critical role in the segmentation process and are attractive candidates to mediate the translation of the periodic signal of the segmentation clock into a periodic spatial pattern(Sparrow, *et al.,* 1998; Sawada, *et al.,* 2000). These genes act upstream of a genetic cascade involving the Notch pathway, which ultimately results in boundary positioning and formation of anterior and posterior somitic compartments (Jen, *et al.,* 1999; Takahashi, *et al.,* 2000). Therefore, it is tempting to speculate that the periodic activation of genes of the Mesp family, which takes place at the determination front level is controlled by the segmentation clock.

Rostrally, the PSM is limited by an epithelial boundary formed during the separation of the last somite. Therefore, the actual making of a somite merely represents a very final step of the epithelialisation process during which the caudal boundary is formed. A key aspect of the process is therefore to properly position the caudal somitic boundary. The wave of cyclic gene expression ends in the rostral PSM and in chick and mouse embryos, this final position appears to define the location of the boundary (Fig. 3)(Del Barco Barrantes, *et al.,* 1999; Dale, *et al.,* 2003). In these embryos, the somitic boundary will form immediately rostral to the *lunatic fringe* expression domain, a situation reminiscent of boundary formation of the fly wing disk(Irvine, 1999). In gain or loss of lunatic fringe function in mouse and chick embryos, boundaries become irregularly positioned in the rostral PSM resulting in somites of irregular size(Evrard, *et al.,* 1998; Zhang and Gridley, 1998; Dale, *et al.,* 2003). Intriguingly, however, whereas no periodic oscillations of *lunatic fringe* is seen in zebrafish, the gene is expressed as a stripe in the rostral PSM suggesting that its role in boundary formation could have been conserved in evolution(Prince, *et al.,* 2001).

A pathway acting downstream of Notch signaling in boundary formation is the cell-communication system EPH-Ephrin (Holder and Klein, 1999). Once the boundary position is properly specified, members of the family including EphA4 and Ephrin B2 have been shown to be expressed in stripes in the rostral-most PSM (Durbin, *et al.,* 1998; Del Barco Barrantes, *et al.,* 1999). The receptor and ligand are located on opposite sides of the forming boundary and are thought to play a role in the clefting process. In zebrafish, gain

or loss of EPH-ephrin function in the PSM can alter the boundary formation process (Durbin, *et al.,* 1998; Durbin, *et al.,* 2000).

At the cellular level, the formation of the boundary can proceed quite differently among species. In the frog, somite formation involves a complex rotation movement of the paraxial mesoderm cells(Hamilton, 1969). In zebrafish, boundary formation is initiated in a rather stochastic fashion (Wood and Thorogood, 1994; Henry, *et al.,* 2000). In the chick embryo, in ovo time lapse experiments have revealed that cells undergo a complex ballet during which the somite gets isolated as a ball from a socket of paraxial mesoderm(Kulesa and Fraser, 2002). Thus whereas, the molecular steps leading to boundary definition are rather conserved among vertebrates, their cellular read-out appears quite diverse between different species.

The clock and wavefront: a paradigm for animal segmentation

Segmentation of the body axis is clearly not a vertebrate characteristic as it is seen in several invertebrates groups, including annelids and arthropods. The evolutionary origin of segmentation has been a subject of intense debate among biologists since at least two centuries(Davis and Patel, 1999). On one hand, segmentation was proposed to have arosen independently in many different animal groups, whereas on the other hand segmentation was proposed to be an ancestral character of Urbilateria, the common ancestor of the bilateria.

For the last three decades, the major paradigm for the study of segmentation has been the fly embryo. The molecular cascade leading to the establishment of the segmental pattern is now well established in this organism(Wilkins, 2001). It is initiated by gradients of maternal effect gene products, bicoid and nanos which are then converted into a series of gap genes (*hunchback, kruppel*) expression domains sequentially organised along the AP axis of the embryo. The combinatorial expression of the gap genes then results in the periodic expression of the pair-rule genes, which include *hairy*, *even-skipped* and *runt* in seven alternate domains that prefigurate (but do not strictly correspond to) the embryonic segments. The combinatorial expression of the pair rule genes in turn activate the segment polarity genes such as *engrailed*, *wingless* or *hedgehog* that establish the definitive segmental pattern of the embryo.

Many of the vertebrate homologues of the fly segmentation genes have now been identified and do not appear to play a role in somitogenesis (Ito and Miyazono, 2003; Kaczynski, *et al.,* 2003). At first glance, such an observation could seem in agreement with the idea that segmentation evolved completely independently in flies and vertebrates. However, among arthropods, flies are very derived insects and exhibit an unusual mode of segmental patterning. In *Drosophila*, the segmental pattern is established simultaneously for all segments in a syncitial embryo, whereas for most other insects and arthropods, segmentation proceeds sequentially, in concert with extension of the body axis as is seen in vertebrates (see Fig. 1B). Thus this progressive segmentation mode which is also seen in annelids, might in fact reflect an ancestral character.

Unfortunately, very little is known about the segmentation of invertebrates other than flies. Much of the focus has been placed on the homologues of the segmentation polarity genes *engrailed* and *wingless*, whose segmental expression appears widely conserved throughout invertebrates(Wilkins, 2001). However, these genes are expressed after the metameric pattern has been established and are not really informative with respect to the segmental patterning process. Pair-rule homologues are more informative because in flies they are the first to materialise the metameric pattern. The expression of the pair-rule homologues *even-skipped* and *hairy* was found to be associated to the segmentation process in a wide variety of species ranging from arthropods to vertebrates but their expression pattern is distinct from that seen in the fly embryo(Bastian and Gruss, 1990; Patel, *et al.,* 1992; Joly, *et al.,* 1993; Patel, 1994; Palmeirim, *et al.,* 1997; Damen, *et al.,* 2000; Hughes and Kaufman, 2002). *even-skipped* homologues are not expressed in a pair-rule fashion in several species of arthropods but share expression in the caudal domain rostral to the growth zone between annelids, arthropods and vertebrates. Other pair-rule homologues such as *hairy, runt* or *Pax* group III genes exhibit a segmental pattern which could be interpreted as pair rule in insects different from *Drosophila* and in chelicerates(Damen, *et al.,* 2000; Davis, *et al.,* 2001; Dearden, *et al.,* 2002). No evidence for pair-rule patterning mechanisms was ever evidenced outside arthropods, suggesting that it might represent a derived character of this branch. Homologues of fly maternal effect or gap genes do not appear to share a conserved role in segmentation outside insects. Therefore, the fly segmentation gene cascade is unlikely to represent a conserved segmental patterning mechanism even among insects. Virtually nothing of this cascade is conserved in vertebrates.

In contrast, if one now looks at invertebrate homologues of the vertebrate segmentation genes, then some striking similarities are found. For instance, the Notch pathway which does not appear to play a role in fly segmentation was recently described to be involved in the segmentation of spiders which share their progressive segmentation mode with vertebrates(Stollewerk, *et al.,* 2003). The spider homologues of the genes *delta, notch* and *hairy* are expressed in a striped pattern which is very reminiscent of the expression of similar genes in the vertebrate PSM. More strikingly, there appear to be a dynamic aspect of their expression in the patterning zone rostral to the growth zone of the spider axis. Whereas it is technically not feasible to demonstrate oscillations of these genes in this type of embryos, these dynamic patterns are consistent with the existence of a clock regulating their expression in spiders. Disrupting Notch and Delta expression by RNAi treatments results in a disorganisation of the segmental pattern, a defect highly reminiscent of mutations in this pathway in mouse and fish embryos(Conlon, *et al.,* 1995; Hrabe De Angelis, *et al.,* 1997; Jiang, *et al.,* 2000). These results open the possibility that the segmentation clock could also act in the invertebrate patterning zone and would thus represent an ancestral segmentation mechanism.

Thus far, no evidence for the conservation of the *fgf8* gradient has been provided outside of vertebrates, and it will be extremely interesting to know about expression of FGF homologues in invertebrate development. A conserved role for FGF signaling in gastrulation of flies and vertebrates has been reported (Ip and Gridley, 2002). Also, the *even-skipped* homologues which are co-expressed with *fgf8* in vertebrates are found to be expressed in the caudal patterning zone of most invertebrate species in which it has been examined(Patel, *et al.,* 1992; Hughes and Kaufman, 2002;

Song, *et al.,* 2002). It remains thus very possible that invertebrates also use a wavefront system similar to that seen in vertebrates to couple extension of the axis to the segmental patterning process.

Altogether, the machinery of the segmentation process appears to be largely conserved among vertebrates. Key players are the segmentation clock which ticks in the PSM and involves the Notch and the Wnt pathway, and the FGF8-based wavefront which defines the level at which the segmental pattern is first established. The segmentation mode adopted by vertebrates which requires a tight coupling between axis elongation and segment formation is also seen in large number of invertebrate species. Recent studies in invertebrates have opened the exciting possibility that the clock and wavefront patterning system characterized in vertebrates might in fact operate also in invertebrates and thus represent an ancestral segmentation mechanism shared by these two phyla. Hopefully, future investigation of the molecular aspects of the segmentation process in invertebrates showing a progressive mode of segment formation, should tell us whether the molecular process at play during vertebrate somitogenesis in fact represents an ancestral segmentation mechanism shared by the common ancestor of vertebrates and invertebrates. The debate on conservation of segmentation that started more than two-hundred years ago might well come to an end in the next few years.

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