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Review

A Notch feeling of somite segmentation and beyond

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Abstract

Vertebrate segmentation is manifested during embryonic development as serially repeated units termed somites that give rise to vertebrae, ribs, skeletal muscle and dermis. Many theoretical models including the "clock and wavefront" model have been proposed. There is compelling genetic evidence showing that Notch–Delta signaling is indispensable for somitogenesis. Notch receptor and its target genes, *Hairy/E(spl)* homologues, are known to be crucial for the ticking of the segmentation clock. Through the work done in mouse, chick, *Xenopus* and zebrafish, an oscillator operated by cyclical transcriptional activation and delayed negative feedback regulation is emerging as the fundamental mechanism underlying the segmentation clock. Ubiquitin-dependent protein degradation and probably other posttranslational regulations are also required. Fgf8 and Wnt3a gradients are important in positioning somite boundaries and, probably, in coordinating tail growth and segmentation. The circadian clock is another biochemical oscillator, which, similar to the segmentation clock, is operated with a negative transcription-regulated feedback mechanism. While the circadian clock uses a more complicated network of pathways to achieve homeostasis, it appears that the segmentation clock exploits the Notch pathway to achieve both signal generation and synchronization. We also discuss mathematical modeling and future directions in the end.

Keywords: Notch; Delta; Fgf; Wnt; Hes1; Hes7; Her1; Her7; Lfng; Somitogenesis; Segmentation clock; Circadian clock; Gradient; Negative feedback; Modeling

> Seul le rythme provoque le court-circuit poétique et transmue le cuivre en or, la parole en verbe.
>
> 'Éthiopiques', 1956: Léopold Sédar Senghor (1906-2001).

Introduction

In vertebrates, the embryonic paraxial mesoderm is transiently composed of serially repeated epithelial segments known as somites that emerge as bilaterally symmetrical pairs flanking the notochord and neural tube, and display a regular arrangement along the anteroposterior (AP) axis (Fig. 1). Each somite eventually matures and gives rise to three compartments: the dermatome, myotome and sclerotome that are the anlagen for their derivatives—dermis, skeletal muscle and axial skeleton, respectively. The metameric pattern afforded by somites thus provides a vital scaffold for the subsequent production of segmental structures in the body plan. Although somites are serially homologous structures, they eventually diverge and undergo regionalization along AP axis (reviewed in Gossler and Hrabe de Angelis, 1998; Pourquié, 2001; Tam and Trainor, 1994). The formation of the ordered array of somites is characterized by the following salient features: first, somites are always generated in a rostral to caudal fashion in vertebrates; second, the number of somite pairs and the time required for each to form is fixed and species-specific; third, segmentation is tightly coupled temporally and spatially to other processes during embryonic morphogenesis such that its onset and termination occur at a set time and location during development (Cooke, 1975; Deuchar and Burgess, 1967). Therefore, accurate and robust mechanisms must operate within the cells of the presomitic mesoderm (PSM) to ensure the fidelity of this complicated, reiterative process and to integrate it into the broad framework of embryonic development.

Model organisms such as amphibians, chick, mouse and zebrafish have served as excellent paradigms to study the

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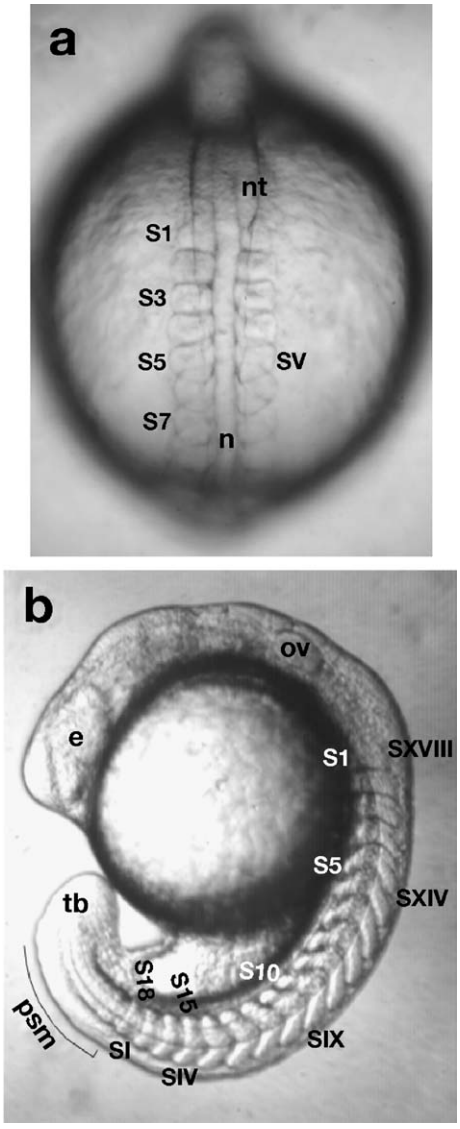

Fig. 1. Zebrafish somite morphology. Dorsal view of a 9-somite (s) stage embryo (a) and lateral view of an 18s stage embryo, showing the chevron-shaped somites (b). Somite numbering system: somites are counted beginning with the anterior-most somite in Arabic numbers and, in addition, numbered according to developmental age in Roman numbers, the most recently formed somite being number I (Christ and Ordahl, 1995). For example, the fifth somite of a 9s embryo would be somite V/5 and the most recently formed somite in the same embryo would be somite I/9. The fifth somite of an 18s embryo would be XIV/5 and the most recently formed somite in the same embryo would be I/18. Note that S1 somite does not have an obviously delineated anterior boundary. Abbreviations: e, eye; n, notochord; nt, neural tube; ov, otic vesicle; psm, presomitic mesoderm; tb, tail bud.

meticulously orchestrated process of somitogenesis. Evidently, sequential segmentation of paraxial mesoderm requires a strategy distinct from those of simultaneous and discrete subdivision of a field of cells into segments as seen in long-germ-band insects and in the formation of rhombomeres in the vertebrate hindbrain, respectively. Furthermore, there are notable differences in the way vertebrates undergo segmentation. For instance, in some anuran amphibians such as *Xenopus*, the paraxial mesoderm becomes morphologically segmented through coordinated turning of blocks of cells such that the length of each cell in the block initially spans the entire length of the metamere. These blocks subsequently change shape to form diagonal "chevrons" (Hamilton, 1969, and reviewed in Keller, 2000). In amniote vertebrates (reptiles, birds and mammals) and fish, there is a growth zone (primitive streak/node/tail bud) that continuously generates cells that enter the PSM (segmental plate in chick) and move anteriorly. Segmentation occurs some time later as groups of cells adhere, become compacted and epithelialized and then form somites that are separated by distinct clefts.

Study of vertebrate segmentation in the pre-molecular era

The regular architecture of somites and the clock-like precision that characterizes their genesis have suggested the existence of an underlying periodicity (i.e. a pre-pattern) in the biochemical state of the cells comprising the PSM. Meier et al. have found in a series of papers that there are segmental units, called somitomeres, existing in the PSM before somites form, when the ectoderm was stripped off and examined with SEM (Meier, 1979 and reviewed in Jacobson and Meier, 1986). Moreover, it was observed in mouse, chick and zebrafish embryos that the cells of the PSM undergo very little movement, suggesting that groups of cells in the PSM are coordinately partitioned into prospective somites by some commonality in their cell state (Jiang et al., 2000; Palmeirim et al., 1997; Stern et al., 1988; Tam and Beddington, 1986). In an effort to elucidate the mechanism by which the PSM becomes segmented, biologists have carried out a variety of tissue excision and grafting experiments. When animal–vegetal sectors were excised from near the ventral meridian of *Xenopus laevis* blastulae to yield embryos much smaller than usual, it was observed that the entire body plan developed normally, though with smaller cell numbers, and there were normal numbers of somites at the correct positions at all stages of development (Cooke, 1975). This experiment provided strong evidence indicating that lengths of somites can be adjusted depending on the total size of tissue available. When lengths of neural tube and somites were excised unilaterally just caudal to the region that had segmented, it was found that tissue posterior to the region of the operation still segmented normally. Moreover, when amphibian embryos were cut into rostral and caudal halves, with the concern that unilaterally operated tissue may receive signal(s) from the other side, the separated caudal half was found to segment normally (Deuchar and Burgess, 1967). It was also observed that a quail node graft can induce PSM tissue in the chick host to develop a secondary axis with segmented somites. Interestingly, the resulting somite pattern depended on the mediolateral position of the quail node graft, which led the authors to propose that there is a morphogen gradient originating in the node (Hornbruch et al., 1979). Taken together, these data lent strong support to

the idea that a graded distribution of positional information is established along the AP axis by the time gastrulation is completed, and that normal segmentation does not require the continual flow or propagation of this information in an anterior-to-posterior direction. Moreover, cells are sensitive to the rate of change of this information (steepness of the gradient, rather than its absolute value) and respond by differentiating accordingly. The identification of some of the molecules distributed in this gradient fashion as well as their vital roles is setting up the somite pattern are described in later sections of this review.

Scientists have also studied the effect of different physicochemical treatments on the embryos from various amphibian and avian species. They found that a single transient heat shock reproducibly produced discrete, repeated somitic disturbances in the embryos. Interestingly, the first anomaly occurred not at the time of heat shock but instead, a few hours later. The successive zones of abnormality were evenly spaced and had a fixed species-specific number of normally formed somites interspersed between them (Pearson and Elsdale, 1979; Primmett et al., 1988; Roy et al., 1999). The multiple and repeated anomalies suggested that an oscillatory process, which coordinates groups of cells during segmentation, was disrupted in the somite precursor cells. It was also observed that within each zone of abnormality, the defect was most severe at the anterior border and gradually became less severe near the caudal margin (Pearson and Elsdale, 1979). It was therefore inferred that heat shock probably disrupts crucial intercellular coordination, and thereby, causes somitic disruptions, and that recovery from heat shock requires a gradual restoration of this coordination. It was later demonstrated that the effects of heat shock were mimicked by treating embryos with cell cycle inhibitors affecting the S and M phases of the cell cycle (Primmett et al., 1989). This intriguing observation led to the notion that the cell cycle somehow impinges on the oscillatory process within the cells of the PSM.

Somitogenesis and models

Several theoretical models were put forth to account for these observations (Collier et al., 2000; Cooke and Zeeman, 1976; Flint et al., 1978; Jaeger and Goodwin, 2001; Kaern et al., 2000; Kerszberg and Wolpert, 2000; Keynes and Stern, 1988; Meinhardt, 1986; Polezhaev, 1992; Schnell and Maini, 2000) but the "clock and wavefront" model posited by Cooke and Zeeman has found widest acceptance and applicability (reviewed in Pourquié, 2001). At the heart of this model is the proposed existence of a molecular clock or biochemical oscillator within the cells of the (unsegmented) PSM. According to this model, neighboring cells are coordinated or entrained with respect to their oscillations. Clock and wavefront model also proposes the existence of a wavefront of cell change (cell determination) that sweeps posteriorly through the PSM, slowing and halting the oscillation and inducing or permitting somite maturation. Current evidence suggests that the wavefront could correspond to a gradient of Fgf–mitogen-activated protein kinase (MAPK) signaling (Dubrulle et al., 2001; Sawada et al., 2001), which may be regulated by or coordinated with Wnt3a signaling (Aulehla et al., 2003). The progress of this smooth wavefront is gated into discrete steps as a direct result of its interaction with the cellular oscillator. When anterior PSM cells (oscillating in synchrony) receive the wavefront signal, it gives rise to stable bands or cohorts of cells of one somite wide, characterized by a specific gene expression pattern. Continued signaling within and between these stabilized cohorts of cells refines the anterior and posterior domains of each somite and induces further cellular differentiation, somite regionalization and finally, boundary formation. It must be mentioned that the idea of a "positional signal system" or gradient that regulates pattern along the AP axis, and whose slope determines the rate of passage of the wavefront, is implicit in this model (Cooke, 1975), although it has probably not been emphasized sufficiently. Slack (1983, 1991) has, in fact, suggested that this model be renamed as the "clock and gradient" model.

Although there are several exciting aspects related to the morphogenesis of somites that are worthy of description (reviewed in Brennan et al., 2002; Gossler and Hrabe de Angelis, 1998; Keller, 2000; Keynes and Stern, 1988; Pourquié, 2001; Stockdale et al., 2000; Tam and Trainor, 1994), we shall limit the scope of this review to a discussion of the molecular clock that acts as a periodicity generator in the PSM cells. We shall describe the elucidation of the dynamic, interdependent and cyclic processes that underlie the ticking of the segmentation clock, its entrainment between cells and its interaction with molecular gradients. We also make a fitting comparison with another fundamental rhythmic biological process, the circadian clock, and discuss perspectives on the future research directions in the field of vertebrate segmentation.

The molecular era

The process of vertebrate segmentation has been studied intensely for several decades. In recent years, with the advent of the molecular era and the development of genetically tractable model systems such as mouse and zebrafish, our understanding of vertebrate segmentation has expanded exponentially. The key transcription factors and signaling modules identified so far are described below.

Cyclic genes

The existence of a molecular oscillator as envisioned by Cooke and Zeeman has been first molecularly evidenced by the discovery that a basic helix–loop–helix (bHLH) transcription repressor, *c-hairy1*, displays cyclic expression patterns in the chick PSM with the same temporal periodicity as that of somite formation—one cycle per 90 minutes

(Palmeirim et al., 1997). Initially, *c-hairy1* expression is found as a broad domain in the posterior PSM and tail bud. As these cells mature anteriorly, the cycles of gene expression are slowed down and finally arrested, and *c-hairy1* expression becomes limited to the posterior half of the formed somite (see Fig. 2 for a similar pattern of *deltaC*). Importantly, the dynamic pattern of *c-hairy1* expression is independent of cell movements and in fact, is an intrinsic property of the PSM tissue as indicated by the following lines of evidence: (i) it is unaffected by the ablation of the caudal part of the PSM including the tail bud; and (ii) the cyclic expression pattern of *c-hairy1* continues in PSM explant cultures devoid of all surrounding tissues (Palmeirim et al., 1997).

Similar cycling genes have since then been discovered in other vertebrates: including *Lunatic fringe* (*Lfng*) (Aulehla and Johnson, 1999; Forsberg et al., 1998), *Hes1* (Jouve et al., 2000), *Hey2* (Leimeister et al., 2000) and *Hes7* (Bessho et al., 2001b, 2003) in mouse; *c-hairy2*, *c-Hey2/HRT2* (Leimeister et al., 2000) and *Lfng* (Aulehla and Johnson, 1999; McGrew et al., 1998) in chick; *her1* (Holley et al., 2000; Sawada et al., 2000), *deltaC* (Jiang et al., 2000) and *her7* (Gajewski et al., 2003; Oates and Ho, 2002) in zebrafish; and *esr9* and *esr10* in *Xenopus* (Li et al., 2003). Importantly, the cycling genes mentioned above oscillate in phase and their expression is driven by Notch signaling (del Barco Barrantes et al., 1999; Jouve et al., 2000; Leimeister et al., 2000; Oates and Ho, 2002; Sieger et al., 2003; Takke and Campos-Ortega, 1999). Some of the cyclic genes have been demonstrated to exploit negative feedback transcriptional regulation to keep the segmentation clock ticking (see below).

Until now, the majority of the cycling genes found in vertebrates reside in Notch signaling: target genes, such as *Hes1*; ligands, such as *deltaC*; and modulators, such as *Lfng*. The first cycling gene found distinct from canonical Notch signaling is *Axin2*, a negative regulator of Wnt signaling (Aulehla et al., 2003 and see below). It would be interesting to see whether *Axin2* is directly involved in somite segmentation.

Notch signaling and mutants

Phenotypic analyses of several mouse and zebrafish mutants have unequivocally demonstrated a vital role for Notch signaling in somitogenesis. Notch signaling involves the binding of transmembrane Delta-Serrate-Lag-2 (DSL) ligands to the extracellular domain of large (approximately 300 kDa) transmembrane Notch receptors on adjacent cells. Thus, the ligands can only influence immediate neighboring cells expressing the receptor. The mature Notch on cell membrane is a heterodimer processed intracellularly by a furin-like convertase (Logeat et al., 1998). Ligand binding makes Notch susceptible to TNFα-converting enzyme (TACE) metalloproteases that cleave it at a second extracellular site (Brou et al., 2000). A third proteolytic cleavage, made in the tramembrane domain by the γ-secretase activity of Presenilin, releases the Notch intracellular domain (NICD) (Fortini, 2001; Kopan and Goate, 2000). The NICD translocates into the nucleus (Kidd et al., 1998; Kopan et al., 1996; Lecourtois and Schweisguth, 1998; Struhl and Adachi, 1998), where it associates with the evolutionarily conserved DNA-binding protein Su(H)/RBPJκ (Jarriault et al., 1995; Lu and Lux, 1996) and converts the latter from a transcriptional repressor to a transcriptional activator. The NICD–Su(H) complex turns on the expression of downstream target genes, such as *Hairy/E(spl)* family of genes, including *c-hairy1*, *Hes1* and *her1*, which in turn regulate the transcription of other gene sets and themselves (Bailey and Posakony, 1995; Bessho et al., 2001a; Hirata et al., 2002; Holley et al., 2002; Lecourtois and Schweisguth, 1995; Oates and Ho, 2002; Ohtsuka et al., 1999). Thus, Notch receptor functions as a membrane-bound transcription factor that turns on specific gene expression patterns in response to ligand binding and allows one cell to be influenced by its immediate neighbors. Ligand–receptor affinity can be modulated by posttranslational modification of the extracellular domain of Notch by the glycosyltransferase Fringe. Fringe can either potentiate or inhibit Notch signaling in a cell-autonomous fashion depending on the developmental context in which it functions (Brückner et al., 2000; Moloney et al., 2000; Panin et al., 1997). *Lunatic fringe* is the only one of the three known mammalian Fringe homologues to be expressed in the PSM (Cohen et al., 1997; Johnston et al., 1997).

The first handle on genes that control vertebrate somite formation came from the analysis of mouse homozygous *Notch1* null embryos where somitogenesis is significantly delayed and disorganized (Conlon et al., 1995). The phenotype of homozygous *Su(H)/RBPJκ* null embryos was found to be slightly more severe with somitogenesis failing earlier than that seen in *Notch1* mutants (Oka et al., 1995). It was also demonstrated that in *Xenopus* and zebrafish embryos, injection of mRNAs encoding proteins that either lead to a deregulated ubiquitous activation of Notch signaling or an inhibition thereof, both cause aberrant somite formation, suggesting that tight control of Notch signaling was crucial for proper implementation of somitogenesis (Dornseifer et al., 1997; Jen et al., 1997; Takke and Campos-Ortega, 1999). Subsequent knock-outs and spontaneous mutations in genes of other core components and modulators of Notch signaling—*Delta-like 1* (*Dll1*), *Dll3*, *Presenilin1*, *Lfng* and *Hes7*—also led to somite phenotypes (Bessho et al., 2001b; Evrard et al., 1998; Hrabe de Angelis et al., 1997; Kusumi et al., 1998; Wong et al., 1997; Zhang and Gridley, 1998). Altogether, these studies clearly implicate Notch signaling in the direct regulation of segmentation.

The concomitant emergence of zebrafish as an important model system allowed the use of forward genetic approaches to study vertebrate development. The first milestone was the isolation and characterization of a wide range

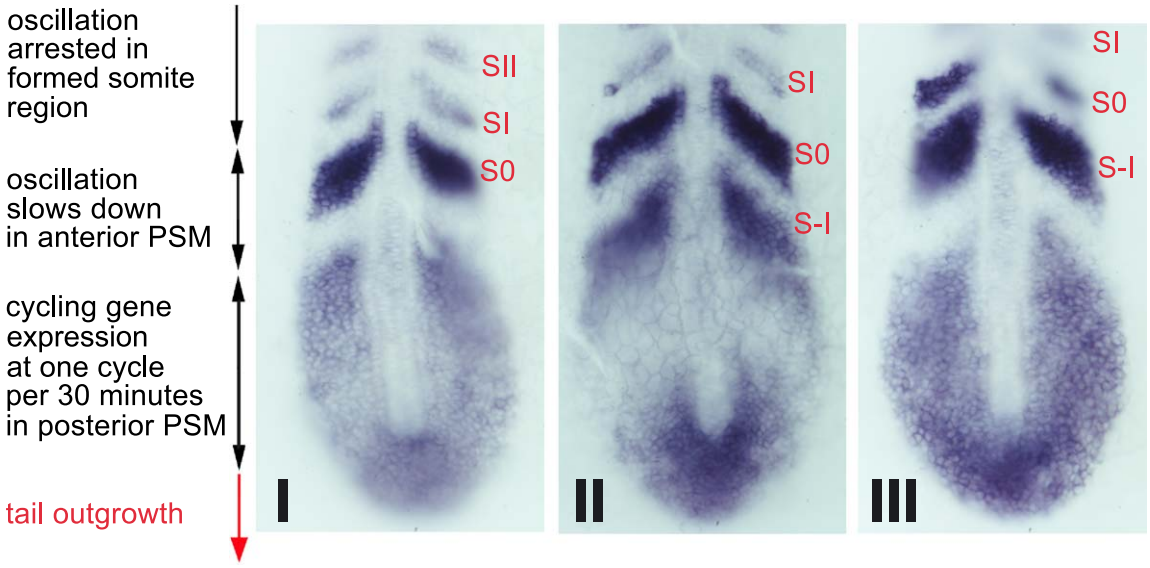

Fig. 2. Cycling expression pattern of zebrafish *deltaC* in 10s stage embryos. The forming somite in the anterior-most PSM is S0, the next one to be segmented is S-I and in this order to the posterior end. Designation of three expression phases (I, II and III) is according to a consensus nomenclature (Pourquié and Tam, 2001). Note the changes of *deltaC* stripes of S0 and S-I somites in three consecutive phases: both appear as broad bands initially and narrow down as time goes by. In addition, only when the oscillation starts to slow down in the anterior PSM, the *deltaC* will be up-regulated and then down-regulated in formed somites, ending in the posterior part thereof (Jiang et al., 2000).

of zebrafish mutants through a large-scale mutagenesis screen including those showing defects in somite formation (Jiang et al., 1996; van Eeden et al., 1996). These mutants again turned the spotlight onto the evolutionarily conserved Notch–Delta signaling pathway. In *after eight (aei)/deltaD*, *deadly seven (des)/notch1a*, *beamter* (*bea*) and *mind bomb* (*mib*) [alias, *white tail* (*wit*), a novel Notch component, encoding a RING E3 ligase] mutants, the anterior somites were formed normally but the posterior somites were profoundly disorganized with weak and irregularly spaced boundaries, as seen in mouse knock-outs (Holley et al., 2000, 2002; Itoh et al., 2003; Jiang et al., 1996; van Eeden et al., 1996). A deficiency mutant with deletion covering *her1* and *her7* showed a similar phenotype (Henry et al., 2002). In addition, antisense morpholino knockdown and drug treatment experiment have shown that *her1*, *her7*, *su(h)* and *presenilin* are essential for somite segmentation (Gajewski et al., 2003; Geling et al., 2002; Henry et al., 2002; Holley et al., 2002; Oates and Ho, 2002; Sieger et al., 2003). Importantly, the expression of cycling genes like *deltaC* and *her1* are disrupted in Notch pathway mutants (Gajewski et al., 2003; Geling et al., 2002; Henry et al., 2002; Holley et al., 2000, 2002; Jiang et al., 2000; Oates and Ho, 2002; Sieger et al., 2003). *deltaC* shows a "salt and pepper" pattern of expression in the entire PSM with the speckled pattern being most obvious in the anterior PSM (Jiang et al., 2000; Oates and Ho, 2002). This tantalizing observation implied that in these mutants, cyclic gene expression was not completely abolished; but rather, cells of the PSM were simply uncoordinated in their expression of these genes. While this data do not necessarily preclude a role for Notch–Delta pathway in the generation of these cell-intrinsic oscillations, it strongly indicates that Notch signaling is crucial for synchronization of oscillations between neighboring cells in the zebrafish PSM.

Gradients—Wnt and Fgf–MAPK signaling pathways

The importance of Fgf signaling in somitogenesis came to the fore with the observation that mouse embryos homozygous null for *Fgfr1* and *Fgf8* usually make no somites due to improper gastrulation (Sun et al., 1999; Yamaguchi et al., 1994) and those lacking the *Fgfr1α* isoforms are deficient in caudal somites (Xu et al., 1999). *Fgfr1* is expressed in migrating embryonic mesoderm and then becomes restricted to the paraxial mesoderm. Subsequently, highest levels of *Fgfr1* are transiently found in the anterior PSM (Yamaguchi et al., 1992). Mice which are homozygous null for both *Notch1* and *RBPJκ* show normal *Fgfr1*expression, suggesting that Notch signaling is perhaps not involved in the regulation of the Fgf pathway (Conlon et al., 1995; Oka et al., 1995).

The direct connection between Fgf signaling and somite segmentation comes from recent studies in chick and zebrafish. It has been shown in chick that the posterior PSM, where the signaling molecule Fgf8 is highly expressed, is undetermined and labile. Inversion of one-somite-length fragments in the posterior PSM leads to normal segmentation since the reversed fragment remains responsive to cues from surrounding tissues. In contrast, tissue inversion in the anterior-determined zone leads to inversion of somite AP polarity (Dubrulle et al., 2001). Segmental determination occurs at the level of the determination front (approximately four somites caudal to the last formed somite, S-IV), where

expression of *Fgf8* and *Fgfr1* overlaps. Down-regulation of Fgf signaling at the level of the determination front is required for PSM cells to enter the maturation phase: compromised or enhanced Fgf signaling by treatment with the drug SU5402, a kinase inhibitor specific to all types of Fgfrs, or by grafting Fgf8-soaked beads in the PSM led to an increased or decreased somite size, respectively (Dubrulle et al., 2001; Sawada et al., 2001). It is intriguing to note that the effect of SU5402 (big somites) does not occur immediately but rather after the formation of several somites. Also, ectopic Fgf8 expression only effects smaller somite size when located between the determination front and the caudal PSM (Dubrulle et al., 2001). The determination front corresponds to the region sensitive to heat shock in chick and zebrafish where somitic anomalies are observed after a time lag following heat shock (Dubrulle et al., 2001; Primmett et al., 1988; Roy et al., 1999; Sawada et al., 2001). It is very likely that this determination front corresponds to the "prior wave" referred to by Pearson and Elsdale (1979) and the "wavefront of cell determination" in the model put forth by Cooke and Zeeman (1976).

The involvement of Wnt-mediated signaling in somite formation was supported by the observation that anomalous somites are induced in chick embryos treated with LiCl (Linask et al., 1998) which is believed to mimic in vivo Wnt signaling (Hedgepeth et al., 1997; Klein and Melton, 1996). Wnt3a was shown to be responsible for the elongation of the body axis (Greco et al., 1996; Takada et al., 1994). In mouse embryos null for *Wnt3a*, *Brachyury*, and *Tbx6*, and in *Lef-1*$^{-/-}$/*Tcf-1*$^{-/-}$ double mutant embryos (Lef-1 and Tcf-1 are downstream components in the transduction pathway of a subgroup of Wnt signals), gross defects in mesoderm formation are apparent (Chapman and Papaioannou, 1998; Galceran et al., 1999; Takada et al., 1994; Wilson et al., 1995; Yamaguchi et al., 1999).

It has recently emerged that Wnt signaling is also connected to segmentation. *Axin2*, which encodes a negative regulator of Wnt3a signaling, shows strong expression in the tail bud and gradually diminishes anteriorly. Expression of *Axin2* cycles and is not in phase with *Lfng* (Aulehla et al., 2003). Misexpression of *Axin2* adversely affects segmentation by leading to an ectopic upregulation of *Lfng*. Conversely, *Lfng* expression was found to be down-regulated and non-oscillating in the *vestigial tail* (*vt*—a hypomorphic allele of *Wnt3a*, Greco et al., 1996) mutant where Wnt signaling was compromised. It had also been previously noted that *Notch1* expression was lost in *Lef-1*$^{-/-}$/*Tcf-1*$^{-/-}$ double mutant embryos (Galceran et al., 1999). Furthermore, mutants for a deficiency in *Dishevelled 2* (*Dvl2*) show an incomplete segmentation and this phenotype is more severe in the *Dvl1*$^{-/-}$/*Dvl2*$^{-/-}$ double mutant mice (Hamblet et al., 2002). Thus, it appears that the Notch pathway may operate downstream of Wnt signaling at least in the posterior PSM. Similar to Fgf8 signaling, transient manipulation of Wnt3a signaling in mouse was able to induce an alteration in somite size (Aulehla et al., 2003), showing that the gradient of Wnt3a-mediated signal constitutes a vital source of positional information for boundary placement. The existence of a similar gradient of Wnt signaling has not yet been established in chick and zebrafish.

Connecting cyclic gene expression to somite boundary formation

For the pre-pattern set up by cyclic gene expression to contribute meaningfully to morphological segmentation, it is imperative that somite maturation be stringently coordinated with oscillating gene expression both spatially and temporally. Members of the T-box (*Tbx*) gene family encode developmentally regulated transcription factors which are vital for embryogenesis and organogenesis (reviewed in Papaioannou, 2001). The zebrafish *fused somites* (*fss*) mutant shows a complete lack of somite boundaries along the entire body axis although the cycling gene expression is normal in the posterior PSM (Holley et al., 2000; Jiang et al., 2000; van Eeden et al., 1996). The *fss* mutant is therefore instrumental in showing that the process of somite boundary formation can be uncoupled from pre-pattern implemented via segmentation clock. The *fss* gene, which encodes a T-box protein Tbx24, is expressed in maturing cells in the intermediate to anterior PSM (Nikaido et al., 2002). Tbx24 is required to stabilize the pattern of oscillating gene expression in the anterior PSM and is also essential for the expression of genes such as *mesp* and *papc* in the anterior PSM—key events in the generation of boundaries at regular intervals during somite maturation (Jiang et al., 2000; Sawada et al., 2000). Genetic analyses of zebrafish segmentation mutants have shown that the Fss and Notch pathways are functionally distinct and perhaps independent of each other (Holley et al., 2000; Jiang et al., 2000). Transcriptional regulation of *tbx24* is also independent of the Notch pathway (Nikaido et al., 2002). At present, the downstream target genes of Tbx24 await identification. The murine Tbx6 has been shown to be essential for the formation of posterior somites (Chapman and Papaioannou, 1998). Interestingly, *Tbx6* genetically interacts with *Dll1*, whose gene expression is lost in the *Tbx6* null mutant, suggesting that *Dll1* could be a target of Tbx6 (Beckers et al., 2000b; White et al., 2003). In zebrafish, it also emerged that the inhibition of synthesis of Foxc1a (a winged-helix transcription factor) blocked the formation of morphological somites although *deltaC* and *deltaD* showed normal oscillatory and dynamic expression in the PSM (Topczewska et al., 2001). It therefore appears that transcription factors like Fss/Tbx24 and Foxc1a are part of the machinery that reads and interprets the cycling gene expression and translates it into a specific pattern of differentiation (maturation). Additionally, it is well documented that Fgf signaling can activate T-box genes and the T-box proteins can interplay among themselves (Griffin et

al., 1998; Latinkic et al., 1997). Since Activin can activate and suppress *Xenopus Brachyury* promoter at low and high concentration, respectively (Latinkic et al., 1997), it will be particularly interesting to examine the regulation of *fss*/*tbx24* in the anterior PSM, where the concentration of Fgf8 is low.

Hox genes—segmentation clock-controlled genes

During embryonic development, *Hox* genes, arranged as a gene cluster in the genome, are activated sequentially along the body axis such that at each axial position, a unique combination of *Hox* genes is expressed and this combinatorial "Hox code" is thus believed to pattern the AP axis (Krumlauf, 1994). As this activation also displays temporal colinearity, *Hox* genes at 3′ end of the cluster are expressed earlier and are responsible for generating anterior structures, whereas *Hox* genes at 5′ end are expressed later and hence function in posterior parts (Duboule, 1994). As part of the AP structure, the specification of somites and their derivatives is influenced dramatically by *Hox* genes. How are somite segmentation and specification coordinated?

It has recently been demonstrated that *Hoxd1* shows a transient dynamic stripe expression pattern in nascent somites (Zákány et al., 2001). The cyclic transcription of the *Hoxd1* stripe is abolished in the $RBPJ\kappa^{-/-}$ mutant but somite segmentation is intact in $Hoxd1^{-/-}$ mice, indicating that *Hoxd1* is temporally controlled by the segmentation clock. Other *Hox* genes, such as *Hoxd3*, also respond to waves of Notch-mediated transcriptional activation in the PSM. The *cis*-regulatory element appears to be located outside the cluster and can control the stripe expression of *Hoxd11* and *Hoxb1*. These data suggest that the segmentation clock may set the pace of temporal colinearity in *Hox* gene expression with tight coupling to somite formation, such that the first burst of cyclic gene expression would activate group 1 genes, the next burst (one somite later) would activate group 1 and group 2 genes and so on (Zákány et al., 2001). Further evidence for this coupling comes from the observation that when somite boundary position is altered by manipulating Fgf8 signaling in chick embryos, *Hox* gene expression is maintained in the appropriately numbered somite rather than at an absolute axial position (Dubrulle et al., 2001). Though it has been shown that the "segmentation stripe enhancer" is a unique and shared regulatory element and is very likely located outside the *Hox* complex, its nature and exact position are still unclear. Furthermore, the activation is unlikely to involve a one-*Hox*-gene-to-one-somite regulation, since the somite number is greater than that of genes in a *Hox* cluster. The scenario is even more complicated by the fact that along the AP axis there are different parts, yet sharing morphological similarities, such as cervical, thoracic, lumbar and sacral regions. It is therefore conceivable that a "regional enhancer" may operate in parallel with or on top of the "segmentation stripe enhancer".

The identity of other genes that are regulated by the segmentation clock remains to be determined and it will be intriguing to uncover what kind of developmental processes these clock-controlled genes may govern. Interestingly, while expression of mouse *Hes1* has been shown to cycle in a Notch-dependent manner (Jouve et al., 2000), the deficiency of *Hes1*, however, did not giver rise to any detectable somite phenotypes (Ishibashi et al., 1995). Moreover, the segmentation clock remained functional in such mutants and cycling *Hes1* expression is lost in *Dll1*- and *Dll3*-deficient embryos (Dunwoodie et al., 2002; Jouve et al., 2000), which suggests that *Hes1* is a readout or output of the segmentation clock. While a role of *Hes1* in somitogenesis cannot be entirely excluded (e.g. genetic redundancy), the periodic surge of *Hes1* controlled by the segmentation clock may be essential for an unknown function.

Integration of the somitogenesis oscillator with tail outgrowth and somitic specification

Vertebrate somite segmentation takes place in an open-end system, the PSM. In the posterior end, cell division generates new cells from the tail bud, while in the anterior end, the mesenchymal cells after several cycles of gene expression finally mature and become epithelialized somites with corresponding AP value. It makes perfect sense that all these processes have to be coordinated. Furthermore, the coordination is necessary and crucial not only for later stages, when a slab of PSM is prominent, but also for gastrulation at early stages, when many morphogenetic movements take place to bring about somitic mesoderm. There are two modes of segmentation pattern control: one functions during gastrulation before the inception of real growth and the other operates after gastrulation with true growth. The existence of these two modes was exquisitely demonstrated in experiments where the size of amphibian embryos was purposely reduced at blastula stage: while the size of anterior 15 or so somites decreased accordingly, that of the posterior ones remains unchanged (Cooke, 1975, 1981). Though recent progress on Fgf8 and Wnt3a gradients and *Hox* gene regulation in somitogenesis do shed light on the molecular basis for the coordination, several questions still remain unanswered.

There may be differences between anterior and posterior somites in terms of molecular gradients that influence them. It is noteworthy that the expression of Fgf8 was drastically diminished in *vt*/*vt* embryos suggesting that Fgf signaling is controlled by the Wnt3a pathway (Aulehla et al., 2003). Intriguingly, the *Wnt3a*-deficient mice still form rostral somites (Takada et al., 1994), whereas the null mutants for *Fgf8* and *Fgfr1* usually make no somites, since they do not gastrulate properly (Deng et al., 1994; Sun et al., 1999; Yamaguchi et al., 1994). These observations do not support the idea that Wnt3a regulates FgF8 in the anterior somites. The relationship between the two signaling cascades is

probably true only for the posterior somites where Wnt3a integrates body axis elongation with segmentation. It is possible that other gradients related to segmentation exist and operate at different stages of development, such as BMP and retinoic acid (RA) signaling pathways. Consistent with this view, it has been shown that early Chordin function is essential for patterning the zebrafish axial skeleton (Fisher and Halpern, 1999); removal of RA in quail embryos by vitamin A deficiency leads to an embryo with somites of half the normal size but normal number of somites (Maden et al., 2000), and BMP signaling is involved in the outgrowth and patterning of the *Xenopus* tail bud (Beck et al., 2001).

It seems that there is, however, only one Notch-dependent oscillatory mechanism at the heart of the segmentation process as suggested by the following observations. First, the segmentation clock is running early at gastrula before any somites are visible, as demonstrated by the cyclic gene expression of Notch components (Jouve et al., 2002, and our unpublished data). Second, although the normal anterior somites seen in mouse and zebrafish Notch pathway mutants may intuitively suggest the existence of two independent segmentation clocks (one residing in the anterior part, which is Notch-independent and the other in the posterior, which is Notch-dependent), these observations can be explained by the desychronization of a Notch-dependent oscillation (Jiang et al., 2000). According to this hypothesis, the PSM oscillator is set into motion synchronously in the PSM precursor cells at some time point early in gastrulation and the cells are kept locally synchronized in their subsequent oscillation cycles by Notch-mediated cell–cell communication. However, if the communication that maintains synchrony is defective, the cells will gradually drift out of synchrony until the lack of coordination causes somitogenesis to fail. How soon this coordination fails depends on the coupling strength for synchronization. Consistent with this notion, double mutants do show more severe somite phenotypes (Donoviel et al., 1999; Henry et al., 2002; Oates and Ho, 2002, and our unpublished data).

What is the biological consequence of the graded distribution of Wnt and Fgf signaling in the PSM? One possible scenario is that the high levels of these signals in the tail bud set the initial value for the cellular oscillators and cause cohorts of cells to be coordinated with respect to their oscillation phase, since most of the key components of the segmentation clock appear to be highly and homogeneously expressed in the tail bud. This implies that the Notch pathway is perhaps not required for initiating the ticking of the segmentation clock but is, instead, responsible for maintaining its oscillation and synchrony. Once the cells move out of the tail bud region (and probably the organizer during gastrulation), they are gradually released from the influence of extremely high concentrations of Wnt and Fgf signals, and the Notch pathway takes over the crucial task of keeping the oscillation synchronized. This scheme would provide a mechanism to explain how zebrafish Notch pathway mutants initially entrain the PSM cells and why they eventually drift out of synchrony (as evidenced by the "salt and pepper" pattern of *deltaC* expression) resulting in a failure of somitogenesis at a later stage (Jiang et al., 2000). It also provides a possible explanation as to why in mouse *Dll1* and *Dll3* mutants, there is residual cyclic expression of *Lfng* at 10.5 days post coitus (dpc), unlike the *RBPjκ* mutant where cyclic *Lfng* expression is absent as early as 9 dpc (Morales et al., 2002). Also in agreement with this line, the genes expressed in PSM, including *c-hairy1*, remain cycling normally in a PSM explant culture without the posterior part and tail bud (Palmeirim et al., 1997, 1998). The other important function of the gradient could be in slowing and/or arresting the segmentation clock, perhaps at a lower threshold, as cells progress from the undetermined zone into the determined zone, to facilitate and/or permit somite maturation and boundary placement (Aulehla et al., 2003; Dubrulle et al., 2001; Sawada et al., 2001).

The clockwork of the segmentation clock

Although recent progress has pointed to the existence of a biochemical clock related to Notch signaling that drives somite segmentation, an important question is how the segmentation clock works, in other words, what is the biochemical mechanism of a clock. The nature of an oscillator is a system that regularly departs from and returns to equilibrium. There are two major ways to sustain an oscillation: (i) positive feedback, a deviation-amplifying process, in which threshold is a common phenomenon, for example, cAMP oscillation in *Dictyostelium amoebae*, and (ii) negative feedback, a deviation-counteracting process, which is necessary but not sufficient for homeostasis, for example, circadian rhythms (Goldbeter, 2002).

Work done so far has highlighted four important features of the segmentation clock: (i) negative feedback loops; (ii) two transcriptional factors as cores; (iii) dual function of Notch signaling and (iv) posttranslational regulations.

Negative feedback regulation—the essence of the segmentation clock

Notch signaling has been shown to be required for oscillatory gene expression in the PSM, implying that this pathway is periodically activated during successive rounds of somite formation. A vital link between Notch signaling and cyclic gene expression emerged with the identification of the Hairy/E(spl)-related proteins as target genes of Notch signaling in various systems (Jarriault et al., 1995, 1998; Kageyama and Ohtsuka, 1999; Tomita et al., 1999). Being transcriptional repressors, this group of bHLH transcription factors can repress the expression of a host of downstream genes and themselves. In *Xenopus*, it was demonstrated that the periodic repression of Notch signaling was mediated by ESR4 and ESR5 (also Notch targets) via a negative feedback loop in

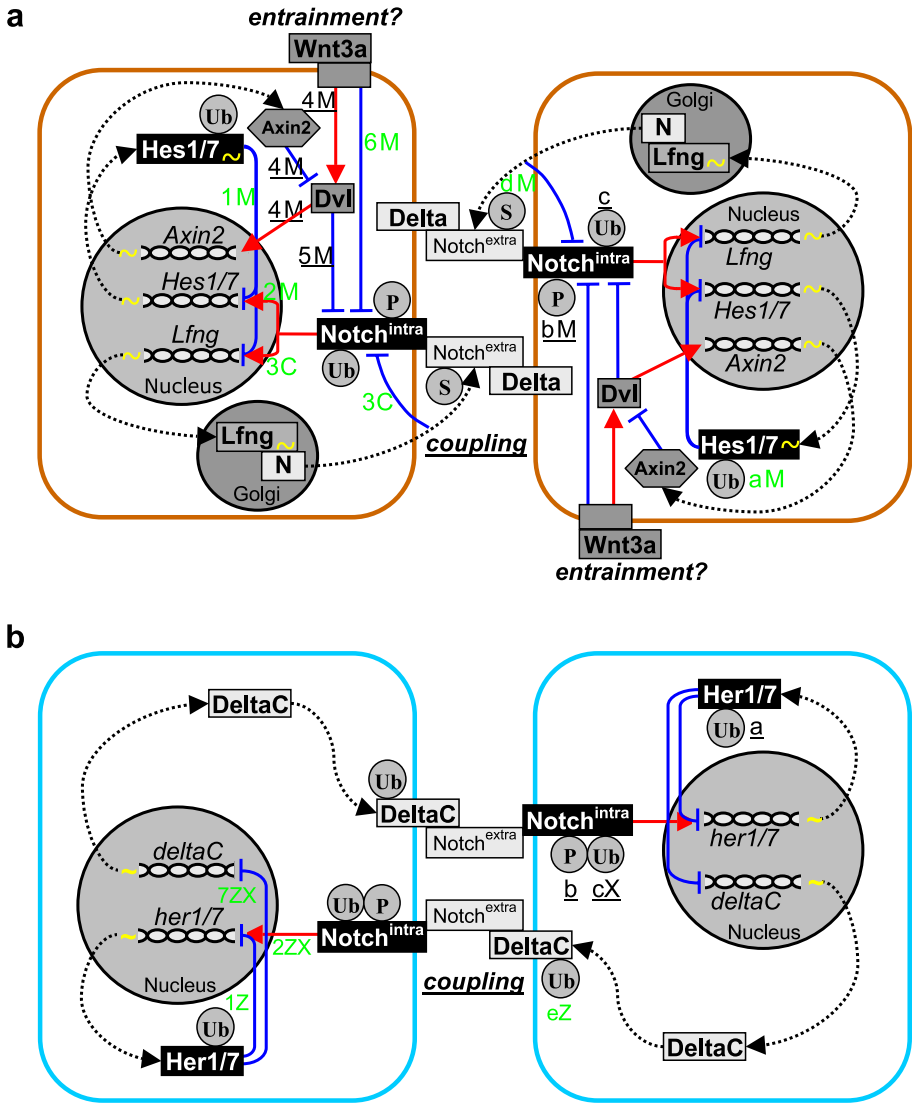

Fig. 3. Summary of feedback loops and posttranslational regulations among components involved in vertebrate segmentation clock of mouse (mainly) and chick (a), and of zebrafish (mainly) and *Xenopus* (b). Numbered footnotes indicate known activation (red arrow line) or inhibition (blue blunt line); lettered footnotes indicate known posttranslational modifications. Ub, P and S stand for ubiquitylation, phosphorylation and glycosylation, respectively. M, C, Z and X mean that results obtained from mouse, chick, zebrafish and *Xenopus*, respectively. Underlined items stand for those regulations and modifications not yet shown to be related to somite segmentation but probably would be; the rest in green have been otherwise shown to be linked to somite segmentation. Dotted lines mean time delay due to transcription, translation, posttranslational modification, translocation and protein turnover; yellow tilde sign indicates the cycling of transcripts or proteins. Hes1/7 and Her1/7 feedback loops are mainly responsible for intracellular oscillation; on the contrary, Lfng and DeltaC loops can additionally couple the oscillation between neighboring cells. Wnt3a/Axin2 could, in theory, behave as an input signal to entrain the oscillation. However, it needs to be investigated further (see text for more details). Footnotes: **1**, Bessho et al. (2001b, 2003); Holley et al. (2002); Oates and Ho (2002); **2**, Bessho et al. (2001a); Jen et al. (1999); Takebayashi et al. (1994); Takke and Campos-Ortega (1999); **3**, Dale et al. (2003); **4**, Jho et al. (2002); Lustig et al. (2002); **5**, Axelrod et al. (1996); **6**, Aulehla et al. (2003); **7**, Holley et al. (2002); Jen et al. (1997); Oates and Ho (2002); Takke and Campos-Ortega (1999); **a**, Bessho et al. (2003); Hirata et al. (2002); **b**, Foltz et al. (2002); **c**, Lamar et al. (2001); **d**, Brückner et al. (2000); Moloney et al. (2000); **e**, Itoh et al. (2003).

which ESR5 represses the expression of *X-Delta-2* and *ESR4* (Jen et al., 1999). This observation led to the hypothesis that negative transcriptional feedback regulation may underlie periodic Notch activation during segmentation.

In mammals, some members of the Hairy/E(spl) family, such as Hes1, were found to bind to their own promoters and shut off their own expression (Takebayashi et al., 1994). A clear demonstration has been done in mouse, where *Hes7* is cycling in PSM and its deficiency results in somite phenotypes and up-regulation of *Hes7* transcript (Bessho et al., 2001b, 2003). Antisense morpholinos (MO) that block translation of zebrafish Hairy/E(spl) proteins, Her1 and Her7, lead to a similar result: dramatic increase in the level of *her1* and *her7* transcript, abolished cyclic expression of both *her1* and *deltaC*, and prominent somite abnormalities (Gajewski et al., 2003; Henry et al., 2002; Holley et al., 2002; Oates and Ho, 2002), suggesting that the Her proteins form part of an autoinhibitory loop. However, the

result details, for example, gene expressions in MO-injected embryos, are not all identical, and sometimes even lead to opposite interpretations (e.g. Her1 as an activator in Gajewski et al., 2003). This is probably due to the differences in concentration, efficacy and sequence of oligos applied (Heasman, 2002, and see a quantitative consideration of the consequence of translational blockade in Lewis, 2003).

Another important target of Notch signaling in mice and chick is the glycosyltransferase *Lfng*, the mRNA of which shows rhythmic oscillations in the PSM of these organisms (Aulehla and Johnson, 1999; Forsberg et al., 1998; McGrew et al., 1998). It was observed that misexpression of *dnRBPJκ* abolished the oscillatory expression of the chick *Lfng*. Conversely, misexpression of NICD caused ectopic expression of *Lfng* (Dale et al., 2003), which is in agreement with the findings that RBPJκ-binding sites are in *Lfng* promoter and it responds to Notch regulation (Morales et al., 2002). Furthermore, there was rapid turnover of Lfng protein in the PSM. The misexpression of *Lfng* resulted in an inhibition of Notch signaling, abolition of cyclic gene expression and irregular positioning of somite boundaries, indicating a profound disruption of the segmentation clock in the chick PSM. This result demonstrated that Lfng can negatively regulate Notch signaling and this feedback loop could potentially underlie the periodic inhibition of Notch signaling during segmentation (Dale et al., 2003). Furthermore, due to its autoinhibitory nature, the oscillating expression of *Lfng*, but not the expression per se, is important for coordinated somite segmentation, and hence constitutive expression of *Lfng* leads to a failure of somite segmentation in chick and mouse (Dale et al., 2003; Serth et al., 2003).

Nrarp, which encodes a small protein with two ankyrin repeat domains, is expressed in the PSM and formed somites (Krebs et al., 2001; Lahaye et al., 2002; Lamar et al., 2001; Topczewska et al., 2003, and our unpublished data). Though *Nrarp* has not yet been shown to be directly involved in somite segmentation, its expression is down-regulated in *Notch1* −/− and *Dll1* −/− mutants (Krebs et al., 2001). Interestingly, it has been demonstrated in *Xenopus* that Nrarp functions in a negative feedback loop within the Notch signaling pathway (Lahaye et al., 2002; Lamar et al., 2001) to attenuate NICD-mediated transcription, probably by triggering proteasome-dependent degradation of NICD (Lamar et al., 2001).

Two transcription factors drive interconnected loops of segmentation clock

Experiments done in a variety of tissues from different organisms have demonstrated that transcriptional feedback regulation is an essential feature of Notch signaling (reviewed in Artavanis-Tsakonas et al., 1999). Of all Notch components, NICD and the Hairy/E(spl) proteins are the two key transcription factors that comprise the prime driving force of the segmentation clock and have the following characteristics in common: (i) they are both intracellular manifestations of cyclic Notch activation (see above, though this has not been shown directly for NICD); (ii) they negatively regulate their own transcript levels directly or indirectly (see below) and (iii) they are short-lived and probably degraded after ubiquitylation (Bessho et al., 2003; Foltz et al., 2002; Hirata et al., 2002; Lamar et al., 2001; Schroeter et al., 1998). Moreover, Notch is a membrane-bound transcriptional factor, whose maturation and activation are tightly regulated within Notch signaling and among other pathways (Artavanis-Tsakonas et al., 1999; Axelrod et al., 1996; Kopan, 2002; Ross and Kadesch, 2001).

The NICD loop and the Hairy/E(spl) loop of the segmentation clock are connected and interdependent. Hairy/E(spl)-related proteins, functioning as transcriptional repressors, can repress their own transcription and hence reduce their protein synthesis. Once the protein level drops, the repression is relieved and transcription rebounds until the protein accumulates to a level that inhibits transcription again (Bessho et al., 2001b; Holley et al., 2002; Jen et al., 1999; Oates and Ho, 2002, and see below). Compared to the Hairy/E(spl) loop, the NICD loop is more complicated and the details may differ among species (see below). Notch activation can induce expression of *Hairy/E(spl)*-related genes, such as *Hes1*, *ESR4* and *her1*, in a Su(H)/RBPjκ-dependent manner (Jen et al., 1999; Takebayashi et al., 1994; Takke and Campos-Ortega, 1999). In zebrafish, it has been demonstrated that Her1 and Her7 can repress expression of *deltaC* and *deltaD*, although it is not yet certain whether this repression is direct or indirect (Holley et al., 2002; Oates and Ho, 2002; Takke and Campos-Ortega, 1999). Similar results were found in *Xenopus* (Jen et al., 1999). Therefore, zebrafish Her proteins and *Xenopus* ESR4 and ESR5 are both effectors and (as repressors of ligand expression) upstream regulators of the Notch signaling cascade, forming a negative feedback loop. In addition, Lfng and Nrarp also form a negative regulatory loop with NICD in chick and *Xenopus*, respectively (see above).

Dual role of Notch signaling in somite segmentation

In the clock and wavefront model, the segmentation clock was described as "an oscillator, shared by all the pre-somite cells, with respect to which they are an entrained and closely phase-organized population, because of intercellular communication" (Cooke and Zeeman, 1976). In other words, the PSM cells are coupled oscillators. From the work done in zebrafish, we proposed that Notch signaling is indispensable for the synchronization of the segmentation clock (Jiang and Lewis, 2001; Jiang et al., 2000; Lewis, 2003). On the other hand, the work done by Dale et al. (2003), Bessho et al. (2001b) and Holley et al. (2000, 2002) argued for a more direct role of Notch signaling in maintaining the ticking of segmentation clock. Nevertheless, a reconciled view of how Notch signaling functions in the clockwork is emerging: Hairy/E(spl)-linked Delta–Notch signaling is the oscillator with dual functions as a clock

generator as well as a clock synchronizer (Oates and Ho, 2002). Interestingly, the data for arguing the role of Notch signaling in signal synchronization and generation are mostly related to NICD and Hes/Her loops, respectively. Lewis (2003) has discussed and demonstrated the effect of different wiring within segmentation clock by modeling.

It seems that Notch signaling pathway performs both functions, at least in zebrafish. This unique capacity of Notch signaling could be due to its very nature as a module that allows cells to talk to each other and alter their behavior accordingly. A perturbation of any one of these functions would probably result in a perturbation of the other to some extent—an important feature of coupled oscillators. We are limited by the degree to which these functions are genetically separable because of technical limitations. Moreover, the circuitry that comprises the clock mechanism may be wired dissimilarly in different organisms (as Fig. 3 shows) and mutations could affect the two functions of the Notch pathway to different degrees (see below). Recent evidence from cell culture experiments has shown that serum shock can induce oscillatory expression of *Hes1* in several cell lines (Hirata et al., 2002). Interestingly, similar periodic *Hes1* expression was observed when the cells were mixed with Delta-expressing S2 cells. This phenomenon once again points to the possibility of dual function of Notch signaling—signal induction and/or signal synchronization—among cells in culture. It would be interesting to determine whether the segmentation clock still runs in individual PSM cells of Notch pathway mutants in vivo using transgenic lines with clock-controlled real-time reporters.

Posttranslational regulation in Notch signaling

The first direct evidence for the involvement of a posttranslational regulation of Notch signaling in somite segmentation is the glycosylation of Notch by Lfng (Brückner et al., 2000; Dale et al., 2003; Evrard et al., 1998; Moloney et al., 2000; Zhang and Gridley, 1998). Then came the molecular identification of a somite mutant, *mib*, which harbors a mutation in a gene that encodes a RING E3 ligase (Itoh et al., 2003; Jiang et al., 1996). Though it has not yet been shown to be responsible for the degradation of any Notch component, Mib has been demonstrated to ubiquitylate Delta and lead to its endocytosis (Itoh et al., 2003), which has been hypothesized to be essential for ligand-dependent Notch activation (Klueg and Muskavitch, 1999; Parks et al., 2000). Additionally, there is indirect evidence suggesting a role for regulated protein turnover in somite segmentation: (i) Hes1 and Hes7 proteins have been shown to hold a short in vivo half-life due to ubiquitin-proteasome-mediated degradation and protein level of Hes1 oscillates every 2 h in cultured cells, matching the time for a somite to form in mouse (Bessho et al., 2003; Hirata et al., 2002); (ii) Lfng protein behaves similarly (Dale et al., 2003) and (iii) NICD is short-lived, if it coexists with Nrarp (Krebs et al., 2001; Lamar et al., 2001).

Along this line, other proteins have been shown to modulate Notch signaling via ubiquitylation, though it remains unclear whether these modifications are truly essential for somite segmentation: Sel-10, Itch, Deltex and Neuralized (Cornell et al., 1999; Deblandre et al., 2001; Izon et al., 2002; Lai et al., 2001; Matsuno et al., 1995; Öberg et al., 2001; Qiu et al., 2000; Takeyama et al., 2003; Wu et al., 2001; Yeh et al., 2001). Other forms of post-translational modification also exist: only a specifically phosphorylated form of NICD interacts with Sel-10 (Gupta-Rossi et al., 2001; Wu et al., 2001); glycogen synthase kinase-3β (GSK-3β) phosphorylates NICD and thereby protects it from degradation by the proteasome (Foltz et al., 2002). It is also known that both Hairy and Hey proteins can form homo- and heterodimers, raising the possibility of combinatorial action and additional levels of regulation (Leimeister et al., 2000, and see Lewis, 2003, for a possible role in changing clock periodicity). bHLH proteins have been shown to be regulated by short-lived HLH proteins of the Id family in mammals (Bounpheng et al., 1999; Jogi et al., 2002) and Extramacrochaetae (Emc) in *Drosophila* (Baonza et al., 2000; Campuzano, 2001). These and other unidentified modulators may be responsible for stabilizing the nexus of interactions around the core segmentation clock machinery and for ensuring that the oscillations are robust and resistant to perturbations. The resiliency, adaptability and fine tuning of the segmentation clock, therefore, could be attributed to regulations of NICD and Hairy/E(spl) proteins at multiple levels.

Comparisons to the circadian clock

Most eukaryotic and several prokaryotic organisms, ranging from bacteria to human, possess circadian clocks that manifest themselves in daily rhythms of behavior, physiology, and biochemistry. Thanks to the rapid development of genetic, molecular and biochemical approaches, together with precise behavioral observations, we have significantly advanced our knowledge of circadian clock in a variety of organisms during the last decades (reviewed in Young and Kay, 2001). This universal and well-known circadian clock shares some striking similarities in its mechanism and organization to the segmentation clock. Knowledge of the former, therefore, would greatly facilitate the understanding of the latter. The comparison between these two clocks is stated in following paragraphs and summarized in Table 1.

Similarities between the two clocks

A common feature of a molecular clock in a multicellular organism is the necessity for it not only to be generated in individual cells, but also for it to be coordinated among different groups of cells within the organism. Dispersed individual neurons derived from the suprachiasmatic nucleus

Table 1
Comparison between two clocks

Features		Circadian clock	Segmentation clock	References
Generation of the clock (transcriptional negative feedback)	Positive elements	CLK/CYC (*Drosophila*), CLK/BMALs (mouse, zebrafish)	Notch activation (Su(H)/NICD), Axin2[a]	Aulehla et al., 2003; Bessho et al., 2001a; Darlington et al., 1998; Gekakis et al., 1998; Jen et al., 1999; Jouve et al., 2000; Sieger et al., 2003; Takke and Campos-Ortega, 1999
	Negative elements	PER/TIM (*Drosophila*), PERs/CRYs (mouse, zebrafish)	Lfng, Hes7 (chick, mouse)[b], Her1, Her7 (zebrafish)[c], ESR4, ESR5 (*Xenopus*)	Bessho et al., 2001b, 2003; Dale et al., 2003; Gajewski et al., 2003; Holley et al., 2002; Jen et al., 1999; Lee et al., 1999; Oates and Ho, 2002; Sangoram et al., 1998
Synchronization of the clock		Various humoral and/or neuronal pathways	Notch signaling[d], Wnt3a/Axin2[a]	Aulehla et al., 2003; Hirata et al., 2002; Jiang et al., 2000; Reppert and Weaver, 2002
Transcriptional activation of clock-controlled genes		Some genes are clustered	HoxD complex is probably regulated as a gene cluster	Etchegaray et al., 2003; McDonald and Rosbash, 2001; Zákány et al., 2001
Posttranslational regulations		Phosphorylation (e.g. DBT, GSK3), ubiquitylation (e.g. Slimb)	Phosphorylation (e.g. GSK3)[e], Ubiquitylation (e.g. Mib), Glycosylation (e.g. Lfng)	Brückner et al., 2000; Duffield et al., 2002; Foltz et al., 2002; Grima et al., 2002; Itoh et al., 2003; Kloss et al., 2001; Martinek et al., 2001; Moloney et al., 2000
Induced by serum shock in vitro		Yes[f]	Yes[f]	Balsalobre et al., 1998; Hirata et al., 2002
Entrainment (input)		Light, etc.	Wnt signal[a]	Aulehla et al., 2003; Reppert and Weaver, 2002
Involvement of gradients		Not yet found	Fgf8 and Wnt3a[g]	Aulehla et al., 2003; Dubrulle et al., 2001; Sawada et al., 2001
Temperature dependency		Temperature-compensated	Temperature-dependent	Jiang et al., 2000; Reppert and Weaver, 2002
Number of cycles		Unlimited number of cycles, covering life span of organisms	Limited number of cycles, arrested in maturing somites	Pourquié, 2001; Reppert and Weaver, 2002

[a] The role of Axin2 through Wnt3a signaling is not yet clear. It has been demonstrated in mouse but not in zebrafish. It negatively regulates Notch through Dishevelled and hence can activate or synchronize and/or entrain the segmentation clock (Aulehla et al., 2003; Axelrod et al., 1996). See text for more details.
[b] Lfng and Hes7 have been shown to negatively regulate Notch activation in chick and transcription of *Hes7* and *Lfng* in mouse, respectively (Bessho et al., 2001b, 2003; Dale et al., 2003).
[c] Gajewski et al. (2003) argued that Her1 behaves as an activator.
[d] There is no direct evidence for the synchronization role of Notch signaling. However, it has been shown indirectly in zebrafish and in mammalian cell culture (Hirata et al., 2002; Jiang et al., 2000).
[e] Only the role of GSK3 in Notch stability has been shown but not its role in somite segmentation (Foltz et al., 2002).
[f] The concentration of serum rose from 5% to 50% and from 0.2% to 5% for circadian and segmentation clocks, respectively (Balsalobre et al., 1998; Hirata et al., 2002).
[g] A direct demonstration at cellular levels is not yet available (Aulehla et al., 2003; Dubrulle et al., 2001; Sawada et al., 2001).

(SCN—the site of central circadian clock in mammals) display circadian rhythms in their firing rate in vitro, but with various phases and even slightly different periods. This variation is significantly decreased when cells are cultured in the form of SCN explants, indicating that a mechanism within the SCN tissue synchronizes circadian oscillations generated by its individual neurons (Herzog et al., 1998). In fact, recent evidence has suggested a role for an inhibitory neurotransmitter, gamma-aminobutyric acid (GABA), in synchronization of SCN neurons (Liu and Reppert, 2000). Another level of coordination is seen in the way the master clock in the SCN synchronizes countless peripheral oscil-

lators ticking in almost every cell of the organism. This process seems to be complex and probably involves both humoral and neuronal pathways (reviewed in Reppert and Weaver, 2002). A similar situation is likely to take place in the PSM of developing vertebrates, where the segmentation clock is composed of two different but probably interconnected processes: generation of oscillations within a cell and their coordination between neighboring cells. A group of cells forming the same part of a future somite would oscillate in the same phase, whereas different cohorts of cells along the AP axis which give rise to different parts of future somites would oscillate with different but coordinated phases. Notch signaling is likely to be involved in both generation and synchronization processes (duality, see above).

The most intriguing and important similarity between circadian and segmentation clocks is that both of them utilize negative feedback loops to generate oscillation. Delayed negative feedback regulation lies at the heart of circadian oscillators in all organisms studied to date, including cyanobacteria (Ishiura et al., 1998), *Neurospora* (Merrow et al., 2001), *Arabidopsis* (Strayer et al., 2000), *Drosophila* (Bae et al., 2000; Darlington et al., 1998; Lee et al., 1999), zebrafish (reviewed in Pando and Sassone-Corsi, 2002), mouse (Gekakis et al., 1998; Griffin et al., 1999) and human. In *Drosophila*, for example, Clock (CLK) and Cycle (CYC) bind directly to E-box elements in the promoters of *period* (*per*) and *timeless* (*tim*) and thereby activate their transcription. This induction of gene expression is antagonized by PER and TIM which bind to CLK–CYC complexes and prevent their binding to DNA. Thus, oscillating expression pattern of clock gene is generated by a periodic inhibition of CLK–CYC activity. Likewise, though less thoroughly demonstrated in the vertebrate segmentation clock, it has been proposed that avian Lfng exercises periodic inhibition on Notch signaling activity, leading to a rhythmic expression of clock genes in the chick PSM (Dale et al., 2003). Similar situation has been observed in *Xenopus* PSM (Jen et al., 1999). Furthermore, Hes7 in mouse and Her1 and Her7 in zebrafish have been demonstrated to repress their own transcription, forming a negative feedback circuit (Bessho et al., 2001b; Holley et al., 2002; Oates and Ho, 2002).

Although transcriptional regulation seems to play an important role in rhythm generation, it may not be sufficient. Various posttranslational mechanisms were shown to have an essential role in circadian clockwork (reviewed in Allada et al., 2001). Presumably, an important function of these posttranslational mechanisms is to delay the action of the repressors concerning the synthesis of their mRNAs. Without such a delay, the system would quickly fall into equilibrium and, therefore, the oscillation would damp (reviewed in Gonze et al., 2000). Specific series of delays would also contribute to determine the period characteristic for each molecular clock. Phosphorylation is a prominent biochemical modification often used by cells to modulate their timing processes. In *Drosophila*, the casein kinase, Doubletime (DBT) phosphorylates and destabilizes PER only when PER is free of TIM, thereby, it retards the initial accumulation of PER. A high concentration of TIM then promotes formation and nuclear entry of the DBT–PER–TIM complex. Once in the nucleus, DBT-dependent phosphorylation of PER frees PER from TIM, and thus helps to advance the clock (Kloss et al., 2001). Mutations in *dbt*, therefore, result in shortened, lengthened or abolished rhythms. Another kinase, Shaggy/GSK-3, was shown to stimulate the nuclear entry of PER–TIM by promoting TIM phosphorylation (Martinek et al., 2001). Recently, several groups have reported an essential role for ubiquitylation in the *Drosophila* circadian clock: (i) Slimb—a member of the F-box/WD40 protein family of the ubiquitin ligase SCF complex—has been shown to bind preferentially to phosphorylated PER and to stimulate its degradation (Ko et al., 2002); and (ii) fly *Slimb* mutants behave arrhythmically (Grima et al., 2002). In addition, microarray analysis done in mammalian cells has implicated a role for ubiquitin-proteasome in the oscillation of the circadian clock (Duffield et al., 2002). In the segmentation clock, posttranslational regulations, such as phosphorylation and ubiqutitylation, are emerging as stated above.

Another common feature of molecular clocks could be the means by which they control expression of output genes (clock-controlled genes). Microarray analysis of circadian clock-regulated genes in *Drosophila* revealed that some of the clock-controlled genes cluster in the genome. It has been proposed that *cis*-acting elements (e.g. E-box) could be involved in this transcriptional co-regulation (McDonald and Rosbash, 2001). Intriguingly, recent evidence has suggested a role for chromosome remodeling in circadian transcription. Thus, transcriptional regulation of clock genes in the mouse liver is accompanied by rhythmic H3 histone acetylation, which appears to be a potential target of the feedback repression action of CRY (an inhibitory factor in a negative feedback loop of mammalian circadian clock) (Etchegaray et al., 2003). Although such a modification of chromosome structure has not yet been reported in the case of the segmentation clock, there is evidence that supports *cis*-acting elements in regulating clock output genes. In fact, *Hoxd1* and *Hoxd3* were shown to express in a Notch-dependent dynamic stripe pattern in the mouse PSM. A putative "segmentation stripe enhancer" from the genomic surroundings of these two genes has been demonstrated to be able to control also the promoters of *Hoxd11*, *Hoxb1*, and perhaps, of other *Hox* genes as well (Zákány et al., 2001).

Surprisingly, a serum shock on cultured cells can induce the oscillation of clock genes of both circadian clock (e.g. *Per1* and *Per2*) and segmentation clock (e.g. *Hes1*) for several cycles, suggesting that some unknown blood-borne substance(s) could act as time-resetting cue(s) in either clock (Balsalobre et al., 1998; Hirata et al., 2002). So far, there is no indication that these two developmentally different biochemical oscillators are linked. However, some

components, such as GSK-3 and casein kinase IIα, have been related to both circardian clock and Notch signaling (Foltz et al., 2002; Lin et al., 2002; Martinek et al., 2001; Trott et al., 2001). Interestingly, *Per1* is located upstream to *Hes7* and both are assigned to a position 37.0 cM from the centromere on mouse Chromosome 11 (Bessho et al., 2001a).

Differences between the two clocks

Circadian and segmentation clocks, however, display several fundamental differences. These are probably because circadian clock is universal and long-evolved and is required during the whole life span of an organism, whereas segmentation clock is probably restricted to vertebrates and is functional only during a certain period of development. It seems obvious that PSM cells are induced to oscillate only for a few cycles before their oscillation slows down and finally arrests in a maturing somite, whereas the circadian clock continues ticking till the demise of the organism. The segmentation clock is temperature-dependent (e.g. shown in Jiang et al., 2000), like most biochemical processes, possibly because it should be in coordination with other developmental events within the same organism. Circadian clock, by contrast, is temperature-compensated, as it should help organisms to be in tune with the external 24-h period of the Earth's rotation, whatever the temperature. Notch signaling seems to be the core of biochemical oscillators in segmentation clock, in charge of both generation and synchronization of the oscillating signal (Dale et al., 2003; Holley et al., 2002; Jiang et al., 2000; Oates and Ho, 2002), while the circadian clock is more complex in terms of these two processes (see above).

Evolutionary considerations

Despite the fact that Notch signaling is the kernel of the vertebrate segmentation clock, there are differences among mammals, birds, amphibians and fish (summarized in Fig. 3 and see its legend for details) as in the circadian clock. Significant differences exist among various circadian systems. Some organisms use completely different clock components (compare the circadian clocks of cyanobacteria, *Neurospora*, *Arabidopsis*, *Drosophila* and mice, reviewed in Dunlap, 1999 and Young and Kay, 2001), while others assign different functions to gene homologues. CRY is used as a circadian photoreceptor in *Drosophila*, while its counterpart acts as a core regulator in mice (Stanewsky et al., 1998; van der Horst et al., 1999); TIM has an essential role in fly but has no obvious clock function in mice (Gotter et al., 2000; Myers et al., 1995).

As for the segmentation clock, the regulation of *Hes7* and its zebrafish homologue, *her1* (Davis and Turner, 2001), seems to be conserved: a 0.9-kb *Hes7* promoter and the sequences between 2.3 and 8.6 kb upstream of the *her1* transcription start can modulate the cyclic expression (Bessho et al., 2003; Gajewski et al., 2003). There is a pair of putative RBPJκ binding sites, two E-boxes and one N-box, target sequences for Hes7 protein, in the *Hes7* promoter (Bessho et al., 2001a). Coexpression of *NICD* and *Hes7* can up-regulate and down-regulate *Hes7* promoter activity, respectively (Bessho et al., 2001a, 2003). Furthermore, Hes7 can override the Notch-induced transcription from the *Hes7* promoter (Bessho et al., 2003).

The expression of *Lfng* has been shown to oscillate during somite formation in chick and mouse but not in *Xenopus* and zebrafish (Aulehla and Johnson, 1999; Forsberg et al., 1998; Leve et al., 2001; McGrew et al., 1998; Prince et al., 2001; Wu et al., 1996). A conserved 2.3-kb region in the promoter of the murine *Lfng* governs the cyclic initiation of its transcription in PSM cells. This region integrates both positive and negative inputs since it includes *cis*-acting elements for both enhancing and repressing factors. Moreover, Notch signaling acts directly via RBPJκ-binding sites to activate *Lfng* expression (Morales et al., 2002). It was also demonstrated that mutation or deletion of E-boxes in the A/2 region of *Lfng* promoter abolishes *Lfng* periodic expression in posterior PSM, suggesting a direct regulation by the cyclically expressed Hes proteins (region A assigned in Morales et al., 2002; region 2 assigned in Cole et al., 2002). Interestingly, in such mutants, *Lfng* is still expressed in anterior PSM and formed somites in a manner similar to that seen in zebrafish (Morales et al., 2002). This observation implies that the A/2 region is responsible for *Lfng* cycling in posterior PSM, whereas the rest of the 2.3-kb region could be an ancestral promoter shared by all vertebrates that controls the expression of *Lfng* in anterior PSM and formed somites.

On the contrary, zebrafish *deltaC* is cycling in PSM, whereas chick *Delta1* and mouse *Dll1* are expressed in PSM but not in a cyclic or dynamic manner (Hrabe de Angelis et al., 1997; Jiang et al., 2000; Palmeirim et al., 1998). Though not cyclically expressed, *X-Delta-2* has been shown to be dynamically expressed within the PSM and mediates somite segmentation, reminiscent of zebrafish *deltaC* and *deltaD* (Jen et al., 1997). The promoter analysis of zebrafish *deltaC* is not yet available, but the analyses done in mouse *Dll1* and zebrafish *deltaD* have shown that their mesodermal elements are more divergent than neural elements during evolution (Beckers et al., 2000a; Hans and Campos-Ortega, 2002), suggesting that the corresponding transcription factors and hence the regulatory circuit are dissimilar as well.

The difference in the expression dynamics of *Fringe* and *Delta* genes among species suggests a different wiring for NICD regulation: in chick and likely in mouse as well, this loop is more "intrinsic", since the feedback loop can happen in individual cells with minimal interactions with their neighbors; in zebrafish, the NICD loop, if not entirely "extrinsic", exploits mutual interactions to certain degree. In other words, the coupling strength between individual PSM oscillators is stronger in zebrafish than that in

amniotes. This may explain the observed differences in expression patterns of key genes among species (e.g. "salt and pepper" pattern in zebrafish, see above). The finding that murine *Axin2* is cycling suggests another possible mechanism in the entrainment of individual PSM oscillators, probably from Wnt3a (extrinsic factor) through Dishevelled (intrinsic factor), which then binds and antagonizes Notch (Axelrod et al., 1996). It would be intriguing to know whether Wnt signaling plays an indispensable role in zebrafish somite segmentation. However, a *wnt5* corresponding mutant, *pipetail* (*ppt*), does not show any obvious segmentation phenotypes except defective tail outgrowth (Rauch et al., 1997).

A minimal model for the segmentation clock-coupled oscillators

The current knowledge about somite segmentation allows us to understand this complex process on a molecular basis. Thus, we can start to use mathematical modeling and simulation based on experimental data to facilitate our understanding. The goal of models is to understand the oscillatory physiology in terms of biochemical components and processes. Reaction kinetic models, in particular, can provide quantitative description of individual components of the oscillator and predictions of unknown aspects, which would assist us in designing new experiments to verify or to refute theories and models.

Intuition is a poor guide for understanding coupled oscillators—the nature of the segmentation clock, whose dynamics can be very complicated in the real embryos. As shown in a simple computer simulation, by changing the parameters, a two-celled oscillator can behave from synchronized, in-phase-locked oscillation to anti-phase-locked and desynchronized cycling (Jiang and Lewis, 2001). Furthermore, similar oscillation kinetics can be achieved within a space domain of parameters (Ariaratnam and Strogatz, 2001), which complicates the analysis of biological systems with limited accessibility. To this aim, there are two main approaches: (i) minimal models, where a composite system is disintegrated into smaller and simpler modules (Hartwell et al., 1999), from which, however, interesting and constructive results can be obtained (e.g. Jiang and Lewis, 2001; Lewis, 2003); and (ii) extensive and large-scale models, intending to incorporate from the outset all known details about the variables and processes of interest (e.g. Meir et al., 2002). It may be a while before the latter approach becomes feasible, since we have not yet acquired a complete picture of vertebrate somite segmentation.

A model for somitogenesis must, at least, account for the following features: (i) a periodic structure that is rostrocaudally generated; (ii) the size of anterior somites (about 10–20, depending on species) that is regulated according to the total size of the organism; (iii) cell–cell communication that is involved in the formation of the periodic structure; (iv) the time when the separation of somites occurs is intrinsically determined; (v) a boundary that is formed after somite formation; (vi) each somite consists of an anterior and a posterior part; (vii) the somites formed in this process are distinct from each other and (viii) a mechanistic explanation can be obtained for most, if not all, observations from perturbation experiments and mutant analyses. We think that coupled oscillators lie at the core of segmentation clock, acting as a module, and can account for many features mentioned above. Gradients certainly interact with the Notch-dependent segmentation clock but the molecular details just start to emerge. An updated network regulation in higher vertebrates (mouse and chick) and lower vertebrates (*Xenopus* and zebrafish) is schematized as a two-celled system in Fig. 3. There are several feedback loops and posttranslational modifications in these two broad systems, which can be taken to formulate an accessible and meaningful model either as a whole or partially (e.g. a Hes-dependent oscillation only or plus a Lfng-dependent feedback loop) for a simple two-celled coupled oscillator (Lewis, 2003, simple as it is but there are some interesting findings and possible mechanistic ways beyond intuition), for a cluster of cells in 2-D region, or even for a group of cells in 3-D space—a more realistic situation. In addition, the modeling and simulation of these two systems may allow us to appreciate the evolutionary constraints in designing the segmentation clock.

Perspectives

Recent advances in in vitro studies could help to shed light on the mechanisms underlying gene oscillation, as it has been done for *Hes1* (Hirata et al., 2002), but final conclusions could only be achieved with the establishment of in vivo reporter transgenic lines. Another interesting issue to be addressed is whether segmentation clock is cell autonomous. Although indirect observations coming from desynchronization model (Hirata et al., 2002; Jiang et al., 2000) are in support of cell autonomy, direct evidence is still missing. The fact that serum treatment induces *Hes1* oscillation in various cell lines in vitro would be a strong argument but this experiment does not rule out the possibility that serum merely triggers Notch activation in these cells. Similar experiments in a Notch-deficient cell line would help to solve the question, but here again, definitive answers would be obtained only with in vivo transgenic reporter lines at the single-cell level.

Recent progress has provided valuable insights into the components and operation of the segmentation clock in the PSM as well as the means by which adjacent cells are coordinated with respect to their oscillations. It would be interesting to explore how cyclic gene expression eventually translates into the series of somites and to understand the interface among dynamic gene expression, cellular differentiation and morphogenesis at the organismal level. To this

end, it would be necessary to identify genes that lie immediately downstream of the segmentation clock (clock outputs), and to study their patterns of expression and functions.

Another outstanding question is the role of Fgf8 and Wnt3a gradients in somite segmentation. These gradients could solely be an input signal to entrain the segmentation clock. Alternatively, Fgf8 and Wnt3a may biochemically interact with Notch signaling to maintain the tempo and coherence of somite formation. The *Axin2* knock-out mouse should be able to answer this question to some extent.

Promoter analysis of cycling genes in different species will shed light on transcriptional regulation underlying the segmentation clock. In addition, it can answer the question of the differences in circuit wiring among different vertebrates.

The evidence from the effect of treating chick embryos with cell cycle inhibitors and other observations suggest a connection between the cell-cycle control machinery and the segmentation clock (Gorodilov, 1992; Primmett et al., 1989). The nature of this link, however, remains to be elucidated.

The nexus of interactions that surrounds the basic segmentation clock is indeed multiplex and complex. Dissecting this gamut of pathways to go beyond simple feedback loops into the realm of molecular networks of astonishing complexity poses a considerable challenge. For such a dynamic and complicated system, mathematical modeling and simulation will definitely complement the experimental methods and facilitate the progress in understanding the clockwork, the perturbation consequences and the evolutionary constraints of the segmentation clock.

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