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The vertebrate segmentation clock

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ABSTRACT

Vertebrate somitogenesis has been shown to be associated with a molecular oscillator, the segmentation clock, whose periodicity matches that of the process of somitogenesis. The existence of such a clock in presomitic mesoderm (PSM) cells was originally proposed in theoretical models such as the 'clock and wavefront'. Molecular evidence for the existence of this clock in vertebrates has been obtained on the basis of the periodic expression of several genes, most of which are related to the Notch signalling pathway. These genes are expressed in a dynamic sequence which appears as a wave sweeping caudo-rostrally along the whole PSM once during each somite formation. Notch-pathway mouse and fish mutants lose the dynamic expression of the cycling genes, indicating that Notch signalling is required for their periodic expression, or is required to coordinate the oscillations between PSM cells. Therefore Notch signalling is either part of the mechanism of the oscillator itself or acts as a cofactor required for cycling gene expression. A further potentially important role for the segmentation clock is to periodically activate Notch signalling in the rostral presomitic mesoderm, thereby generating the periodic formation of somite boundaries.

Key words: Chick embryo; *hairy*; somite; Notch signalling; segmentation clock.

A MOLECULAR CLOCK LINKED TO VERTEBRATE SEGMENTATION

Periodic expression of hairy*-like genes in the presomitic mesoderm identify a molecular clock linked to somitogenesis*

Several models such as the 'clock and wavefront' model (Cooke, 1998), or the cell cycle model (Stern et al. 1988), have proposed the existence of an oscillator or clock in the PSM cells to generate a temporal periodicity, which would be turned into the spatial periodicity of the somites. The identification of *c-hairy1*, an avian homologue of the fly pair-rule gene *hairy*, has provided molecular support for the existence of such a clock linked to segmentation (Palmeirim et al. 1997). *c-hairy1* is strongly expressed in the presomitic mesoderm (PSM), where its mRNA exhibits cyclic waves of expression whose temporal periodicity corresponds to the formation time of one somite (Palmeirim et al. 1997). In vitro studies suggest that these waves result from an intrinsic property of the PSM and do not rely on cell migration or on extrinsic signal propagation. Prospective somitic cells express pulses of *c-hairy1* mRNA while they are in the PSM and stop oscillating around the time of somite formation. Since in a 10–20 somite-stage embryo, the PSM contains 12 prospective somites in the chick, cells will undergo 12 *c-hairy1* expression cycles before their incorporation into a somite (Fig. 1).

Two more genes related to *Drosophila hairy*, called *c-hairy2* and *c-Hey2* exhibit a similar dynamic expression pattern in the chick PSM. Whereas *c-hairy2* protein is very similar to that of *c-hairy1*, its expression lags slightly longer in the caudal PSM, suggesting that its mRNA is more stable (Jouve et al. 2000). Furthermore, *c-hairy2* becomes expressed in the rostral part of the somite whereas *c-hairy1* is found in the caudal somite. *c-Hey2* is more distantly related to the *hairy* family of transcription factors and exhibit a modified C-terminal sequence containing a YRPW tetrapeptide instead of the canonical VRPW sequence found in hairy proteins (Leimeister et al.

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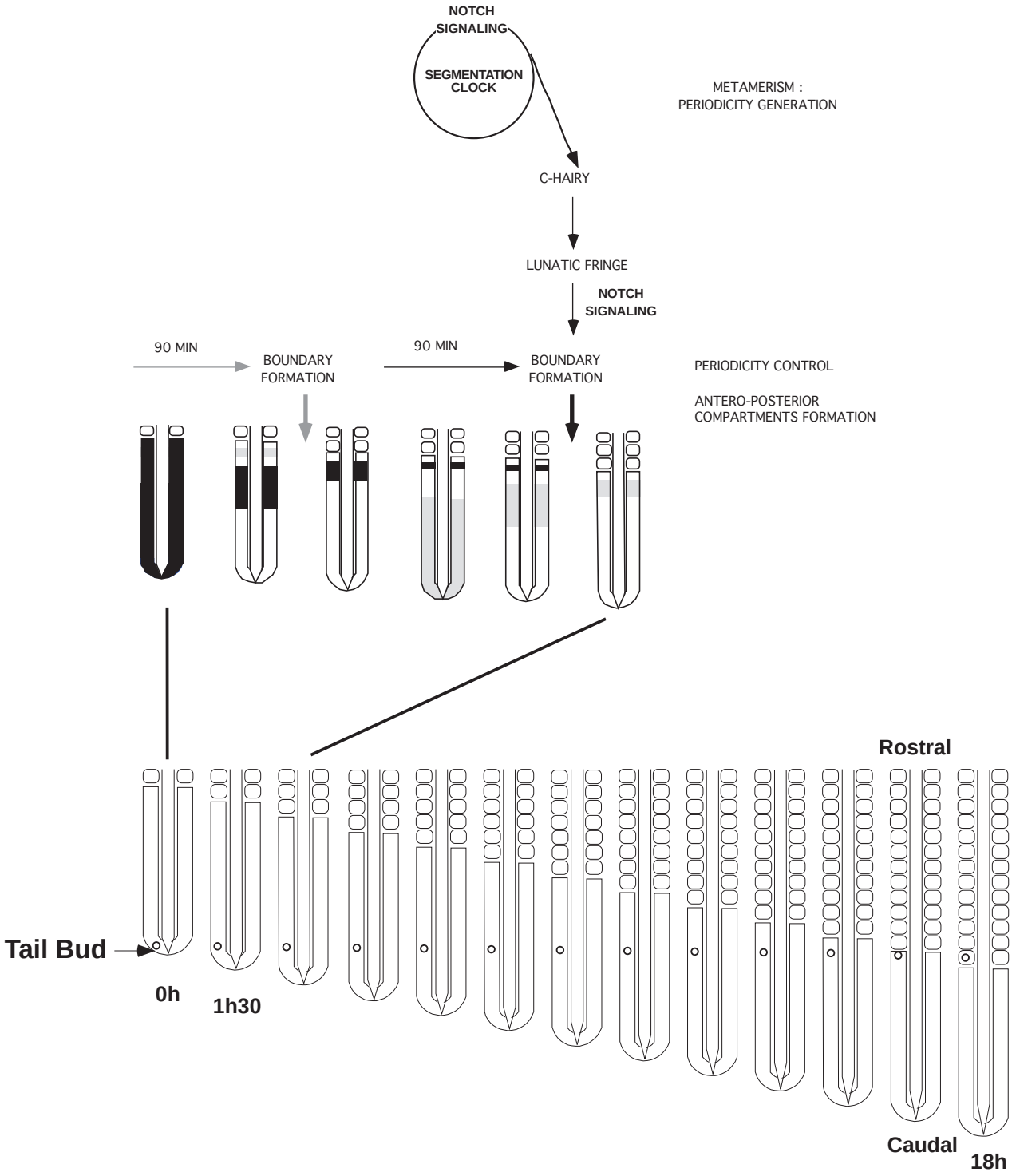

Fig. 1. Expression of *c-hairy1* in the presomitic mesoderm defines a clock linked to vertebrate segmentation *c-hairy1* expression (in black) appears as a wave which arises from the posterior region of the embryo and progresses in a rostral direction. Between the time that a PSM cell (white circle) becomes first specified after gastrulation (Oh) and the moment it becomes incorporated into a somite (18 h), 12 somites will form. Thus, any cell in the PSM will experience 12 *c-hairy1* waves. These oscillations of the *c-hairy1* mRNA occur in every cell of the PSM and define a clock linked to somite segmentation.

2000). *c-Hey2* shows an expression pattern very similar to that of *c-hairy1* and is also expressed in the caudal part of the somites. Both hairy and Hey proteins are able to homo- and hetero-dimerise suggesting that a combinatorial action of these proteins might be important for segmentation (Leimeister et al. 2000). Mammalian homologues of these *hairy* and *Hey* genes have been identified but

only in the case of *Hes1*, a mouse homologue of *c-hairy2*, was a periodic pattern of expression evidenced (Jouve et al. 2000; Leimeister et al. 2000).

Functional relation between hairy *genes and the segmentation clock*

The *c-hairy1* homologue in the fly is a primary pair-rule gene which encodes a basic helix-loop-helix transcriptional repressor (Barolo & Levine, 1997). Similarly, the murine *Hes1* gene, which is structurally highly related to the *c-hairy1* gene and was demonstrated to oscillate in the mouse PSM, encodes a transcriptional repressor, and has been shown to bind to its own promoter (Takebayashi et al. 1994; Jouve et al. 2000). Thus one might imagine that *c-hairy1* may regulate its own transcription and regulation of the somitogenesis clock would resemble that of other well studied biological clock systems, implicating that *c-hairy1* might itself be acting as a crucial clock component. However, inhibiting protein synthesis does not arrest cyclic *c-hairy1* expression, indicating that such a transcriptionally based mechanism is unlikely (Palmeirim et al. 1997).

Furthermore, implication of *c-hairy2* and *c-Hey2* in such a simple feedback mechanism is also unlikely since their dynamic expression is similarly insensitive to cyclohexamide (Jouve et al. 2000; Leimeister et al. 2000). This interpretation is also supported by genetic evidence in the mouse given that in the *Hes1-/-* mutant, no effect on the segmentation clock is observed (Jouve et al. 2000). These experiments therefore do not favour a simple model of negative transcriptional regulation by hairy proteins. They rather suggest the existence of a mechanism acting at the post-transcriptional level, by an as yet unknown means, to regulate the transcription of *hairy*-like genes.

NOTCH SIGNALLING ACTS UPSTREAM AND DOWNSTREAM OF THE SEGMENTATION CLOCK

Periodic expression of genes of the Notch signalling pathway provide a link between Notch signalling and the segmentation clock

Notch signalling has been shown to be critically implicated in somitogenesis (Pourquie, 1999). Notch-pathway is a large transmembrane receptor, which is able to recognise 2 sets of transmembrane ligands, Delta and Serrate. Upon ligand binding, Notch undergoes a proteolytic cleavage at the membrane level leading to the translocation of its intracytoplasmic domain into the nucleus where, together with the transcription factor Su(H)/RBPjk, it activates the transcription of downstream genes such as those of the *Enhancer of split* complex in the fly or *Hes1/Hes5* in vertebrates (Artavanis-Tsakonas et al. 1999). In the fly, Notch signalling has been implicated in several distinct developmental processes such as lateral inhibition or boundary formation between compartments. Many of the genes of this pathway are strongly expressed in the PSM and mutation studies in the mouse, as well as overexpression experiments in the frog, have established their role in the control of somitogenesis (Pourquie, 1999).

Studies of the dynamic expression of *Lunatic Fringe* in the chick and mouse PSM and of *DeltaC* in the zebrafish have provided a link between the segmentation clock and the Notch signalling pathway (Forsberg et al. 1998; McGrew et al. 1998; Aulehla & Johnson, 1999; Jiang et al. 2000). In the fly, *fringe* has been implicated in compartment boundary definition in the wing and the eye disk (Irvine, 1999). *fringe* encodes for a glycosyltransferase (Moloney et al. 2000; Munro & Freeman, 2000), which acts by modifying Notch signalling. It can potentiate the interaction with Delta while preventing that with Serrate. In the developing wing imaginal disk, the expression of Fringe in the cells of the dorsal compartment which also express Notch and Serrate, prevents their responding to the Serrate ligand whereas the same cells retain the capacity to respond to the Delta ligand which is expressed in the ventral compartment. This system results in a localized activation of Notch at the interface between the *Fringe* positive and negative compartments, which will ultimately become the wing margin cells.

Lunatic Fringe is the only one of the 3 *Fringe* vertebrate homologues to be expressed in the PSM (Johnston et al. 1997). In the PSM of mouse and chick embryos, *Lunatic Fringe* is expressed in a dynamic expression sequence, which is highly reminiscent of that reported for *c-hairy1*. *Lunatic Fringe* expression appears as a wave, which sweeps along the PSM and narrows as it moves anteriorly. In the chick anterior-most PSM and the newly formed somites, *Lunatic Fringe* is expressed in the anterior compartment, i.e. complementary to *c-hairy1* (McGrew et al. 1998; Aulehla & Johnson, 1999). Surprisingly, however, in the mouse, *Lunatic Fringe* is found in the posterior half of the forming somite in the rostra1 PSM and newly formed somites (Forsberg et al. 1998) indicating that some properties have been inverted between the mouse and chick anterior and posterior somatic compartments.

Expression domains of *c-hairy1* and *Lunatic Fringe* have been compared in the chick PSM and were found to be similar indicating that the 2 genes oscillate in synchrony (McGrew et al. 1998; Aulehla & Johnson, 1999). Experiments involving explant culture similar to that performed to study *c-hairy1* regulation have demonstrated that the dynamic expression of both genes is an intrinsic property of the PSM and does not involve signal propagation (McGrew et al. 1998; Aulehla & Johnson, 1999). In contrast to *c-hairy1*, however, the *Lunatic Fringe* dynamic expression sequence was disrupted by cyclohexamide treatment, indicating that it requires protein synthesis (McGrew et al. 1998). This observation led to the suggestion that *Lunatic Fringe* might act downstream of *c-hairy1*. The hairy proteins have been demonstrated to act as transcriptional repressors both in fly and vertebrates (Takebayashi et al. 1994; Barolo & Levine, 1997). These results therefore suggest that although they oscillate synchronously, the 2 genes may be regulated differently. The regulatory links between them, however, remain unclear.

In the zebrafish, *Lunatic Fringe* is not expressed in the PSM. However, the *DeltaC* mRNA was reported to be expressed in a dynamic fashion resembling the dynamic wave of expression of *hairy*-like genes in the PSM (Jiang et al. 2000). Demonstration of the cyclic expression of *DeltaC* in the zebrafish was achieved by exposing the 2 sides of the embryos to different temperatures thus desynchronizing the somitogenesis speed between the 2 sides. In addition, *Her1* is an *enhancer of split*-like gene shown to act downstream of the Notch pathway in zebrafish which was also recently demonstrated to exibit such a cyclic behaviour (Takke & Campos-Ortega, 1999; Holley et al. 2000; Sawada et al. 2000).

These observations provided a link between the segmentation clock and the Notch signalling pathway and suggest that one role of the clock could be the rhythmic control of Notch activation. Such periodic control of Notch signalling could result directly in the periodic specification of cells endowed with boundary properties, like the fly wing margin cells. The somitic boundary forms immediately rostra1 to the *Lunatic Fringe* expression domain and could in principle result from an interaction at the boundary between *Lunatic Fringe* positive and negative domains. It is however unlikely that the apposition model between Serrate and Delta expressing compartments such as that described in the fly wing disk is directly transposable to somite boundary formation. First, both chick and mouse *Serrate1* and *Dll1* expression domains overlap (our own observations and those of del Barco Barrantes et al. 1999). Secondly, Notch activation as evidenced by expression of downstream response genes such as *Hes5* is not strictly limited to the boundary domain (de la Pompa et al. 1997). Alternatively, periodic Notch signalling in the somites could drive the specification of alternating domains (such as prospective anterior and posterior), secondarily leading to boundary formation. The clock regulation of *Lunatic Fringe* expression could lead to expression of this gene in an appropriate expression domain to initiate these events. A further possibility is that the level of Lunatic Fringe protein accumulates during each cycle of expression until it reaches a threshold leading to boundary formation.

Mice homozygous null for the *Lunatic Fringe* gene exhibit gross segmentation defects (Evrard et al. 1998; Zhang & Gridley, 1998). In one allele of this mutant, irregularly distributed epithelial somites are still observed. Segmentation of the paraxial mesoderm derivatives such as muscles and skeleton was also perturbed but a metameric pattern was retained. In addition, although *Notch1*, *Delta1* and *Delta3* gene expression is maintained, their boundaries of expression appear more diffuse than in the wild type. The *Hes5* gene, a downstream target of the Notch pathway that belongs to the hairy family of transcription factors, is downregulated in the *Lunatic Fringe* mutant, indicating that Notch signalling is impaired. Therefore both segmentation and Notch signalling are clearly affected in the *Lunatic Fringe* null mouse mutant, thereby confirming its requirement during somite boundary formation. However, the mutants retain a remarkable degree of segmentation of PSM derivatives which indicates that clock control over *Lunatic Fringe* expression cannot solely account for the mechanism of metamery. Such a degree of segmentation is retained in all Notch pathway mutants indicating that Notch signalling is also unlikely to represent the only mechanism responsible for metamerisation of the vertebrate embryo. This pathway is more likely to govern the regularity and the coordination of the segmentation process. Whether the clock is essentially devoted to this latter process or whether it may also play an important role in the generation of metamery remains to be determined.

Is Notch signalling part of the core oscillator?

Notch function might not only lie downstream of the clock. Recent studies suggest that Notch signalling might also participate to control the expression of

cycling genes and could thus be part of the oscillator itself. In the mouse, the *Hes1* gene has been well characterised as a downstream target of the Notch pathway (Jarriault et al. 1995). *Hes1* is a murine orthologue of *hairy2*, a novel cycling gene identified in the chick (Jouve et al. 2000). A reinvestigation of the *Hes1* expression pattern in the mouse PSM reaveled it to be cyclical, like its chick counterpart. The dynamic expression of *Lunatic Fringe* in the PSM of *Hes1* null mutant mice is not altered indicating that *Hes1* is more likely to act downstream of the segmentation clock as has been proposed for the *hairy1* gene in the chick. The lack of a somitic phenotype in *Hes1* mutants could also be due to functional redundancy with the mouse homologue of the chick *hairy1* gene. However, extensive efforts for cloning the *hairy1* homologue in the mouse have proven unsuccessful suggesting that rodents might only have one oscillating *hairy*-like gene.

Taken together, the absence of an obvious somitogenic phenotype in *Hes1* mutant mice, and the existence of 2 different *hairy/E*(*spl*)-like genes in the chick raise the possibility that they may exist a second *hairy/E*(*spl*)-like gene in the mouse. We therefore decided to look for such a gene in the mouse. The first strategy employed was the RT-PCR technique using degenerate primers, corresponding to the bHLH domain of the protein, on total RNA samples extracted from E10.5 embryos. After cloning the PCR fragments, the DNA was sequenced and analysed. All the clones obtained corresponded to the *Hes1* sequence. This is probably due to the fact that analysis of the *Hes1* expression profile in mouse reveals a very wide expression. To bypass this *Hes1* expression problem, we decided to screen a genomic DNA library at low stringency using *c-hairy1* as a probe. After 3 rounds of screening, we were able to isolate 7 clones. After analysis of these clones, we found that 3 of the 7 corresponded to the *Hes1* gene, and 4 were cloning artifacts. In the chick, the *c-hairy2* gene could be identified because it cross-reacts with the *c-hairy1* probe. Under this premise we decided to screen a mouse E13.5 embryonic cDNA library at low stringency using m*Hes1* as a probe. After 3 rounds of screening we isolated 87 clones. These 87 clones were hybridised with the m*Hes1* probe at high stringency to distinguish the *Hes1* clones. Fifty clones were eliminated, in this way, the 37 remaining clones were analysed by sequencing. It appeared each of these 37 clones were cloning artifacts. Taken together, these results demonstrate that we were unable to clone anything except *Hes1* using these strategies. In an attempt to bypass this problem, the last strategy adopted was to try an RT-PCR strategy on RNA extracted from caudal regions of *Hes1*-/-embryos, using degenerate primers. After cloning and sequencing, we found that all the clones obtained using this strategy corresponded to the m*Hes5* gene, another member of the HES family. All together, these results suggest that there is not a homologue of *c-hairy1* in the mouse.

The fact that *Hes1* oscillates in the PSM suggests that Notch activation might play a role in this process. This was directly tested by examining the expression of *Lunatic Fringe* and *Hes1* in mouse Notch pathway mutants. In mutants such as *Delta1*-/- in which Notch signalling in the PSM is thought to be altered, the dynamic expression of both *Lunatic Fringe* and *Hes1* is lost. Similarly, analysis of *Lunatic Fringe* pattern in several mouse mutants for the Notch signalling pathway such as *Dll1* or *RBPJk* reveals a severe down-regulation and a loss of its dynamic expression in these animals (del Barco Barrantes et al. 1999). Since these genes are not expressed according to a dynamic expression sequence, a simple direct link such as that proposed between *c-hairy1* and *Lunatic Fringe* is unlikely.

This suggests that Notch signalling does not only act downstream of the segmentation clock but appears to be required for the periodic expression of the cycling genes in the mouse. Therefore Notch may be a part of the oscillator or it could act as a necessary cofactor for the expression of the cycling genes. The Notch pathway could play an important role via a negative feedback loop in the coordination of gene expression in neighbouring cells during the progression of the *c-haily1* and *Lunatic Fringe* wavefront in the PSM.

A role for Notch signalling in coordinating oscillations between PSM cells?

In the zebrafish Notch pathway mutants *mindbomb* and *after eight*, the dynamic expression of *Her1* and *DeltaC* is lost (van Eeden et al. 1998; Jiang et al. 2000). However, the 2 genes are still expressed in the rostral PSM in a large static band of expression in which a salt and pepper expression pattern is observed. Lower expression is seen in the rest of the PSM. Jiang et al. (2000) recently proposed that this static expression pattern did not result from an arrest of the dynamic expression of the *DeltaC* mRNA but was due to a loss of synchronisation between *DeltaC*-expressing cells in these mutants. They proposed that Notch-signalling was not necessarily required for the

function of the oscillator but to coordinate the expression of the cyclic genes between PSM cells. An attractive consequence of this hypothesis is that it could explain why in both mouse and fish Notch pathway mutants the formation of the first somites is spared whereas the caudal somites do not segment. If one postulates the existence of a Notch-independent oscillator, at the onset of somitogenesis this oscillator could be set going synchronously in PSM precursor cells. In these mutants, in the absence of the Notch coordinating system, cells would progressively drift out of synchrony until somitogenesis fails.

CONCLUSION

In all vertebrate species examined thus far, somites are produced bilaterally in a periodic fashion from a mesenchymal zone located immediately anterior to the gastrulation site. Evidence for genes expressed in a periodic fashion in the PSM has now been provided in distantly related species such as fish, birds and mammals (Pourquie, 1999; Holley et al. 2000; Jiang et al. 2000; Sawada et al. 2000), indicating that the segmentation clock has been conserved throughout vertebrate evolution. It is particularly striking to note that all the oscillating genes identified thus far are related to the Notch pathway whereas in insects such as Drosophila, this pathway does not play an important role in the segmentation process. Therefore, in contrast to other patterning systems such as the Homeotic genes which have been well conserved during evolution, it is possible that the segmentation mechanisms arose largely independently in invertebrates and vertebrates.

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