Developmental regulation of the Hox genes during axial morphogenesis in the mouse

Jacqueline Deschamps* and Johan van Nes

Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands
*Author for correspondence (e-mail: jacqueli@niob.knaw.nl)

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Summary

The Hox genes confer positional information to the axial and paraxial tissues as they emerge gradually from the posterior aspect of the vertebrate embryo. Hox genes are sequentially activated in time and space, in a way that reflects their organisation into clusters in the genome. Although this co-linearity of expression of the Hox genes has been conserved during evolution, it is a phenomenon that is still not understood at the molecular level. This review aims to bring together recent findings that have advanced our understanding of the regulation of the Hox genes during mouse embryonic development. In particular, we highlight the integration of these transducers of anteroposterior positional information into the genetic network that drives tissue generation and patterning during axial elongation.

Introduction

The regulation of the patterning of the trunk and tail as they develop is a function of the homeobox-containing Hox gene family, which has been evolutionarily conserved among the metazoans. The conservation of the structure and function of these genes may lie in their ability to adequately provide an identity to the anteroposterior (AP) structures during embryogenesis. An excellent example of this property is the ontogeny of the vertebral column. During this process, pairs of mesodermal blocks are established sequentially on either side of the neural tube as the vertebrate embryo develops. Although morphologically very similar, these blocks will differentiate into distinct mesodermal tissues, depending on their axial level. The identity of these blocks, or somites, is specified by their unique combinatorial expression of the Hox genes. For example, the first trunk somites to form will give rise to the most anterior prevertebrae. Their anterior (or rostral) identity is achieved through the exclusive expression of the 3′ Hox genes (Fig. 1). The next somites to form acquire a more posterior (or caudal) identity through the expression of these 3′ Hox genes, together with the following more 5′ Hox genes. All axial and paraxial tissues between the middle of the hindbrain and the tip of the tail acquire differential and combinatorial Hox expression patterns, irrespective of whether they are segmented. Lateral plate mesoderm and spinal cord cells, for example, also express a differential combination of Hox genes depending on their ultimate axial level.

The combination of Hox genes expressed in a specific AP region has been called its ‘Hox code’ (Kessel and Gruss, 1991). The correspondence between the order of the Hox genes on their chromosome and the anterior-to-posterior sequence of the structures that express them has been called ‘spatial co-linearity’ (for reviews, see Krumlauf, 1994; Kmita and Duboule, 2003). Furthermore, in mammals and in short germ-band insects, which, unlike *Drosophila*, extend their axis progressively by adding new tissues from their posterior end, 3′ Hox genes are expressed first, whereas more 5′ Hox genes are expressed later and sequentially. This latter phenomenon has been called ‘temporal co-linearity’ (reviewed by Kmita and Duboule, 2003) (see Fig. 1). The intimate relationship between the co-linear timing of Hox gene expression and morphogenesis may initially have played an evolutionary constraining role in maintaining the Hox genes in their chromosomal clusters (Kmita and Duboule, 2003; Ferrier and Minguillon, 2003). However, an extensive analysis of the molecular mechanisms that modulate axial Hox gene expression strongly suggests that coordinated expression is achieved through a variety of species-dependent mechanisms. The strategy by which these genes are expressed in a correct spatiotemporal pattern at the molecular level appears not to matter too much, providing that the proper Hox protein distribution is achieved (Kmita and Duboule, 2003).

This review focuses on recent data on Hox gene regulation that shed new light on the integration of AP patterning into the morphogenetic programme that drives embryogenesis. We evaluate the importance of the early transcriptional activation of these genes in the posterior primitive streak, as well as the role of the node region in modulating the Hox gene expression domain during the laying down of axial and paraxial tissues. We survey recent work on how the signalling molecules that have a crucial role in the onset of patterning in the neural tube and mesoderm influence Hox gene transcription. We then focus on a class of Hox regulators, the Cdx transcription factors, that participate in tissue generation during axial extension, as well as in AP patterning, and discuss how these processes are intimately linked. Regulatory events that ensure the long-term memory of the transcriptional states of the Hox genes are also discussed, together with recent findings that some epigenetic marks that ensure the inheritance of the expression status of the Hox genes also act as a chromatin-editing system. This system differentially sensitises the Hox genes to transcription at early stages of development in tissues that will only later

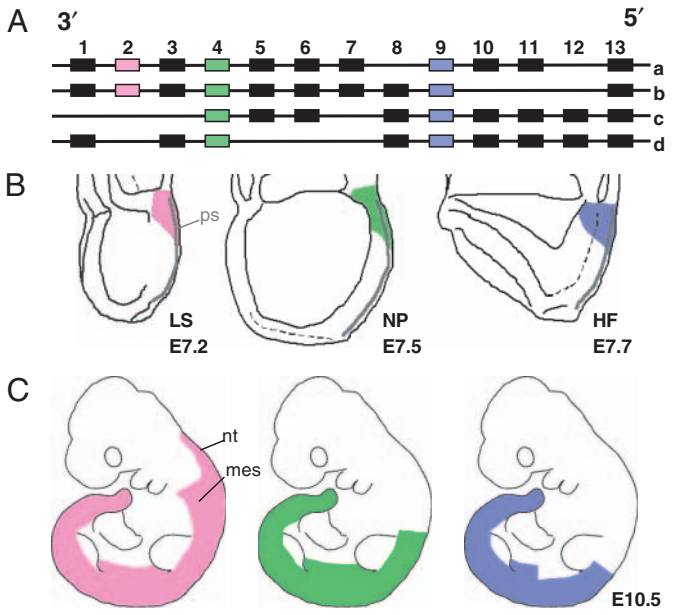

Fig. 1. Mouse Hox genes and their early temporal and spatial co-linearity of expression. (A) The four Hox clusters (a to d). Hox genes with the same number (1 to 13) are called paralogs. Three paralog groups are shown (2, 4 and 9) in colour to illustrate the early temporal co-linearity of their expression, as shown in B. (B) Mouse embryos at: embryonic day (E) 7.2, late streak (LS) stage; E7.5, neural plate (NP) stage; and E7.7, head fold (HF) stage. (C) An E10.5 embryo, showing the spatial co-linearity of Hox gene expression. *Hox*2 paralogs begin to be expressed earlier, and *Hox*4 and *Hox*9 paralogs progressively later, in the posterior part of the primitive streak (ps, indicated by a grey line on the posterior side of the embryos in B). At E10.5, the expression domains of the 3′ genes extend to more anterior positions than that of the more 5′ genes. For each gene, the expression boundary is more anterior in the nervous system than in the mesoderm. mes, mesoderm; nt, neural tube. Actual widths of embryos at widest point: LS, 0.26 mm; NP, 0.44 mm; HF, 0.60 mm; E10.5, 4.1 mm.

express these genes. We also discuss other parameters that correlate with Hox gene transcription. Finally, we consider how the genomic area that surrounds a Hox cluster contributes to controlling tissue-specific Hox gene expression in the limbs and, independently, spatiotemporal expression in the trunk. [Other aspects of the regulation of the Hox genes are discussed elsewhere (Krumlauf, 1994; Rijli et al., 1998; Trainor and Krumlauf, 2000; Kmita and Duboule, 2003).]

Initiating Hox gene expression

In mice, temporal co-linearity of Hox gene expression is observed from the very first transcription initiation event in the posterior primitive streak (see Figs 1 and 2), an area that is fated to become extra-embryonic mesoderm. The regulation of the Hox genes at this very early stage in the posterior-most epiblast does not directly concern the future axial and paraxial embryonic structures, as these are derived from more anterior cells within and around the anterior streak (Lawson et al., 1991). However, the sequential initiation of Hox transcription will determine the time at which the expression domains successively reach the anterior primitive streak, or node region, from where the embryonic axis mainly extends (Fig. 3) [Forlani et al. (Forlani et al., 2003) in mice; Wacker et al. (Wacker et al., 2004) in *Xenopus*]. The precise temporal activation of the Hox genes at the initial stages in the generation of their expression domains is therefore crucial for establishing regional identity. For example, in the mouse, a *Hoxc8* regulatory mutation affects skeletal patterning by causing a transient delay in the initial transcription of the Hox gene. This mutation phenocopies many of the axial defects of the *Hoxc8*-null mutant (Juan and Ruddle, 2003).

Initial Hox transcription and the early rostral expansion of Hox expression domains are regulated by events that are connected to the emergence and extension of the primitive streak (Forlani et al., 2003). Wnt signals that regulate the formation and function of the primitive streak may modulate Hox gene expression during its anteriorward spreading (Forlani et al., 2003). Fgf signalling that modulates the morphogenetic movement of the mesoderm at the primitive streak (Ciruna and Rossant, 2001) may regulate the Hox genes as well. A role for retinoic acid (RA) in initiating Hox gene expression has also been suggested because endogenous RA has been detected in the posterior part of early post-implantation embryos (Hogan et al., 1992). This role is more difficult to ascertain, however, because at early developmental stages, embryos with impaired RA biosynthesis exhibit rather normal initial 3′ Hox gene expression domains (Niederreither et al., 1999).

After expanding anteriorly in and along the primitive streak, the Hox expression domains continue to spread and sweep through the node region (Figs 2 and 3). This region has proved to be crucial for the generation of axial and paraxial structures (Lawson et al., 1991; Beddington, 1994) (reviewed by Joubin and Stern, 2001), and to constitute an ‘organizing’ area of gene expression that is traversed by cells that contribute to the extending axial tissues (Joubin and Stern, 1999). However, cell lineage analysis in mouse embryos has shown that the Hox codes are not fixed in the node region (Forlani et al., 2003), as Hox gene expression appears to be modified after nascent mesoderm and neurectoderm have been generated there. This modulation occurs independently in mesoderm and neurectoderm (Forlani et al., 2003). Below, we discuss how a variety of regulatory influences progressively modulate Hox gene expression in the interval between the emergence of cells from the primitive streak and the time when they acquire their definitive Hox identity.

Generating and patterning embryonic mesoderm

The cohorts of cells that leave the primitive streak at the neural plate (E7.5) stage to contribute to future rostral somites express 3′ Hox genes exclusively. The next cohorts to leave the streak express these 3′ Hox genes together with more 5′ Hox genes. This early Hox expression program does not correspond to the definitive Hox codes of the axial and paraxial descendants of these cells, which will only be fixed later on, upon receiving additional regulatory influences. Paraxial mesoderm cells receive patterning signals, including positional information, as they transit in the presomitic mesoderm (PSM), after they have emerged from a zone just posterior to the node that has been suggested to contain stem cells for the elongating axis (see the stem cell zone shown in Figs 2 and 3) (Nicolas et al., 1996; Cambray and Wilson, 2002; Eloy-Trinquet and Nicolas, 2002). It is in this crucially important phase that cells are exposed to threshold values of the caudorostral gradients of Fgf (Dubrulle

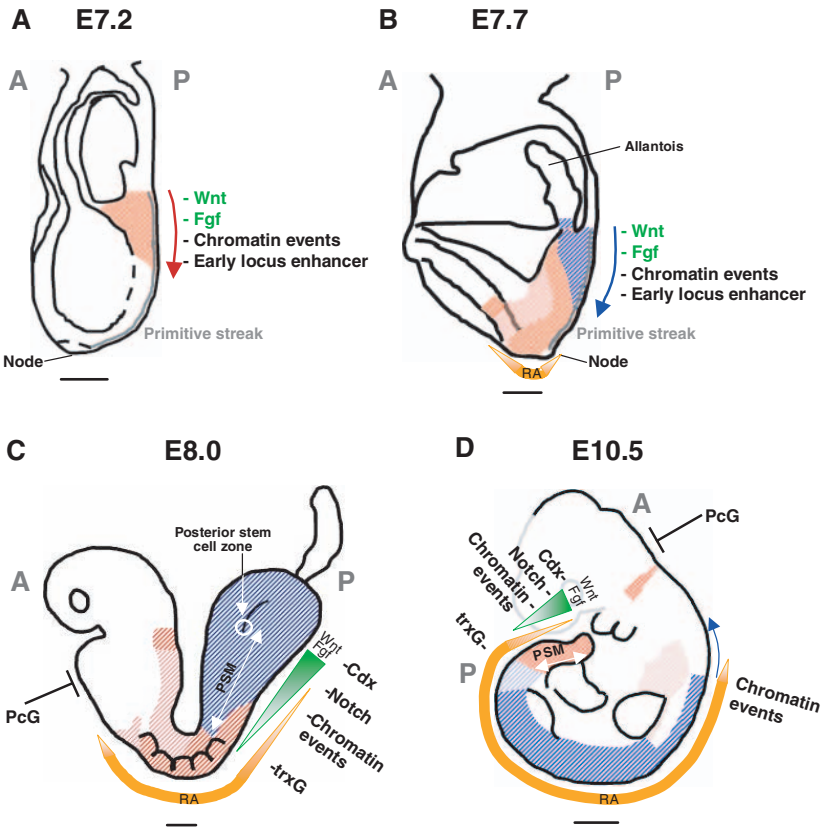

Fig. 2. Regulation of Hox gene expression throughout mouse embryogenesis. Expression of a 3′ Hox gene, *Hoxb1* (red), and of a 5′ Hox gene, *Hoxb8* (blue), at different developmental stages in mouse embryos. Posterior (P) is towards the left of the E10.5 embryo, owing to axial rotation, which mouse embryos undergo between E8.5 and E9.0. (A) E7.2, late primitive streak stage embryo, with the primitive streak (grey) reaching the node at the distal tip of the embryo. The red arrow shows the direction of the anterior expansion of the *Hoxb1* expression domain. (B) E7.7, late head fold stage embryo, showing a maximally extended *Hoxb1* expression domain (red) and an early expression field for *Hoxb8* (in blue overlaying *Hoxb1* expression). The blue arrow indicates the anteriorwards spread of the *Hoxb8* expression domain; the orange line indicates retinoic acid (RA) in the mesoderm. (C) E8.0, five-somite stage embryo; the remnant of the primitive streak is shown in black medioposteriorly, with the node region at its anterior end. White circle indicates posterior stem cell zone. (D) E10.5 embryo; the remnant of the primitive streak is in the tailbud. *Hoxb1* expression is downregulated and remains strong in rhombomere 4 anteriorly, and in the tailbud posteriorly. *Hoxb8* expression is about to be induced by RA to extend rostrally into the posterior hindbrain (blue arrow). The role of Wnt and Fgf at the early stages (A,B) is assumed, but not definitively documented. See Fig. 8 for more detail on the role of the early locus enhancer, and Fig. 7 for that of the chromatin events. PSM, presomitic mesoderm. Green triangle indicates posterior-to-anterior Fgf and Wnt gradients. trxG and PcG, trithorax group and polycomb group protein complexes, which activate and repress Hox gene expression, respectively. Scale bars: 100 μm for A-C; 75 μm for D.

et al., 2001) and Wnt signals (Aulehla et al., 2003), and to the rostrocaudally decreasing RA gradient (reviewed by Dubrulle and Pourquié, 2004a). These signals couple the speed of paraxial mesoderm production during axial elongation to the rate of somite formation (Dubrulle and Pourquié, 2004a). The mechanism that generates the Fgf gradient was recently elucidated (Dubrulle and Pourquié, 2004b). Fgf gene transcription is exclusively restricted to cells in the ‘stem cell zone’ of the node region, and transcript levels decrease thereafter in their descendants (which are carried away during the extension of the axis). Fgf and Wnt proteins, key players in the maturation and commitment of paraxial mesoderm to its segmental fate, are molecules that have all been shown to regulate Hox genes either directly or indirectly: thus, *Wnt3a* and *Fgfr1* hypomorphic mutations restrict Hox gene expression domains to more posterior positions and give rise to vertebral anterior transformations along the AP axis (Ikeya and Takada, 2001; Partanen et al., 1998). Thus, cells at the node do not have their definitive Hox code yet, and will acquire it upon receiving graded signals in regions anterior to the node at later developmental stages (Fig. 4).

Retinoids, which did not seem to be involved in the initial activation of the Hox genes in the primitive streak, do regulate mesoderm segmentation (Moreno and Kintner, 2004). The precise distribution of RA in the PSM is likely to be crucial for proper Hox regulation in this tissue because abnormal RA dose or signalling, which impairs mesodermal patterning (Niederreither et al., 1999; Abu-Abed et al., 2001; Sakai et al., 2001), also leads to AP patterning and Hox regulation defects (Kessel and Gruss, 1991; Lohnes et al., 1993).

In addition to the influence of these morphogens, Hox gene expression in the anterior PSM is affected by mutations in genes that function in the segmentation program itself. Loss- or gain-of-function mutations in genes of the Notch pathway, or mutations that alter the temporal periodicity of their expression, affect Hox gene expression in the PSM (Zákány et al., 2001; Cordes et al., 2004), and subsequently vertebral patterning (Cordes et al., 2004). The effect on Hox gene expression caused by altering Wnt signalling or by mutating genes of the oscillatory ‘clock mechanism’ are, in fact, related, as recent work has shown that the gradient of Wnt signals that controls PSM maturation directly crossregulates the fluctuating expression of the segmentation genes in the Notch pathway (Hofmann et al., 2004; Galceran et al., 2004) during somitogenesis. The very first link between the segmentation genetic program and Hox gene expression came from the discovery that discrete stripes of expression of the Hoxd genes exist that closely correlate with the segmentation process caudal to the last-formed somite (Zákány et al., 2001). When the Hoxd cluster was replaced with a *lacZ* reporter under the control of a Hox promoter, the reporter gene also showed bursts of reinforced gene expression, indicating that regulatory sequences outside of the Hoxd cluster account for the dynamic expression of the genes in the PSM, in phase with the segmentation process. These data indicate that transient bursts of Hox gene activation occur each time cells approach the

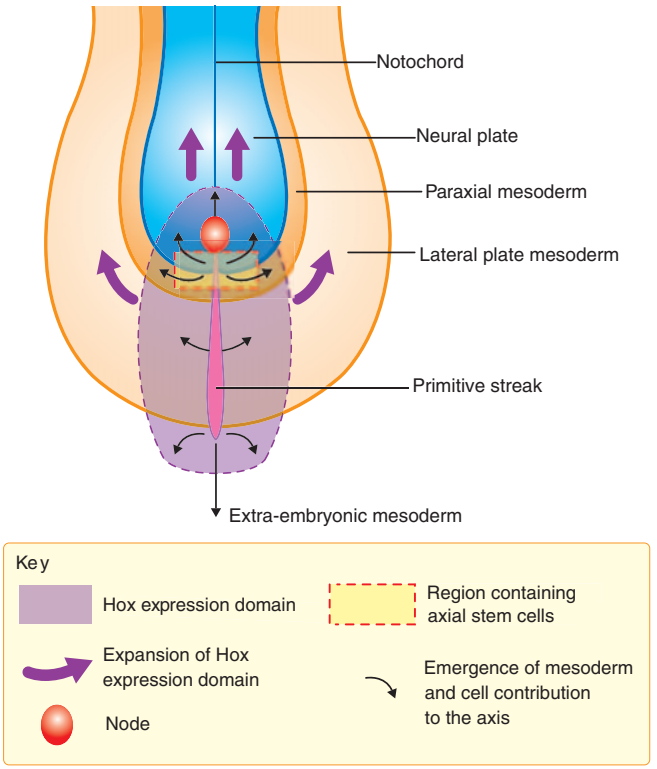

Fig. 3. Rostral progression of Hox expression through the primitive streak region. The rostrally extending expression domain (purple) of a Hox gene (from the middle of a Hox cluster) in the posterior region of an E7.7 presomitic mouse embryo (anterior is towards the top). This Hox expression domain encompasses the area where cells emerge from the primitive streak and from the axial stem cell zone (see black arrows). It also crosses the posterior region of the axis, which is undergoing morphogenesis. Extra-embryonic mesoderm is produced from the posterior levels of the streak. Lateral plate and paraxial mesoderm emerge from more anterior levels. The approximate position of the axial stem cells is shown (yellow). The descendants of these stem cells contribute to the extending paraxial mesoderm and neural plate (black arrows). The node produces axial mesoderm (notochord), endoderm and the ventral midline of the neural plate (not shown). Extra-embryonic mesoderm production is indicated for the sake of completeness, although it has stopped by this stage.

PSM/somite transition. These bursts may provide the cells with specific PSM 'instructions', which add to the information already received by their progenitors during early Hox gene activation in the primitive streak. This would provide these new somites with at least a component of their AP identity. A mutation that inactivates *RBPjk* (*Rbpsuh* – Mouse Genome Informatics), the effector of the Notch pathway, abolishes these stripes and leads to the downregulation of mesodermal Hox gene expression (Zákány et al., 2001). Another link between the segmentation genetic program and Hox gene expression has been highlighted in a recent study (Cordes et al., 2004), which found that reduced Notch signalling caused by a dominant-negative form of the Notch ligand *Dll1* results in the anterior transformation of vertebral identity and a posterior shift of the expression domain of several Hox genes. In addition, the loss of the oscillatory character of lunatic fringe (*Lnfg*) expression, a crucial modulator of Notch function in the PSM, led to vertebral transformations and to a shift of the expression domain of the same Hox genes. Rather than observing a general drop in Hox gene expression levels in the PSM, Cordes and co-workers found that reduced Notch signalling caused a shift in the rostral extension of the expression domains of the Hox genes (Cordes et al., 2004). These two studies could appear to produce different results because of the different ways in which the Notch pathway has been affected in these experiments. Inactivating RBPjk, which is essentially required by the Notch intracellular domain to transcriptionally activate its targets, totally abolishes Notch signalling and therefore drastically affects gene expression. Weakening the action of Dll1 or altering the cyclic expression of a modulator of the Notch interaction with its ligands only partially affects the Notch signalling pathway, possibly leading to a delay, rather than to a decrease, in Hox gene expression in the PSM. In any case, although the interactions between Hox genes and the Notch pathway at the molecular level remain unclear, these two studies underscore the existence of a link between the acquisition of positional identity by Hox gene expression and the activity of the genetic cascade that drives somitogenesis.

It is becoming clear that somitogenesis is tightly coupled to the generation of posterior tissues (reviewed by Dubrulle and Pourquié, 2004a), as it is the balance between the two processes that modulates axial extension. The studies cited above thus suggest that a link exists between mesoderm maturation and segmentation, the specification of AP identity via the Hox genes and the growth of the axis (Figs 2 and 4).

In addition to patterning the paraxial mesoderm (as discussed here) and the lateral mesoderm (such as the emerging limb bud mesenchyme, which is not dealt with in detail in this review), Hox genes supply AP identity to the neurectoderm between the middle of the hindbrain and the caudal end of the embryo. We will see in the next section that the regulation of patterning in the mesoderm and neurectoderm is tightly coordinated and uses common morphogenetic signalling. And so is the control of the expression of the Hox genes in these tissues.

Regulating Hox gene expression in the neurectoderm

A distinction must be made here between the regulation of the Hox genes in the anterior part of their neural expression domains in the forming hindbrain, and the regulation of these genes in the posterior spinal cord, where the axis elongates by the production of new tissue from the node region. The hindbrain neurectoderm is generated from a small region of the epiblast that is located anterolaterally to the node at the late streak stage (Lawson et al., 1991; Forlani et al., 2003). The anterior rhombomeres (r3 and r4) in the neurectoderm, which will form the rostral part of the expression domain of the 3′ most Hox genes, are laid down sequentially at the neural plate (E7.5) and subsequent stages, as the axis extends. At that time, the expression domains of the Hox genes are still located more posteriorly (Forlani et al., 2003). RA is present at these stages in chick embryos at AP positions just posterior to the forming hindbrain, where it diffuses from the underlying mesoderm (Blentic et al., 2003) (Fig. 2B and Fig. 4). It has been proposed that this signalling molecule provides the hindbrain rhombomeres with AP positional identity by inducing 3′ to

more 5′ Hox genes (Gavalas and Krumlauf, 2000; Dupé and Lumsden, 2001) in the hindbrain. Two main conditions are essential for the normal expression of the Hox genes in the hindbrain: the distribution of the inducing signals and the sensitivity of the promoter region of the Hox genes to these signals. The decreasing concentration of RA diffusing from the boundary of RA production in the mesenchyme, combined with the increasing sensitivity to RA of 5′ to 3′ Hox genes, generate the unique combinations of Hox genes expressed in r3 to r8 that define rhombomere identity (Gould et al., 1998; Gavalas and Krumlauf, 2000; Dupé and Lumsden, 2001). Increasing levels of retinoids are required for the Hox-mediated specification of the identity of chick rhombomeres 3 to 8 (Dupé and Lumsden, 2001). In the mouse, the anterior expression boundaries of 3′ to 5′ Hox genes in the hindbrain directly depends on endogenous retinoids (Niederreither et al., 2000; Oosterveen et al., 2003) and on functional retinoic acid responsive elements (RAREs) around some of the Hox genes. These RAREs are active in sequential, co-linear time windows; this time window is earlier for the *Hoxb1* RAREs than for the *Hoxb4* RARE, and for the RARE located between the *Hoxb4* and *Hoxb5* (reviewed by Gavalas and Krumlauf, 2000; Oosterveen et al., 2003). The expression of the Hox genes in the hindbrain therefore undergoes a spatially and temporally co-linear regulation by RA. In addition to the mesoderm-derived signals such as RA, rhombomere-specific transcription factors modulate the expression of Hox genes in the neurectoderm itself, including the r3- and r5-specific Krox20 (Egr2 – Mouse Genome Informatics), the r5- and r6-specific kreisler, and *Hoxb1* and *Hoxb4*, which act in autoregulatory loops in r4 and r6, respectively.

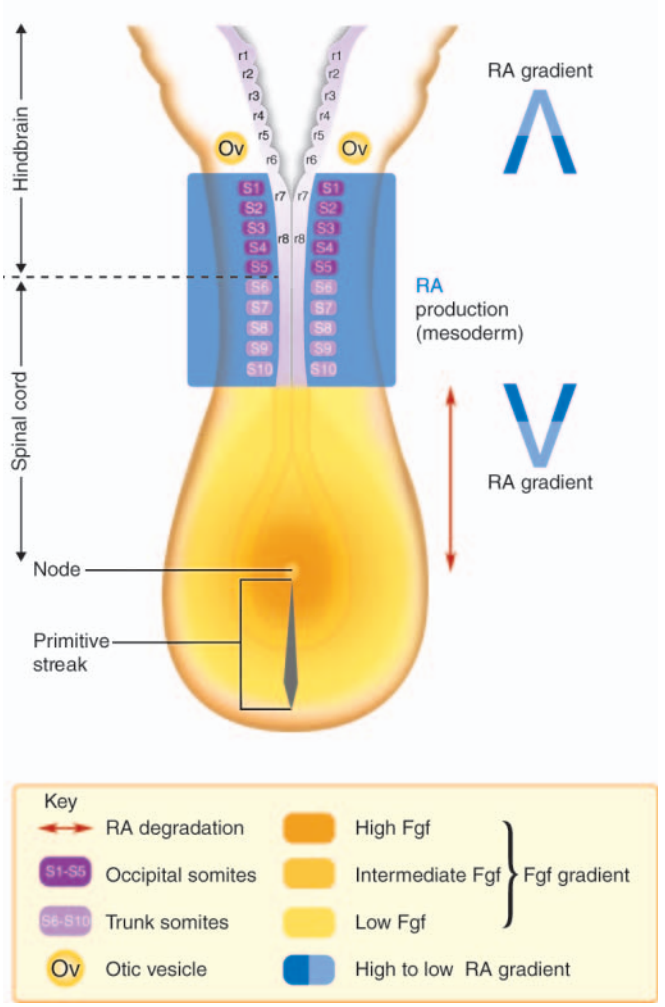

Fig. 4. Signalling molecules that affect Hox gene expression along the AP axis. An E8.5 (10 pairs of somites) stage mouse embryo, showing the hindbrain and spinal cord in the neural tube, and the occipital and trunk somites in the paraxial mesoderm. The distribution of RA is indicated in blue. RA is synthesized by Raldh2 in the somites. Anteriorly, RA diffuses into the hindbrain, where the Hox genes are differentially sensitive to RA. For example, rhombomeres (r) 3 and 4, where RA concentration is low, express only the most 3′ Hox genes; r6 to r8 express the 3′ plus more 5′ Hox genes. Posteriorly to somite levels, the concentration of diffusing RA decreases more sharply because of the activity of a RA-degrading enzyme, Cyp26 (see red double-headed arrow, which also shows the extent of the presomitic mesoderm). Other signalling molecules present posteriorly are Wnt (not shown) and Fgf. Fgf signals (orange/yellow) are abundant around the node region and decrease gradually to fade out in the neurectoderm and in the mesoderm at the level of the last-formed somite. The node region and its nearby pool of stem cells (see Fig. 3) are exposed to high Fgf concentrations. The mesoderm and neurectoderm cells exposed to low Fgf concentrations are maturing. As the axis extends, ‘younger’ cells come to experience this decreasing Fgf concentration.

In the posterior part of the embryo, the elongation of the embryonic trunk and spinal cord occurs in a process that continues gastrulation. As discussed in the previous section, this process involves the maintenance of a posterior zone of self-renewing stem cells that contributes descendants to the elongating neural tube and mesoderm (Mathis et al., 2001; Dubrulle and Pourquié, 2004a; Diez del Corral and Storey, 2004). Complex regulatory interactions modulate the expression of the Hox genes in the interval between the emergence of new cells from the posterior stem cell zone and their final contribution to the elongating neural tube. In addition to the diffusion of RA from the somitic mesoderm at AP levels anterior to the newly generated neural cells, Fgf produced in an area centred around the node region modulates axial extension in the neurectoderm and regulates the expression of the Hox genes (Fig. 4). Fgf signalling has been shown in the chick to be essential for the maintenance of the progenitor cell population throughout the period of spinal cord elongation (Mathis et al., 2001). Gradually decreasing Fgf concentrations at more anterior positions are thought to regulate the transition between the proliferating progenitor cells (high Fgf) and the maturing neurons escaping from the stem cell zone (low Fgf) (Mathis et al., 2001). Interestingly, RA and Fgf have an opposite effect on the cells: while mesoderm-derived RA stimulates the maturation and differentiation of cells in the young spinal cord, Fgf produced by both the ectoderm and mesoderm in the node region prevents this differentiation. The integration of both signals acts as a switch that coordinates the patterning of the extending spinal cord with that of the mesoderm (reviewed by Diez del Corral and Storey, 2004). The mutually inhibitory action of RA and Fgf signalling ensures that neuronal maturation progresses in concert with the generation of new somites (Diez del Corral and Storey, 2004). It is likely that the combined action of RA and Fgf coordinately regulate the expression of the Hox genes in both tissues, while additional

regulatory inputs also act on neurectoderm and mesoderm independently.

In addition to regulating the release of cells from the stem cell zone and their neural differentiation when they become flanked by somites, RA and Fgf modulate a subsequent phase of neural differentiation. They are essential for the rostrocaudal modulation of Hox gene expression during neuronal cell fate specification in the ventral spinal cord (Liu et al., 2001). It has been demonstrated that graded Fgf signals from Hensen's node region and retinoids from the cervical paraxial mesoderm both contribute to the establishment of the rostrocaudal pattern of Hoxc gene expression in the progenitors of chick motoneurons (Liu et al., 2001). At stages later than E10.5, the action of Fgf is enhanced at posterior levels by the TGFβ family member Gdf11, which diffuses from the paraxial mesoderm and induces expression of 5′ Hoxc genes at thoracolumbar levels (Liu et al., 2001). These successive episodes of signalling that regulate Hox gene expression in nascent, maturing and differentiating neurectoderm during embryogenesis are brought together in Fig. 4. The following section focuses on the role of the Cdx transcription factor family, which regulates the Hox genes and integrates several posterior signalling pathways, in the genetic network that links AP patterning to the extension of the body axis.

The Hox regulator Cdx and development of axial structures

The Cdx genes are relatives of the Hox genes. Both gene families are believed to derive from a common *ProtoHox* ancestral cluster (Pollard and Holland, 2000) (see Box 1). The products of the three Cdx genes directly regulate vertebrate Hox genes in mesoderm and neurectoderm in a dose-dependent way (Subramanian et al., 1995; Charité et al., 1998; Pownall et al., 1996; Isaacs et al., 1998; Gaunt et al., 2004), and modulate the morphogenesis of vertebrae. The transcriptional stimulation of the Hox clusters by Cdx proteins occurs at Cdx-binding sites, which are often found in clusters throughout the Hox complexes. Although recent data have suggested that the Cdx proteins normally do not affect all Hox genes to the same extent (van den Akker et al., 2002; Bel-Vialar et al., 2002; Houle et al., 2003), a complete picture of how normal Cdx inputs increase the expression levels of the different 3′ to 5′ Hox genes remains to be established. The expression of the three Cdx genes at two stages of mouse embryogenesis is shown in Fig. 5. The data derived from the effects of Cdx mutations on anterior-to-posterior vertebral patterning suggest that Cdx genes affect the Hox code at 'cervical' to 'caudal' axial levels (van den Akker et al., 2002; Houle et al., 2003) (reviewed by Lohnes, 2003). Although *Cdx2* is not expressed more rostrally than the PSM, *Cdx2* mutations do alter Hox gene expression and the identity of vertebrae at cervical levels, implying that the molecular interactions between Cdx proteins and Hox genes occur early in the PSM (van den Akker et al., 2002). Several other aspects of the Hox/Cdx regulatory interaction are worth highlighting. First, the effect of combined Cdx mutations tested so far on Hox gene expression is modest. Whether this is due to the existence of other simultaneous regulatory pathways affecting Hox gene expression, and/or functional redundancy between the three Cdx genes, remains an unresolved issue. Second, it is not yet clear whether the Fgf and Wnt morphogenetic signals are transmitted to the Hox

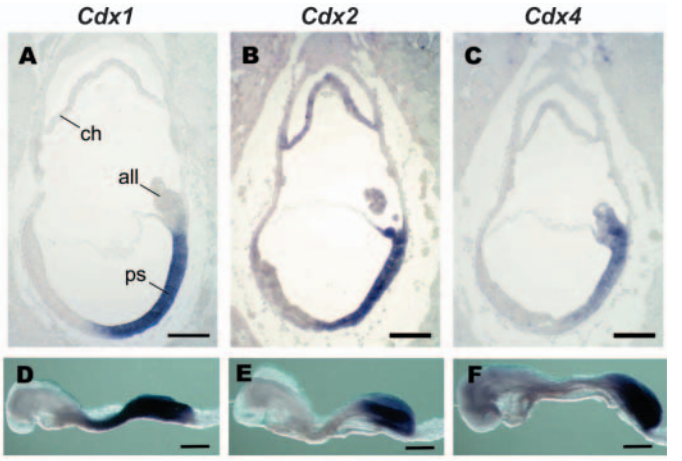

Fig. 5. Hox-like Cdx expression in embryos at gastrulation and early somite stages (A-C). Expression of the three Cdx genes in the primitive streak (ps) resembling that of 3′ Hox genes (see Fig. 2). *Cdx2* and *Cdx4* are also expressed at the base of the allantois (all), and *Cdx2* is expressed in the chorionic ectoderm (ch). *Cdx2* is expressed earlier in the trophectoderm, where it is required for implantation (Chawengsaksophak et al., 1997; Strumpf et al., 2005). (D-F) The three Cdx genes are expressed strongly in posterior embryonic tissues (all three germ layers) at somite stages (anterior is towards the left). (D) The expression of *Cdx1* extends to more anterior positions than that of *Cdx2* (E) and *Cdx4* (F). At later stages, Cdx genes are expressed in gene-specific patterns in the gut endoderm (not shown) (see Beck et al., 2000). Scale bars: 100 μm. ps, primitive streak.

Box 1. Cdx genes and the ancestral mechanism of axial extension

Vertebrates, arthropods and short germ-band insects develop their axial structures in strikingly similar ways, even though their somites and segmental metameres differ substantially from each other (reviewed by Tautz, 2004). In these phyla, axial tissues are produced sequentially from a posterior presegmental 'growth zone', and they acquire their AP identities as they emerge from this zone. During germ band elongation, the expression of *Caudal* (*cad*) homologs is restricted to the posterior growth zone in the coleopteran insect *Tribolium* and in the arthropod *Artemia*, until all body segments are formed (Copf et al., 2004). Cdx expression persists in the vertebrate embryonic tailbud and presomitic mesoderm during axial elongation. Recent work makes it clear that a functional role for Caudal in posterior axial elongation has been conserved in modern short germ-band arthropods (Copf et al., 2004), in intermediate germ-band insects (Shinmyo et al., 2005) and in vertebrates (van den Akker et al., 2002; Chawengsaksophak et al., 2004). The Cdx genes, which belong to the *ParaHox* gene family, share common ancestry with the Hox genes (Pollard and Holland, 2000). Of the three paralogous Cdx genes, only *Cdx2* has retained its location on a cluster with two other *ParaHox* genes. It is not clear which Hox paralog class the mammalian Cdx genes are most closely related to. The three Cdx genes found in birds, amphibians, fish and mammals possess a hexapeptide motif (van den Akker et al., 2002), are initially expressed early in the posterior primitive streak or its equivalent, and extend their expression domains rostrally (see Fig. 5). This gives Cdx genes a rather 3′ 'Hox signature'.

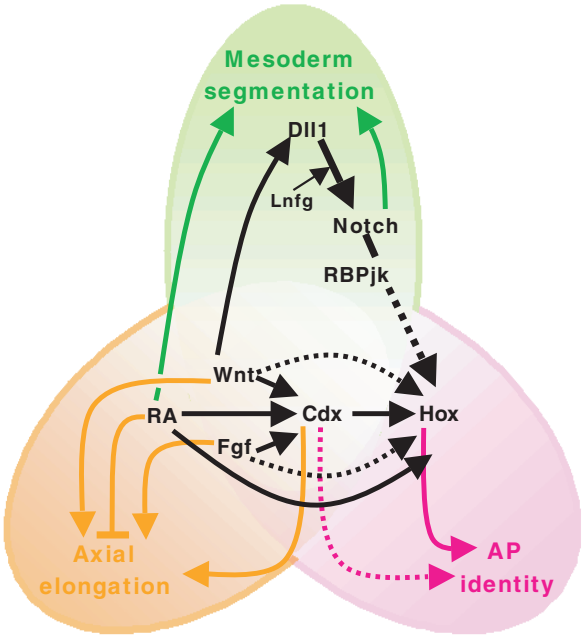

Fig. 6. Hox genes and the genetic network driving axial extension, mesoderm segmentation and AP patterning. Fgf, Wnt and RA signalling are functionally involved in axial extension (orange), somitogenesis (green) and AP patterning (purple). The relationship between the Hox genes, the Cdx genes, the segmentation genes of the Notch pathway and the three morphogenetic processes are indicated. Unbroken lines indicate established interactions; broken lines represent documented interactions that have not yet been established at the molecular level.

genes via the Cdx genes exclusively [as suggested for exogenous Fgf (Isaacs et al., 1998)] or whether Hox genes also respond to these signals independently of Cdx regulation.

A striking novel property of the Cdx transcription factors has recently emerged. In addition to their role in transducing AP positional information, they also play a dominant role in embryonic axial elongation, a function that has been evolutionary conserved (Box 1). *Cdx2* homozygote mutant embryos, when rescued from their implantation defect, fail to complete the extension of their body axis and are severely truncated posteriorly (Chawengsaksophak et al., 2004), while an earlier analysis of compound *Cdx1*/*Cdx2* mutant embryos had already revealed that these genes have a role in axial elongation (van den Akker et al., 2002). The posterior body truncations of Cdx mutants are very similar to the phenotype of loss-of-function *Wnt3a* (Ikeya and Takada, 2001) and *Fgfr1* mutants (Partanen et al., 1998). This finding suggests that a genetic interaction exists between the Wnt and Fgf pathways and the Cdx transcription factors in axial extension. *Cdx2*-null mutant embryos also have irregular and often smaller somites, particularly in the posteriormost region, a feature that possibly relates to an imbalance between mesoderm generation and the recruitment of PSM cells into somites, as discussed in the previous section. The Hox regulator Cdx thus plays a role in the balance between tissue generation, mesodermal segmentation and AP patterning, clearly demonstrating that Cdx genes belong to the constellation of genes that form an integrated genetic network for these three processes (Fig. 6).

In addition to the molecular genetic interactions that regulate the Hox genes during morphogenesis and patterning, a higher level of gene control modulates the expression of these genes by acting on the structure of the chromatin. This is dealt with in the following section.

Chromatin modifiers: prelude to Hox expression and transcriptional memory

Polycomb group (PcG) and trithorax group (trxG) proteins play an important role in maintaining the spatially restricted silenced and active transcriptional states of the Hox genes, respectively, in both flies and mice (reviewed by Ringrose and Paro, 2004). Histone methylation has recently been implicated in the long-term maintenance of gene silencing by the PcG complex (Cao et al., 2002; Ringrose et al., 2004) (see Box 2). Another recent study has demonstrated that the mono-ubiquitylation on lysine 9 of histone H2A (U-H2A K9) plays an essential role in chromatin-mediated heritable gene silencing (Wang et al., 2004; de Napoles et al., 2004). Histone lysine modification therefore plays a central role in the stability of chromosomal states and ensures that a transcriptionally inactive, condensed chromatin state is inherited by the progeny of a cell.

In addition to their role in the epigenetic maintenance of transcriptional states of their target genes, PcG and trxG protein complexes probably regulate the transcription of their targets in *Drosophila* (Breiling et al., 2001; Saurin et al., 2001), as well as in early mouse embryos (de Graaff et al., 2003). A recent study (Milne et al., 2002) elegantly showed that the binding of SET domain methyl transferase activity to the proximal promoter of human *HOXC8* in cultured fibroblasts was crucially required for transcription of the gene. The transcriptionally repressive, mono-ubiquitylated form of histone H2A (U-H2A K9) is recruited to the Hox promoters by the main PcG protein complex (Wang et al., 2004; de Napoles et al., 2004). These two studies link histone K9 and K27 methylation and ubiquitylation to Polycomb-mediated transcriptional repression, and histone K4 methylation to active transcription.

A particularly interesting issue in the genetics of AP patterning during embryonic development concerns the role that chromatin events play in the early co-linear activation of the clustered Hox genes during early embryogenesis (Duboule and Dollé, 1989; Kmita et al., 2000; Kmita and Duboule, 2003). Recently, the sequential activation of clustered Hox genes was followed in mouse embryonic stem (ES) cells (Chambeyron and Bickmore, 2004) and in early developing embryos (Chambeyron et al., 2005). Chromatin modifications were scored across the *Hoxb* locus in ES cells during RA-mediated differentiation. Acetylation at lysine 9 and dimethylation at lysine 4 of histone H3, both marks of actively transcribed chromatin, were increased in both *Hoxb1* and *Hoxb9* at an early time point, when only *Hoxb1* was expressed. These histone tags therefore are not tightly coupled to gene transcription, but rather indicate that the genes are in a 'poised' state, ready for transcription. Another recent study of the chromatin changes that occur during the initiation of Hox gene expression examined the relationship between histone modification and *Hoxd4* activation in *Hoxd4*-expressing and non-expressing embryonic tissues (Rastegar et al., 2004). This study concluded that *Hoxd4* acquires the marks of active

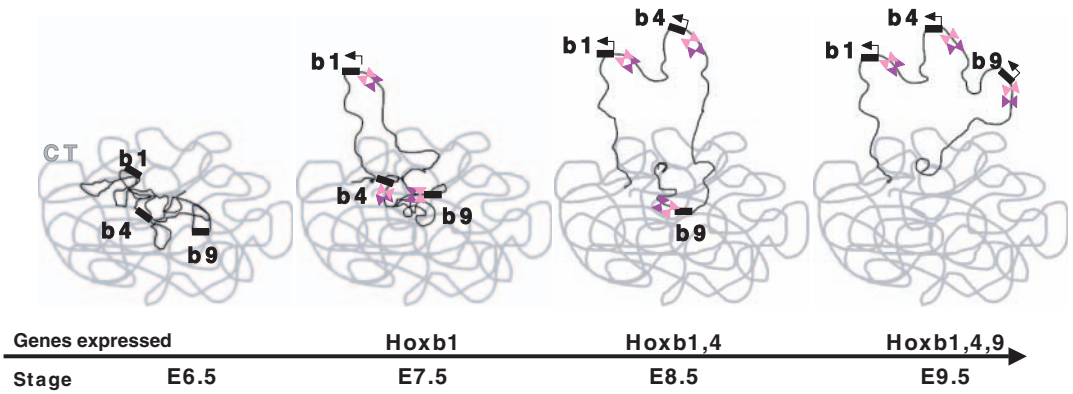

Fig. 7. Histone marks and nuclear reorganisation during co-linear Hox activation. Schematic representation of histone marks and changes in the subnuclear position of Hox genes before (E6.5) or at the time of their first expression [E7.5 for *Hoxb1* (b1), E8.5 for *Hoxb4* (b4) and E9.5 for *Hoxb9* (b9)]. The histone marks on histone H3, methylated lysine 4 (pink) and acetylated lysine 9 (purple), poise the genes for transcription from the moment the first Hox gene of the cluster (*Hoxb1*) is activated. Individual genes loop out of their chromatin territory (CT, grey line) at the time of their expression. Figure modified, with permission, from Chambeyron and Bickmore (Chambeyron and Bickmore, 2004), incorporating data from Chambeyron et al. (Chambeyron et al., 2005) and Rastegar et al. (Rastegar et al., 2004). The looping out of *Hoxb4* is a personal extrapolation of the data on *Hoxb1* and *Hoxb9*.

chromatin at a stage earlier than its transcriptional activation, exclusively in the posterior embryonic territories where it will later become expressed. Again, these histone modifications seem to confer selective transcriptional 'awareness' to the locus in the presumptive Hox expression domain.

Recent studies suggest that the spatial localization of genes in the cell nucleus is not random, but rather specifically facilitates the orchestrated regulation of the activity of a gene in specific cellular functions or fates (reviewed by Misteli, 2004). This is particularly true for gene clusters, the coordinated regulation of which is essential for development and tissue differentiation. Chromosome territories (CTs), the discrete structures formed by individual chromosomes in the interphase nucleus, constitute one of the subnuclear 'compartments' in which a gene can reside or from which it can be extruded (reviewed by Kosak and Groudine, 2004). Bickmore and colleagues studied the sequence of events accompanying the sequential activation of Hoxb genes during their RA-mediated stimulation in mouse ES cells (Chambeyron and Bickmore, 2004) and during the onset of the expression of the Hox genes in gastrulating embryos (Chambeyron et al., 2005). Upon short exposure to RA, sufficient to induce a general decondensation of the *Hoxb* locus in ES cells at a time when only *Hoxb1* is activated, a selective looping out of *Hoxb1* from its CT and towards the centre of the nucleus was observed. *Hoxb1* also looped out of its CT in the posterior part of the primitive streak at the time at which the gene is first expressed. The non-expressed *Hoxb9* did not loop out at this point, but did upon longer RA induction of the ES cells, and in the posterior neural tube of a E9.5 embryo, where and when it is expressed. Both *Hoxb1* and *Hoxb9* looped out of their CT in the tailbud tissues of E9.5 embryos, where both are expressed (Chambeyron et al., 2005) (Fig. 7). These data therefore demonstrate that gene exclusion from the CT is tightly coupled to gene transcription. Whether the selective looping out of a gene from its CT is a cause or a consequence of transcription is not yet known. Other coordinately regulated gene arrays that have been studied did not all reveal a consistent correlation between elevated transcription activity and looping away from the CT. The genes from the major histocompatibility complex (MHC) and the immunoglobulin heavy chain (IgH) loci also loop away from their CT during robust transcription, but the genes of the β-globin cluster loop out prior to transcriptional induction in erythroid cells (reviewed by Kosak and Groudine, 2004). Looping out in this case thus represents a 'poised' state for transcription. Importantly, replacing the enhancers of the β-globin locus control region (LCR) with a regulatory element that represses its transcription still leads to a looping out of the genes from their CT, but this time towards the transcriptionally inactive pericentric heterochromatin. The conclusion from these studies is that extrusion from the CT probably plays a role in transcriptional activation (or repression) by localizing genes to subnuclear positions that are associated with structures

Box 2. Chromatin histone marks

Chromatin organisation depends on a dynamic, higher-order structuring of the nucleosomes, which consist of a histone H3/H4 tetramer and two histone H2A/H2B dimers. Discoveries over the past 3 years have revealed a general epigenetic marking system that helps to regulate transcription (reviewed by Schotta et al., 2004; Peterson and Laniel, 2004), which involves histone modifying enzymatic activities, such as acetylases, methyl transferases and ubiquitin ligases. Some of these activities are encoded by Hox epiregulators themselves, and others are recruited into complexes targeting them to Hox loci. Among the many amino acid modifications of histones that are associated with chromatin remodelling, the most extensively studied has been the methylation of lysines K4, K9 and K27 on histone H3 (Jenuwein and Allis, 2001; Peterson and Laniel, 2004). Although H3 K9 and H3 K27 methylation are associated with transcriptionally repressive chromatin, H3 K4 is a mark of active transcription. Polycomb-mediated transcriptional repression is brought about by methylation at H3 K9 and H3 K27 (Ringrose et al., 2004). By contrast, the trx/Mll SET domain methylates H3 K4 in the promoter region of some Hox genes (Nakamura et al., 2002). The fact that trx/Mll-mediated H3 K4 methylation is associated with transcriptional activation and PcG-mediated H3 K9 and H3 K27 methylation is associated with transcriptional repression might account for the antagonistic effect of the PcG and trxG proteins on Hox gene expression (Lachner and Jenuwein, 2002).

facilitating gene transcription (or silencing). The findings of Koseki and colleagues of a direct interaction between PcG proteins and an essential spliceosomal protein can possibly be interpreted in this light (Isono et al., 2005).

Interestingly, the looping out of the clustered β-globin genes specifically depends on regulatory sequences around the genes (Kosak and Groudine, 2004). The importance of global regulatory regions for the control of clustered genes has been recognized (Grosveld et al., 1987; Spitz et al., 2003). We will see in the next section that such global control regions play essential roles not only in the recruitment of a set of clustered genes to common functions but also in the differential regulation of the gene members of a Hox cluster.

Balanced regulatory inputs from inside and outside Hox clusters

The sequentially and spatially co-linear expression of the Hox genes has to be orchestrated in concert with morphogenesis. This harmony is realised in part through molecular regulatory interactions that have an impact on subsets of the Hox genes. The cluster from which most information regarding this enigmatic regulatory process has been obtained is the Hoxd cluster. A regulatory element on the 3′ side of the Hoxd cluster has been proposed to account for early co-linear Hoxd gene expression in the lateral plate mesoderm of the emerging limb field along the AP axis. The existence of this element, called the early limb control region (ELCR), has been inferred from the effects of experimentally inverting and deleting parts of this Hox cluster in the mouse (Zákány et al., 2004). Expression of the Hoxd genes in the mesenchyme of the nascent limb bud follows the same co-linearity rules as the early expression of the Hoxd genes in axial and paraxial tissues, and the molecular regulatory interactions occurring through the ELCR therefore must be intimately linked to the mechanism of spatiotemporal co-linearity of expression during embryogenesis (Zákány et al., 2004) (Fig. 8).

Although the integrity of the Hox clusters has been maintained in mammals, they have not behaved through evolution as isolated islands in the genome. The discovery of a global control region (GCR), which coordinately regulates gene expression over large chromosomal domains (Spitz et al., 2003), confirmed the hypothesis (Spitz et al., 2001; Kmita et al., 2002a) that the Hoxd genes recently acquired a novel function in limb development, in addition to their ancestral function along the main axis. This GCR, localized about 240 kb upstream of Hoxd, contains the long-predicted remote control element that coordinately regulates the expression of 5′ Hoxd genes in the distalmost part of the limb buds (Spitz et al., 2003). This element controls the 5′ Hoxd genes and two other genes, lunapark (*Lnp*) and *Evx2*, both of which are located in the intervening region that separates the GCR from the most 5′ Hoxd gene. The GCR harbours, in addition to the digit enhancer, a small cluster of neural enhancers. These neural enhancers, which are conserved in mammals, drive *Lnp* and *Evx2* expression in patterns that differ from those of the Hoxd genes, which are insulated from these enhancers (Kmita et al., 2002b) (see Fig. 8). In the ancestral scenario, which is still present in the genome of cartilaginous fish, the GCR contains only the neural enhancers (Spitz et al., 2003). The generation of a digit enhancer within the GCR allowed the 5′ Hoxd genes to be strongly expressed in the distal-most limb mesenchyme, where their activity probably allowed the emergence of the digits, which have been conserved ever since.

Global control regions regulate the expression of all or groups of the clustered Hox genes, adding their effects to those of the local, Hox-proximal regulatory elements. A last potential element to add in this survey of the regulatory circuits that modulate Hox gene expression are microRNAs, which interfere with gene expression at the post-transcriptional level and have target sequences within the Hox clusters (see Box 3 for more).

Conclusions

Significant new findings have emerged in the past few years that allow us to integrate the regulation of Hox-mediated positional information with the morphogenetic processes of gastrulation, axial extension, somite formation and AP

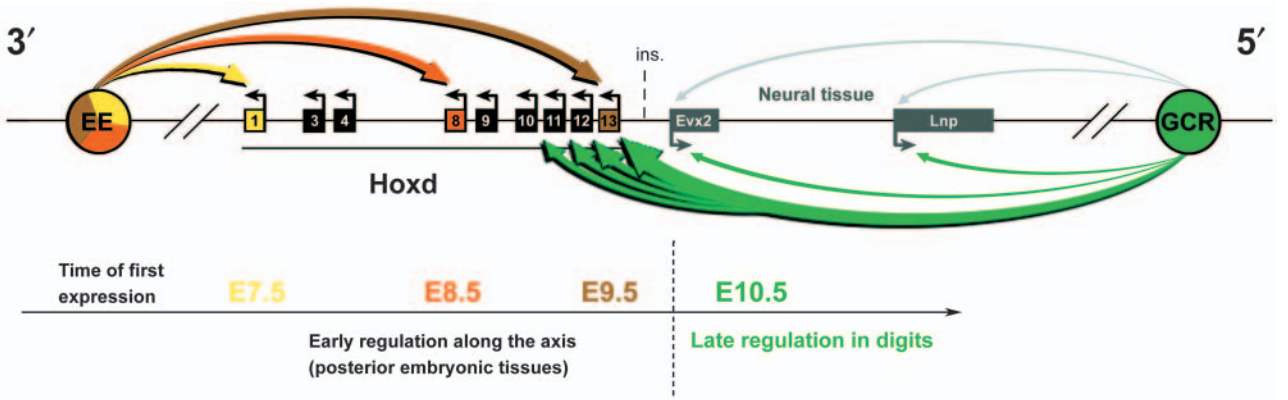

Fig. 8. Global regulation of Hoxd gene cluster. (Left) The sequential activation of 3′ to 5′ Hoxd genes is shown from the hypothesised global early enhancer (EE) that mediates the temporally co-linear activation of the Hoxd genes along the main axis. The EE in the scheme corresponds to the ELCR (early limb control region) postulated by Zákány and colleagues (Zákány et al., 2004). (Right) The regulatory influence of the 5′ global control region (GCR) on the Hoxd cluster and neighbouring genes in the digits (green) and neural tube (grey). A timescale is depicted below. The activation times of the Hoxd genes is shown for only three genes: *Hoxd1* (yellow), *Hoxd8* (orange) and *Hoxd13* (brown). The action of the GCR is stronger on the most 5′ gene *Hoxd13* (thicker green arrow) than on *Hoxd12* to *Hoxd10*. ins, insulator in neural tissues. *Lnp*, *lunapark*; *Evx2*, mouse *even-skipped* homolog 2.

Box 3. MicroRNAs: fine-tuning co-linear Hox gene expression during embryogenesis?

The post-transcriptional regulation of Hox genes during embryogenesis has been documented (Nelson et al., 1996; Brend et al., 2003). MicroRNAs (miRNAs) might regulate Hox genes post-transcriptionally. The recent discovery that two conserved miRNA loci exist at two positions in the Hox clusters and that their target sequences are present in the 3′ UTR of neighbouring Hox genes, indicates that these molecules might fine-tune the maximal extension of Hox expression domains along the AP axis (Yekta et al., 2004). As previously observed, the presence of miRNA targets in the 3′ UTR of Hox genes causes their translational inhibition (Yekta et al., 2004). *Hoxb8* RNA is also cleaved by a microRNA, as visualised at the Hox UTR using specifically designed transgenic sensors (Mansfield et al., 2004). As miRNAs are also present in embryos from early stages (Yekta et al., 2004), these data together indicate that miRNAs might regulate Hox genes in vivo. However, the spatial restriction of miRNA expression during development should not by itself be considered as an indication of a specific function, as it results from the fact that these miRNA loci reside inside the Hox clusters and are regulated accordingly. Whether miRNAs contribute functionally to Hox regulation during development awaits demonstration by genetic loss- and gain-of-function experiments.

patterning along the main embryonic axis (summarised in Fig. 2).

During axial extension, cells emerging from the posterior stem cell zone do not have or receive their Hox code when leaving the node region, but the transcription of the Hox genes in these cells is regulated thereafter by multiple mechanisms. Despite recent progress, key issues remain unresolved. These include the genetic and cellular mechanism of cell generation from the stem cell zone, its relation to Hox gene expression and the control of the arrest of axial extension at later stages. The live imaging of cells released from the stem cell zone in cultured wild-type and mutant mouse embryos, coupled to the visualisation of gene expression at the cellular level, are just some approaches that promise to shed more light on these processes in the future.

Even if the molecular mechanism that underpins 3′ to 5′ co-linear expression of the Hox genes has so far been elusive, a corner of the veil has been lifted. It will be exciting to discover the molecular mechanism underlying the action of the global regulatory element that drives early co-linear Hoxd gene expression in the emerging limb buds (ELCR) (Zákány et al., 2004), given that this element probably controls the early spatiotemporal co-linearity of expression of the clustered genes along the axis, as hypothesised in Fig. 8. In addition, the relationship between this early spatiotemporally acting enhancer and the regulatory element presumed to generate sequential transient bursts of Hox gene expression in the anterior PSM (Zákány et al., 2001) is intriguing. Whether and how signalling by RA, Fgf and Wnt is involved in this regulation is another puzzling issue. It will be interesting to uncover the mechanism of action and the relationship between these various episodes of co-linear Hox gene control during embryogenesis.

Another largely unachieved goal is the deciphering of the numerous gene interactions that involve the Hox genes in tissue generation and patterning during elongation of the axis (see Fig. 6). The emerging view suggests that the Hox genes belong to the common constellation of genes that orchestrate morphogenesis in an integrated way during embryogenesis. But much of the functional network involving Wnt, Fgf and Cdx in axial extension and patterning remains elusive. Even the issue of whether Cdx proteins affect posterior axial elongation by regulating the Hox genes remains to be addressed. The availability of many mutants and gene array technology should soon bring more order to this puzzle.

Finally, in addition to the molecular interactions between signalling effectors and the cis-acting responsive elements that lie proximal or more distal to the Hox genes, chromatin modification also prepares the genes for transcription. The physical looping out of Hox genes from their CT correlates with their co-linear expression in time and space in the embryo. It is therefore possible that chromatin events play the important role proposed long ago in setting the prerequisites for initial co-linear Hox gene expression. These events might start much earlier than the maintenance of the Hox transcription status by PcG and trxG proteins. Among the issues that remain to be resolved about these processes is whether the nuclear repositioning of the Hox genes facilitates or results from their transcriptional activation, and how gene extrusion itself is regulated at the molecular level. Exciting new discoveries in this field will surely come.

Note added in proof

Four papers have recently revealed that molecular links exist between the generation and transmission of left-right (LR) asymmetry to body organs, and the bilaterally symmetrical extension and patterning of the anteroposterior axis (AP) axis. Tanaka et al. (Tanaka et al., 2005) report that the AP patterning signals Fgf and retinoic acid are key components of a novel mechanism that generates LR asymmetry by unidirectionally transporting morphogens across the mouse node. Retinoic acid signalling is subsequently needed to shield forming somites from these LR asymmetrical cues (Vermot et al., 2005; Kawakami et al., 2005; Vermot and Pourquié, 2005). In the absence of retinoic acid, the coordination between left and right somite formation is transiently disturbed, following delayed Fgf8 front regression on one side and the desynchronization of Notch-dependent oscillation patterns of clock gene expression (see Hornstein and Tabin, 2005).

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