Initiation, establishment and maintenance of *Hox* gene expression patterns in the mouse

JACQUELINE DESCHAMPS*, ERIC VAN DEN AKKER, SYLVIE FORLANI,
WIM DE GRAAFF, TONY OOSTERVEEN, BERNARD ROELEN and JEROEN ROELFSEMA

Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Utrecht, The Netherlands

ABSTRACT Spatially and temporally restricted expression of the *Hox* genes along the main and appendicular axes is essential for correct patterning of vertebrate embryos. In this overview we discuss the latest data that shed light on the mechanisms underlying the generation of the expression domains of the *Hox* genes. The molecular genetic interactions governing initial transcription of the *Hox* genes in the posterior part of the primitive streak during mouse and chick gastrulation remain enigmatic. But the recent discovery by Kondo and Duboule (Cell, 97, 1999, 407-417) of a «cluster repressive regulation», will undoubtedly lead to a better understanding of the molecular genetic mechanism underlying colinear and sequential initiation of *Hox* gene transcription. Recently progress has been booked in characterizing the basal processes driving progression of the *Hox* expression domains during their establishment. *Hox* expression is still labile while being established. The transcriptional state of *Hox* genes in anterior tissues can be reprogrammed under the influence of more posterior locations. Posteriorizing activity may involve RA and FGF signaling. It is only when these interactions and, in some cases at least, regulatory interactions with *Hox* and *cdx* gene products occur appropriately, that the *Hox* expression domains would be correctly established. After the *Hox* expression domains have been established, regulatory processes involving the products of *Polycomb* and *trithorax*- Group genes start operating, perpetuating the transcriptional state of the *Hox* genes within and outside the expression domains. Whether control at the level of chromatin structure, believed to operate during the late maintenance phase of *Hox* gene expression, is also involved in regulating concerted initial expression of these genes, is a possibility that has been suggested.

KEY WORDS: *Hox genes, antero-posterior patterning, transcriptional regulation*

The *Hox* genes are key regulators of regional pattern formation along the antero-posterior (A-P) and other embryonic axes. These genes have been involved in transducing positional information to the precursors of embryonic axial and paraxial structures since before the ancestors of insects and vertebrates diverged from each other. In vertebrates as in insects, the precise restricted localization of each *Hox* gene product is crucial for correct patterning of tissues surrounding the rostral expression boundary of this gene. The tight transcriptional regulation of the *Hox* genes is therefore essential to the proper orchestration of embryonic morphogenesis.

This overview focuses on particular aspects of the regulatory processes involved in controlling the expression of the *Hox* genes. The paper deals primarily with the mouse, with some excursions to chick, frog, *Amphioxus*, nematode and insect embryos. Our objective is to discuss a number of current issues regarding the ontogeny of the definitive *Hox* expression domains

in relation with the embryological processes occurring concomitantly. We subdivided the genesis of the *Hox* expression domains in the mouse in three periods: the *initiation (I)* of *Hox* transcription in the posterior primitive streak region during gastrulation, the *establishment (II)* of the transcription domains, from the time cellular precursors of embryonic tissues are expressing the genes till the definitive expression boundaries are reached in the embryo, and the *maintenance (III)* of transcriptional states thereafter (see Fig. 1). Relatively more will be said about the modulation of the establishment phase of the expression domains: particular attention will be devoted to aspects of the ontogenesis of the *Hox*

*Address for reprints: Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands. FAX: 31 30 2516464. e-mail: jacqueli@niob.knaw.nl
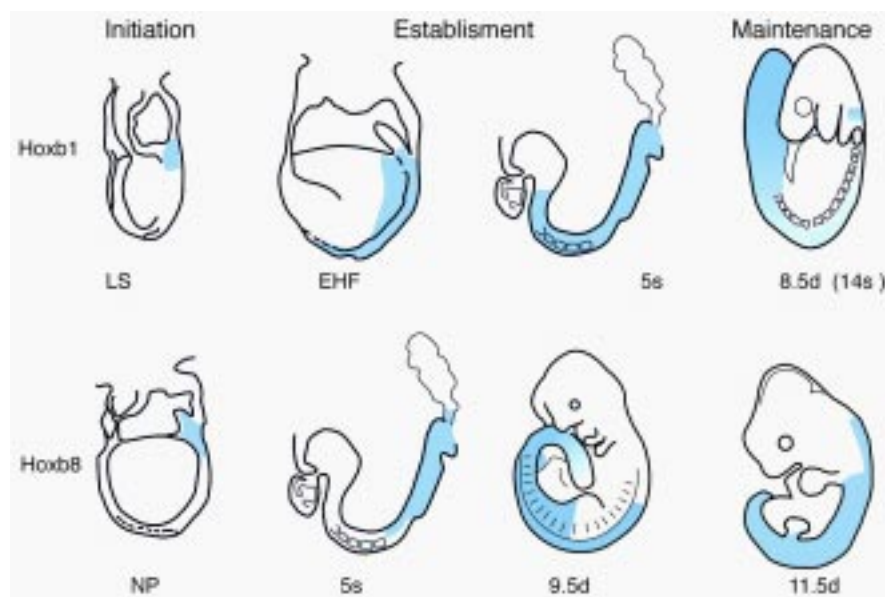
Fig. 1. Schematic sagittal representation of Hox expression domains at different stages of mouse embryonic development. *Ontogenesis of the expression pattern of a 3' (Hoxb1) and of a 5' (Hoxb8) Hox gene is depicted. Transcript distribution is shown in blue at the time (from left to right) expression is initiated, at two time points during the establishment of the definitive expression domains, and when maintenance of transcription within these domains is occurring. Hoxb1 expression starts at the late streak (LS) stage, in the posterior part of the primitive streak, at the boundary with extraembryonic tissues, when the anteriormost part of the streak, the node, is at the distal tip of the embryo; at the early headfold stage (EHF), expression has extended rostrally and passed the node, still located at the distal tip of the embryo; at the early somite stage, the rostralmost extension of the expression domain is reached at the level of the boundary between rhombomeres 3 and 4. In the particular case of Hoxb1, only rhombomere 4 expression will be maintained at later stages, expression at trunk and tail levels decreasing progressively. Hoxb8 starts being expressed later, at the neural plate (NP) stage, at the boundary between the posterior streak and extraembryonic tissues; at early somite stage, when expression in the neural tube is at the level of somite 5, expression in the mesoderm is at a more posterior presomitic level; at later stages, expression boundaries in the neurectoderm and mesoderm are clearly apparent, at the level of somite 5 and 11, respectively. A rostral shift of the expression boundary of Hoxb8 in the neural tube takes place between day 9.5 and 11.5, from the level of somite 5 up to the posterior hindbrain. As discussed in the text, we estimate it likely that maintenance of transcriptional states operates earlier for a 3' than for a 5' gene. LS, late primitive streak stage; NP, neural plate stage; EHF, early headfold stage; 5s, five somite stage. For LS till 5s stages, anterior is to the left, and posterior to the right; for LS, EHF and NP, extraembryonic tissues are the above moiety of the conceptus, and embryonic tissues the bottom cylinder; stage 8.5 day (8.5d), 9.5 day (9.5d) and 11.5 day (11.5d) represent embryos at 8.5, 9.5 and 11.5 days post-coitum, respectively; for the latter embryos, anterior (head) is above. Squares at stages 5s and 8.5 day represent somites, and dashes (numbers are not accurately drawn) at day 9.5 and 11.5, represent boundaries between the prevertebrae.*

1989; Izpisua-Belmonte *et al.*, 1991; Gaunt and Strachan, 1996; Van der Hoeven *et al.*, 1996). The earliest (3') *Hox* genes start to be expressed in the mesoderm, and more weakly in the primitive ectoderm (epiblast) of the posterior primitive streak of mouse and chick embryos (Gaunt, 1988; Frohman *et al.*, 1990; Sundin and Eichele, 1992; Gaunt and Strachan, 1994), in late streak stage embryos. Transcription initiation of more 5' genes occurs in the same region, overlapping the junction between extraembryonic and embryonic ectoderm (Deschamps and Wijgerde, 1993; Gaunt and Strachan, 1994), at progressively later stages but not in the same cells, since gastrulation movements have then brought more lateral epiblast cells into this position, exposing them to the "*Hox* induction field". This *Hox* induction field in the posteriormost part of the embryo, traversed by cells fated to become extraembryonic mesoderm (Tam and Beddington, 1987; Lawson *et al.*, 1991), must therefore inherently bear a transcription inducing mechanism.

Among the potential *Hox* inducers present during gastrulation in this embryonic region are fibroblast growth factors and their receptors, some of which (i.e. eFGF, Pownall *et al.*, 1996; Isaacs *et al.*, 1998; FGF8, Crossley and Martin, 1995; Meyers *et al.*, 1998; FGFR1, Deng *et al.*, 1994; Yamaguchi *et al.*, 1994) have been proposed to play a role in the control of mesoderm organization and antero-posterior (A-P) patterning. Modulation of *Hox* gene expression by altered FGF signaling has been observed in *Xenopus* (Isaacs *et al.*, 1998) and in the mouse (Partanen *et al.*, 1998). Whether initial *Hox* gene expression is affected in these conditions is not known yet. Evidence has accumulated to strongly support a role of retinoic acid (RA), or a related retinoid, as a factor affecting *Hox* gene early transcription. RA was shown to turn on the *Hox* genes according to a colinear sequence from 3' to 5' in differentiating human EC cells (Simeone *et al.*, 1990). RA has been shown to be present in the gastrulating chick embryo (stage 4/5, definitive streak stage; Maden *et al.*, 1998). Higher RA levels were measured in the posterior part than in the rest of the embryo including the node, even though the absolute values of RA concentration were rather low. A number of *Hox* genes were found to carry functional RA response elements (RAREs) (see section II.C.3). Inactivation of the RARE 3' to *Hoxa1* leads to a delay in transcriptional initiation in mutant embryos, pointing to a role of RA signaling in correctly setting the timing of the initial expression of this gene (Dupé *et al.*, 1997). Recent experiments (Niederreither *et al.*, 1999) preventing early RA biosynthesis by inactivating Retinaldehyde Dehydrogenase 2 (Raldh2), suggest that RA is involved in the earliest *Hox* gene expression, but that it does not totally account on its own for *Hox* gene initiation. *Hoxa1* was expressed at lower levels, and seem-

patterns during this phase, such as dynamic forward spreading of the transcript distribution, plasticity of the gene expression state, role of retinoids in establishing the expression domains, regulation in the hindbrain versus spinal cord/trunk, regulation during limb development, and concerted regulatory events within *Hox* complexes.

I. Initiation of *Hox* gene transcription

Mouse *Hox* genes, homologs of *Drosophila* homeotic genes (*HOM/Hox* genes) (reviewed by McGinnis and Krumlauf, 1992) start to be expressed in an ordered sequence correlating with the localization of the genes from 3' to 5' in the clusters (Dollé *et al.*,

ingly with a delay in time, in the primitive streak of headfold-stage *Raldh2* mutant embryos (Niederreither *et al.*, 1999).

The essential link between the timing of initial *Hox* gene expression and the position of the genes in their cluster was initially demonstrated by van der Hoeven *et al.* (1996) who genetically transposed a *Hoxd* transcription unit to a more 5' position in its cluster, and observed a delay in the initial expression of the corresponding gene. More recently, Kondo and Duboule (1999) brought the evidence to light that a cis-regulatory element is located at the 5' side of the *Hoxd* cluster, some 40 kb from *Hoxd13*, which is necessary to set up colinear and sequential activation of the *Hoxd* genes. Deletion of the region containing this remote element leads to a release of a repressive mechanism normally preventing posterior genes to be activated prematurely. This results in the complete loss of colinearity and sequentiality of gene expression. However, the ectopic expression of the prematurely expressed 5' *Hoxd* genes in the anterior part of these embryo is transient, probably because the lack of adequate molecular interactions in anterior tissues, where 5' *Hoxd* genes normally are not expressed, does not allow full establishment and maintenance of gene expression at these anterior levels (Kondo and Duboule, 1999). A mechanism linking the correct sequential timing of initial *Hox* gene expression and genetically controlled modulation of chromatin structure has been proposed by Kondo *et al.* (1998) and Kondo and Duboule (1999). Recently, Bel *et al.* (1999) showed that 5' *Hoxd* genes are initially expressed earlier in a null mutant for the Polycomb-Group gene Pc-G *M33* than in control embryos (Bel *et al.,* 1999). Furthermore, a given *Hox* gene was shown to exhibit premature competence to respond to RA activation in *M33* null mutant embryos. These data strongly suggest that the Pc-G gene product M33 may play a role in the initiation of *Hox* transcription and in the time-restricted competence of *Hox* genes to respond to RA. This will be further discussed in section III.

The mechanism underlying the anterior progression of the initial *Hox* expressing domains along and lateral to the streak, successively in presumptive extraembryonic, lateral plate and paraxial mesoderm (Fig. 2), is still enigmatic. Early expression is not clonally transmitted (Deschamps and Wijgerde, 1993) but extends in spite of lateral migration of newly formed extraembryonic and embryonic mesoderm leaving the primitive streak (see Fig. 2). Experiments following the expression of *Hoxd4* in the chick have shown that rostral spreading of the *Hox* expression domain along the primitive streak was neither relying on cell movement, nor on the diffusion of inducers from posterior to more anterior positions, at least from the time *Hoxd4* was expressed posteriorly (Gaunt and Strachan, 1994).

Tomihara-Newberger *et al.* (1998) have found that *amnionless (amn)* is required for proper formation of this middle streak region as the streak extends. Correct functioning of this gene was found to rely on its expression in the visceral endoderm. A role of the endoderm overlying the primitive streak as a guide/instructor in the process of forward spreading of the *Hox* expression domains from the posterior to the anterior streak at the late streak stage appears as an interesting possibility.

Importantly, recent work by Liu *et al.* (1999) has made it clear that Wnt signaling is essential for primitive streak and mesoderm formation, and for epiblast differentiation and A-P patterning. The *Wnt3* expression pattern and the *Wnt3* null phenotype are compatible with Wnt signaling being involved in a genetic cascade turning on the *Hox* genes at the late streak stage.
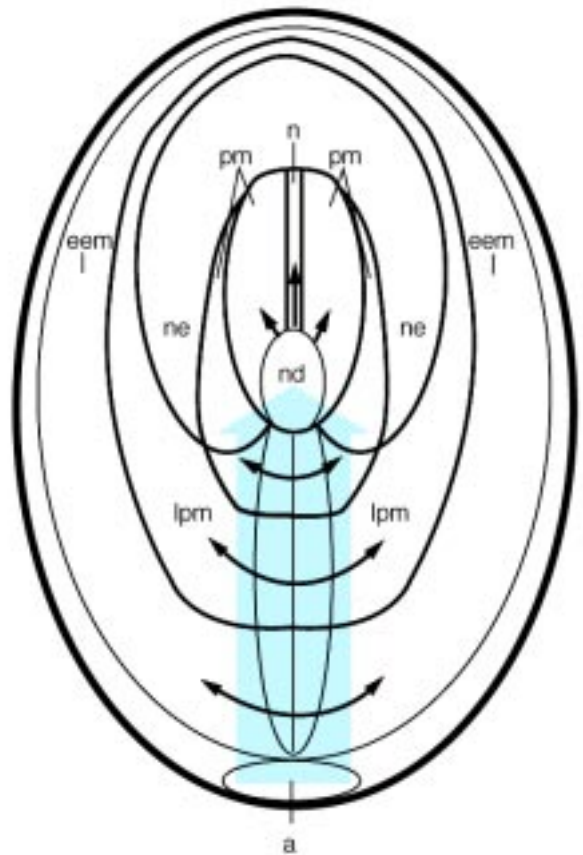

Fig. 2. Diagram showing the progression of early Hox expression domains at the late streak to neural plate stages. *The two dimension representation of the fate map is a projection of the cup-shaped embryo on the plane perpendicular to the one shown in Figure 1. Posterior is at the bottom, and anterior at the top; midline from a (allantois) to nd (node) represents the primitive streak, and midline from nd to the top, the elongating embryonic axis. The representation of the fate map is adapted from Smith et al. (1994). Transcripts (in blue) are detected along the primitive streak from its posterior side connected to the allantois (a) towards its rostral side, the node (nd) region. The wide blue arrow indicates the progression of the Hox transcription domains within and adjacent to the streak (Hoxb1 is taken as an example). Black arrows indicate that cells that have ingressed from the primitive streak into mesoderm migrate sideways; eem, extraembryonic mesoderm; lpm, lateral plate mesoderm; pm, paraxial mesoderm; n, notochord (axial mesoderm); ne, neurectoderm. Note that at rostral primitive streak levels, the progressing Hox expression domain encounters a field of overlap between pm and ne; this means that progenitors of both neurectoderm and mesoderm express Hox genes in areas lateral to the node (see text for discussion).*

II. Establishment of the *Hox* domains in axial and paraxial embryonic tissues and in the limbs

After being initially observed in the posterior primitive streak region, the *Hox* transcript domains spread anteriorwards in and along the streak, meet and pass the node region and extend further rostrally to reach their anteriormost position (see Fig. 2). Initiation and establishment of the *Hox* expression domains are therefore a
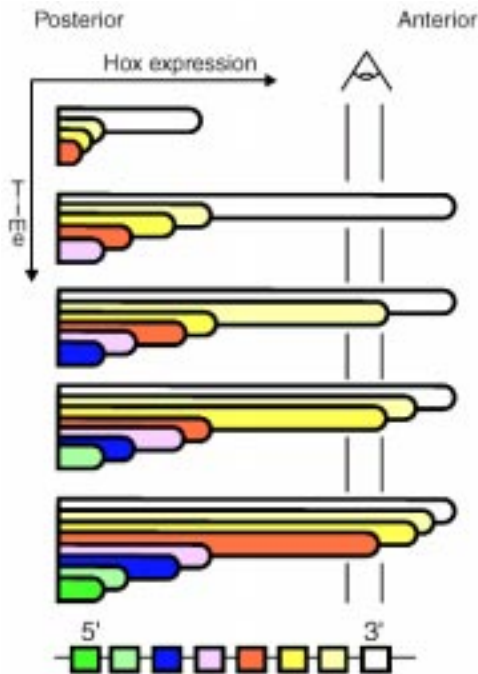
Fig. 3. Schematic representation of the establishment of vertebrate Hox expression domains in one germ layer according to the "morphogen timing and spreading model" *(Gaunt and Strachan, 1996; Gaunt, 1999, Gaunt et al., 1999). This scheme is adapted from a figure from Gaunt and Strachan (1996). Expression of 3' to 5' Hox genes is depicted with colors. Only relatively 3' genes are expressed at early, primitive streak stages (earliest time shown in the scheme); the more 5' genes become transcriptionally active at later time points (see for example the blue and green domains). Note that the recent findings of Kondo and Duboule (1999) make it clear that a cluster-involving repressive regulatory mechanism is essentially controlling initial sequential and colinear transcription of the Hox genes (see text for discussion). With time (y axis), Hox expression spreads forward along the embryonic axis (second to fifth time point represented). The definitive rostral expression boundary will be reached earlier for a 3' gene (e.g. white domain) than for a more 5' gene (e.g. yellow domain). Spreading of the Hox expression domains is proposed to result from a time-dependent, intracellular cascade of Hox induction in posterior to anterior cells, either corresponding to differential sensitivity of the Hox genes for a posterior diffusing morphogen, or possibly corresponding to a directional opening of chromatin structure (see the progressive expression of more and more genes -from 3' to 5'- at a given axial position, seen by the symbolic eye on the scheme). The definitive Hox expressing territories become established progressively for 3' to 5' genes. Once the definitive anterior expression boundary is reached for any gene, the rostrally flanking cells will never express this gene, and the cells at the boundary will never induce more 5' Hox genes (see text for more details).*

continuous process, differing essentially by the fact that initiation occurs in cells that will not contribute to the embryo proper (presumptive extraembryonic mesoderm), while subsequent expression involves cells that will contribute descendants in embryonic tissues. The first embryonic tissue to express a *Hox* gene following transcriptional initiation is presumptive lateral plate mesoderm, and subsequently paraxial mesoderm, the cellular precursors of which are located at progressively more anterior positions, lateral to the streak, at the late streak stage (Tam and Beddington, 1987) (see Fig. 2). The area lateral to the node containing paraxial mesoderm progenitors also contains neurectoderm progenitors of

hindbrain and spinal cord (Tam and Beddington, 1987; Quinlan *et al.,* 1995; Lawson and Pedersen, 1992 and K. Lawson, personal communication). In this area, inducing signaling events may therefore operate on precursors of both neural and mesodermal *Hox* expression domains.

The relationship between initial *Hox* gene expression in the primitive streak region and gene expression in the definitive expression domains in the three embryonic germ layers is not clearly understood so far. In any case, correction of early spatial and temporal delay in initial expression of the *Hoxa1* RARE mutant (Dupé *et al.*, 1997) and of genetically transposed *Hox*d11 (van der Hoeven *et al.*, 1996), and lack of maintenance of premature *Hoxd* gene expression upon deletion of a "locus repressive element" (Kondo and Duboule, 1999) suggests that control mechanisms are superimposed onto the initial *Hox* gene regulation.

### II.A. The rostral Hox expression boundaries are established gradually

The definitive expression domains of the most 3' *Hox* genes along the antero-posterior (A-P) axis of the embryo extend up to rostral limits located at rhombomeric boundaries in the anterior hindbrain in the neurectoderm and rostrally to the first somite in the mesoderm (the expression level decreasing considerably in this germ layer in the case of *Hoxb1*, Murphy *et al.*, 1989; Wilkinson *et al.*, 1989). More 5' genes are expressed up to rostral boundaries located at successively more posterior positions. It has become clear that these definitive boundaries are not acquired at once as soon as the expression domains invade embryonic neurectoderm and mesoderm during embryogenesis. *Hoxb8*, for instance, is and remains expressed from day 8.5 on in a domain extending up to a rostral boundary at the level of somite 11 and its derivatives in the mesoderm, while the expression boundary in the neurectoderm, initially at the axial level of somite 5, shifts from this to a more anterior level in the posterior hindbrain by day 11 (Deschamps and Wijgerde, 1993). Gaunt and Strachan (1994) have clearly shown that the expression domain of chick *Hoxd4* in the neurectoderm spreads anteriorwards into initially negative tissue: the expression boundary moves forward from a position located at a level anterior to the node (in the 5-somite embryo) to a position anterior to somite 1, passing a landmark of DiI-stained mesodermal cells at the level of somite 4. Gaunt and colleagues more recently showed that, at least for some *Hox* genes, *Hox* expression domains at somite stages also spread forward into initially negative cells within lateral plate mesoderm (Gaunt, 1999) and paraxial mesoderm (Gaunt *et al.*, 1999). Rostral extension of the *Hox* expression domains in these tissues clearly takes place independently of cell movement, since there is no intersomitic exchange of cells (Stern *et al.*, 1988).

Gaunt and colleagues propose a model accounting for these observations, assuming the existence of an early morphogen gradient with a maximum located posteriorly, and a decrease in sensitivity to this morphogen of the genes from 3' to 5'. For example, the paraxial mesoderm emerging from the streak would carry a morphogen gradient leading to the expression of each gene up to the axial level where the concentration of the morphogen corresponds to its sensitivity, the latter correlating with its position in the cluster (Gaunt and Strachan, 1996; Gaunt *et al.*, 1999). A time-linked mechanism would lead to later induction of each gene at slightly more anterior levels, causing the expression domains to progress further rostrally (Fig. 3). Signaling in the paraxial mesoderm could diffuse into the adjacent neural tube to induce *Hox*

Fig. 4. Schematic representation of the dynamic spreading of cdx and Hox transcripts (in blue) during neural plate/ early headfold (day 7,5 represented) to later stages (day 11.5 represented) of mouse development. *For cdx2, the only cdx gene to be expressed before gastrulation, a sagittal view of an early egg cylinder embryo is depicted, with cdx2 expression in extraembryonic ectoderm (Ext.ect.) all around the boundary with embryonic ectoderm (Emb.ect.). All other schemes show a dorsal view of the stretched embryonic axis. This representation is based on a scheme of cdx1 expression by Meyer and Gruss (1993), and the data are from the work cited and from our own published and unpublished data. Hoxb2 is taken as an example of a 3' Hox gene, Hoxb8 as an example of a more 5' Hox gene, and the particular Hoxb8/lacZ expression is shown because the Hox regulatory region driving lacZ expression contains 4 functional CDX binding sites (see Charité et al., 1998). Note that cdx expression decays from anterior to posterior from early stages on, and that*

it is maximal only at the earliest time point shown (the anterior expression boundary at day 8.5 being in fact more fuzzy already than suggested here). cdx expression is almost gone at day 11,5, while Hox genes are still expressed at high levels during this maintenance phase of transcriptional states (see Fig. 1); from P (posterior) to 0 (node region): primitive streak; from the node to A (anterior): extending embryonic axis; pos, preotic sulcus; r, rhombomere; s1, somite 1; pv1, prevertebrae 1; flb, forelimb bud; hlb, hindlimb bud; paraxial rows of circles represent the somites (up to 9.5 day), and the prevertebrae at day 11.5.
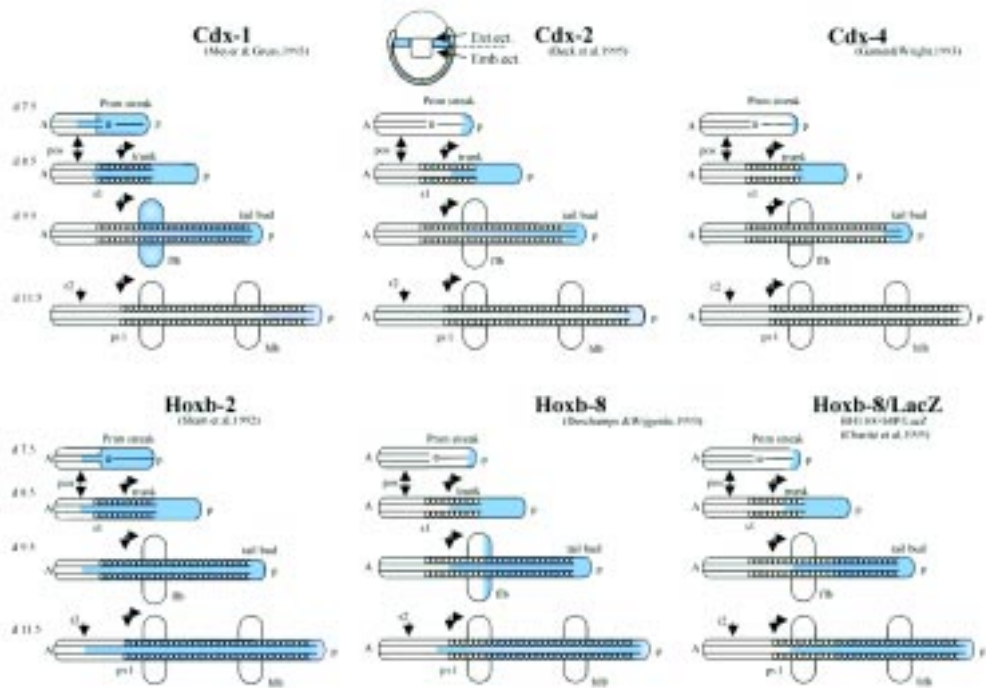
genes in this tissue. Alternatively, the acquisition of positional values could occur within the neurectoderm as well, provided that it carries an autonomous morphogen gradient in the plane of the neurepithelium. At some point, and by a still unknown mechanism, the definitive expression boundary of a *Hox* gene would be reached and the *Hox* expression domains would stop spreading more anteriorly. The transcriptional state of the *Hox* loci at all A-P positions would then be "frozen", and further maintained by another molecular mechanism (see section III). A striking feature of mouse and chick *Hox* expression domains, which is not dealt with in the model, is the difference between the axial position of the rostral expression boundary in neurectoderm and mesoderm. This difference is not likely to be due to a slower anterior spreading of mesoderm cells initiated in the node region: descendants of cells labeled in the node region at a given stage spread to the same anterior level in the ventral neural tube and in the paraxial and lateral mesoderm (K. Lawson, personal communication).

II.B. Plasticity of Hox gene expression state during the establishment phase

Experiments with chick–quail chimeras (Grapin-Botton *et al.*, 1995, 1997) and with chick-mouse chimeras (Itasaki *et al.*, 1996) have brought to light the evidence that posterior signals are able to induce expression of given *Hox* genes in the neurectoderm where they were not, and would normally never be, expressed. Thus *Hox* genes in neural tissues are reprogrammed upon transposition of rhombomeric neurepithelium to a more posterior position in the neural tube or upon transposition of posterior somites to more anterior positions until at least the 10-somite stage in the chick. Posteriorly transposed rhombomeres were shown to turn on more

5' *Hox* genes under the influence of their landing position, sensing a *Hox*-inducing posterior signal stronger in this location than at the original site, and the more posterior the position the stronger the signal (Itasaki *et al.*, 1996; Grapin-Botton *et al.*, 1997). These *Hox*-inducing signals were proposed to be of two types, as shown by additional experiments. On the one hand, transposition of posterior rhombomeric neurepithelium to a more anterior position did not alter its *Hox* expression status, but gave rise to the induction of more posterior *Hox* genes in the host epithelium surrounding the graft. "Planar" signals, traveling within the neurepithelium plane, were thus capable of inducing 5' *Hox* genes in anterior neural tissue (Grapin-Botton *et al.*, 1997). On the other hand, posterior somites transposed more anteriorly were shown to lead to *de novo* induction, in the adjacent neural tube, of *Hox* genes normally corresponding to the more posterior position of the explanted somites (Itasaki *et al.*, 1996; Grapin-Botton *et al.*, 1997). Moreover, more posterior somites induced stronger *Hox* gene expression in the adjacent neural tube, suggesting the existence of a caudo-rostral gradient of inducer activity in the paraxial mesoderm, vertically transmitted to induce novel *Hox* gene expression in the neurepithelium (Itasaki *et al.*, 1996; Grapin-Botton *et al.*, 1997). In independent experiments, Gould *et al.* (1998), studied the interactions between paraxial mesoderm signaling and *Hoxb4* expression in the neurepithelium in explanted mouse tissues in coculture. They obtained results consistent with the reprogramming of *Hox* gene expression upon tissue transposition *in vivo*. In addition, they could identify that a RA-mediated process, conserved between mouse and chick embryos, was crucially involved in the signaling event.

It seems therefore clear that the *Hox* gene expression state can be changed in a particular neural cell provided that this cell is

competent to express *Hox* genes (this competence slowly decreasing rostrally to r7) and that its new surrounding corresponds to axial positions posterior enough to induce more 5' *Hox* genes (caudally to the r6/r7 border, or adjacent to a somite more posterior than somite 5) (Itasaki *et al.,* 1996; Grapin-Botton *et al.*, 1997). This plasticity of gene expression has been described at developmental stages that are believed to precede the time when the maintenance mode of *Hox* gene regulation is stabilizing the *Hox* transcriptional states (day 9.5, according to Yu *et al.*, 1998, looking at mouse *Hoxa7*, and to Koseki *et al.*, personal communication, looking at mouse *Hoxc6*).

Considering these experimental data together with the results from Gaunt and colleagues (Gaunt and Strachan, 1994, 1996; Gaunt *et al.*, 1999), it is tempting to speculate that the molecular mechanism responsible for de novo induction of more posterior *Hox* genes after tissue transposition, is actually the same as the mechanism accounting for rostral extension of the *Hox* expression domains in the normal situation (see II.A).

II.C. Upstream regulators and effectors in the establishment phase: transduction of positional information to the Hox genes

Signals mediating A-P positional information in the embryonic germ layers have to be transduced to the *Hox* transcriptional machinery. Several transcription factors have been shown to contribute to controlling the spatially restricted expression of the *Hox* genes. Some of these factors seem to modulate rostral spreading of *Hox* gene expression along most of the rostrocaudal embryonic axis while others confine their effect to specific hindbrain compartments (rhombomeres). One can distinguish between these regulatory processes on a time basis as well, i.e. between the early establishment phase of the *Hox* expression domains from posterior regions to their maximum anterior extension, and the later definition and reinforcement of rhombomeric positional identity. *Hox* gene expression in the hindbrain seems to be subject to specific, additional regulatory circuits restricting and reinforcing gene expression in very specific territories, individual rhombomeres or subsets of rhombomeres and associated neural crest (see section II.C.3).

II.C.1. Molecular genetic interactions presiding to the establishment of the Hox expression domains in spinal cord and trunk mesoderm

II.C.1.a) Trans-acting regulatory factors: the case of caudal

The patterning action of *Drosophila* homeotic genes is prepared by several categories of genes that are partitioning the blastoderm along the A-P axis into progressively smaller compartments: the maternal and the segmentation (gap, pair-rule and segment polarity) genes, respectively (reviewed by St Johnston and Nüsslein Volhard, 1992). However, no correspondence between flies and vertebrates has been uncovered at this level, probably reflecting the fact that the strategies of development differ considerably between them. Work of several laboratories, though, seems to involve the homologs of *Drosophila caudal*, an upstream regulator of regional A-P patterning (MacDonald and Struhl, 1986; Mlodzik *et al.*, 1990), in the control of *Hox* gene expression in mice, chick and frog embryos. *Drosophila* maternal *caudal*, the expression of which is shaped into a posterior to anterior protein gradient by maternal *bicoid* gene products (Rivera-Pomar *et al.*, 1995), and

later on zygotic *caudal,* are known to positively regulate posterior gap and pair-rule gene expression (Dearolf *et al.*, 1989; Rivera-Pomar *et al.*, 1995; Schulz and Tautz, 1995), the latter turning on selected *Hox* genes (Levine and Harding, 1989).This function of *caudal,* contributing to shape the expression patterns of the *Hox* genes, was shown to be conserved in the short germ band insect *Tribolium* (Wolff *et al.*, 1998).

Among the three mouse *caudal* homologs, *cdx2* seems to be expressed the earliest, transcripts being detected in extraembryonic ectoderm along the junction with adjacent proximal epiblast at the expanded blastocyst stage (Beck *et al.*, 1995). The three mouse *cdx* genes begin to be expressed in the embryonic part of the conceptus during gastrulation, in domains initially very posterior within and along the primitive streak, spreading forward to a rostral limit in neurectoderm and mesoderm which is gradually more anterior for *cdx4* (Gamer and Wright, 1993), *cdx2* (Beck *et al.*, 1995) and *cdx1* (Meyer and Gruss, 1993). Transcription of these three *cdx* genes therefore generates nested sets of expression domains overlapping posteriorly, together creating a stepwise gradient of *cdx* gene products with a posterior maximum (see also Marom *et al.*, 1997, describing a similar situation in the chick). In each case, the expression domain in the mesoderm is slightly more posterior than in the neurectoderm. The characteristics of initiation and early forward spreading of *cdx* gene expression, and the maximal extension of the *cdx* transcription domains from the caudal part of the embryos up to the preotic sulcus -r2/r3- for *cdx1* at the head fold stage (Meyer and Gruss, 1993), are very reminiscent of the properties of the *Hox* genes (Fig. 4). Recent findings from experiments on *Amphioxus* are possibly revealing major clues to this resemblance. The *Amphioxus cdx* gene belongs to the same cluster as other homeobox genes, this cluster most likely being a paralog of the *Amphioxus* proper *Hox* cluster, the ParaHox cluster (Brooke *et al.*, 1998). Ancestor *Hox* and *cdx* genes were therefore most presumably close relatives of each other. Recent experimental work in *Drosophila* in fact reinforces the hypothesis of a close functional likeness between *Hox* and *cdx* genes, making it clear that *caudal (cad)*, in addition to modulating early segmentation, behaves as a canonical *Hox* gene (Moreno and Morata, 1999). It is expressed exclusively in the most posterior segment of the fly (A10) and its derivatives, the analia; it is required in this segment for normal analia development; it suppresses in A10 the expression of the immediately more anteriorly acting gene, *Abdb,* and is able to induce ectopic analia development when expressed in ectopic places. *Cad* behaves as a *Hox* gene located immediately 5' to *AbdB.* Since *cad* homologs are not physically linked to the *Hox* genes in any known animal group, *cad* would be a paralog of a 5' neighbor of *Abdb* on the ParaHox cluster, as shown for its *Amphioxus* homolog. *caudal* would therefore have two functions in *Drosophila* and in short germ band insects: early on, it would regulate segmentation of the blastoderm under syncitial conditions (in the long germ band *Drosophila* embryos and in the syncitial part of the short germ band embryos); later on, it would pattern the terminal structures, conferring its identity to the most posterior body segment of the fly (Moreno and Morata, 1999). The expression pattern of *caudal* in this second phase, in short germ band *Tribolium* embryos which produce abdominal segments in a secondary growth process, is in fact very reminiscent of that seen in vertebrates (Schulz *et al.*, 1998).

It is striking, in the light of these findings, that the mouse *cdx* gene products are found to regulate the *Hox* genes. Null muta-

tions in mouse *cdx1* (Subramanian *et al.*, 1995) and *cdx2* (Chawengsaksophak *et al.*, 1997) genes have been shown to lead to anterior homeotic-like transformations in the vertebral column, and *Hox* gene expression was shown to be altered in *cdx1* null mutants (Subramanian *et al.*, 1995). Moreover, overexpression of each of the three *cdx* genes in the mouse has been shown to be capable of rostrally shifting *Hoxb8lacZ* transgene activity as well as endogenous *Hoxb8* expression upon interaction with CDX binding sites in the upstream proximal region of the *Hoxb8* gene (Charité *et al.*, 1998), indicating that *cdx* gene products are capable of directly stimulating *Hox* gene expression, and that the three *cdx* genes have overlapping functions when coexpressed (Charité *et al.*, 1998). Independently, *Xenopus caudal* (Isaacs *et al.*, 1998) was shown to control *Hox* gene expression, and the *C. elegans caudal* homolog was found to regulate the early expression of the *Hox* gene *mab-5* in a particular cell (Hunter *et al.*, 1999). The discovery of a late, very conserved function of *caudal* as a homeotic-like posterior determinant makes the interpretation of the *cdx/Hox* interactions documented *in vivo* in mouse embryos less straightforward: do the latter reflect the evolutionary conservation of the early function of *caudal* in insects, regulating early A-P patterning and *Hox* gene expression (indirectly in the case of insects, and directly in *C. elegans* and vertebrates), or do they correspond to positive cross-regulatory interactions between related genes? A definitive answer to this question is not available at the moment.

A direct involvement of FGF in the control of *Hox* gene expression and A-P patterning mediated by a *caudal* homolog has been reported in *Xenopus* (Pownall *et al.*, 1996; Isaacs *et al.*, 1998). FGF was shown to be involved in controlling *Hox* genes also in the mouse (Partanen *et al.,* 1998). The latter authors studied the phenotypes of FGFR1 receptor mutants, and observed homeotic-like transformations in the vertebral column and alterations of *Hox* gene expression domains. It is possible that the FGF effect on mouse *Hox* genes operates via the *cdx* genes as well, although no evidence pointing in this direction has been obtained so far (see Partanen *et al.*, 1998).

II.C.1.b) cis-acting Hox regulatory elements

Proximal regulatory elements have been uncovered for many *Hox* genes. The emerging picture is that some of these enhancers autonomously confer *Hox*-like expression properties to a reporter transgene (see for example Marshall *et al.*, 1994), that some enhancers influence the transcription of the genes flanking them on both sides (see for example Sharpe *et al.*, 1998), and that several enhancers of a particular gene often cooperate to direct more anterior expression than each of them alone (see for example Charité *et al.*, 1995). Unlike it is the case with paralog groups 1 to 4, single or combined proximal regulatory regions of paralog groups 6 to 13 generally do not suffice in driving expression of a *Hox lacZ* transgene to the correctly restricted A-P pattern, transgene
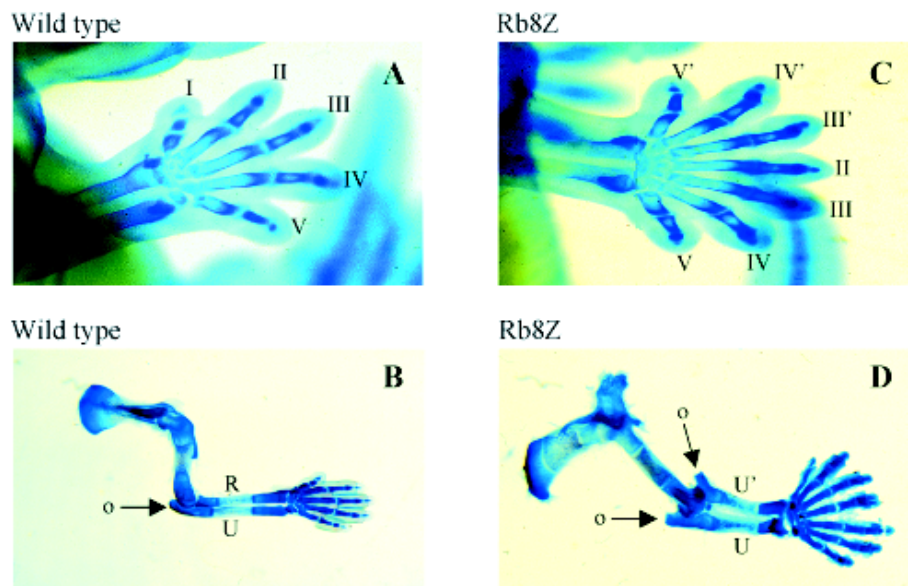

Fig. 5. Alcian blue stained control (wild type) and Hoxb8 overexpressing transgenic (Rb8Z) forelimbs. *Control* **(A)** *and transgenic* **(C)** *forelimbs of day 12.5 embryos show mirror-image duplication of posterior digits and carpal bones of the transgenic limb; Control* **(B)** *and transgenic* **(D)** *forelimbs of 14.5 day embryos reveal mirror-image duplicated ulna (u) and olecranon process (o) as well as mirror-image duplicated posterior digits in the transgenic limb (see text and Charité et al., 1994).*

expression being usually more posterior than that of the endogenous gene. This implies either that remote cis-acting sequences are involved in specifying endogenous *Hox* expression domains, or that a higher level of *Hox* gene control is operating (i.e. at the level of the whole cluster), or possibly both. In three cases recently, involvement of a remote control element has been invoked to account for *Hox* gene expression features. Firstly a *Hoxd* regulatory element responsible for expression of *Hoxd9* to *Hoxd13* in overlapping domains in distal limb buds is believed to reside very far from the corresponding transcription unit (van der Hoeven *et al.*, 1996; Hérault *et al.*, 1999). This element would correspond to a unique function of these genes in patterning the autopod (see section II D). Secondly, a regulatory element essential for maintaining the anterior expression boundary of *Hoxc8* in neural tube and mesoderm has been localized between 11 and 19 kb downstream of *Hoxc8* (Bradshaw *et al.*, 1996). Thirdly, a control element located 30 kb downstream of *Hoxb8* may contribute, together with other proximal *Hoxb8* control elements, to the specification of the definitive rostral expression boundary of this *Hox* gene in neural tube and mesoderm (Valarché *et al.*, 1997). This element is also a candidate for regulating the definitive expression boundary of several neighbor *Hoxb* genes.

Among the mechanisms presumed to account for the balance between the contribution of different enhancers in properly regulating particular *Hox* genes, selectivity, competition and sharing of regulatory elements have been put forward and experimentally documented (Gould *et al.*, 1997; Sharpe *et al.*, 1998). These interactions between the control sequences of neighbor *Hox* genes would account at least in part for the fact that the genes have remained clustered during evolution. However, *Drosophila* homeotic genes do not appear to share enhancers (discussed by Duboule, 1998). Loss of selective pressure to maintain integrity of the cluster

in this organism may be causally linked to changes in the regulatory interactions to more appropriately fit the strategy of development in this long germ band insect.

II.C.2. RA and the establishment of Hox gene expression domains

A possible role of RA in modulating the expression domains of the *Hox* genes during embryogenesis was suggested by Kessel and Gruss (1991). These authors exposed embryos *in utero* to exogenous RA, and observed posterior homeotic-like transformations in the vertebral column concomitantly with rostral shifts in *Hox* expression domains. Depletion of endogenous RA has now confirmed that this or a related compound plays a crucial role during the establishment of the *Hox* expression domains: while the initial expression of *Hoxa1* in the Retinaldehyde dehydrogenase (Raldh2) null embryos was present at a significantly reduced level, complete loss of *Hoxa1* expression was observed in 8.5 and 9.5-day mutant embryos (Niederreither *et al.*, 1999). The significant increase in the level of RA generation at the beginning of somitogenesis in the chick (Maden *et al.,* 1998) might, at least in part, correlate with the role of RA in stimulating *Hox* gene expression in the rostrally extending axial and paraxial expression domains. Strikingly enough, spinal cord and somites were shown to generate very high levels of RA at early somite-stages, while the CNS anterior to the brain/spinal cord junction (r1 to r6) did not (Maden *et al.*, 1998). RA is present homogeneously within a large domain with a sharp cutoff at the level of r6/r7, rather than as a gradient with a posterior maximum. In order to account for the expression of the most 3' *Hox* genes such as *Hoxb1* whose transcription is controlled by a RARE-containing regulatory element, at levels of the hindbrain which do not produce RA (anterior to r7), diffusion of RA from the spinal cord into the presumptive hindbrain in the plane of the neuroepithelium was proposed as a possibility, although no such diffusion could be observed when assaying the presence of RA in the tissue (Maden *et al.,* 1998). Alternatively, because the establishment of the early expression domain of *Hoxb4* in the neurectoderm depends on a retinoid-based mechanism in the flanking paraxial mesoderm (Gould *et al.,* 1998*)*, there is a possible role of the RA-producing hindbrain mesenchyme in the induction of 3' *Hox* genes in the rostral hindbrain neurepithelium. However, again, no diffusing RA could be visualized in the hindbrain neurectoderm anterior to r7 (Maden *et al.*, 1998). Gould and colleagues elegantly showed that a mesoderm-derived factor and a retinoid signal are necessary for *Hoxb4* to be induced in the hindbrain in explant cocultures. The influence of vertical signals from the flanking paraxial mesoderm was shown to induce *de novo* expression of more 5' *Hox* genes in rhombomeres surgically transposed more posteriorly at spinal cord levels in the chick (Itasaki *et al.*, 1996; Grapin-Botton *et al.*, 1997). The *Hox* genes shown to be induced in posteriorly transposed rhombomeres were not only the genes known to be directly RA-sensitive such as paralog groups 1, 2 and 4 (see next section), but all genes belonging to paralogous groups 1 to 8 (Grapin-Botton *et al.,* 1997). Whether the competence to respond to RA is direct or indirect in all cases is not known.

II.C.3. Molecular interactions controlling Hox gene expression in the hindbrain

Several transcription factors have been shown to participate in the regional control of *Hox* gene expression in the hindbrain. The zinc finger protein Krox-20 directly regulates the expression of

Hoxb2 (Sham *et al.*, 1993; Vesque *et al.*, 1996) and *Hoxa2* (Nonchev *et al.*, 1996) in rhombomeres 3 and 5 upon binding to specific sequences in their control regions. Targeted inactivation of *Krox20* resulted in the progressive loss of r3 and r5 territories, which then also affected the development of even-numbered rhombomeres (Schneider-Maunoury *et al.*, 1993, 1997). Interestingly, recent analysis of *Hoxa2⁻ᐟ⁻/Hoxb2⁻ᐟ⁻* mutants revealed normal generation and maintenance of rhombomeric territories, indicating that the progressive elimination of r3 and r5 in *Krox-20⁻ᐟ⁻* embryos must be mediated through different downstream targets (Davenne *et al.*, 1999). The product of the *kreisler* gene, a basic domain-leucine zipper transcription factor (Cordes and Barsch, 1994), was shown to directly activate *Hoxb3* and its paralog *Hoxa3* in r5, via conserved binding sites (Manzanares *et al.*, 1997, 1999). The kreisler-dependent activation of *Hoxb3* in r5 occurs in combination with an Ets-related transcription factor that binds to a kreisler neighbor site. While a similar Ets-dependent motif is also present in the *Hoxa3* enhancer, this site does not appear to be required for *Hoxa3* spatially restricted expression (Manzanares *et al.*, 1999). In contrast, kreisler also directs *Hoxa3*-specific upregulation in r6 through the same high-affinity binding site controlling r5 expression (Manzanares *et al.*, 1999). Altogether these data highlight the common origin of paralogous *Hox* gene regulatory regions from an ancestral *Hox* cluster and their subsequent evolutionary divergence following *Hox* cluster duplications, leading to subtle changes in paralogous *Hox* gene expression domains. Consistent with a role in initiating r5 and r6 segmental identities, *kreisler* mutants lack r5- and r6-derived structures, including cranial nerves and their associated ganglia (McKay *et al.*, 1994; Manzanares *et al.*, 1997; McKay *et al.*, 1997).

As mentioned above, retinoic acid receptors RARs and RXRs (Kastner *et al.*, 1997) and their ligands are also directly involved in the regulation of *Hox* genes expressed in the hindbrain. Retinoic Acid Response Elements (RAREs) have been found proximally to promoter regions of *Hox* gene paralog groups 1 and 4. *Hoxb1* is flanked by two distinct RARE containing sequences, a 3' element shown in transgenic studies to be essential for gene expression in the early neural tube (Marshall *et al.*, 1994), and a 5' element involved in restricting later expression to r4 (Studer *et al.*, 1994) (see also Ogura and Evans, 1995). *Hoxa1* also carries a 3' RARE containing sequence (Frasch *et al.*, 1995), the inactivation of which has been shown to lead to a delay in the establishment of the anterior expression boundary, resulting in abnormal hindbrain patterning at later developmental stages (Dupé *et al.*, 1997). Double mutant analysis with *Hoxb1* 3' RARE, *Hoxa1* 3' RARE and *Hoxa1* and *Hoxb1* null alleles revealed the requirement of *Hoxa1*, *Hoxb1*, and endogenous retinoids to initiate *Hoxb1* expression, as well as strong genetic interactions between *Hoxa1* and *Hoxb1* in establishing r4 identity and in patterning r4-derived structures (Gavalas *et al.*, 1998; Studer *et al.*, 1998). Maintenance of *Hoxb1* expression in r4 is mediated through an autoregulatory element to which HOXB1 binds together with the product of the *pbx* gene (homolog of the *Drosophila extradenticle* gene) as a cofactor (Popperl *et al.*, 1995). Furthermore, Maconochie *et al.* (1997) reported that cross-regulation by HOXB1 and PBX proteins is also involved in the control of *Hoxb2* expression in r4.

In paralog group 4, a 5' RARE containing sequence contributes to setting *Hoxd4* expression patterns in paraxial and lateral plate mesoderm (Zhang *et al.*, 1997), and a RARE modulates *Hoxa4*

gene expression as well (Packer *et al.*, 1998). A RARE containing enhancer is required, together with a mesoderm signal, to induce early expression of *Hoxb4* in the neural tube up to an anterior boundary at r6/r7 (Gould *et al.*, 1998) (see section II.B.). It is noteworthy that swapping the *Hoxb4* RARE sequence (a DR5-type) with the 3' RARE of *Hoxb1* (a DR2-type) results in a shift of the rostral expression boundary from r6/r7 to r3/r4, indicating that these sequences bear information necessary for correct positioning of the anterior boundaries of *Hoxb4* and *Hoxb1* expression (Gould *et al.*, 1998). Maintenance of *Hoxb4* expression in its rostralmost domain at later developmental stages is controlled through a distinct rhombomeric enhancer (Gould *et al.*, 1998). This late enhancer contains a *Hox*-responsive element which was shown to bind the HOXB4 protein and its paralogs, together with PBX, in a way that appears to be conserved between mammals and *Drosophila* (Gould *et al.*, 1997). Interestingly, HOXB5 can also bind the *Hoxb4* autoregulatory element, resulting in a positive cross-regulation which reinforces *Hoxb4* expression in the region of overlap between the *Hoxb4* and *Hox*b5 expression domains (Gould *et al.*, 1997).

II.D. Control of Hox gene expression in emerging and developing limb buds

The regulation of *Hox* gene expression is thought to play an important role during two distinct phases of limb development: during the positioning of the limb fields along the A-P axis, and during later control of growth and morphogenesis of the developing limbs (reviewed by Johnson and Tabin, 1997). It is difficult to describe the molecular regulation of the *Hox* genes during limb outgrowth and patterning without a simultaneous description of the embryological events associated with the (changes in) gene expression features. Early *Hox* gene expression in the lateral plate mesoderm presumably contributes to specifying the axial position of the future forelimb bud (reviewed by Cohn and Tickle, 1996). Regulation of *Hox* expression domains in lateral plate mesoderm is believed to have been crucial in the sequential appearance of the two pairs of limbs which are fundamentally non-equivalent (Tabin and Laufer, 1993; Coates and Cohn, 1998), leading to the emergence of tetrapods. Control of *Hox* gene expression in the lateral plate mesoderm is likely to have operated independently from gene control in paraxial mesoderm (Gaunt, 1994; Burke *et al.*, 1995; Cohn *et al.*, 1997; Coates and Cohn, 1998). Control of *Hox* expression in the presumptive fore- and hindlimb and during limb emergence is likely to be exerted by growth factors and their receptors, important modulators of growth and morphogenetic patterning. FGF10 in particular has been shown to be required for development of both pairs of limbs (Min *et al.*, 1998). The A-P position of the rostral expression boundary of a number of *Hoxb* genes in lateral plate mesoderm has been proposed to contribute crucial information to positioning the limb field (Rancourt *et al.*, 1995; Cohn and Tickle, 1996; Cohn *et al.*, 1997), and to position the posterior part of the limb field and its signaling center, the Zone of Polarizing Activity (ZPA) (Charité *et al.*, 1994 regarding the mouse; Stratford *et al.*, 1997, and Lu *et al.*, 1997 for chick studies). The molecular-genetic relationship between *Hox* gene expression and the generation of the ZPA is still enigmatic. The ZPA concept itself has evolved with time to optimally fit the biological observations: it was first defined as the "polarizing activity" present in the posterior limb margin, that could induce development of supernumerary

digits and mirror-image symmetry after being transplanted into the anterior margin of a host chick limb bud (Saunders and Gasseling, 1968); this polarizing activity was found to be present at earlier stages all along the flank mesoderm from the node region up to the limb levels, and to peak at the level of the posterior part of the limb bud at the time limb buds are about to emerge (Hornbruch and Wolpert, 1991). More recently, the ZPA was found to be an organizer, site of very specific gene expression centered on the activity of *Sonic hedgehog* (*Shh*), considered to primarily mediate the polarizing activity (Echelard *et al.*, 1993; Krauss *et al.*, 1993; Riddle *et al.*, 1993). The posterior limb margin was also proposed to be an area of stage-dependent differential growth.

A role of early *Hoxb* gene expression in the lateral plate mesoderm would be to supply positional information specifying and polarizing the limb fields along the A-P axis. *Hoxb* genes, and *Hoxb8* in particular, could possibly confine early asymmetric expression to potential key regulators of *Shh* such as *Gli3* (Schimmang *et al.*, 1992; Masuya *et al.*, 1995) and *Alx4* (Qu *et al.*, 1997; Takahashi *et al.*, 1998), resulting in the posterior restriction of the *Shh*- expressing ZPA. *Hoxb8* expression in the lateral plate mesoderm during forelimb bud emergence overlaps the ZPA and precedes *Shh* expression (Charité *et al.*, 1994). *Hoxb8* gain of function mutants exhibit mirror-image duplications of the posterior part of the autopod and zeugopod (see Charité *et al.*, 1994). This phenotype is more severe than that of *Gli3 (Xt)* (Hui and Joyner, 1993), and *Alx4 (Lst)* (Qu *et al.*, 1997) null mutants, which present mirror-image duplications in the autopod exclusively, suggesting that *Hoxb8* is hierarchically higher/operating earlier than the asymmetrically expressed *Gli3* and *Alx4* genes in the control of limb bud patterning. Moreover, no change in *Hoxb8* expression boundary was observed in *Gli3* (*Xt*) (Zúñiga and Zeller, 1999) and *Alx4* (*Lst*), (Chan *et al.*, 1995) polydactyl null mutants.

The AbdB-type 5' *Hoxd* genes are coordinately expressed in the limb buds after emergence (Dollé *et al.*, 1989). In addition to being involved in patterning axial and paraxial structures at relatively caudal levels in the embryo (reviewed by Duboule, 1992), these genes have been shown to play a role during limb morphogenesis. They are sequentially expressed in overlapping domains centered on the posterior margins of fore- and hindlimb buds (Dollé *et al.*, 1989) where *Shh* will be expressed later. In a subsequent phase, the expression domains of these 5' *Hoxd* genes rotate and extend distally into the presumptive digit territory. This distal shift/rotation in the expression domains correlates with the bending of the proximo-distal limb axis, the metapterygial axis, in digited tetrapods. This bending of the axis, and the resulting differential growth of the postaxial mesoderm are believed to have allowed the generation of the digits (Sordino *et al.*, 1995; Sordino and Duboule, 1996; Shubin *et al.*, 1997). Experimental evidence strongly suggests that a common 5' *Hoxd* enhancer mediates gene expression in the neomorphic digit territory (Van der Hoeven *et al.*, 1996; Hérault *et al.*, 1999). This novel regulatory mechanism would have been acquired by a *Sarcopterygian* fish in the Devonian period, marking the transition between finned and digited tetrapods (Sordino and Duboule, 1996; Shubin *et al.*, 1997). Bending of the metapterygial axis modifies the asymmetries in the limb, the proximo-distal polarity being transformed into apparent posterior-anterior polarity in the autopod (hand and foot). The fish axis does not undergo bending and remains proximo-distally oriented. Re-

garding A-P regionalization, the metapterygial axis runs through the stylopod element, through the most posterior element of the zeugopod (ulna and fibula), and through the posterior carpal and tarsal bones (in the arm and leg autopod, respectively), before it bends in the anterior direction, leaving the digits on the postaxial side (see Sordino and Duboule, 1996). Digit morphogenesis therefore is the result of the extended growth phase in distal limb buds, accounted for by the extended *Hoxd13* expression domain driven by the "digit" enhancer (Hérault *et al.*, 1999). Not surprisingly, *Hoxd13* null mutants exhibit truncated digits rather than A-P patterning defects (Dollé *et al.*, 1993). The A-P polarity of the early limb bud, foreshadowing the A-P polarity of the complete limb, is unlikely to rely on *Shh* expression from the beginning on since this gene is only expressed relatively late (in quite developed forelimb buds at about the 30-somite stage in the mouse, Charité *et al.*, 1994). Expression boundaries of early *Hoxb* genes are candidates to supply positional information polarizing the early limb bud long before *Shh* is expressed at the posterior margin. *Shh* would be involved later, both in controlling A-P polarity and growth of the autopod proximo-distally. The phenotype of the *Shh* null mutant confirms the involvement of this gene in distal growth of the limb buds rather than in the specification of posterior identity exclusively (Chiang *et al.*, 1996).

Expression of *Abdb*-type *Hoxa* and *Hoxd* genes (of paralog groups 9 to 13) in developing limbs is spatially and temporally coupled to limb bud outgrowth (Dollé *et al.*, 1989; Yokouchi *et al.*, 1991). Strikingly, the phenotypes of loss of function mutants in these genes affect limb elements in a way that is colinear with the position of the genes in the cluster (reviewed by Rijli and Chambon, 1997). Recent data reveal an additional level of complexity in the regulation of *Hoxd* genes in the limbs: *Hoxd* promoters would differentially integrate the influence of a remote, common 5' distal "digit enhancer" simultaneously exerted on all promoters, and the influence of a repressor exerted in a colinear way on the different genes (Hérault *et al.*, 1999; Kondo and Duboule, 1999).

II.E. Concerted gene regulation within the Hox complexes

The organization of the *Hox* genes in clusters has long been proposed to be intrinsically linked to the sequential expression of the genes along the embryonic axis. Colinearity is observed between the order in which the genes reside in their cluster and the rostral extensions of their expression domains (Duboule and Dollé, 1989; Graham *et al.*, 1989). A colinearity between the genes from 3' to 5' and their time of first activation during embryogenesis has been described as well (reviewed by Duboule and Morata, 1994). This temporal colinearity rule, was found in short germ band insects (see Kelsh *et al.*, 1994, studying *schistocerca*) and is thought to have been lost during evolution to the highly specialized *Drosophila*, where rapid and simultaneous partitioning of the blastoderm into parasegments has abolished temporal sequentiality of events. In the abdominal part of short germ band insect embryos and in the vertebrate embryonic territories patterned by the *Hox* genes, each *Hox* gene is assumed to be initially expressed earlier than its 5' neighbor in the complex (although differences in relative expression levels, and occurrence of positive autoregulation of certain genes make it hard to document this point in each case). The latter colinearity in fact reflects the chronological progression of embryogenesis, anterior structures (patterned by 3' *Hox* genes) being laid down before posterior structures (patterned by more 5' *Hox* genes) (reviewed

by Duboule, 1994). A major recent finding (Kondo and Duboule, 1999) suggests that the colinear and sequential initiation of *Hoxd* gene expression is essentially controlled by a repressive mechanism operating via a regulatory element located upstream of the complex. Concerted, directional regulation of the genes within the clusters would thus take place during initiation of gene expression. It would essentially depend on a general repressive mechanism, preventing the promoters from becoming active prematurely, and making them accessible sequentially in time in a way that is not understood so far. Whether this repressive regulation and sequential activation involves the Polycomb and trithorax gene products, known to maintain a repressive or active transcriptional state (respectively) by acting at the level of chromatin remodeling (see section III) is not known yet.

Molecular interactions occurring through binding of transacting factors to gene-specific enhancers would be a lower operating system, active during the establishment phase of the *Hox* domains. These interactions are nevertheless essential for the patterns to be maintained at later embryonic stages: after deletion of the "upstream *Hoxd* repressive element", genes that were prematurely initiated were ectopically expressed in anterior structures, where the normal interactions with regulators during the establishment phase will not take place, resulting in the absence of maintenance of transcription. The late *Hoxd* expression patterns in the embryos where the "general repressive element" has been deleted are very similar to that of control embryos.

Enhancers common to several genes would contribute an additional level of integration, either supplementing additional "safety" control, or introducing a regulatory loop to insure specific, locally restricted expression to a subset of the clustered genes, (e.g. the distal limb element in the *Hoxd* cluster, van den Hoeven *et al.*, 1996; Hérault *et al.*, 1999).

III. Long-term maintenance of the spatially localized expression of the *Hox* genes

Hox gene functions in *Drosophila* are required not only for the early acquisition of positional identity along the A-P axis, but also for the maintenance of this identity after the early *Hox* inducers, i.e. maternal and segmentation genes, have been turned down. Regulatory mechanisms allowing maintenance of spatial patterns of gene expression have been conserved between insects and mammals: in both types of organisms the products of two classes of regulator genes come into play to insure cellular memory of the transcriptional states. These genes control the expression of numerous loci, among which the *Hox* complexes. The Polycomb group (Pc-G) genes maintain the position of the rostral *Hox* expression boundaries by repressing transcription outside the normal expression domains; the trithorax group (trx-G) genes contribute to maintaining the spatial *Hox* expression domains by sustaining gene activation within their established transcription domains. These negative and positive "executors" of cellular transcriptional memory have been supposed to exert their action on gene expression by affecting chromatin structure and accessibility of cis-control elements to trans-acting factors (reviewed by Paro, 1995; Gould, 1997; Pirrotta, 1997a,b, 1998; Schumacher and Magnuson, 1997).

The Pc-G gene family encodes proteins acting in multimeric complexes to repress expression of multiple loci among which the

Hox genes, by some "silencing" mechanism. These Pc-G protein complexes are thought to act upon binding chromatin at Polycomb Response Elements (PREs), found in the regulatory regions of target genes. Recently, a conserved sequence motif was detected between a number of PREs (Mihaly *et al.*, 1998). So far, only one of the known Pc-G proteins binds to DNA in a sequence specific way: the *Drosophila Pleiohomeotic* (PHO) gene product directly interacts with a PRE from the *engrailed* gene. Interestingly, the core DNA binding sequence recognized by PHO largely overlaps the conserved motif found in many PREs. It has been proposed that PHO acts to anchor Pc-G protein complexes to DNA (Brown *et al.*, 1998). Nevertheless, the molecular mechanism allowing the Pc-G complexes to affect transcription of the target loci is still not understood. The current hypothesis is that the Pc-G multimeric complexes interact with the regulatory regions of inactive *Hox* genes and promote a local remodeling of chromatin leading to sustained repression of transcription.

Because Pc-G mutations lead to homeotic-like transformations and ectopic expression of homeotic genes in *Drosophila*, mutants in these genes could be easily recovered, and some 15 Pc-G genes have been identified and cloned, a number of which have been found to have homologs in mice and humans (reviewed by Gould *et al.*, 1997, and Schumacher and Magnuson, 1997). A number of fly and mammalian Pc-G gene products were found to share a common motif (see Schumacher and Magnuson, 1997), the chromodomain, also found in the heterochromatic protein HP1, and essential for the functional activity of this protein. Others share different essential conserved motifs, some of which involved in protein-protein interactions (specialized zinc finger domains such as the Ring finger motif, the SET domain, also found in a heterochromatin protein and in the trithorax protein). The ESC protein and its mouse homolog EED bear a set of WD40 repeats, a motif found in other proteins.

Drosophila Pc-G mutations do not equally affect A-P patterning and lead to derepression of homeotic genes in a varying extent. The homozygous *Pc* mutant, for example exhibits derepression of all homeotic genes along the entire A-P axis, and all segments are transformed into the eighth Abdominal type (Busturia and Morata, 1988). Most other Pc-G mutants show weaker homeotic phenotypes than *Pc*, affecting only a few segments. But heterozygous combinations of different Pc-G mutations result in more severe phenotypes, (Jurgens, 1985) suggesting that these genes act synergistically.

In the mouse, Pc-G mutations in most cases cause local derepression of a subset of *Hox* genes in the region anteriorly flanking the expression domains, giving rise to posterior transformations of the embryonic mesodermal (Akasaka *et al.*, 1996; Van der Lugt *et al.*, 1996; Coré *et al.*, 1997) and neurectodermal (polyhomeotic, Takihara *et al.*, 1997) structures at that level. Recent data reporting the synergistic effect of the mouse *bmi1* and *M33* (Bel *et al.*, 1998), and *bmi1* and *mel18* (H. Koseki and A. Berns, personal communication) null mutations document the validity of this feature in mammals as well. The mouse *eed* Pc-G gene seems to be required for earlier essential embryonic processes, similarly to its *Drosophila* counterpart *esc*, the mutation of which leads to severe phenotypes (Simon *et al.*, 1995). Homozygous *eed* null mutants die early during gastrulation, failing to correctly pattern their mesoderm which is almost integrally diverted to the posterior extraembryonic region of the conceptus

(Faust *et al.*, 1998). Posterior homeotic-like transformations are observed in the vertebral column of heterozygous *eed* null mutants and homozygous mutants carrying a hypomorphic allele (Schumacher *et al.*, 1996). As expected, an anterior shift of the expression domain of *Hoxc8* was observed in heterozygous *eed* null embryos (J. Wang and T. Magnuson, personal communication).

The fact that mutations in a Pc-G gene like *eed (esc)* arrest development before the time *Hox* genes are even starting to be transcribed is very intriguing. The *Drosophila* homolog of *eed, esc* is also required early, but at the time *Hox* silencing is thought to be established, suggesting that it mediates the recruitment of Pc-G proteins in the initial stage of complex formation. Is the mouse gene exerting two consecutive, independent functions during embryogenesis, and is the regulation of *Hox* gene expression the latest of those, or does this and possibly other Pc-G gene products exert a general basal function crucially requested at different time points and in different cells during embryogenesis? Frequent association between loss of function of Pc-G genes and impaired lymphoid cell proliferation (reviewed in Schumacher and Magnuson, 1997), and recent work establishing the link between Pc-G gene function and control of cell cycling versus senescence (Jacobs *et al.*, 1999) may well point towards the latter hypothesis. It has become a fact that growth and patterning during vertebrate development are for a large part tightly associated.

Drosophila trithorax -Group (trx-G) genes have been genetically defined by the fact that their mutations suppress the phenotype of Pc-G mutants (Capdevila and Garcia-Bellido, 1981; Ingham, 1983). In addition, mutations in *trithorax* genes do not affect early expression of the homeotic genes, but result in a late decrease in the expression level with varying degrees of severity depending on the trx mutation and the *Hox* gene analyzed (reviewed by Paro, 1995; Gould, 1997; Schumacher and Magnuson, 1997). Similarly, the mouse *trx* homolog *Mll* has been shown not to affect early *Hox* gene expression, but to lead to a decrease in the expression of tested *Hox* genes within their expression domains from day 9.5 on (Yu *et al.*, 1998). According to these data, maintenance of *Hoxa7* expression would take place between day 8.5 and day 9.5 post-fertilization. Since there is about a two day-difference between first expression of the most 3' and the most 5' genes, a difference between the operation time of the maintenance process is expected as well between 3' and 5' genes (see Fig. 1).

A subgroup of trx-G gene products is binding DNA through specific sequences (i.e. the GAGA factor, encoded by *trithorax-like, trx-l,* Farkas *et al.*, 1994, and the product of *Zeste*, Rosen *et al.*, 1998). The GAGA factor contributes to the NURF complex, presumed to act by stimulating nucleosome mobility in promoter regions (reviewed by Gellon and McGinnis, 1998). The products of some trx-G genes (*Drosophila brm*, mammalian *brm1* and *brg1*, see review by Gould *et al.*, 1997) are homolog to components of the SWI/SNF complex. This complex is capable of remodeling chromatin structure, presumably by regulating the accessibility of cis-control sequences to activator molecules. Analysis of *brm1* minus mice has shown that the BRM1 gene product has an essential role in cell proliferation that cannot be compensated fully by mouse BRG1, in spite of the fact that BRM1 and BRG1 have amino acid sequences that are for more than 70% identical (Reyes *et al.*, 1998). Recently, the *Drosophila* Brahma chromatin remodeling factor was shown to genetically interact with the trx-

G gene *osa* to regulate transcription of the *Antennapedia* P2 promoter at specific 5' sequences (Vazquez *et al.*, 1999). The OSA protein contains a DNA binding motif that may target the BRM complex to some of its regulatory targets (discussed in Vazquez *et al.*, 1999). The mode of action of the product of *trithorax* itself is not known.

It is becoming clear, in *Drosophila*, that different trx and Pc-G genes functionally interact, and that the Pc-G and trx-G mediated control of *Hox* gene expression mutually interfere: PREs bind trx-G gene products as well as Pc-G proteins (Chinwalla *et al.*, 1995). Furthermore, the binding of Pc-G and trx-G proteins to the bithorax complex was shown to be dynamic during early *Drosophila* development (Orlando *et al.*, 1998). A major challenge remains to elucidate the molecular interactions leading to specific silencing and positive maintenance of *Hox* gene expression by the Pc-G and trx-G complexes. It seems clear that Pc-G repression of homeotic genes utilizes a mechanism that is very similar to the silencing mechanism responsible for Position Effect Variegation (PEV). Both Pc-G silencing and PEV involve many proteins, the level of which determines the extent of the silenced region and the degree or stability of silencing (discussed by Pirrotta, 1997a). Moreover, gene silencing in *Drosophila* has been shown to use a mechanism related to the one used in mammals to prevent expression of an imprinted allele inherited from one of the parents (Lyko *et al.*, 1997).

IV. Discussion: are cluster-involving regulatory mechanisms at the chromatin level modulating both initiation and maintenance of *Hox* transcription?

An intriguing question arises from considering the latter section on the relatively late role of Pc-G and trx-G mediated chromatin remodeling in propagating the memory of *Hox* transcriptional states, and the hypothesis mentioned in section I of an involvement of a chromatin-mediated control as a mechanism underlying the colinear initiation of the *Hox* genes in time and space. Van der Hoeven *et al.* (1996), Kondo *et al.* (1998) and Kondo and Duboule (1999) propose that the *Hox* clusters undergo a 3' to 5' opening at the level of chromatin structure: progressively, more 5' genes would become available for transcription as development progresses from the time the earliest *Hox* gene is initially expressed (day 7.5, later primitive streak stage), till the time the most 5' gene is turned on (day 9.5, tail bud stage). At a specific stage, later on, which is likely to be reached earlier for the 3' than for more 5' *Hox* genes, "freezing" the transcriptional state of the different genes of the clusters at the different A-P levels, would involve the same or an independent chromatin remodeling mechanism. An involvement of the Pc-G gene products in the initial "opening" of the *Hox* chromatin has been proposed by Bel *et al.* (1999) on the basis of their experiments with *M33* null mutants. Their hypothesis is that Pc-G gene products control time-restricted initiation of the clustered *Hox* genes by limiting access of transcription factors, some of which they suggest to be RA mediators (Bel *et al.*, 1999), a model in agreement with the hypothesis of Van der Hoeven *et al.* (1996), Kondo *et al.* (1998) and Kondo and Duboule (1999). The seducing aspect of an involvement of chromatin remodeling in the molecular process presiding over the concerted initiation of *Hox* gene expression is the following: there would be no need for RA (and/or the inducing morphogen) to be distributed in a gradient if the different RARE elements are "wrapped up" into a higher chromatin structure that would only gradually and sequentially make promoters and regulatory elements accessible to the transcription machinery. This would fit with the lack of evidence for any gradient of RA production/distribution in the vertebrate embryo, whether it is assayed by tracing RALDH2 protein, visualizing RA produced (Maden *et al.*, 1998), or following *RARbeta lacZ* expression in embryos (Mendelsohn *et al*, 1991; Charité *et al.*, 1994).

Summary

Spatially and temporally restricted expression of the *Hox* genes is essential for correct patterning of vertebrate embryos. In this overview we discuss the latest data that shed light on the mechanisms underlying the generation of the expression domains of the *Hox* genes. The molecular genetic interactions presiding over initial transcription of the *Hox* genes in the posterior part of the primitive streak during mouse and chick gastrulation remain enigmatic. But the recent discovery by Kondo and Duboule (1999) of a "cluster control region" mediating a repressive regulation, will undoubtedly lead to a better understanding of the molecular genetic mechanism underlying colinear and sequential initiation of *Hox* gene transcription. Recently, progress has been booked in elucidating the basal processes driving progression of the *Hox* expression domains during their establishment. The *Hox* expression domains are still labile while being established. The transcriptional state of *Hox* genes in anterior tissues can be reprogrammed under the influence of more posterior locations. RA, a posteriorly distributed endogenous morphogen, appears to be crucial for setting the expression domains of 3' *Hox* genes such as *Hoxa1* and *Hoxb1* in early embryonic tissues. In addition, a RA-related signal from the paraxial mesoderm is required for a 3' *Hox* gene like *Hoxb4* to be expressed in the early neuroepithelium. RA signaling seems to directly control 3' *Hox* genes via RAREs, and to play an important role in patterning the hindbrain. More 5' *Hox* genes, the expression boundaries of which are crucial for patterning the trunk and caudal tissues, are also responsive to posterior signals during the establishment of their expression domains. These signals are likely to act at specific cis-acting sequences, some of which have been identified. Sequential initial expression of the *Hox* genes is normally followed by regulatory events probably including RA and FGF signaling, and auto and cross regulatory interactions with *Hox* and *cdx* gene products. It is only when these interactions occur appropriately that the expression domains would be correctly established and maintained. After the *Hox* expression domains have been established, regulatory processes involving the products of *Polycomb* and *trithorax*- Group genes start operating; these perpetuate the transcriptional state of the *Hox* genes within and outside the expression domains. Plasticity of *Hox* gene expression would then be lost, presumably due to changes to the chromatin structure of the *Hox* clusters. Whether control at the level of chromatin structure, believed to operate during the late maintenance phase of *Hox* gene expression, is also involved in regulating concerted initial expression of the *Hox* genes, is a possibility that has been suggested. The expression of the *Hox* genes along the appendicular axes is regulated in part concomitantly with, and in part independently from the way these genes are controlled along the main A-P axis.

Early *Hoxb* genes might be involved in positioning the limb fields along the rostro-caudal axis, while *Abdb*-type *Hoxa* and *Hoxd* genes modulate later limb growth and morphogenesis along the proximo-distal limb axis.

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