

Acquisition of Hox codes during gastrulation and axial elongation in the mouse embryo

Sylvie Forlani*, Kirstie A. Lawson and Jacqueline Deschamps†

Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands

*Present address: Unité de Biologie Moléculaire du Développement, Institut Pasteur, 25 rue du Dr Roux, Cedex 15, 75724 Paris, France

†Author for correspondence (e-mail: jacqueli@niob.knaw.nl)

Accepted 1 May 2003

SUMMARY

Early sequential expression of mouse Hox genes is essential for their later function. Analysis of the relationship between early Hox gene expression and the laying down of anterior to posterior structures during and after gastrulation is therefore crucial for understanding the ontogenesis of Hox-mediated axial patterning. Using explants from gastrulation stage embryos, we show that the ability to express 3' and 5' Hox genes develops sequentially in the primitive streak region, from posterior to anterior as the streak extends, about 12 hours earlier than overt Hox expression. The ability to express autonomously the earliest Hox gene, *Hoxb1*, is present in the posterior streak region at the onset of gastrulation, but not in the anterior region at this stage. However, the posterior region can induce *Hoxb1* expression in these anterior region cells. We conclude that tissues are primed to express Hox genes early in gastrulation, concomitant with primitive streak

formation and extension, and that Hox gene inducibility is transferred by cell to cell signalling.

Axial structures that will later express Hox genes are generated in the node region in the period that Hox expression domains arrive there and continue to spread rostrally. However, lineage analysis showed that definitive Hox codes are not fixed at the node, but must be acquired later and anterior to the node in the neur ectoderm, and independently in the mesoderm. We conclude that the rostral progression of Hox gene expression must be modulated by gene regulatory influences from early on in the posterior streak, until the time cells have acquired their stable positions along the axis well anterior to the node.

Key words: Mouse, Hox genes, *Hoxb1*, *Hoxb4*, *Hoxb8*, Gastrulation, Clonal analysis, AP patterning

INTRODUCTION

Hox genes are key players in anteroposterior (AP) patterning, and their crucial role in this process is reflected in their extremely strong conservation among phyla (Gellon and McGinnis, 1998). The conserved chromosomal organisation of Hox genes in clusters (four in vertebrates) is intimately linked to regulatory constraints, which couple Hox gene expression to the progression of embryogenesis. In mammals, the laying down of anterior to posterior structures within the territories patterned by the Hox genes is accompanied by a sequential activation of these genes from 3' to 5' in the clusters. As a result, early structures are given an anterior identity with 3' Hox genes as key determinants, while progressively later structures start expressing more 5' Hox genes and acquire a more posterior identity. The remarkable correlation between the spatiotemporal expression of the genes and their linear order in the Hox clusters has been called spatiotemporal colinearity (reviewed by Duboule and Morata, 1994).

The function of Hox genes in AP patterning in the mouse has been most clearly demonstrated at levels close to the rostral boundary of their definitive expression domains. Homeotic transformations of segmented structures developing from these

boundary regions were observed in loss-of-function mutants (Krumlauf, 1994). Recently, transient abolition of early colinearity, which is evident during gastrulation, by deletion of a 5' located *Hoxd* regulatory region was shown to result in skeletal homeotic transformations in embryos and neonates (Kondo and Duboule, 1999). These results first demonstrated that the concerted colinear control of Hox expression from its onset at primitive streak stages is absolutely required for a correct Hox patterning function.

Most efforts to elucidate the molecular mechanisms that underlie regionalised Hox gene expression have focussed on relatively late developmental stages (reviewed by Deschamps et al., 1999). The few studies addressing the early establishment of the Hox domains have highlighted the difficulty in correlating these early patterns with cell behaviour during morphogenetic movements at gastrulation (Deschamps and Wijgerde, 1993; Gaunt and Strachan, 1994). Hox genes are activated when the primitive streak is almost fully extended, and then in the most posterior (caudal) part of the streak which is generating extra-embryonic mesoderm and not contributing to the embryo proper. The early transcription domains subsequently spread rostrally to reach the anterior part of the streak by an unknown, non-lineage-related mechanism (Deschamps and Wijgerde, 1993).

After the early Hox expression domains reach the node region, they continue to spread more rostrally to reach their definitive rostral boundaries in neurectoderm, mesoderm and endoderm in axial and paraxial structures. Previous work suggested that mesoderm acquires its positional information when emerging from the primitive streak (Tam and Beddington, 1987), and Frohman et al. (Frohman et al., 1990) proposed that differential Hox gene expression is established at that moment. The fate map of the presumptive neurectoderm at late gastrulation (Tam, 1989) similarly indicates that the epiblast near the anterior end of the streak contain progenitors of hindbrain and spinal cord; retrospective lineage analysis indicates that spinal cord is laid down at the node, sequentially from anterior to posterior (Mathis and Nicolas, 2000). Analysis of the evolution of the early Hox gene expression patterns suggested that the successive rostral boundary regions could be fixed at the node and carried by lineage transmission as the axis was laid down and the node 'regressed' (Deschamps and Wijgerde, 1993).

We have investigated the mechanism by which Hox gene expression is initiated and propagated along the streak towards the node, using embryonic explants, and show that the conditions for autonomous Hox expression are already present posteriorly but not anteriorly, at the beginning of gastrulation, more than 12 hours before overt Hox gene expression. We also show that this primed but non expressing posterior tissue can induce *Hoxb1* expression in non primed and non expressing anterior streak and epiblast tissue. Second, we examine the possibility that Hox expression boundaries are carried rostral to the node by lineage transmission, and show that this is not so in the neurectoderm, because the precursors that will occupy the future rostral expression boundary region are already anterior to the node when Hox expression reaches the node. No consistent relationship between the Hox gene expression status of cells at the node and the destination of their anteriormost mesoderm descendants was found. We conclude that the rostral progression of Hox gene expression must be modulated by gene regulatory influences from early on in the posterior streak, until the time cells have acquired their stable positions along the axis well anterior to the node.

MATERIALS AND METHODS

Mice

Embryos at E6.5-8.0 (E0.5 is defined as noon on the day of vaginal plug detection after overnight mating) were recovered from (C57BL/6J×CBA) F1 matings, and from a transgenic line containing a *Hoxb1-LacZ* construct (with 18 kb of genomic DNA from the *Hoxb1* locus, described by Marshall et al. (Marshall et al., 1994) on a similar background. This reporter construct perfectly mimics the early and late patterns of the endogenous *Hoxb1* expression in mesoderm/neurectoderm and in rhombomere 4 (Marshall et al., 1994; Studer et al., 1994). The heterozygous *Hoxb1-lacZ* embryos analysed were produced by crossing homozygous males with (C57BL/6J×CBA) females.

Embryonic explants

Embryos were isolated from the decidua at the desired gestational stage and Reichert's membrane was removed with tungsten needles (Hogan et al., 1994). Embryos were staged according to morphology (Downs and Davies, 1993), modified for C57BL/6×CBA embryos

(Edinburgh Mouse Atlas Project; <http://genex.hgu.mrc.ac.uk>; K.A.L., unpublished) and according to size (Lawson and Pedersen, 1992). Explants spanning the length of the primitive streak (Fig. 2) were excised with a glass needle while restraining the embryo by the extra-embryonic part with a tungsten needle. The posterior part of the primitive streak (posterior streak region, PSR) was separated from the extra-embryonic tissue at the level of the junction between embryonic and extra-embryonic ectoderm. The anterior part of the primitive streak designated ASR (anterior streak region, see Fig. 2) included the distal part of the embryo, because the anterior part of the streak alone did not grow well in culture. All explants contained the three germ layers and were about 100×100 µm in size, as measured with a micrometer. An intermediate piece between the most proximal and distal pieces of the streak was also taken from older stages ensuring that explants of similar size were taken from embryos of different age. Explants were transferred individually to small depression wells made in bacteriological dishes with a darning needle, covered with 60 µl drops of Dulbecco's modified Eagle's medium (DMEM) plus 15% fetal calf serum (FCS) under mineral oil and further cultured according to Ang and Rossant (Ang and Rossant, 1993). Recombinants were made by aggregating two explants. The culture period was 24 hours unless otherwise stated. Growth and survival of the explants were verified by measuring size with a micrometer and viability with Trypan Blue staining.

Embryo culture and cell labelling

The conditions for embryo culture and iontophoretic injection into single epiblast cells were as described (Beddington and Lawson, 1990; Lawson et al., 1991; Perea-Gomez et al., 2001). One epiblast cell/embryo was injected with 7.4% HRP (Horseradish peroxidase, ~1000 U/mg, Boehringer) and 2.6% lysinated rhodamine dextran (10³ Mr, Molecular Probes) in 0.05 M KCl, as described by Perea-Gomez et al. (Perea-Gomez et al., 2001) for endoderm, except that, for epiblast, depolarising current pulses were applied for 15 to 20 seconds. The fluorescent label served to confirm injection into an epiblast cell and was used to record the position of the cell.

Identification of the position of labelled cells

HRP-containing cells were identified after culture by staining the embryos for 1-1.5 hours with Hanks Yates reagent (Polysciences) as described (Lawson et al., 1991) before fixing with 2.5% glutaraldehyde in PBS, dehydrating, clearing in 1:2 benzyl alcohol:benzyl benzoate (BABB), embedding in glycolmethacrylate (Technovit 1700) and cutting 7 µm serial sections followed by staining with Methylene Blue. The number and position of the labelled cells, in relation to identifiable landmarks along the AP axis, were recorded in embryos in BABB before embedding, and crucial embryonic dimensions noted. The embedded embryos were sectioned in the appropriate orientation to identify the position orthogonal to the midline of labelled cells i.e. DV position in the neurectoderm and whether labelled mesoderm was axial, paraxial or lateral plate.

For comparison of clones in the hindbrain and spinal cord the distance along the midline between the most anterior member of a clone and the boundary between the first and the second somite was measured on a sagittal view of the cleared embryo. The initial axial position of the progenitors was measured along the midline from the anterior junction of epiblast and extra-embryonic ectoderm.

Gene expression

Whole-mount in situ hybridisation was performed as described by Roelen et al. (Roelen et al., 2002).

Digoxigenin-labelled (Boehringer Mannheim) antisense probes were as follows. The *Hoxb1* probe was a T7 polymerase transcript from a 800 basepairs (bp) *EcoRI* fragment (Wilkinson and Krumlauf, 1990). The *Hoxb8* probes were a 1:1 mix of a SP6 polymerase transcript from a 350 bp 3' untranslated *SacI-KpnI* cDNA fragment and a SP6 polymerase transcript from a 420 bp *SacI* fragment

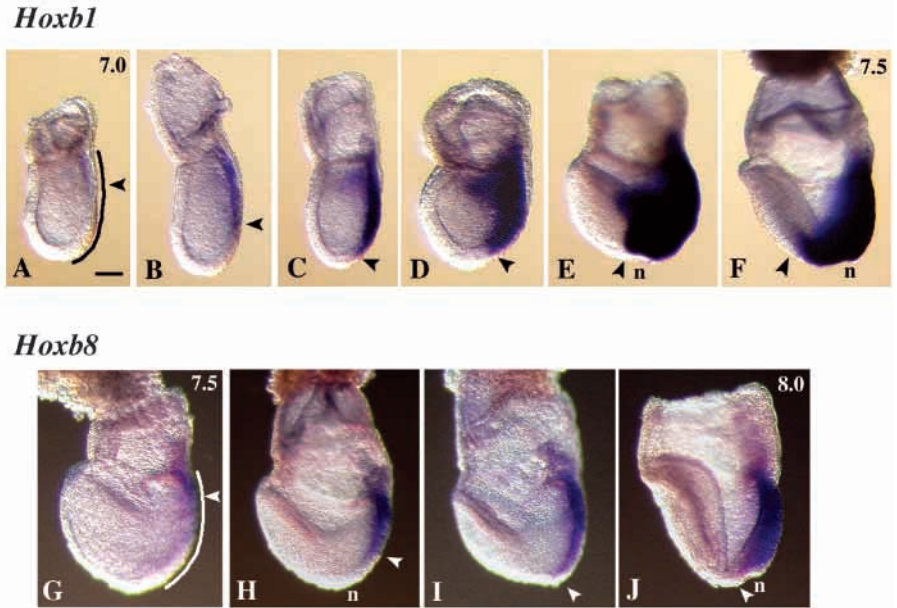


Fig. 1. Hox gene activation in the mouse primitive streak. Expression of *Hoxb1* (A-F) and *Hoxb8* (G-J) at E7.0 to 8.0 assayed by in situ hybridisation. (A,G) Onset of expression in the proximal part of the primitive streak. (B-D,H,I) Rostral and lateral spreading of Hox expression domains towards the distal part of the streak (D,I), containing the organiser or node (n) and beyond (E,F,J). Arrowhead, rostral expression front. Lines in A and G, primitive streak. Late midstreak/late streak (A), late streak (B), late streak early bud (C), late streak early bud/neural plate (D), late neural plate (G), early headfold (E,H,I), headfold (F), late headfold (J). Anterior is towards the left, posterior towards the right. Scale bar: 100 µm.

containing the first exon of the gene (Charité et al., 1994). The T/Brachyury specific probe was a T7 polymerase transcript from a 2kb *EcoRI* fragment (clone pSK75) (Herrmann et al., 1990). The chordin probe is described in Bachiller et al. (Bachiller et al., 2000). Probes were tested on embryos before use on explants.

For β-galactosidase activity, explants were fixed in 1% formaldehyde, 0.2% glutaraldehyde (Sigma) in phosphate-buffered saline (PBS) for 5 minutes, rinsed twice in PBS and stained with X-gal as described (Charité et al., 1994).

Statistics

The anterior limits of clones at different stages or in different germ layers at the same stage were compared with the Wilcoxon rank test. The relationship of the anterior limit of clones with progenitor position was obtained by linear regression analysis (Snedecor and Cochran, 1967).

RESULTS

Initial activation of *Hoxb1* and *Hoxb8* in the primitive streak region

The spatiotemporal patterns of activation of the 3' gene *Hoxb1* and the 5' gene *Hoxb8* were compared. Expression of *Hoxb1* began in the most caudal part of the primitive streak, at the junction between extra-embryonic and embryonic tissues, at the late midstreak (LMS) stage (E7.0) (Fig. 1A). Dynamic rostral expansion of the transcription domain followed. This domain spread along and lateral to the primitive streak (Fig. 1B-D) and later beyond the node (Fig. 1E,F). Transcription of *Hoxb8* started about 12 hours later than *Hoxb1* (late neural plate, E7.5, Fig. 1G). The spreading of the expression domains of either Hox gene from posterior to the node region took less than 8 hours. *Hoxb1* and *Hoxb8* transcripts reached the anterior end of the streak at the late streak early bud (LSEB)/neural plate (NP) stage (Fig. 1D), and early headfold/headfold (EHF/HF) stage, respectively (Fig. 1I). Variation between embryos in the time of arrival of the expression boundary at the node is shown in Table 1 (with *Hoxb4* included for comparison). During this phase,

Hoxb8 transcripts remained mainly restricted to the primitive streak and nascent mesoderm (Fig. 1G-I), while *Hoxb1* was transcribed more widely laterally and anteriorly in the mesodermal wings (Fig. 1D-F). This distribution was confirmed in histological sections (Frohman et al., 1990; Deschamps and Wijgerde, 1993). Therefore, late streak nascent lateral and paraxial mesoderm only expresses *Hoxb1*, while mesoderm born later at the EHF stage expresses both *Hoxb1* and *Hoxb8*. Both ectoderm and mesoderm at the level of the node, but not the axial mesoderm or endoderm express *Hoxb1* at the NP stage and both *Hoxb1* and *Hoxb8* at the HF stage.

Upstream inducing interactions are set much earlier than actual Hox gene expression

Earlier work had made it clear that the spread of the Hox expression domains along the primitive streak was not by proliferative expansion of the initially expressing cell population (Deschamps and Wijgerde, 1993) and did not involve diffusion of inducers from posterior to anterior in the streak (Gaunt and Strachan, 1994). In order to investigate whether inducing molecules were involved earlier than at the stages analysed by Gaunt and Strachan for *Hoxb4*, we analysed the autonomy of expression of one of the earliest Hox genes, *Hoxb1*, in explants of anterior and posterior streak regions.

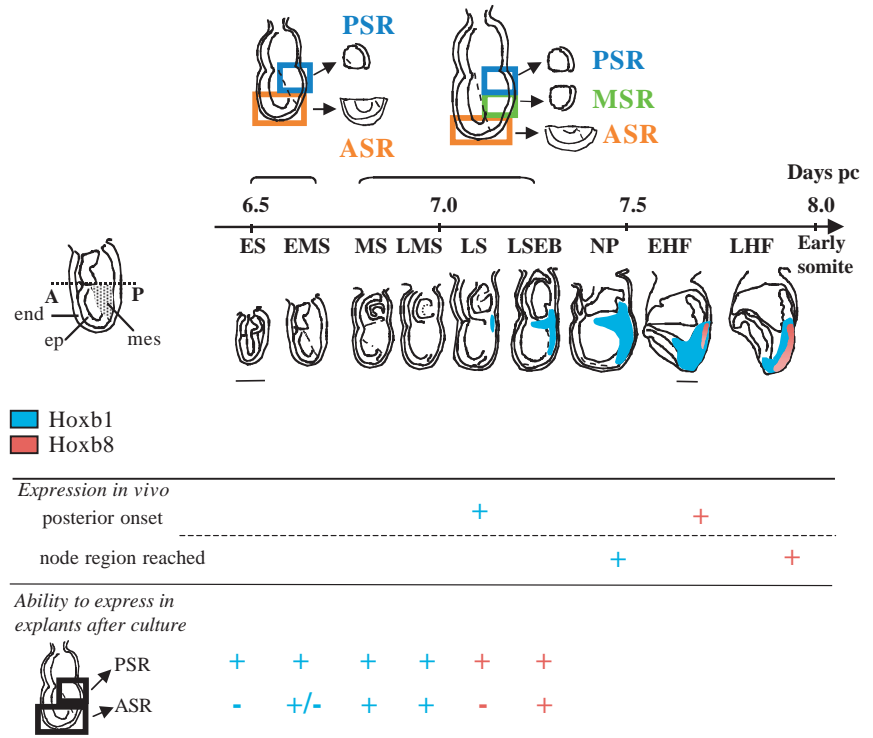
We investigated whether *Hoxb1* was activated autonomously in embryonic tissues at stages preceding initial gene

Table 1. Passage of anterior boundary of Hox expression through the node region

	LSOB	LSEB	NP	EHF	HF	LHF
<i>Hoxb1</i>	5 0 0	2 6* 0	0 1 0	0 0 4	0 0 6	0 0 1
<i>Hoxb4</i>			1 0 0	0 3 1	0 0 5	0 0 7
<i>Hoxb8</i>			4 0 0	4 3 0	0 3 0	0 1 4

*Anterior expression boundary at posterior edge of node. Numbers of embryos at different stages are shown. Regular type, posterior to the node; bold, at the node; italic, anterior to the node.

Fig. 2. Schematic representation of early mouse embryogenesis and *Hoxb1* and *Hoxb8* expression in vivo and in explants. Under the time axis (6.5 to 8.0 days post coitus), embryonic stages (ES to LHF) are drawn, with the expression of *Hoxb1* and *Hoxb8* in cyan and red, respectively. Top two panels: the explants from different regions of the primitive streak are indicated above the corresponding stages. Anterior is towards the left. Posterior streak region, PSR, blue; anterior streak region, ASR, orange; median streak region, MSR, green. Lower two panels: pattern of activation of *Hoxb1* and *Hoxb8* in vivo and in explants. All explants contained three germ layers: the epiblast (ep), mesoderm (mes) and endoderm (end). The gastrulation stages are designated as follows: early streak (ES), early midstreak (EMS), midstreak (MS), late midstreak (LMS), late streak (LS), late streak early allantoic bud (LSEB), neural plate (NP), early headfold (EHF) and late headfold (LHF). Scale bars: 200 μ m.



expression. To do this, we cultured posterior streak region (PSR) and anterior streak region (ASR) (Fig. 2, upper panels) separately at different primitive streak stages, and examined the expression of *Hoxb1* and *Hoxb1-lacZ*. In control experiments, explant culture conditions supported normal Hox and marker gene expression at the different stages, as shown by the maintenance of *Hoxb1* and *Hoxb8* expression in culture in 100% of PSR and ASR explants from headfold stage embryos (E7.5-7.75): such pieces already express the genes at the time of excision (data not shown). The 24-hour culture period was chosen because this time is sufficient for Hox gene expression to progress from the posterior to the anterior streak region both in vivo and in longitudinally bisected egg cylinders in vitro (Fig. 2, second row and data not shown).

PSR explants excised at different stages between early streak (ES) and late midstreak (LMS) stages expressed *Hoxb1* (Fig. 3A-D) and *Hoxb1-lacZ* (not shown) after culture, with the proportion of positive explants rising from 54% for ES explants to 80-100% from the early mid streak (EMS) to the LMS stages (Fig. 3O). Likewise, 80-100% median streak region (MSR) explants from mid streak (MS) and LMS stages had activated the *Hoxb1* gene and transgene (Fig. 3E,F,O) after culture. By contrast, ES ASR explants failed to express *Hoxb1* and *Hoxb1-lacZ* (Fig. 3G,O). The proportion of ASR explants expressing the gene rose with increasing age, from 18% at the EMS stage (with a low number of positive cells in this latter case) (Fig. 3H,O), to 36% at the MS stage (Fig. 3I,O), and to 57% for the LMS embryos (Fig. 3J,O). The absence of *Hoxb1* expression in the youngest material was not due to inappropriate culture conditions because the explants expressed the Hox-independent genes brachyury (*T*) (Fig. 3K,L) and chordin (Fig. 3M,N). Hox gene expression could not be induced by increasing the culture period of ES ASR explants to 30-32

hours (data not shown), whereas 100% MS ASR explants were positive after a similar culture period (versus 36% after 24 hours culture, data not shown).

In summary, these results show that *Hoxb1* expression can start autonomously in PSR explants cultured from the ES stage onwards. ASR explants were only able to activate *Hoxb1* autonomously at the EMS/MS stage and later. This suggests that, early in gastrulation and more than 12 hours before the first *Hoxb1* expression appears in the embryo, the underlying molecular genetic interactions have occurred in proximo-posterior embryonic tissues. Alternatively, cell interactions within the explants might set these instructions up in vitro.

The activation pattern of *Hoxb8* in explants was also examined. A similar time period (about 12-16 hours) was found to separate permissiveness to 'autonomous' activation in explanted tissues (Fig. 4), and effective activation in the intact embryo in vivo (Fig. 1). PSR explants can autonomously activate *Hoxb8* expression after culture from the LMS stage onwards (E7.0) (Fig. 4A,B) but not earlier, and the MSR and ASR explants from the late streak early bud (LSEB) stage on (E7.25) (Fig. 4C-F), although *Hoxb8* is only expressed in the embryo from late neural plate/head fold (LNP/HF) stages (E7.75) (Fig. 1G-J). The dynamics of activation of *Hoxb1* and *Hoxb8* in the explant system suggest that, like Hox expression itself, the process which anticipates this expression in the primitive streak takes place sequentially (for the 3' genes earlier than the 5' genes) in a proximal (posterior) to distal (anterior) sequence (compiled in Fig. 2, bottom panel).

The proximal posterior region of the primitive streak has Hox-inducing capacity

As the PSR appears to be instructed for *Hoxb1* expression before the ASR, and very young ASRs were unable to activate

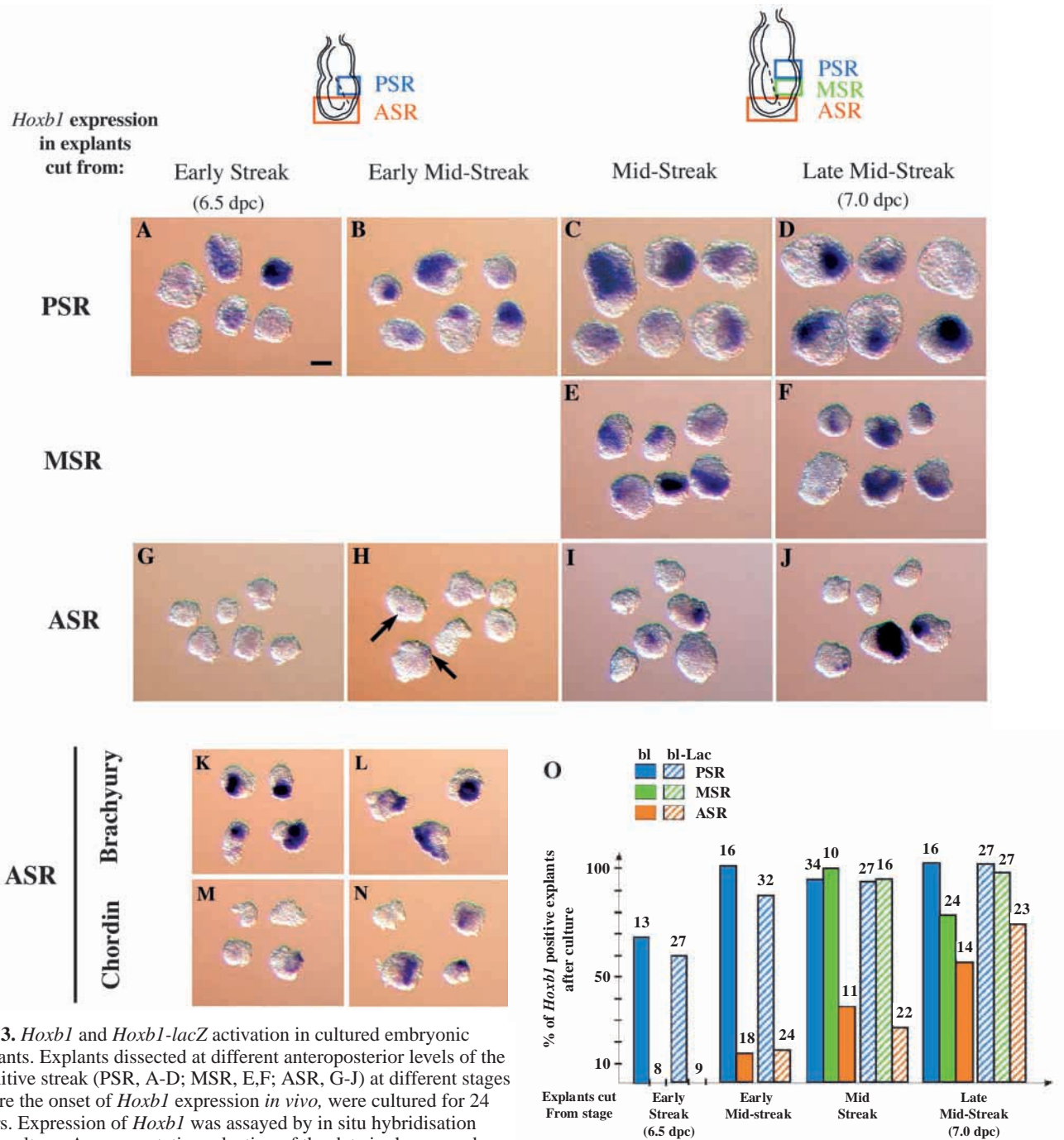


Fig. 3. *Hoxb1* and *Hoxb1-lacZ* activation in cultured embryonic explants. Explants dissected at different anteroposterior levels of the primitive streak (PSR, A-D; MSR, E,F; ASR, G-J) at different stages before the onset of *Hoxb1* expression *in vivo*, were cultured for 24 hours. Expression of *Hoxb1* was assayed by in situ hybridisation after culture. A representative selection of the data is shown, and a quantitative (total numbers above bars) representation of all data obtained with *Hoxb1* and *Hoxb1-lacZ* is given in O. Results for gene and reporter transgene were similar. (K,L) Brachyury/T and (M,N) chordin expression in ES and EMS ASR. Scale bar: 100 μ m.

Hoxb1 autonomously, we asked whether a PSR explant would induce *Hoxb1* in an ES ASR explant when recombined. We combined ASR explants from *Hoxb1-lacZ* ES embryos with PSR explants from non transgenic embryos at different stages (E6.5 to E7.5) and analysed the *lacZ* expression after 24 hours of co-culture (Fig. 5A).

Of the ES ASR/PSR recombinates containing PSR explants from ES to HF stage embryos, 92% (11/12) showed *lacZ* expression, which indicates that *Hoxb1* expression was

induced in the ASR explant tissues [Fig. 5A (early streak) and Table 2]. Combination with a PSR also strongly increased *Hoxb1-lacZ* expression in cells of ASR explants from EMS and MS embryos (78% and 100% of positives, respectively), compared with expression seen in ASR explants cultured alone (17% and 27%) (Table 2 and Fig. 3). However, PSR explants were not able to induce *Hoxb1-lacZ* expression in explants from the extra-embryonic part of ES/EMS embryos, a region that never expresses Hox genes during *in vivo* development

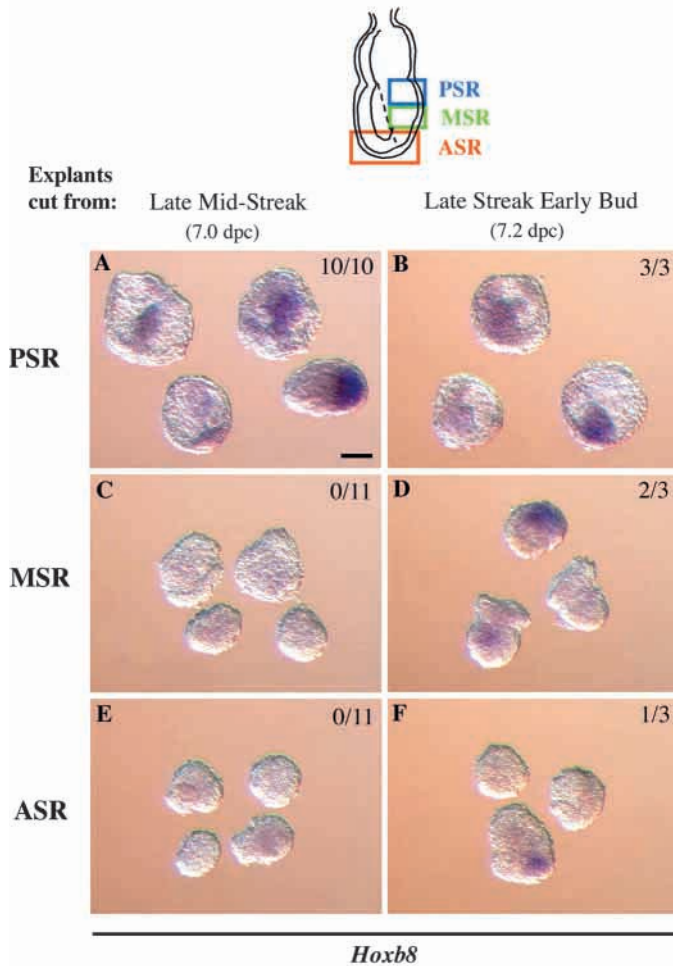


Fig. 4. *Hoxb8* activation in cultured embryonic explants. Posterior streak region, PSR, blue; anterior streak region, ASR, orange; median streak region, MSR, green. *Hoxb8* expression in PSR (A,B), MSR (C,D) and ASR (E,F) explants cut at two different stages preceding the onset of gene expression in vivo and cultured for 24 hours. The fraction of positive explants of each type is indicated in the top right-hand corner.

(Table 2). In contrast to the induction observed in ASR/PSR recombinates, no *lacZ* expression was scored in ASR/ASR control recombinates when the non transgenic ASR was taken from ES to MS embryos (E6.5-E7.0) (Fig. 5B; Table 2). Interestingly though, we observed *Hoxb1-lacZ* expression in 67% (2/3) of recombinates between transgenic ES ASR explants and the node region of E7.5 headfold stage embryos [Fig. 5B (HF) and Table 2], showing the emergence of a *Hoxb1*-inducing capacity in late ASRs. The Hox-inductive capacity therefore is present in the PSR early during gastrulation (ES stage, E6.5) until at least the HF stage (E7.5), a time when it can also be identified in the anterior streak region.

These results indicate that the posterior part of the primitive streak region is capable of producing signals leading to the induction of *Hoxb1* expression in competent anterior streak tissues. Regardless of whether this anterior tissue still represents anterior streak, or is differentiating as neurectoderm and mesoderm, the results imply that rostral spreading of Hox

Table 2. *Hoxb1-lacZ* expression in recombinates between two explants from the primitive streak region of gastrulating embryos

Explants and embryonic stage of cutting*	Proportion of β -gal positive recombinates (number of positives/total number) [†]
ES ASR Lac (from <i>Hoxb1-lacZ</i> embryos)	0 (0/9)
ES ASRLac + ES PSR	100 (2/2)
ES ASRLac + EMS PSR	80 (4/5)
ES ASRLac + MS PSR	100 (1/1)
ES ASRLac + HF PSR	100 (4/4)
ES ASRLac + ES ASR	0 (0/4)
ES ASRLac + EMS ASR	0 (0/2)
ES ASRLac + MS ASR	0 (0/1)
ES ASRLac + HF ASR	67 (2/3)
EMS ASRLac	17 (4/24)
EMS ASRLac + ES PSR	60 (3/5)
EMS ASRLac + EMS PSR	100 (3/3)
EMS ASRLac + MS PSR	100 (1/1)
EMS ASRLac + ES ASR	0 (0/4)
EMS ASRLac + EMS ASR	0 (0/6)
MS ASRLac	27 (6/22)
MS ASRLac + EMS PSR	100 (3/3)
MS ASRLac + EMS ASR	33 (1/3)
ES ExtrEmbLac	0 (0/7)
ES ExtrEmbLac + ES PSR	0 (0/3)

*See Fig. 5 for experimental procedure and legend to Fig. 2 for abbreviations. ExtrEmb, explant of extra-embryonic tissues cut on the posterior side. Explants not designated as 'Lac' are from non transgenic embryos.

[†]Recombinates were cultured for 24 hours before fixation and X-gal staining procedure.

expression from the posterior streak to the node region requires cell-cell interactions.

The spread of Hox expression domains rostral to the node is not lineage related

Rostral spread of Hox expression continues beyond the node (Fig. 1) during a period when the presumptive hindbrain and spinal cord territories are expanding anterior to the node (Tam, 1989) and the node 'regresses' while generating spinal cord (Mathis and Nicolas, 2000). Given this coincidence, a plausible mechanism for the continued spread of the expression front along the AP axis is that a cell acquires a Hox code and positional specification while in the node region and its descendants retain it after leaving the node region (Deschamps and Wijgerde, 1993). Descendants remaining (temporarily) in the node region would acquire a new Hox code as the more 5' genes are expressed there. If this hypothesis is valid, predictions about the final position of the most rostral descendants of cells at the Hox gene expression front at the node at different stages can be made on the basis of the later rostral expression limits of different Hox genes in neurectoderm and mesoderm at E8.5-9.5. Both a general prediction with regard to neurectoderm and mesoderm descendants, and specific predictions can be made.

The general prediction is that the anterior limit of mesodermal clones will be several somite lengths posterior to neurectodermal clones generated in the node region at the same stage (Gaunt et al., 1988; Frohman et al., 1990). Clones

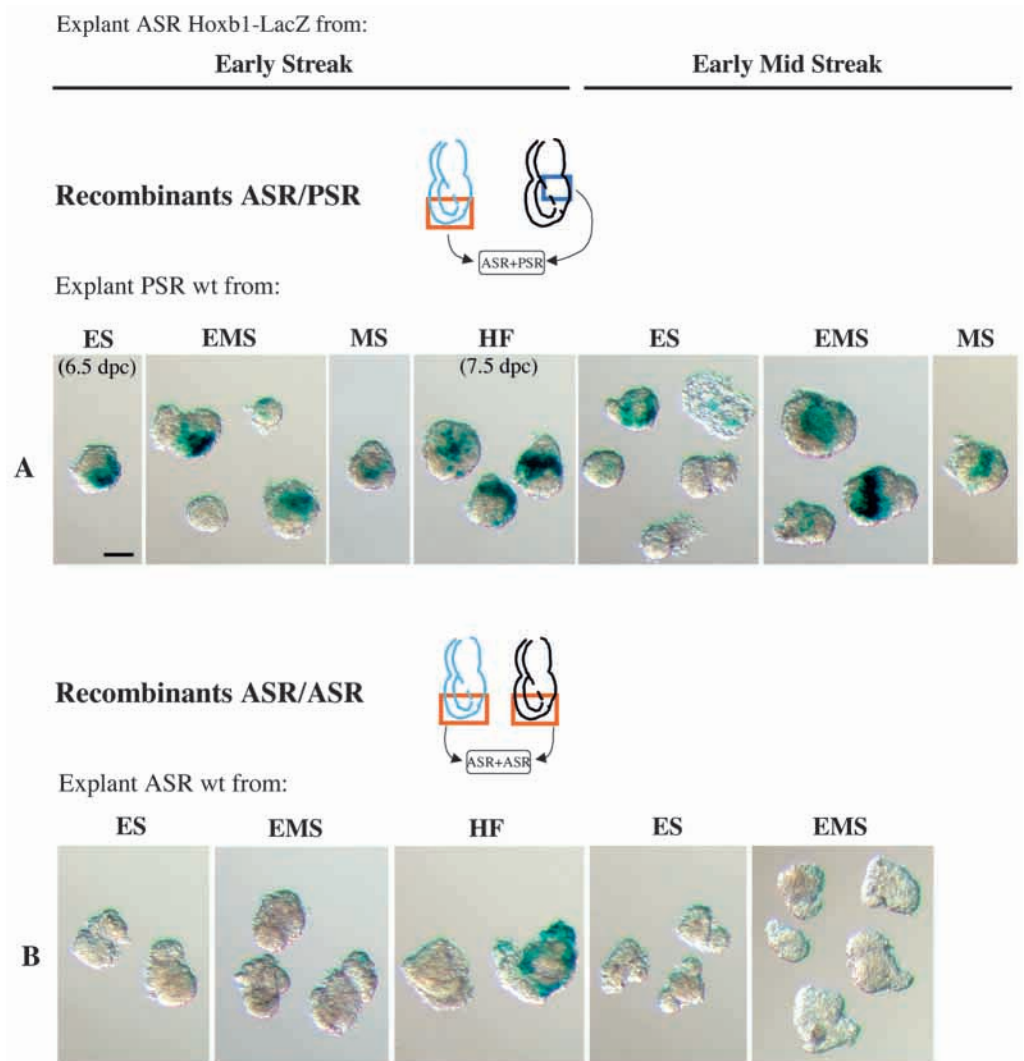


Fig. 5. Recombination with a PSR induces *Hoxb1-lacZ* expression in a non-expressing ASR from ES and EMS stages. *Hoxb1-lacZ* expression in ASR explants cut from transgenic embryos (drawn in blue) at ES or EMS stages (E6.5-6.7), cultured for 24 hours in combination with either a PSR (A) or another ASR (B) cut from wild-type embryos (E6.5, ES, EMS and MS; E7.5, EHF). Combination with a PSR explant resulted in a strong induction of transgene expression in early ASR explants. ASRs, except for the oldest (HF stage), were unable to activate *Hoxb1-lacZ* in transgenic ES ASRs. See Table 2 for explant numbers. Scale bar: 100 μ m.

labelled with HRP were generated in epiblast at the node region from LS to HF stages (E7.0-E7.7), and the embryos cultured for 1 day (Fig. 6). Some clones were also generated in the axial epiblast anterior to the node at LSEB and older stages. Most (93%) of the clones generated at the node after the LS stage and contributing to neurectoderm were restricted to the ventral half of the neural tube; 78% of the clones contributing to non axial mesoderm anterior to the node were in paraxial mesoderm. The anterior limit of neurectodermal descendants in clones generated at the node was progressively more posterior with advancing initial stage (LS versus LSEB, $P < 0.01$; NP versus HF, $P = 0.01$) with the exception that LSEB and NP did not differ significantly (Fig. 7, upper set). A similar progression was seen in the mesoderm (LS stages versus HF, $P < 0.01$). This trend confirms the sequential addition of neural and mesodermal material from the node region. At no stage was the anterior limit reached by mesodermal clones generated at the node posterior to the anterior limit of neurectodermal clones (Wilcoxon rank test). At the HF stage, mesoderm clones were even slightly more rostral to neurectoderm ones ($P = 0.05$). Clones initiated in the mesoderm layer did not differ from those initiated in the epiblast. Axial mesoderm, which does not express Hox genes in the mouse (Deschamps and Wijgerde,

1993), behaved differently: it remained associated with the node, and therefore relatively posterior (Fig. 7). Therefore axial extension from the node progresses at similar speed in neurectoderm and paraxial mesoderm and the general prediction from the hypothesis was not fulfilled.

If Hox codes are acquired at the node, specific predictions about neurectoderm and mesoderm separately are not necessarily dependant on the validity of the general prediction about relative dispersion of neurectoderm and mesoderm from the node region. The specific predictions for neurectoderm, based on later anterior expression boundaries of *Hoxb1*, *Hoxb4* and *Hoxb8*, and the stage at which expression reaches the node (Table 1), are that the colonising population of rhombomeres 3 and 4 (r3/r4, for the anterior limit of *Hoxb1*) (Wilkinson et al., 1989) is generated at the node at LSEB/NP stages, that of r6/r7, at the level of the first somite (S1), (*Hoxb4*) (Gould et al., 1998) is generated at the node at the EHF stage, and that of neurectoderm at the level of S5/S6, in the anterior spinal cord, (*Hoxb8*) (Deschamps and Wijgerde, 1993; Charité et al., 1998) is generated at the node at the HF stage. Analysis of neurectoderm descendants of epiblast clones generated in the node region (Fig. 7, upper set) and anterior to it (Fig. 7, lower set) showed that the most anterior limit of any neurectoderm clone derived from the node region at LSEB and

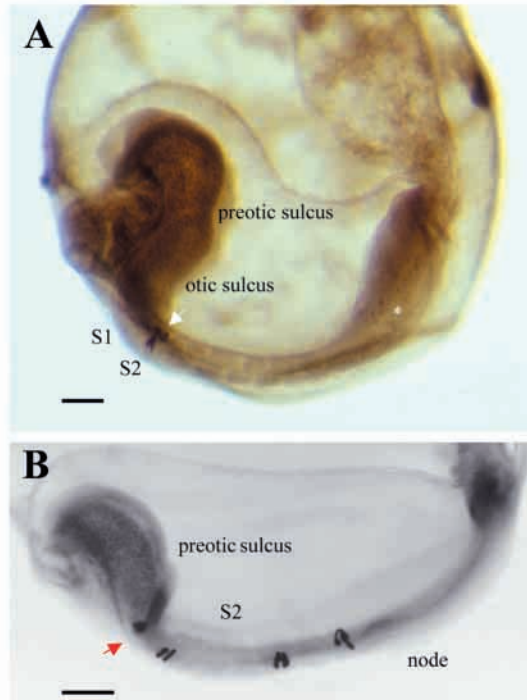


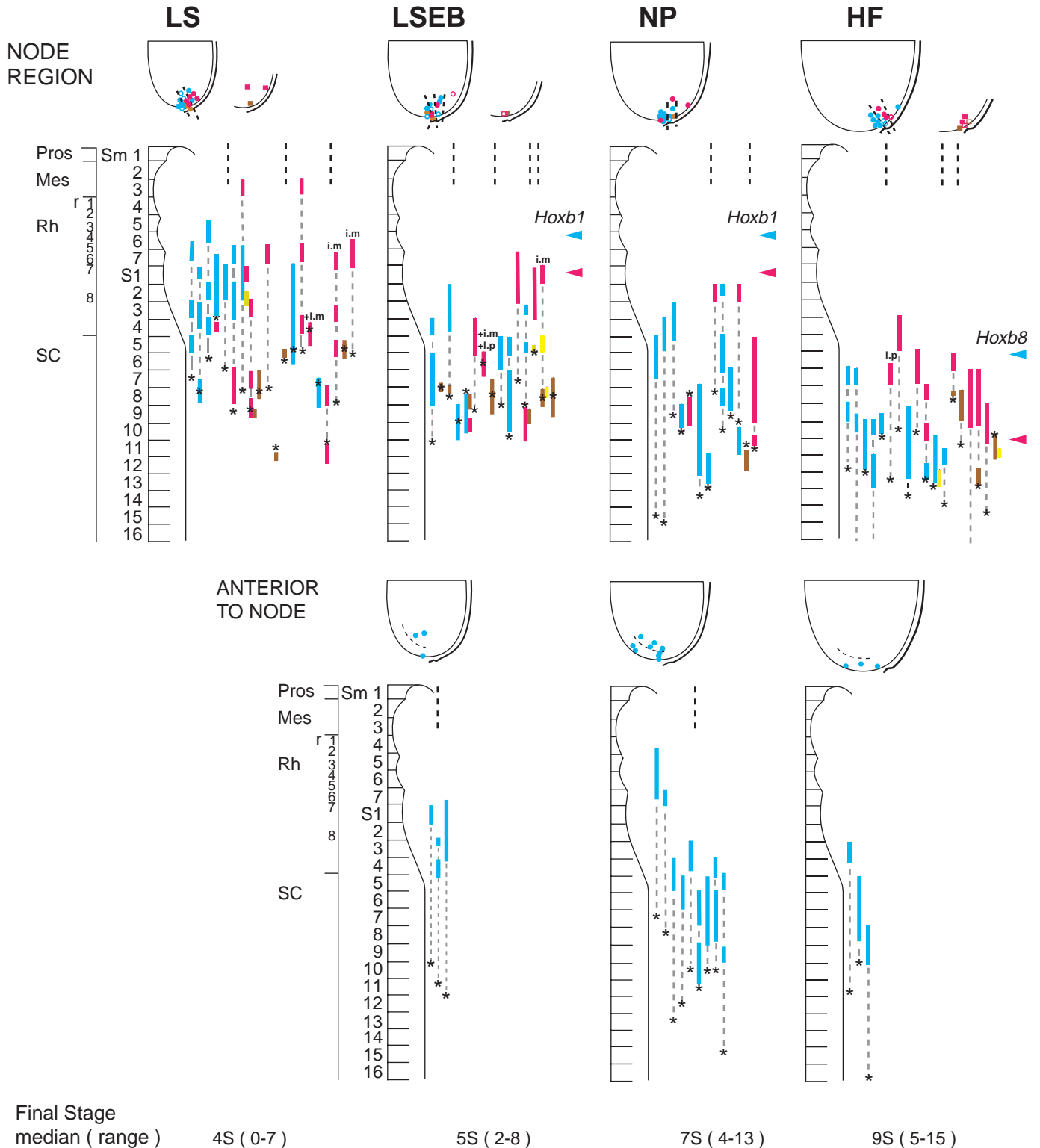
Fig. 6. HRP-labelled clones in cultured embryos. (A) Seven somite embryo labelled in the node region at the NP stage. There are seven mesoderm descendants (arrow) (not all in focus) in the second somite and a further seven lightly labelled descendants in postnodal ectoderm (star) (not all visible), which were presumably derived from a sibling still in cytoplasmic connection with the labelled cell at the time of injection. (B) Four somite embryo labelled at the anterior edge of the node region at the LS stage. Twelve labelled neurectoderm descendants are distributed in small clusters from prospective r5/r6 (arrow) towards the node. Scale bars: 100 µm.

NP stages was at the level of S1 and S2, respectively (median at S5), and not more anteriorly in r3,r4,r5, as expected from *Hoxb1* expression. Contribution to r3,r4,r5 came from the most anterior descendants of clones generated in the node region at the earlier LS stage, and from a region ~100 µm anterior to the node at the NP stage. Contribution to neurectoderm at the level of S1 by anterior descendants of epiblast near the node came not from LNP/EHF stage embryos as expected from *Hoxb4* expression, but from the earlier LS stage and from ectoderm anterior to the node at the LSEB stage. At the HF stage, the most anterior contribution of epiblast at the node region to the neurectoderm was at the level of S7 (median at S9/S10), not at S5/S6 as predicted from the *Hoxb8* expression pattern. Contribution to the S5 level by anterior clonal descendants came from the node region of younger LSEB (median at S4/S5) and NP stage (median at S5) embryos as well as from epiblast anterior to the node at NP and HF stages (Fig. 7). Therefore cells that will eventually occupy the anterior boundary regions of *Hoxb1* and *Hoxb4* expression in the hindbrain or *Hoxb8* in the spinal cord, are already in positions anterior to the node when the anterior expression boundaries reach the node region (Fig. 8).

The specific prediction with regard to mesoderm is that the precursors of the first somite, which is the anterior limit of *Hoxb1* expression in the mesoderm (Murphy and Hill, 1991) are present at the level of the node at the LSEB/NP stage, those

Fig. 7. Anteroposterior clone distribution after labelling epiblast at different stages. The upper set of figures shows results after labelling at or near the node, the lower set after labelling anterior to the node. In each set, the upper figure shows the positions of the progenitor cells projected on a sagittal section of average dimensions. The extent of the primitive streak is indicated by a curved line. A part of the sagittal section to the right of LS, LSEB and HF shows the position of progenitors initially labelled in the mesoderm layer. The positions of the clone progenitors are divided by broken lines into subregions, comprising three or four subregions from anterior to posterior in the node region and, anterior to the node, anterior axial (within 55 µm of the midline) and paraxial. Filled blue circles, progenitors with only ectoderm descendants; open blue circles, progenitors with ectoderm and mesoderm/endoderm descendants; filled red circles and squares, progenitors with only mesoderm descendants; open red circles and squares, progenitors with paraxial mesoderm and axial mesoderm or endoderm descendants; filled brown circles and squares, progenitors with only axial mesoderm descendants. The lower figures in each set show the lineal AP distribution of each clone on a schematic representation of the neural tube; the regions of the neural tube and the corresponding 'segments' in the paraxial mesoderm are shown on the left. The position of the node is indicated by an asterisk. The presence of one of more labelled cells in a 'segment' is indicated by a coloured line, absence of labelled cells by a broken line connecting the clone to the node. Blue, ectoderm; red, paraxial mesoderm (unless otherwise indicated); brown, axial mesoderm (notochordal plate); yellow, endoderm. The clones generated by individual progenitors can be identified as follows: for the node region, anterior precede posterior subregions and, within subregions, proximal precedes distal. Anterior towards the node, the anterior axial subregion precedes the paraxial subregion, and anterior or proximal precedes posterior or distal within each subregion. The subregional divisions are indicated by broken vertical lines on the clonal distribution scheme. Clones initiated in mesoderm follow the last epiblast subregion. Anterior clonal limits predicted by the hypothesis that Hox codes are fixed in the node region and carried further by lineage transmission are indicated by blue (neurectoderm) and red (paraxial mesoderm) arrowheads at the appropriate stages for *Hoxb1* and *Hoxb8*. i.m., intermediate mesoderm; l.p., lateral plate mesoderm; Mes, mesencephalon; Pros, prosencephalon; r, rhombomere; Rh, rhombencephalon; S, somite; SC, spinal cord; Sm, somitomere (Meier and Tam, 1982); +, additional to paraxial mesoderm.

of S5/6 (*Hoxb4*) (Gould et al., 1998) at the node at the EHF stage, and those of S10/11 (*Hoxb8*) (Charité et al., 1998; Deschamps and Wijgerde, 1993) at the node at the HF stage. Although the anterior limits of mesoderm clones generated near the node became progressively more posterior with advancing initial stage, there was no general agreement of prediction with the levels at which anterior descendants were found. The anterior limit of two mesodermal clones was in S1 and one was just anterior, as predicted for *Hoxb1* expression after labelling at the LSEB stage, and two clones derived at the NP stage were in S2, but the anterior limits of the other four clones at LSEB and NP stages were more posterior (median of nine clones at S2). The eight mesoderm clones generated at the HF stage had anterior limits between S4 and S9 (median at S6/7), instead of more posteriorly at S10/11 as expected from the AP level of the definitive anterior expression boundaries of *Hoxb8*. Therefore, although lineage transmission of *Hoxb1* expression in paraxial mesoderm has not been excluded, mesoderm generated at the node does not behave as predicted if anterior Hox boundaries were being



consistently established by the lineage transmission of specific Hox codes acquired sequentially at the node between LS and HF stages.

Hindbrain and anterior spinal cord elongate both by addition from the node and by internal growth

Sequential addition of material into the hindbrain and spinal

cord from the node is indicated by the progressively more caudal position of the anterior limits of clones generated in the node region between LS and HF stages (Fig. 7, upper set). Of the clones contributing to neurectoderm, 45% (14/31) extended to the node, whereas no (0/9) clones generated in the axial region anterior to the node did (Fig. 7, lower set). This is supporting evidence of a self maintaining pool of

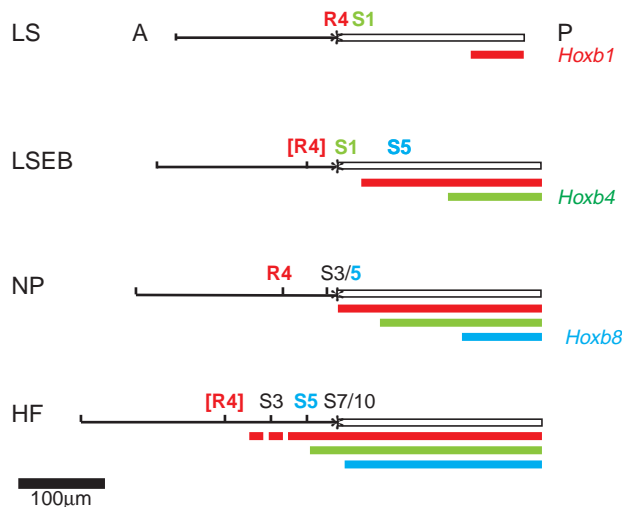


Fig. 8. Rostral spread of Hox expression in relation to the precursors of cells that will eventually occupy the rostral expression boundaries in the neurectoderm. The anteroposterior (A,P) axis is shown at different stages. The node is represented by a star, the primitive streak as an open bar. The extent of *Hoxb1*, *Hoxb4* and *Hoxb8* expression is indicated by coloured lines below the axis. The position of precursors of rhombomere 4 (R4, definitive anterior boundary of *Hoxb1*) and different somite (S) levels (S5 level, definitive anterior boundary of *Hoxb8*) in the neural axis are shown above the axis.

precursors in the node region for the spinal cord (Mathis and Nicolas, 2000; Mathis et al., 2001) and also for part of the hindbrain.

Comparison of the anterior limits of neurectoderm clones shows a consistency in result in clones generated anterior to the node (Fig. 7, lower set) compared with those generated in the node region (Fig. 7, upper set): the anterior limit is well correlated with the distance from the node of anterior axial progenitors (those anterior to the node and within 55 μm either side of the midline) at all stages. The AP position of the anterior limits of the clones was compared with the position of their anterior axial progenitors by linear regression analysis in order to assess quantitatively whether the neural axis anterior to the node at LSEB to HF stages, representing prospective r3 to spinal cord at the level of S8, is stabilised or is continuing to grow within itself (Fig. 9). The value of the regression coefficient (b) was 2.769 (s.e.=0.463, $n=9$, $P<0.001$). This value is also significantly greater than 1 ($P<0.01$), implying that the part of the axis representing precursors of hindbrain and spinal cord to the level of S8, anterior to the node at LSEB to HF stages, increases in length within 24 hours. The axis is therefore extending within itself anterior to the node, and not only at the node by the sequential insertion of new material. In addition, the posterior displacement of the anterior limits of paraxially generated clones relative to anterior axial ones (LSEB and NP, Fig. 7, lower set) suggests that convergence and extension occur in the more lateral ectoderm until at least the NP stage. These results could explain why labelling epiblast just anterior to the node at the HF stage gives clones with anterior limits in the neurectoderm that are more

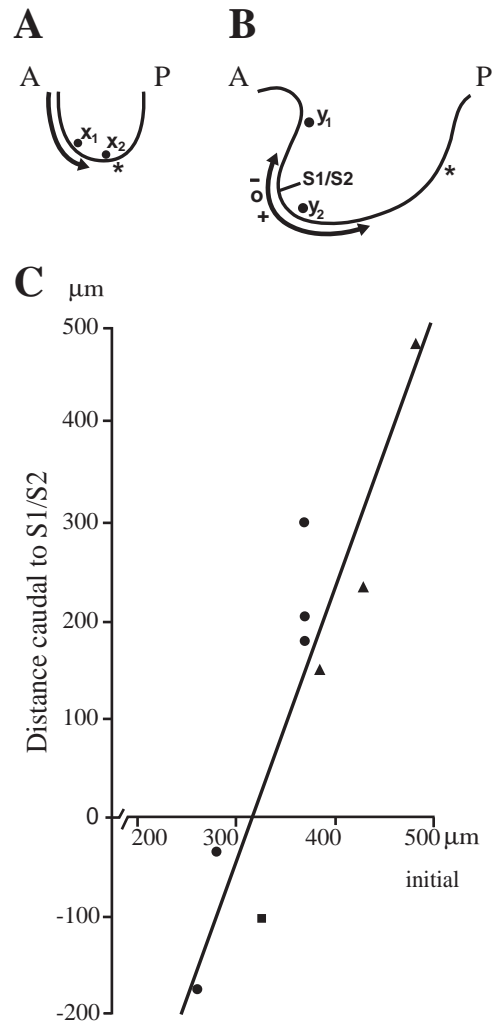


Fig. 9. AP extension of hindbrain and spinal cord. (A,B) Schematic representation of the curved AP axis at the time of injection (A) and after culture (B). The position of the node is indicated by a star. For regression analysis, the position of the injected cell (x) was measured from the anterior embryonic/extra-embryonic junction (thick curved line in A). The position of the most anterior descendant (y) in the resulting clone was measured along the midline (thick curved line in B) from the boundary between the first and second somites (S1/S2). Values of y anterior to S1/S2 are negative. (C) Regression of the AP position of the most anterior descendant ($y_1, y_2 \dots$) on progenitor position ($x_1, x_2 \dots$). Initial stages: Square, LSEB; dots, NP; triangles, HF. Only clones with anterior axial progenitors (see Fig. 7) were used, and were suitable for this approach because there is no significant increase in length or cell mixing along the midline of prospective forebrain, midbrain and anterior hindbrain between the LS and 4S stage (K.A.L., unpublished). The values in the regression equation $Y=a + bX$ are $137.11=-867.60 + 2.7686(362.89)$.

posterior than might have been expected from the shape of the embryo and the space apparently available for the first somites [compare Fig. 1F with Fig. 7 (HF)]. The results also underline the dynamic nature of the relative AP positions of cells that are leaving, and have recently left, the node during a period when Hox expression domains are traversing rostrally through the region.

DISCUSSION

Initiation of Hox gene expression is anticipated by earlier events in the posterior early streak and spreads by cell-cell signalling

Transcriptional initiation of the earliest Hox gene is anticipated by genetic interactions occurring in the posterior streak, much earlier than actual Hox gene expression, perhaps coincident with the generation of the primitive streak. Permissiveness of the presumptive posterior streak region for precociously induced *Hoxb1* expression is present at the pre-streak stage (Roelen et al., 2002), also pointing to a link between Hox inducibility and streak formation. Inducibility was present earlier for 3' than for 5' genes, sequentially in posterior and anterior streak tissues. The conditions for eventual *Hoxb1* expression were not present in the isolated anterior streak of ES stage embryos, and did not develop autonomously in culture. Re-establishing tissue continuity in recombinates restored the expansion of Hox gene inducibility from posterior to anterior tissues. This demonstrates that the mechanism of anterior spreading of Hox expression through the streak (Deschamps and Wijgerde, 1993; Gaunt and Strachan, 1994) (see also Gaunt, 2001) operates by cell to cell signalling, much earlier than suspected, coincident with streak extension. Among possible signalling molecules involved in these interactions, and acting as Hox inducers, are members of the Wnt and Fgf families. Some of these, such as Wnt3 and Fgf8 are strongly expressed in the posteriormost embryonic tissue at prestreak stages, and in the streak at streak stages, and they are required for primitive streak formation and gastrulation (Liu et al., 1999; Sun et al., 1999). Wnt molecules have been shown to induce Hox genes in other contexts in *C. elegans* (Malooof et al., 1999) and *Drosophila* (Riese et al., 1997), and loss of function mouse mutants in the Wnt and Fgf pathways exhibit homeotic phenotypes accompanied by decrease in Hox gene expression (Ikeya and Takada, 2001; Partanen et al., 1998). Preliminary data (S.F. and J.D., unpublished) suggest that a Wnt signal is able to induce *Hoxb1* in ES anterior explants. In addition, early sequential expression of 3' to 5' Hox genes in the primitive streak involves the progressive release of a repression mechanism operating at the level of the cluster from a remote 5' cis-acting element, as demonstrated for the *Hoxd* cluster (Kondo and Duboule, 1999).

Neurectoderm cells acquire their Hox code after they have left the node region

The regions of the neural tube that will be positionally specified by the Hox genes are formed from the LS stage onwards by a combination of the sequential addition of cells from the node region (Fig. 7), convergence and extension of paraxial ectoderm anterior to the node (Fig. 7 and data not shown) and subsequent longitudinal extension within the neurectoderm (Fig. 9). Comparison of the clonal behaviour of neurectoderm generated at the node and the spread of Hox gene expression past the node showed that nascent neurectoderm does not acquire and fix its Hox code at the node: cells whose descendants will contribute to definitive anterior boundary regions of the Hox domains (both 3' and 5' genes) are already anterior to the node when the waves of Hox expression arrive there. Therefore the early Hox domains have to 'catch up' with the cells that will later occupy the rostral boundary domains.

In addition, the relative AP positional values in the neurectoderm are changing anterior to the node as the axis elongates and while Hox expression domains are spreading rostrally through this region, implying that positional specification in terms of a stable Hox code must be acquired later. The delay before Hox codes are fixed may correspond to the time required for stabilisation of the relative AP positions of cells in the neurectoderm and mesoderm, when clonal growth in the neurectoderm changes from an AP to a DV mode (Mathis and Nicolas, 2000), and when cell mixing stops in unsegmented paraxial mesoderm (Tam, 1988).

Clonal expansion and Hox gene expression in paraxial mesoderm versus neurectoderm

Although the rostralmost neurectoderm descendants of cells at the node when the expression domains arrived there ultimately occupy more posterior positions than the anterior expression boundary of the genes considered, it is not so for the mesoderm. The most anterior mesodermal descendants of epiblast cells near the node when the *Hoxb1* expression domain arrived there did, in some embryos, contribute to paraxial positions at or near the anterior boundary region of this Hox gene, and in others were more posterior. By contrast, the most anterior mesodermal descendants of cells near the node when the expression domain of *Hoxb8* reached the node were found at positions much more rostral than the expression boundary of this gene. A conclusion from these data is that the regulatory interactions responsible for generating the sequential 3' to 5' Hox expression boundaries are different in the neurectoderm and in the mesoderm and must involve down regulation in the mesoderm at least of *Hoxb8*. This is unsurprising in the light of the recent findings about mesoderm-specific modulation of Hox gene expression in the segmental plate (Zakany et al., 2001). The Hox codes may well be reset by the oscillatory mechanism in the mesoderm descendants of cells near or anterior to the node, after their ingress through the streak.

Axial extension anterior to the node progresses at similar speed in neurectoderm and mesoderm. The offset of rostral expression boundaries of Hox gene expression in the mesoderm compared with the neurectoderm can not be accounted for by germ layer specific clonal distribution of descendants from progenitors around or anterior to the node, but must also result from differential gene regulation including transcriptional induction in the neurectoderm and downregulation in the mesoderm. The anterior progression of the Hox expression domains in both neurectoderm and mesoderm appears to be modulated by gene regulation from early on until at least early somite stages. Transcriptional regulation of Hox genes, although usually studied at later stages, has indeed been shown, in several cases, to depend on germ layer-specific regulatory elements (Gilthorpe et al., 2002; Gould et al., 1998; Marshall et al., 1994; Sharpe et al., 1998).

A single continuous phase of induction of the Hox expression domains between initial transcription and establishment of the rostral expression boundaries

The data suggest that the Hox expression domains are established gradually from the posterior streak to their definitive rostral boundaries anterior to the node, by cell-cell signalling driving transcriptional modulation. Induced by

genetic interactions occurring at the time of primitive streak formation and extension, Hox gene expression may continue to spread anteriorly beyond the node under the influence of the same or a similar gene regulation mechanism, and does not rely on proliferative expansion of Hox-expressing cells. The explant and lineage experiments therefore suggest that a single continuing process drives the rostral extension of the Hox domains both posterior and anterior to the node. The node region itself, around which the laying down of tissues along the extending axis is coordinated, would not be specifically involved in instructing newly generated cells as to their AP identity and Hox code, but seems to be passively traversed by the progressing Hox domains.

Although some of the candidate Hox-inducing molecules, such as Wnt3, Wnt3a and Fgf8, could be acting early in the primitive streak, as already discussed, additional inducers might come into action around and anterior to the node during the axial extension phase which we studied. Such possible inducers are Wnt8 (Bouillet et al., 1996), the Cdx transcription factors (van den Akker et al., 2002) and effectors of the oscillatory mechanism of the Notch pathway in the paraxial mesoderm (Zakany et al., 2001). During this period and later, retinoic acid signalling has been shown to sequentially shift the definitive expression boundaries of 3' to 5' Hox genes rostrally in the neurectoderm (Marshall et al., 1994; Studer et al., 1994; Gould et al., 1998; Oosterveen et al., 2003). Stabilisation of the Hox expression domains would only take place subsequently, possibly by the epigenetic polycomb and trithorax maintenance system taking over the control of the restricted Hox expression domains, thus putting an end to the rostral spreading of gene expression in the neurectoderm and mesoderm (Yu et al., 1998; Tomotsune et al., 2000; Akasaka et al., 2001). Disruption of the regulatory interactions would, at any stage, lead to altered Hox expression and patterning defects; for example, precocious Hox expression in the primitive streak (Kondo and Duboule, 1999) would result in the disruption of the sequential arrival of the Hox expression domains at the level of the cells to be instructed in the prospective axial and paraxial structures, causing aberrant AP instruction and patterning alteration that cannot be corrected by subsequent regulation.

We thank Rob Krumlauf and Heather Marshall for the *Hoxb1-lacZ* transgenic construct; Wim de Graaff for the generation and maintenance of the *Hoxb1-lacZ* transgenic mouse line; Jeroen Korving for histology and some in situ hybridisation; Eddy de Robertis, Siew-Lan Ang and Bernard Hermann for probes; Bernard Roelen for initiating S.F. into dissecting early embryos; and Jaap Heinen and Ferdinand Vervoordeldonk for artwork. We also thank Rolf Zeller and his group for three months hospitality (to S.F.) when our laboratory moved. This work was supported by a grant from the French 'Association pour la Recherche sur le Cancer' (ARC) (to S.F.) and from the Dutch NWO-ALW (809.38.05) (to J.D.).

REFERENCES

Akasaka, T., van Lohuizen, M., van der Lugt, N., Mizutani-Koseki, Y., Kanno, M., Taniguchi, M., Vidal, M., Alkema, M., Berns, A. and Koseki, H. (2001). Mice doubly deficient for the Polycomb Group genes *Mel18* and *Bmi1* reveal synergy and requirement for maintenance but not initiation of Hox gene expression. *Development* **128**, 1587-1597.

Ang, S. L. and Rossant, J. (1993). Anterior mesendoderm induces mouse *Engrailed* genes in explant cultures. *Development* **118**, 139-149.

Bachiller, D., Klingensmith, J., Kemp, C., Belo, J. A., Anderson, R. M., May, S. R., McMahon, J. A., McMahon, A. P., Harland, R. M., Rossant, J. et al. (2000). The organizer factors Chordin and Noggin are required for mouse forebrain development. *Nature* **403**, 658-661.

Beddington, R. S. P. and Lawson, K. A. (1990). Clonal analysis of cell lineages. In *Postimplantation Mammalian Embryos – A Practical Approach* (ed. A. J. Copp. and D. L. Cockcroft), pp. 267-291. Oxford: Oxford University Press.

Bouillet, P., Oulad-Abdelghani, M., Ward, S. J., Bronner, S., Chambon, P. and Dollé, P. (1996). A new mouse member of the Wnt gene family, mWnt-8, is expressed during early embryogenesis and is ectopically induced by retinoic acid. *Mech. Dev.* **58**, 141-152.

Charité, J., de Graaff, W., Shen, S. and Deschamps, J. (1994). Ectopic expression of Hoxb8 causes duplication of the ZPA in the forelimb and homeotic transformation of axial structures. *Cell* **78**, 589-601.

Deschamps, J. and Wijgerde, M. (1993). Two phases in the establishment of HOX expression domains. *Dev. Biol.* **156**, 473-480.

Deschamps, J., van den Akker, E., Forlani, S., de Graaff, W., Oosterveen, T., Roelen, B. and Roelfsema, J. (1999). Initiation, establishment and maintenance of Hox gene expression patterns in the mouse. *Int. J. Dev. Biol.* **43**, 635-650.

Downs, K. M. and Davies, T. (1993). Staging of gastrulating mouse embryos by morphological landmarks in the dissecting microscope. *Development* **118**, 1255-1266.

Duboule, D. and Morata, G. (1994). Colinearity and functional hierarchy among genes of the homeotic complexes. *Trends Genet.* **10**, 358-364.

Frohman, M. A., Boyle, M. and Martin, G. R. (1990). Isolation of the mouse Hox-2.9 gene; analysis of embryonic expression suggests that positional information along the anterior-posterior axis is specified by mesoderm. *Development* **110**, 589-607.

Gaunt, S. J. (2001). Gradients and forward spreading of vertebrate Hox gene expression detected by using a Hox/lacZ transgene. *Dev. Dyn.* **221**, 26-36.

Gaunt, S. J., Sharpe, P. T. and Duboule, D. (1988). Spatially restricted domains of homeo-gene transcripts in mouse embryos: relation to a segmented body plan. *Development* **104**, 169-179.

Gaunt, S. J. and Strachan, L. (1994). Forward spreading in the establishment of a vertebrate Hox expression boundary: the expression domain separates into anterior and posterior zones, and the spread occurs across implanted glass barriers. *Dev. Dyn.* **199**, 229-240.

Gellon, G. and McGinnis, W. (1998). Shaping animal body plans in development and evolution by modulation of Hox expression patterns. *BioEssays* **20**, 116-125.

Gilthorpe, J., Vandromme, M., Brend, T., Gutman, A., Summerbell, D., Totty, N. and Rigby, P. W. (2002). Spatially specific expression of *Hoxb4* is dependent on the ubiquitous transcription factor NFY. *Development* **129**, 3887-3899.

Gould, A., Itasaki, N. and Krumlauf, R. (1998). Initiation of rhombomeric *Hoxb4* expression requires induction by somites and a retinoid pathway. *Neuron* **21**, 39-51.

Herrmann, B. G., Labeit, S., Poustka, A., King, T. R. and Lehrach, H. (1990). Cloning of the T gene required in mesoderm formation in the mouse. *Nature* **343**, 617-622.

Hogan, B., Beddington, R. and Constantini, F. (1994). *Manipulating the Mouse Embryo: A Laboratory Manual*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Ikeya, M. and Takada, S. (2001). Wnt-3a is required for somite specification along the anteroposterior axis of the mouse embryo and for regulation of *cdx-1* expression. *Mech. Dev.* **103**, 27-33.

Kondo, T. and Duboule, D. (1999). Breaking colinearity in the mouse HoxD complex. *Cell* **97**, 407-417.

Krumlauf, R. (1994). Hox genes in vertebrate development. *Cell* **78**, 191-201.

Lawson, K. A., Meneses, J. J. and Pedersen, R. A. (1991). Clonal analysis of epiblast fate during germ layer formation in the mouse embryo. *Development* **113**, 891-911.

Lawson, K. A. and Pedersen, R. A. (1992). Early mesoderm formation in the mouse embryo. In *Formation and Differentiation of Early Embryonic Mesoderm* (ed. R. Bellairs, E. J. Sanders and J. W. Lash), pp. 33-46. New York: Plenum Press.

Liu, P., Wakamiya, M., Shea, M. J., Albrecht, U., Behringer, R. R. and Bradley, A. (1999). Requirement for Wnt3 in vertebrate axis formation. *Nat. Genet.* **22**, 361-365.

- Maloo, J. N., Whangbo, J., Harris, J. M., Jongeward, G. D. and Kenyon, C.** (1999). A Wnt signaling pathway controls hox gene expression and neuroblast migration in *C. elegans*. *Development* **126**, 37-49.
- Marshall, H., Studer, M., Popperl, H., Aparicio, S., Kuroiwa, A., Brenner, S. and Krumlauf, R.** (1994). A conserved retinoic acid response element required for early expression of the homeobox gene Hoxb-1. *Nature* **370**, 567-571.
- Mathis, L. and Nicolas, J. F.** (2000). Different clonal dispersion in the rostral and caudal mouse central nervous system. *Development* **127**, 1277-1290.
- Mathis, L., Kulesa, P. M. and Fraser, S. E.** (2001). Fgf receptor signalling is required to maintain neural progenitors during Hensen's node progression. *Nat. Cell Biol.* **3**, 559-566.
- Meier, S. and Tam, P. P.** (1982). Metameric pattern development in the embryonic axis of the mouse. I. Differentiation of the cranial segments. *Differentiation* **21**, 95-108.
- Murphy, P. and Hill, R. E.** (1991). Expression of the mouse labial-like homeobox-containing genes, Hox 2.9 and Hox 1.6, during segmentation of the hindbrain. *Development* **111**, 61-74.
- Oosterveen, T., Niederreither, K., Dolle, P., Chambon, P., Meijlink, F. and Deschamps, J.** (2003). Retinoids regulate the anterior expression boundaries of 5' Hoxb genes in posterior hindbrain. *EMBO J.* **22**, 262-269.
- Partanen, J., Schwartz, L. and Rossant, J.** (1998). Opposite phenotypes of hypomorphic and Y766 phosphorylation site mutations reveal a function for Fgfr1 in anteroposterior patterning of mouse embryos. *Genes Dev.* **12**, 2332-2344.
- Perea-Gomez, A., Lawson, K. A., Rhinn, M., Zakin, L., Brulet, P., Mazan, S. and Ang, S. L.** (2001). Otx2 is required for visceral endoderm movement and for the restriction of posterior signals in the epiblast of the mouse embryo. *Development* **128**, 753-765.
- Riese, J., Yu, X., Munnerlyn, A., Eresh, S., Hsu, S. C., Grosschedl, R. and Bienz, M.** (1997). LEF-1, a nuclear factor coordinating signaling inputs from wingless and decapentaplegic. *Cell* **88**, 777-787.
- Roelen, B. A., de Graaff, W., Forlani, S. and Deschamps, J.** (2002). Hox cluster polarity in early transcriptional availability: a high order regulatory level of clustered Hox genes in the mouse. *Mech. Dev.* **119**, 81.
- Sharpe, J., Nonchev, S., Gould, A., Whiting, J. and Krumlauf, R.** (1998). Selectivity, sharing and competitive interactions in the regulation of Hoxb genes. *EMBO J.* **17**, 1788-1798.
- Snedecor, G. W. and Cochran, W. G.** (1967). *Statistical Methods*, 6th edn. Iowa: Iowa State University Press.
- Studer, M., Popperl, H., Marshall, H., Kuroiwa, A. and Krumlauf, R.** (1994). Role of a conserved retinoic acid response element in rhombomere restriction of Hoxb-1. *Science* **265**, 1728-1732.
- Sun, X., Meyers, E. N., Lewandoski, M. and Martin, G. R.** (1999). Targeted disruption of Fgf8 causes failure of cell migration in the gastrulating mouse embryo. *Genes Dev.* **13**, 1834-1846.
- Tam, P. P. L.** (1988). The allocation of cells in the presomitic mesoderm during somite segmentation in the mouse embryo. *Development* **103**, 379-390.
- Tam, P. P. L.** (1989). Regionalisation of the mouse embryonic ectoderm: allocation of prospective ectodermal tissues during gastrulation. *Development* **107**, 55-67.
- Tam, P. P. L. and Beddington, R. S. P.** (1987). The formation of mesodermal tissues in the mouse embryo during gastrulation and early organogenesis. *Development* **99**, 109-126.
- Tomotsune, D., Shirai, M., Takihara, Y. and Shimada, K.** (2000). Regulation of Hoxb3 expression in the hindbrain and pharyngeal arches by *rae28*, a member of the Polycomb group of genes. *Mech. Dev.* **98**, 165-169.
- van den Akker, E., Forlani, S., Chawengsakshophak, K., de Graaff, W., Beck, F., Meyer, B. and Deschamps, J.** (2002). Cdx1 and Cdx2 have overlapping functions in anteroposterior patterning and posterior axis elongation. *Development* **129**, 2181-2193.
- Wilkinson, D. G., Bhatt, S., Cook, M., Boncinelli, E. and Krumlauf, R.** (1989). Segmental expression of Hox-2 homoeobox-containing genes in the developing mouse hindbrain. *Nature* **341**, 405-409.
- Wilkinson, D. G. and Krumlauf, R.** (1990). Molecular approaches to the segmentation of the hindbrain. *Trends Neurosci.* **13**, 335-339.
- Yu, B. D., Hanson, R. D., Hess, J. L., Horning, S. E. and Korsmeyer, S. J.** (1998). MLL, a mammalian *trithorax*-group gene, functions as a transcriptional maintenance factor in morphogenesis. *Proc. Natl. Acad. Sci. USA* **95**, 10632-10636.
- Zakany, J., Kmita, M., Alarcon, P., de la Pompa, J. L. and Duboule, D.** (2001). Localized and transient transcription of Hox genes suggests a link between patterning and the segmentation clock. *Cell* **106**, 207-217.