Nuclear re-organisation of the Hoxb complex during mouse embryonic development

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Summary

The spatial and temporal co-linear expression of Hox genes during development is an exquisite example of programmed gene expression. The precise mechanisms underpinning this are not known. Analysis of Hoxb chromatin structure and nuclear organisation, during the differentiation of murine ES cells, has lent support to the idea that there is a progressive ‘opening’ of chromatin structure propagated through Hox clusters from 3′ to 5′, which contributes to the sequential activation of gene expression. Here, we show that similar events occur in vivo in at least two stages of development. The first changes in chromatin structure and nuclear organisation were detected during gastrulation in the Hoxb1-expressing posterior primitive streak region: Hoxb chromatin was decondensed and the Hoxb1 locus looped out from its chromosome territory, in contrast to non-expressing Hoxb9, which remained within the chromosome territory. At E9.5, when differential Hox expression along the anteroposterior axis is being established, we found concomitant changes in the organisation of Hoxb. Hoxb organisation differed between regions of the neural tube that had never expressed Hoxb [rhombomeres (r) 1 and 2], strongly expressed Hoxb1 but not b9 (r4), had downregulated Hoxb1 (r5), expressed Hoxb9 but not Hoxb1 (spinal cord), and expressed both genes (tail bud). We conclude that Hoxb chromatin decondensation and nuclear re-organisation is regulated in different parts of the developing embryo, and at different developmental stages. The differential nuclear organisation of Hoxb along the anteroposterior axis of the developing neural tube is coherent with co-linear Hox gene expression. In early development nuclear re-organisation is coupled to Hoxb expression, but does not anticipate it.

Key words: Chromatin, Embryonic development, Hox genes, Nuclear organisation, Rhombomere, Mouse

Introduction

In mammals, Hox genes are organised in four clusters, each subject to tight transcriptional regulation. The co-linear expression of Hox genes is a remarkable example of spatial and temporal control of gene expression, and is essential for correct embryonic development (Kmita and Duboule, 2003). Genes located at one (3′) end of a Hox cluster are activated earlier, and in more anterior structures of the embryo, than genes lying progressively toward the 5′ end of the cluster. Transgenic experiments in the mouse have revealed both local (e.g. Marshall et al., 1994), and more distant (Spitz et al., 2003) cis-regulatory elements involved in the control of Hox gene expression. In addition, experiments that transpose Hox genes within and between clusters have led to the suggestion that there is a progressive change in chromatin structure propagated through Hox clusters from 3′ to 5′, that prevents posterior genes from being expressed early during embryonic development. The precise nature of the underlying mechanisms are still unknown, but might involve a progressive ‘opening’ of chromatin, or a progressive relief from a silencing mechanism (Kmita and Duboule, 2003).

We have previously investigated nuclear organisation and chromatin structure changes at the murine Hoxb cluster using a retinoic acid (RA) induced ES cell differentiation model (Chambeyron and Bickmore, 2004). We showed that the progressive transcriptional activation of Hoxb genes is associated, not only with a visible decondensation of Hoxb, but also with the choreographed extrusion of the genes out of their chromosome territory (CT). These observations lend support to a model in which there is progressive change in large-scale chromatin structure, initiating at the 3′ end, which contributes to the sequential activation of gene expression from this Hox cluster (Kmita and Duboule, 2003; Roelen et al., 2002; Bickmore et al., 2005).

Although the ES cell system seems to recapitulate the temporal activation of Hoxb genes (Simeone et al., 1990), it remains unclear whether this activation mechanism, and the concomitant chromatin and nuclear re-organisation, reflect the mechanisms that operate in vivo (Duboule and Deschamps, 2004). Co-linear Hox regulation occurs several times during embryonic development. The first wave of Hoxb expression is early in gastrulation, initiating in the most posterior (caudal) part of the primitive streak (PS) (Forlani et al., 2003). Later, towards mid-gestation, the establishment of restricted domains of Hoxb expression in the neural tube also depends on co-linearity.

Here, we have used fluorescence in situ hybridisation (FISH) on mouse embryo tissue sections in order to determine whether
the chromatin changes seen at Hoxb during ES cell differentiation also occur during different stages of embryogenesis. We show that at the onset of gastrulation the transcripational activation of Hoxb1 in the posterior PS is accompanied by chromatin decondensation of Hoxb and extrusion of Hoxb1 from its CT. These events are not seen in the non-expressing extra-embryonic (EE) cells of the same embryos. Decondensation and looping out of Hoxb1 is also seen in cells of rhombomere 4 (r4) at E9.5, but not in rhombomeres 1 or 2, anterior to r4, or in r5. By contrast a more significant chromatin decondensation is also seen in cells of rhombomere 4 (r4) at E9.5, but not in the non-expressing extra-embryonic (EE) cells of the same stage embryos according to criteria previously published (Downs and Davies, 1993).

Materials and methods

Mouse embryo sectioning and staging

Embryos were collected from crosses between Det-LacZ homozygous transgenic CD1×CD1 mice (MacKenzie et al., 1997). We confirmed, by FISH, that the Det-LacZ transgene is on a different mouse chromosome from Hoxb (data not shown). The day on which the vaginal plug was detected was considered 0.5 days of gestation (E0.5).

E6.5 and E7.5 embryos in the decidua, and E9.5 embryos, were fixed with 4% formaldehyde overnight at 4°C, dehydrated through a graded ethanol series, cleared in xylene, and embedded in paraffin blocks. Adjacent serial sections were cut at 4 µm and used for FISH, immunohistochemistry or staining with Haematoxylin and Eosin (HE). HE stained sections from E6.5 and E7.5 embryos were used to stage embryos according to criteria previously published (Downs and Davies, 1993).

Immunohistochemistry

Immunohistochemistry on 4 µm paraffin sections was performed with Vectastain peroxidase staining kit (Vector Laboratories) according to manufacturer’s instruction using a 1:200 dilution of a polyclonal antibody against Hoxb1 (Covance). For epitope unmasking, sections were treated by microwave (900 W) for 20 minutes in 100 mM TrisHCl pH 10 and subsequently blocked in 90% FCS blocking solution. Sections were counterstained with 0.75% Eosin for 4 minutes and mounted in Histomount.

Whole-mount in situ hybridisation

Antisense probes for Hoxb1 and Hoxb9 were prepared from cDNAs kindly provided by Robb Krumlauf. The Hoxb1 probe was a T3 transcript from a 0.9 kb EcoRI fragment. The Hoxb9 probe was a T7 polymerase transcript from a 1.3 kb EcoRI fragment. The probes were labelled with digoxigenin by in vitro transcription (Roche).

FISH

The protocol for FISH on mouse tissue sections was adapted from (Newsome et al., 2003). Briefly, 4 µm sections were laid on Superfrost slides and were heated to 60°C for 20 minutes, washed four times in xylene for 10 minutes each before dehydrtation through an ethanol series (100%, 90%, 70%). They were then microwaved for 20 minutes in 0.1 M citrate buffer, pH 6.0. Slides were cooled in buffer for 20 minutes, washed and stored in water. Before use, they were rinsed in 2×SSC, incubated in 2×SSC for 5 minutes at 75°C, denatured for 3 minutes at 75°C in 70% formamid/2×SSC, plunged into ice-cold 70% ethanol for 3 minutes, dehydrated through an alcohol series and air-dried.

Probes were labelled for FISH as described previously (Mahy et al., 2002b; Chamleyron and Bickmore, 2004). Approximately 200 ng of chromosome paint, and 250 ng BAC or plasmid were used per slide with 15 µg mouse Cot1 DNA (GibcoBRL) and 5 µg sonicated salmon sperm DNA (ssDNA).

Image capture and analysis

A focus motor was used to collect image stacks of sections on slides, at 0.5 µm intervals along the z-axis, using a Zeiss Axiosplan fluorescence microscope. Images were captured using a Princeton Instruments Micromax CCD camera, and deconvoluted using Hazebuster software (Scanalitics). A three-dimensional (3D) image was reconstructed and analysed using IPLab (Scanalitics).

The distance (d) between two probes in 3D was determined by a more automated approach than previously described (Chamleyron and Bickmore, 2004). Previously, a region containing both probe signals was selected and their segmentation was performed manually. Here, each probe signal is individually selected and a script then determines the segmentation levels. Briefly, a maximum pixel projection was made from the deconvoluted image stack and the two probe hybridisation signals were manually delimited. The xyz coordinates of the weighted signal centroid were determined for each probe, and d was the distance between the two centroids at opposite corners of a cuboid (Chamleyron and Bickmore, 2004).

Probe localisation relative to the CT in the z stack was determined visually. When the probe was not in the same z panel as the CT, it was considered outside the CT, and was attributed an arbitrary value of –0.5 µm (the interval between two z frames). Where the probe and the CT were in the same z-plane, the position of the probe relative to the nearest edge of the CT was determined as previously described for 2D analyses (Mahy et al., 2002a; Chamleyron and Bickmore, 2004).

A two-tailed distribution Student’s t-test was used to test the statistical significance of differences in chromatin compaction and probe position with respect to CTs. Where there was clearly a non-normal distribution of data, chi-square analysis was used.

Results

Chromatin decondensation of Hoxb occurs upon the initiation of transcription in the primitive streak

In our previous ES cell differentiation study we showed that there is a transient decondensation of Hoxb, between Hoxb1 and Hoxb9, upon induction of transcription with RA
(Chambeyron and Bickmore, 2004). During mouse embryogenesis proper, Hoxb1 expression first appears at the late midstreak stage (~E7.0) (Roelen et al., 2002). Expression begins in the nascent mesoderm associated with the most caudal part of the primitive streak (PS), at the boundary between extra-embryonic (EE) and embryonic tissues, and then extends rostrally along, and lateral to, the PS (Forlani et al., 2003). Hoxb9 expression begins at headfold stage (~E7.5) in the posterior embryonic ectoderm just lateral to the PS (Conlon and Rossant, 1992).

To determine if there is also a decondensation of Hoxb chromatin upon the induction of transcription in vivo, we used FISH to determine the interphase separation between Hoxb1 and Hoxb9 in tissue sections of E7.5 embryos. According to the published literature, Hoxb1 expression should be seen from late streak stage onwards, whereas Hoxb9 expression should not appear until late neural plate/early head fold stage (Conlon and Rossant, 1992). To ensure that only embryos expressing Hoxb1, and not Hoxb9, were analysed, we selected late streak early bud and neural plate stage embryos. We confirmed the patterns of Hoxb1 and Hoxb9 expression by RNA in situ hybridisation (Fig. 1A–C) (Table 1). Hoxb1 protein was also analysed in sagittal sections of E7.5 embryos using a Hoxb1 antibody (Fig. 1F). This confirmed the expression of Hoxb1 in the cells of the primitive streak and the adjacent mesoderm (PSM) (Forlani et al., 2003).

Hoxb1 and Hoxb9 are separated by 90 kb of DNA (Chambeyron and Bickmore, 2004). We measured the distance d (in µm) between Hoxb1 and Hoxb9 FISH signals in nuclei from E7.5 embryo tissue sections adjacent to those used for expression analysis (Fig. 1G). Chromatin condensation was assessed by a comparison of d² values (van den Engh et al., 2003). Indeed, we re-measured Hoxb1–Hoxb9 interphase distances in ES cells after 2 days of differentiation with RA (Chambeyron and Bickmore, 2004), with the script used for analysis of embryo sections described here. The resulting <d²> (0.25±0.03 µm²) (Fig. 2B) is remarkably similar to that measured in cells from the E7.5 PSM.

**Movement outside of the chromosome territory accompanies Hoxb1 expression in the primitive streak region**

Induction of Hoxb expression in ES cells is accompanied, not only by a decondensation of the locus, but also by a choreographed looping out from the CT of the expressed genes (Chambeyron and Bickmore, 2004). To determine if this nuclear re-organisation also occurs during Hoxb expression in early embryogenesis, we performed FISH on E7.5 embryo tissue sections, adjacent to those used for whole mount in situ hybridisation

**Fig. 1.** Expression of Hoxb1 and Hoxb9 during mouse embryogenesis. Whole-mount in situ hybridisation to detect Hoxb1 (A) or Hoxb9 (B,C) expression in E7.5 embryos. Hoxb1 expression in the primitive streak and adjacent mesoderm (PSM) is seen in a late streak/early bud embryo (A). No Hoxb9 expression is detected in late neural plate stage embryos (B), but is detected in PSM of headfold stage E7.5 embryos (C). Whole-mount in situ hybridisation to detect Hoxb1 (D) or Hoxb9 (E) expression in E9.5 embryos. Hoxb1 expression is seen in the tailbud (Tb) and in a segment of the hindbrain (D). A sagittal section of this embryo shows that this latter region is rhombomere 4 (r4) (shown in inset at higher magnification). There is no Hoxb1 expression in the anterior region of the spinal cord (SC). Hoxb9 expression (E) is seen in the SC and Tb, but not in the hindbrain. (F) Immunohistochemistry staining of a near sagittal section from an E7.5 (neural plate stage) embryo, with antibody that recognises Hoxb1, and counterstained with Eosin. Hoxb1 is seen in the PSM. There is no staining in extra-embryonic mesoderm (EEM). (G) DAPI staining of an adjacent E7.5 section that was used for FISH analysis. (H) Immunohistochemistry staining for Hoxb1 on a sagittal section from an E9.5 embryo. The positions of rhombomeres 1 to 4 (r1–4) are indicated. (I) DAPI staining of an adjacent E9.5 section that was used for FISH analysis. Scale bars: 200 µm.
Table 1. Expression patterns of Hoxb1 and Hoxb9 during embryogenesis

<table>
<thead>
<tr>
<th>Embryonic stage</th>
<th>Tissue</th>
<th>Expression</th>
<th>Hoxb1</th>
<th>Hoxb9</th>
</tr>
</thead>
<tbody>
<tr>
<td>E6.5</td>
<td>Distal region</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td></td>
<td>Posterior streak region</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td></td>
<td>Anterior epiblast</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td></td>
<td>Posterior epiblast</td>
<td>(autonomous expression in explants)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>E7.5 (late streak and early bud stage)</td>
<td>Extra-embryonic; anterior yolk sac mesoderm; extra-embryonic ectoderm</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Primitive streak + adjacent embryonic mesoderm</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>E9.5</td>
<td>Rhombomere 1 or 2</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td></td>
<td>Rhombomere 4</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Rhombomere 5</td>
<td>Repressed</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Spinal cord</td>
<td>Repressed</td>
<td>–</td>
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<td></td>
<td>Tailbud</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Expression patterns of Hoxb1 and Hoxb9 in different tissues of the mouse embryo from E6.5 to E9.5. –, not expressed; +, expressed; repressed, no longer expressed.

Hoxb1 hybridisation signals were at the edge of, or inside of, the CT (Fig. 3C). However in the PSM, 47% of Hoxb1 hybridisation signals were outside of the CT (Fig. 3C). A comparison of the mean positions of Hoxb1 in EEM and EEE, with that in the PSM, shows that there is a significant ($P=0.025$) movement of Hoxb1 outside of its CT in the expressing PSM tissue (Fig. 3D). By contrast, Hoxb9 is not expressed anywhere in pre-late neural plate stage E7.5 embryos, and most (66 and 73%) Hoxb9 alleles remain inside of the CT in both the EEM and PSM ($P=0.8$) (Fig. 3C,D).

Therefore, as in ES cells, there is a specific extrusion of the 3’ end (Hoxb1) of the Hoxb cluster out of the MMU11 CT when Hoxb1 expression initiates in embryogenesis. Another similarity that we observed with the ES system is that, even in non-expressing tissues (EEM and EEE), Hoxb1 is located closer to the CT surface than Hoxb9 is (Fig. 3D).

### Chromatin decondensation and nuclear organisation of Hoxb during hindbrain segmentation

Later in embryogenesis, Hoxb genes show spatially restricted patterns of gene expression within segments of the developing hindbrain. Expression of Hoxb1 mRNA (Murphy et al., 1989; Wilkinson et al., 1989) and protein (Ferretti et al., 1999; Arenkiel et al., 2004) is restricted to rhombomere 4 (r4) of the hindbrain at E9.5 (Fig. 1D,H). However, these cells are not descendants of the expressing cells from the E7.5 PS (Forlani et al., 2003). Rather, Hoxb1 expression in r4 is the result of a separate induction of expression. We therefore determined whether Hoxb1 expression in r4 is accompanied by chromatin and nuclear re-organisation events similar to those seen in ES cells and in the PSM.

The interphase separation of Hoxb1 and Hoxb9 in rhombomeres 1 or 2 (r1/r2), where neither gene is expressed, is very similar to that in EEM of E7.5 embryos (Fig. 4A). There was decondensation of Hoxb chromatin in r4, but interestingly the distribution of $d^2$ values in these cells did not follow a normal distribution. Instead, 21% of the loci have a highly decondensed chromatin fibre ($<d^2> = 1 \mu m^2$) whereas 80% remain more condensed ($<d^2> 0.02 \mu m^2$) (Fig. 4A). This suggests that not all cells of r4 were transcribing Hoxb1 at the time of analysis. A chi-squared test showed that the difference
in the distribution of $d^2$ values between r1/r2 and r4 is statistically significant ($P<0.0001$). Chromatin decondensation in r4 is also accompanied by a significant ($P=0.015$) re-localisation of Hoxb1 outside its CT, compared with r1/r2 (Fig. 4B).

There is no expression of Hoxb1 in r5, immediately posterior to r4, at E9.5 (Fig. 1C). Hoxb1 chromatin is recondensed in this rhombomere, and in particular the population of highly decondensed loci that were seen in r4, is not present (Fig. 4A). Hoxb1 also re-localises back towards the surface of the CT in r5 cells (Fig. 4B).

Therefore, even though separate events lead to the induction of expression of Hoxb1 in the PS early in development (E7.5), and in r4 later on (E9.5), both appear to be accompanied by chromatin decondensation and an extrusion of Hoxb1 out of the CT. By contrast, Hoxb9 is not expressed anywhere in the hindbrain, and we found that in both r4 and r5, most (66%) Hoxb9 hybridisation signals are located within the CT (Fig. 4C).

**Nuclear organisation of Hoxb in the spinal cord**

During RA-induced ES cell differentiation, we were able to activate the more 5' Hoxb gene, but at a later time (day 10) than Hoxb1. At that time point we saw movement of Hoxb9 out of the interior of the CT to a position at the CT surface, or just beyond it. However, there was no accompanying visible decondensation of Hoxb1 chromatin (Chambeyron and Bickmore, 2004). In the E9.5 embryo, Hoxb9 is strongly expressed along the neural tube, posterior to the level of somites 7-8 (Chen and Capecchi, 1997) (Fig. 1E), but Hoxb1 is not expressed there (Fig. 1D). By contrast, Hoxb1 (Gofflot et al., 1997) and Hoxb9 are both expressed in the tailbud region at E9.5 (Fig. 1D,E).

Similar to the situation in Hoxb9-expressing differentiated ES cells, we do not detect gross decondensation of Hoxb1 chromatin in the spinal cord (SC); the $<d^2>$ (0.11 $\mu$m$^2$) is not significantly different from that in r5 ($P=0.5$), which is anterior to the limit of Hoxb9 expression. However, the $<d^2>$ measured in the spinal cord of the tail bud region (Tb) (0.15 $\mu$m$^2$), was also not significantly larger than that in r5 ($P=0.18$) (Fig. 5A). This is the first situation in either ES cells or in the embryo, where Hoxb1 expression has not been accompanied by cytological levels of chromatin decondensation.

One explanation for this might be that extreme levels of chromatin decondensation are a consequence of the movement of Hoxb1 far out of the CT, while Hoxb9 is still resident within it. In the SC we found that, compared with r5, most Hoxb9 loci had moved away from the CT interior and towards, or just beyond ($P=0.01$), its edge (Fig. 5B). Hoxb1 remains within the CT in SC cells (Fig. 5C). As in ES cells, Hoxb9 movement out of the CT is not as large as that of Hoxb1 in expressing cells such as r4 (Fig. 4). In the Tb we found that, on average, both Hoxb1 and Hoxb9 are located just beyond the edge of the CT (Fig. 5D), but the distribution of positions for both these genes is bimodal (Fig. 5B,C).

**Chromatin organisation in cells capable of autonomous or precocious activation of Hoxb1**

More than 12 hours before overt Hoxb1 expression, cells from the posterior streak region (PSR) of early streak stage embryos...
(E6.5) can initiate Hoxb1 expression autonomously in explants (Forlani et al., 2003). By contrast, cells from the distal region (DR) alone cannot. This might suggest some prior opening of Hoxb chromatin structure in PSR cells at the onset of gastrulation, which anticipates Hoxb expression itself. Therefore, we analysed chromatin condensation and nuclear organisation of Hoxb in cells from the PSR (epiblast and mesoderm) and the DR (epiblast) in early streak stage E6.5 embryos (Fig. 6A). Examination of Hoxb1-Hoxb9 d2 values indicated that the locus remains condensed in both groups of cells (<d2>=0.09±0.02 and 0.10±0.01 µm², respectively), similar to EEM cells at E7.5 (<d2>=0.08±0.02 µm²) (Fig. 6B). Similarly, Hoxb1 remains within its CT in nuclei from both regions at E6.5 (Fig. 6C). However, as in the E7.5 EE, at E6.5 Hoxb1 is located closer to the surface of the CT than Hoxb9 (Fig. 6D).

Roelen et al. (Roelen et al., 2002) have shown that, in the E6.5 embryo, a short pulse of exogenous RA can stimulate the precocious expression of Hoxb1 in the presumptive posterior epiblast (PEP) at the junction between embryonic and EE ectoderm (Fig. 6A). However, we did not find any decondensation of Hoxb1 chromatin in these cells of the embryo either (Fig. 6B). Hoxb1 was also localised within the CT of these cells (data not shown). We conclude that there is no nuclear re-organisation of Hoxb in cells that are destined to express, or that are capable of expressing, Hoxb1 in the very early embryo. Rather, chromatin opening is tightly coupled to the actual activation of Hoxb transcription during gastrulation.

Discussion
Chromatin changes at Hoxb during ES cell differentiation recapitulate those that occur early in embryogenesis
We have shown that induction of Hoxb1 expression in the posterior primitive streak and adjacent mesoderm (E7.5), is accompanied by chromatin decondensation of Hoxb (Fig. 2), and by an extrusion of the 3′ end of the cluster (Hoxb1) from the chromosome territory (Fig. 3). No remodelling of Hoxb9 chromatin is seen in cells from pre-late neural plate stages (Fig. 3). This mirrors the polarised Hoxb chromatin organisation after 2-4 days of RA-induced ES cell differentiation, when Hoxb1, but not Hoxb9, is expressed (Chambeyron and Bickmore, 2004). No such re-organisation of Hoxb is seen in cells of the EE yolk sac mesoderm, where Hoxb1 is not expressed. Nor do we detect any evidence of decondensation, or Hoxb1 movement, in cells of the E6.5 posterior streak region (Fig. 6), a region that does not yet express Hoxb1, but that can initiate Hoxb1 expression autonomously in cultured explants (Forlani et al., 2003). Therefore we conclude that, at the onset of gastrulation, there is no prior chromatin re-modelling at
Development

Fig. 5. Nuclear re-organisation of Hoxb in the spinal cord. (A) The distribution of squared interphase distances ($d^2$) in $\mu m^2$ measured between probes for Hoxb1 and Hoxb9, in rhombomeres 5 (r5), spinal cord (SC), and tailbud (Tb) from E9.5 embryos. (B,C) Histogram showing the position of Hoxb9 (B) or Hoxb1 (C) hybridisation signals, relative to the inside, edge or outside of the MMU11 territory, in nuclei from r5 (grey bars), SC (hatched bars) and Tb (black bars) of two E9.5 embryos from different litters. Negative distances indicate signals localised beyond the visible limits of the detectable CT ($n\geq 60$). (D) Positions (mean±s.e.m.) of Hoxb1 (black squares) and Hoxb9 (white circles) relative to the inside, edge or outside of the MMU11 territory in nuclei located along the anteroposterior axis of the neural tube at E9.5, from r1/r2 down to the tailbud (Tb) ($n\geq 60$).

Hoxb in cells from the region that is destined to later express Hoxb1.

However, as in undifferentiated ES cells, Hoxb1 occupies a position closer to the surface of the CT than Hoxb9 in early streak stage embryos (Fig. 6). This aspect of Hoxb nuclear organisation may have a role in the polarised response of the 3' end of the cluster to activation in early embryogenesis.

**Differences in chromatin structure at Hoxb in the developing hindbrain**

It is perhaps not surprising that ES cells, which are derived from the inner cell mass of the blastocyst, recapitulate the events of Hoxb1 expression that occur at the primitive streak during gastrulation. However, most of the interest in Hoxb expression has been in the anteroposterior spatially restricted segmental expression patterns in the neural tube, which occur later in development. Hoxb1 is expressed in r4 of the hindbrain of the E9.5 embryo. However, this cannot be the simple result of lineage transmission from the PS cells that first initiate Hoxb1 expression, because the r4 precursors are already anterior to the node when Hoxb1 expression spreads caudally from the PS towards the node (Forlani et al., 2003).

Nevertheless we do find some aspects of Hoxb nuclear re-organisation replayed in r4 cells. There is movement of Hoxb1, but not Hoxb9, out from the CT in nuclei from r4. This did not occur in more anterior (r1/r2) segments of the hindbrain (Figs 4 and 5). There is also a decondensation of chromatin at Hoxb that is specific to r4, but qualitatively different from that seen in PSM at E7.5. In the latter case, there is a generalised shift in the Hoxb1-Hoxb9 $d^2$ to larger values (Fig. 2B), indicating a relatively uniform response of the cell population. This is similar to the decondensation seen in RA-induced ES cells (Fig. 2B). However, the distribution of $d^2$ values in r4 cells is bimodal (Fig. 4B). Twenty percent of loci are hyper-condensed ($<d^2>=1.1\pm 0.01 \mu m^2$, but the other alleles are as, or more, condensed as those in r1/r2 ($<d^2>=0.02\pm 0.02 \mu m^2$ and $0.07\pm 0.02 \mu m^2$, respectively). We think this means that only a proportion of cells in r4 will be expressing Hoxb1 at a given time, perhaps owing to stochastic fluctuation in transcription (Levsky and Singer, 2003; Osborne et al., 2004), or due to changing levels of Hoxb1 expression in differentiating subpopulations, e.g. motoneurons (Arendel et al., 2004). Further investigation of this will necessitate developing assays (e.g. RNA FISH) that allow both transcription and chromatin structure to be analysed in the same nucleus of the embryo.

These data suggest that the precise chromatin changes that occur at Hoxb1 differ between the early phase of activation at E7.5, and the later segment-restricted phase of expression in...
the hindbrain. This would be consistent with the behaviour of a Hoxb1 transgene when transposed to a 5′ position within Hoxd. In this case, there was no expression of the transposed transgene in r4, but there was still early mesodermal expression (Kmita et al., 2000). It will now be interesting to analyse chromatin condensation and nuclear organisation of the transposed Hoxb1 during embryogenesis.

Repression of Hoxb1 outside of r4
The maintenance of Hoxb1 expression in r4, and its repression outside r4, depends in part on auto- and cross-regulatory Hox-mediated mechanisms (Popperl et al., 1995; Gavalas et al., 1998; Studer et al., 1998). Specific cis-acting elements and other trans-acting regulators also serve to repress Hoxb1 transcription in rhombomeres 3 and 5 (Fox, 2000; Giudicelli et al., 2003). It is interesting that although in r5 Hoxb1 is closer to the edge, or just outside, of the CT, compared with its position in r1/2 cells (Fig. 5D) – perhaps reflecting the history of past Hoxb1 expression in cells destined to be part of r5 – Hoxb1 chromatin may be more tightly condensed in r5, than in r1/h2 (Fig. 4A) (P=0.09). In addition, the chromatin configuration in r5, may relate to the expression in this hindbrain segment of Hoxb2, located only 12 kb 5′ of Hoxb1 (Wilkinson et al., 1989).

Nuclear organisation of Hoxb along the anteroposterior axis of the neural tube. We did not find any nuclear re-organisation of Hoxb9 in b1-expressing cells at E7.5, and we were careful to restrict our analysis to pre-late neural plate stage embryos that do not express Hoxb9 (Fig. 1B). However, we do see nuclear re-organisation of Hoxb9 later in development, at E9.5, in the part of the spinal cord that expresses this gene (Fig. 1E). Compared with r5, in cells from the SC there is a re-localisation of Hoxb9 loci away from the interior of the CT, to a position at, or just outside, its edge (Fig. 5). This is reminiscent of the situation in ES cells differentiated for 10 days with RA (Chambeyron and Bickmore, 2004).

The tailbud contains descendants of the node and anterior primitive streak (Cambray and Wilson, 2002), and examination
of Hoxb nuclear organisation in the spinal cord of this region at E9.5 allowed us to analyse a situation not seen in our ES differentiation system – Hoxb1 and b9 co-expression. We do not see Hoxb chromatin decondensation in these cells (Fig. 5A) and both Hoxb1 and b9 have a similar mean position at the edge of the CT (Fig. 5D). Therefore, there seems to be a different relationship between Hoxb expression and nuclear organisation in this part of the neural tube, compared with more anterior regions. In this regard it is interesting to note that the expression of Hoxb13, the most 5′ member of the Hoxb cluster, and located ~100 kb from b9, is restricted to the tailbud region (Zeltser et al., 1996).

We conclude that there is differential nuclear organisation of Hoxb along the anteroposterior axis of the neural tube, in a pattern that parallels the spatial pattern of gene expression. To our knowledge, this analysis represents one of the first studies of nuclear organisation in situ within solid tissues of the developing mouse embryo. Combined with the use of mutant embryos it may now provide the opportunity to dissect the mechanisms that bring about chromatin decondensation and nuclear gene movement during mammalian development.

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