Ectopic nuclear reorganisation driven by a *Hoxb1* transgene transposed into *Hoxd*

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Summary

The extent to which the nuclear organisation of a gene impacts on its ability to be expressed, or whether nuclear organisation merely reflects gene expression states, remains an important but unresolved issue. A model system that has been instrumental in investigating this question utilises the murine *Hox* gene clusters encoding homeobox-containing proteins. Nuclear reorganisation and chromatin decondensation, initiated towards the 3′ end of the clusters, accompanies activation of *Hox* genes in both differentiation and development, and might be linked to mechanisms underlying colinearity. To investigate this, and to delineate the cis-acting elements involved, here we analyse the nuclear behaviour of a 3′ *Hoxb1* transgene transposed to the 5′ end of the *Hoxd* cluster. We show that this transgene contains the cis-acting elements sufficient to initiate ectopic local nuclear reorganisation and chromatin decondensation and to break *Hoxd* colinearity in the primitive streak region of the early embryo. Significantly, in rhombomere 4, the transgene is able to induce attenuated nuclear reorganisation and decondensation of *Hoxd* even though there is no detectable expression of the transgene at this site. This shows that reorganisation of chromosome territories and chromatin decondensation can be uncoupled from transcription itself and suggests that they can therefore operate upstream of gene expression.

Key words: Chromatin condensation, Chromosome territory, Colinearity, Hox, Nuclear organisation

Introduction

Several facets of nuclear organisation have been correlated with gene expression both at constitutively active regions of the genome and at regions subject to coordinate regulation. Firstly, when active, these regions appear decondensed at a cytological level (Chambeyron and Bickmore, 2004; Christova et al., 2007; Goetze et al., 2007). Secondly, even though transcription and transcription factories can be seen within chromosome territories (CTs) (Abranches et al., 1998; Branco and Pombo, 2006; Verschure et al., 1999), many active genomic loci have been seen outside of their CTs (Brown et al., 2006; Chambeyron et al., 2005; Chambeyron and Bickmore, 2004; Mahy et al., 2002; Morey et al., 2007; Volpi et al., 2000; Williams et al., 2002). A key issue remains regarding whether these forms of nuclear organisation are just a consequence of transcriptional activation or whether they have a causative role (Heard and Bickmore, 2007). The fact that the incidence of localisation outside of CTs decreases when transcription is blocked (Mahy et al., 2002) suggests that relocalisation is, at least in part, driven by transcription itself. By contrast, looping out from the CT is not just a downstream consequence of the activation of a specific gene. Along the primary (rostro-caudal) axis of the developing mouse embryo, activation of murine homeobox *Hoxb* and *Hoxd* loci is accompanied by both their decondensation and looping out from the CT (Chambeyron et al., 2005; Morey et al., 2007). However, in the limb bud, *Hoxd* activation and chromatin decondensation occur without relocalisation of this locus to the outside of its CT. We had suggested that this difference in nuclear behaviour of the same locus, when it is activated in different developmental contexts, might be due to the different regulatory pathways and cis-acting sequences acting upon it (Morey et al., 2007).

This latter example illustrates how useful the study of the *Hox* gene clusters is in providing insights into the dynamic repositioning of a locus in the nucleus during spatial and temporal patterning of gene expression. Within mammalian *Hox* clusters, there is a correspondence between the linear order of the genes and their sequence of activation in development, which is termed 'colinearity' (Kmita and Duboule, 2003). Genes located at the 3′ end of the clusters are expressed earlier, and more anteriorly, than genes located in a more 5′ position. It has been proposed that a transition from an inactive to an active chromatin state, propagated through *Hox* clusters from 3′ to 5′, might underlie colinear activation (Kondo and Duboule, 1999; Roelen et al., 2002; van der Hoeven et al., 1996). The relocalisation of *Hox* genes outside of their CTs does indeed initiate towards the 3′ end of the clusters (Chambeyron et al., 2005; Chambeyron and Bickmore, 2004; Morey et al., 2007).

Transgenic experiments have revealed both local (e.g. Marshall et al., 1994; Gould et al., 1997) and more distant (Gonzalez et al., 2007; Spitz et al., 2003; Spitz et al., 2005; Tarchini and Duboule, 2006; Zákány et al., 2004) cis-regulatory elements involved in the control of *Hox* gene expression. Manipulation of mouse *Hox* clusters, transferring genes from one position to another, has also indicated that part of this regulation depends on the position of a given gene in the cluster (Kmita et al., 2000; Kmita et al., 2002). To determine whether cis-acting elements responsible for initiating large-scale changes in chromatin structure are located at the 3′ end

of *Hox* clusters, here we have analysed the nuclear behaviour of the anterior (3′) *Hoxb1* gene transposed to the posterior (5′) end of *Hoxd*. We used transgenic mouse embryos carrying a *Hoxb1-LacZ* reporter inserted by homologous recombination upstream of *Hoxd13* (Kmita et al., 2000) (Fig. 1A). This transgene has the regulatory elements necessary for its autonomous expression when randomly integrated in the genome (Graaff et al., 2003), including 5′ and 3′ retinoic acid response elements (RAREs) and the rhombomere 4 (r4) autoregulatory enhancer (Marshall et al., 1994; Popperl et al., 1995; Studer et al., 1994). When transposed, this transgene breaks the colinearity of *Hoxd*, inducing ectopic *Hoxd13* expression in the primitive streak region (PS) of embryos at embryonic day 7.5 (E7.5) in a manner reminiscent of endogenous *Hoxb1* expression (Kmita et al., 2000). However, ectopic activation of the *Hoxb1-lacZ* transgene occurs in the distal part of the E10.5 developing limb bud, where *Hoxb1* is not normally expressed. Conversely, expression of the transgene is absent in r4 of the E9.5 embryo, a site where both endogenous *Hoxb1* and *Hoxb1-lacZ* transgenes inserted randomly into the genome are expressed (Table 1).

Using fluorescence in situ hybridisation (FISH) on wild-type and transgenic embryos, we show that the transposed *Hoxb1-LacZ* recapitulates some of the behaviour of endogenous *Hoxb1* in the PS of E7.5 embryos, suggesting that the transgene contains the minimal DNA elements necessary to initiate nuclear reorganisation and chromatin decondensation early in embryonic development, and that these elements can initiate nuclear reorganisation at an inappropriate genomic location (the 5′ end of *Hoxd*) that is normally still silent at this early stage of embryogenesis. Moreover, the transgene can also initiate these movements when activated ectopically in the limb bud later in development, where the rest of *Hoxd* does not loop out from the CT even though the *Hoxd* cluster is now active. In both of these cases (early embryo and limb bud), the nuclear organisation of the transposed *Hoxb1-Lacz* transgene completely correlates with its expression, so that transcription and nuclear organisation cannot be uncoupled from one another. However, in r4, the transgene is able to induce some attenuated, but still significant, nuclear reorganisation and chromatin decondensation of surrounding *Hoxd* regions, even though it is not itself expressed detectably. This suggests that reorganisation of CTs is not, in this case, simply a downstream consequence of transcription of the transgene.

Results

The *Hoxb1-LacZ* transgene recapitulates endogenous *Hoxb1* nuclear reorganisation in E7.5 embryos

The earliest events of nuclear reorganisation at *Hoxb* have been seen in the PS of E7.5 embryos, where *Hoxb1* expression is accompanied by a looping out of the gene from its CT (Chambeyron et al., 2005). To determine whether a similar nuclear movement also occurs upon activation of the *Hoxb1-LacZ* transgene relocated within *Hoxd*, we performed 3D DNA-FISH on sagittal sections of E7.5 transgenic embryos at late-streak early-bud and neural-plate stages when *Hoxb1* is expressed (Fig. 1B). Nuclear distances between a *Hoxb1-LacZ* (nr33) probe and the edge of the mouse chromosome 2 (MMU2) CT, where *Hoxd* is located, were determined as described previously (Chambeyron et al., 2005; Chambeyron and Bickmore, 2004; Morey et al., 2007). In this analysis, a probe signal is considered to be outside the CT when the distance to the CT edge is negative. Distances >0 correspond to probe signals inside the CT.

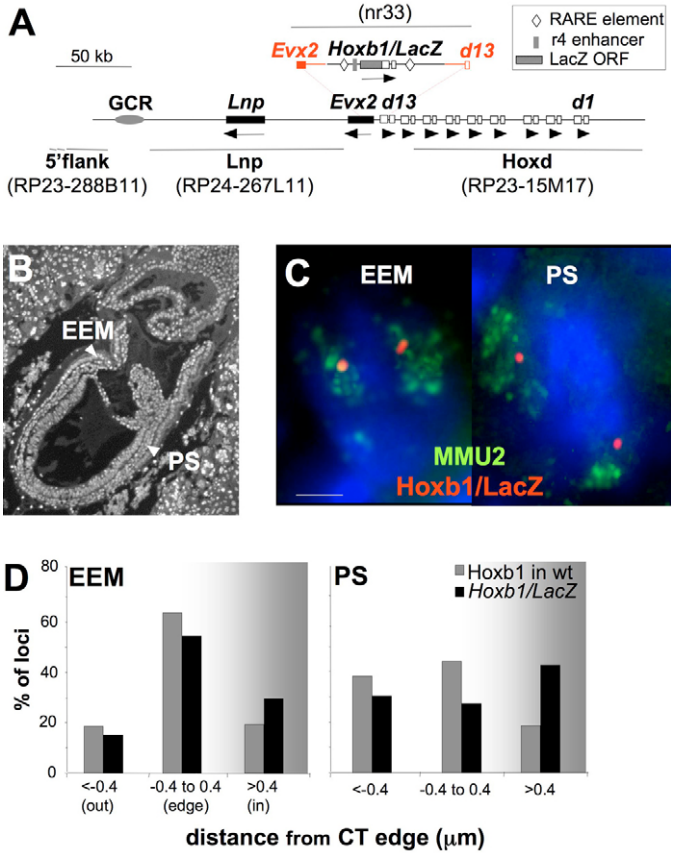

Fig. 1. Looping out of *Hoxb1-LacZ* from the chromosome territory occurs in the primitive streak (PS) of E7.5 transgenic embryos. (A) Map of the *Hoxd* locus on MMU2 showing the structure and the integration site of the *Hoxb1-LacZ* transgene. The extent of the homology arms used for recombination is shown in red and the *Hoxb1* cis-regulatory retinoic acid response elements (RAREs) and the rhombomere r4 enhancer included in the transgene are depicted (Kmita et al., 2000). Exons of *Hoxd* genes from *Hoxd1* (*d1*) to *Hoxd13* (*d13*) are shown as unfilled boxes; other genes in the region are shown as black boxes. The orientation of transcription is indicated by arrows underneath the genes. The grey oval locates a region of noncoding sequence conservation that overlaps the global control region (GCR) (Spitz et al., 2003). The locations of the BACs and the *Hoxb1-LacZ* plasmid used as FISH probes are shown in grey. (B) DAPI staining of a 4 μm E7.5 embryo section used for the FISH analysis showing the nuclei of the PS and extra-embryonic yolk sac mesoderm (EEM). (C) Maximal projection image after deconvolution of 3D FISH using the *Hoxb1-LacZ* probe (red) hybridised together with a MMU2 chromosome paint (green) on DAPI-counterstained nuclei of the EEM or the PS of E7.5 *Hoxb1-LacZ* embryos. Bar, 2 μm. (D) Histograms showing the 3D position of hybridisation signals for the *Hoxb1-LacZ* transgene relative to the MMU2 chromosome territory (CT) edge (black bars), or, for endogenous *Hoxb1*, relative to the edge of the MMU11 CT (grey bars) in nuclei from the EEM and PS of E7.5 transgenic or wild-type (wt) embryos, respectively. Loci are defined as outside of the CT if the distance measured is >0.4 μm beyond the visible limits of the CT hybridisation signal.

As a control, we analysed nuclei from anterior extra-embryonic yolk sac mesoderm (EEM) that does not express either *Hoxb1* or any *Hoxd* genes (Table 1). In this tissue, hybridisation signals from the transgene were mainly located at the edge of the CT. By contrast, in nuclei from the PS, where endogenous and transgenic *Hoxb1* are expressed, the transgene is more frequently relocated towards the outside of the CT (P=0.03 compared with EEM) as is endogenous *Hoxb1* on mouse chromosome 11 (MMU11) in embryos wild-type

Table 1. Expression patterns of Hoxd genes, Hoxb1 and Hoxb1-LacZ transposed at Hoxd during embryogenesis

		Wild-type embryos			*Hoxb1-LacZ* embryos	
Embryonic stage	Tissue	3′ *Hoxd (d1-d10)*	5′ *Hoxd (d11-d13)*	*Hoxb1*	*LacZ*	*Hoxd13*
E7.5	Extra-embryonic; anterior yolk sac mesoderm	–	–	–	–	–
	Primitive streak and adjacent embryonic mesoderm	–	–	+	+	+
E9.5	Rhombomeres 1 and 2	–	–	–	–	–
	Rhombomere 4	–	–	+	–	–
	Tail bud	+	+	+	+ (extends anteriorly)	+ (reinforced in posterior embryo)
	Limb and genital buds	+	–	–	ND	–
E10.5	Rhombomeres 1 and 2	–	–	–	–	–
	Rhombomere 4	–	–	+	–	–
	Tail bud	+	+	+	+ (extends anteriorly)	+
	Limb and genital buds	+	+	–	+ (distal part of the buds)	+

–, Not expressed; +, expressed; ND, not determined.

for *Hoxd* (Chambeyron et al., 2005) (Fig. 1C,D and Table 2). However, the extent of localisation outside of the CT is less for the transgene than for endogenous *Hoxb1* (Fig. 1D). This suggests that the *Hoxb1-LacZ* transgene contains the cis-regulatory elements necessary to initiate some of its autonomous nuclear reorganisation during gastrulation.

Looping out is restricted to the transgene itself

The looping out from CTs, initiated towards the 3′ ends of endogenous *Hoxb* and *Hoxd*, spreads to adjacent genomic regions (Morey et al., 2007). To determine whether this is also the case for the ectopic looping out induced by the *Hoxb1-lacZ* transgene, we measured the nuclear position of FISH signals from probes *Lnp* (BAC RP24-267L11) to the 5′ side of the transgene and *Hoxd* (BAC RP23-15M17) in the 3′ direction (Fig. 1A). In E7.5 transgenic embryos, the *Lnp* region localised well inside of the MMU2 CT, both in the EEM and the PS regions (Fig. 2A and Table 2). The 3′ flanking *Hoxd* region was also located within, or at the edge of, the CT (Fig. 2B). No significant differences in the nuclear behaviour of *Lnp* or *Hoxd* could be detected between wild-type and transgenic embryos ($P>0.05$ for all four distributions). Therefore, *Hoxb1-LacZ* looping out from the CT is a localised event and does not propagate to flanking genomic regions.

The transgene induces chromatin decondensation in the primitive streak

Looping out of endogenous *Hoxb1* in the PS is also accompanied by visible chromatin decondensation (Chambeyron et al., 2005). However, these two facets of nuclear organisation can be dissociated from one another, at least at *Hoxd* (Morey et al., 2007). Changes in chromatin condensation can be assessed by measuring the interprobe distance (d) between the signals from two FISH probes of known genomic separation (Yokota et al., 1997), and we have previously shown that this method can be used to detect chromatin

Table 2. Kolmogorov-Smirnov test applied to distances from chromosome territory (CT) edge and to interphase separations in E7.5 and E9.5 wild-type and Hoxb1-LacZ embryos

		P for comparison of distance from CT edge			P for comparison of interphase separation			
			Tissue			Tissue		
Embryonic stage	Embryo	Genomic region	PS vs EEM		Genomic region	PS vs EEM		
E7.5	wt	*Lnp*	0.895		*Hoxb*	**0.011**		
		Hoxb1	**0.02**		*Hoxd*	0.321		
		Hoxd	0.082					
	Hoxb1-LacZ	*Lnp*	0.493		5′ of *Hoxb1-LacZ*	**0.005**		
		Hoxb1-LacZ	**0.03**		3′ of *Hoxb1-LacZ*	**0.001**		
		Hoxd	0.999					
E9.5			r4 vs r1/r2	Tail bud vs r1/r2	Limb bud vs r1/r2		r4 vs r1/r2	Tail bud vs r1/r2	Limb bud vs r1/r2
	wt	*Lnp*	0.169	**0.018**	0.482	*Hoxb*	**<0.001**	**0.009**	ND
		Hoxb1	**<0.001**	**0.013**	ND	*Hoxd*	0.852	**<0.001**	**<0.001**
		Hoxd	0.313	**0.021**	0.28				
	Hoxb1-LacZ	*Lnp*	**0.006**	**<0.001**	0.992	5′ of *Hoxb1-LacZ*	0.254	**<0.001**	**0.02**
		Hoxb1-LacZ	0.28	**<0.001**	**<0.001**	3′ of *Hoxb1-LacZ*	**0.036**	**<0.001**	**<0.001**
		Hoxd	**0.004**	**<0.001**	0.149				

Significant *P* values are shown in bold. In E7.5 embryos, data distribution in the PS is compared with data distribution in the EEM. In E9.5 embryos, data distributions in the r4, tail bud and limb bud is compared to the distribution in r1/r2 control tissue.

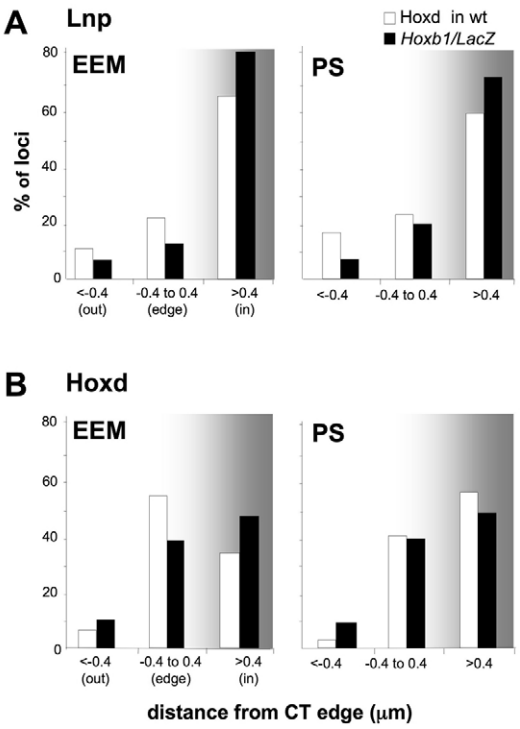

Fig. 2. *Hoxb1-LacZ* nuclear reorganisation at E7.5 does not spread to flanking regions. Histograms showing the 3D positions of hybridisation signals for (A) the *Lnp* and (B) the *Hoxd* probe in nuclei from the EEM and the PS of wild-type (endogenous *Hoxd*, white bars) or transgenic (black bars) E7.5 embryos.

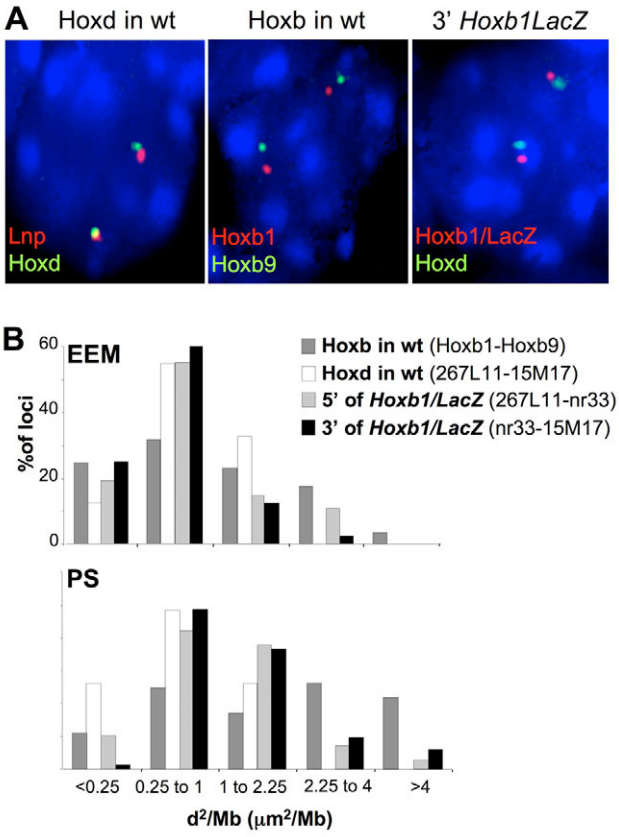

Fig. 3. Chromatin decondensation in E7.5 transgenic embryos. (A) 3D DNA-FISH of: (left panel) *Lnp* and *Hoxd*, (middle panel) *Hoxb1* and *Hoxb9* probes on nuclei from the PS of E7.5 wild-type embryos, and of (right panel) *Hoxb1-LacZ* and *Hoxd* probes on nuclei from the PS of an E7.5 *Hoxb1-LacZ* embryo. Nuclei were counterstained with DAPI. Images are maximal projections of 3D stacks after deconvolution. (B) Distributions of mean-squared interphase distances (d^2 in μm^2) normalised to genomic separation (in Mb) measured between the *Hoxb1* and *Hoxb9* probes (dark-grey bars) at endogenous *Hoxb* and between *Lnp* and *Hoxd* probes (white bars) at endogenous *Hoxd* in EEM and PS nuclei from E7.5 wild-type embryos, or between the *Lnp* and *Hoxb1-LacZ* probes (light-grey bars) and between the *Hoxb1-LacZ* and *Hoxd* probes (black bars) in EEM and PS nuclei from E7.5 *Hoxb1-LacZ* embryos.

decondensation at *Hox* loci in mouse embryos (Chambeyron et al., 2005; Morey et al., 2007). To analyse chromatin condensation around the transgene, we measured the interphase distance in E7.5 transgenic embryos between FISH signals for *Hoxb1-LacZ* and for probes either 5′ (*Lnp*) or 3′ (*Hoxd*) of the transgene. The distribution of these distances was compared with those obtained at endogenous *Hoxb* (Chambeyron et al., 2005) and *Hoxd* clusters in wild-type E7.5 embryos (Fig. 3A). Interphase distances measured in this way follow a Rayleigh distribution, and there is generally considered to be a linear relationship between the mean-squared interphase distances (d^2 in μm^2) and genomic separation for probes that are less than ~1 Mb apart (Chambeyron and Bickmore, 2004; Yokota et al., 1995). Therefore, to normalise for differences in the genomic separations being compared between *Hoxb* and *Hoxd*, and between the wild-type and transgenic *Hoxd* loci, we divided the d^2 value by the genomic separation (in Mb) of the probe pairs being analysed (Fig. 3B). In EEM, the distribution of interphase distances, and so the level of chromatin condensation, 5′ of *Hoxb1-LacZ* is similar to that at endogenous *Hoxb* and *Hoxd* (P=0.095 and P=0.173, respectively). The region 3′ of *Hoxb1-LacZ* also showed a not dissimilar chromatin condensation level to that of *Hoxd* (P=0.052) and was significantly more condensed than endogenous *Hoxb* in this tissue (P=0.005), with an absence of alleles separated by large interphase distances (d^2/Mb >2.25; Fig. 3B). This suggests that the chromatin condensation in non-expressing tissue is determined by the genomic context of the transgene. By contrast, in PS cells, there was a significant shift in interphase separation to larger values, indicative of chromatin decondensation both 5′ and 3′ of *Hoxb1-LacZ*, as compared with the situation in the EEM or with the endogenous *Hoxd* region in wild-type PS (Fig. 3B and Table 2). However, decondensation did not reach the levels seen at endogenous *Hoxb* ($P<0.001$ for both 5′ and 3′ *Hoxb1-LacZ*). Therefore, other elements from the *Hoxb* locus, absent from the transgene, might be necessary to recapitulate full levels of chromatin decondensation. Nevertheless, these results suggest that the *Hoxb1-LacZ* transgene contains the elements that can mediate some long-range decondensation at *Hoxd*.

Ectopic nuclear reorganisation of the transgene in the limb bud

Data in Figs 1, 2 and 3 suggest that the *Hoxb1-lacZ* transgene has a dominant effect on chromosome organisation and decondensation at the integrated *Hoxd* locus in the E7.5 embryo. We have previously shown that changes in nuclear organisation at *Hoxd* later on in development (E9.5) are dependent on the developmental context (Morey et al., 2007). In particular, 3′ *Hoxd* genes are first induced in the emerging limb bud of E9.0 embryos, *Hoxd13* expression is detected in the limb after E10.0 (Tarchini and Duboule, 2006) and *Hoxb1* is never expressed in the limb (Table 1). Activation of *Hoxd* genes in E9.5 forelimbs is associated with chromatin decondensation

but not with significant looping out of *Hoxd* from its CT as compared with control non-expressing tissues of the embryo (Morey et al., 2007). Similar to the behaviour of endogenous *Hoxd*, in the limb bud of E9.5 transgenic embryos, we also saw significant decondensation of the regions both 5′ and 3′ of the transgene in the limb bud compared with control tissue from r1 and r2 (Fig. 4B and Table 2). However, the *Lnp* and *Hoxd* regions flanking the transgene remain well inside of the CT, as at endogenous *Hoxd*, whereas the transgene significantly relocalises to the outside of the CT in the limb bud compared with its position in control cells from r1 and r2 (Fig. 4A and Table 2).

Hoxd nuclear organisation along the rostro-caudal axis in transgenic embryos

In the E7.5 embryo, and later on in the limb bud, ectopic nuclear reorganisation of the *Hoxb1-lacZ* transgene correlates with its state of gene expression. In the first situation, transgene expression is occurring in the context of an otherwise normally silent *Hoxd* locus, and, in the second case, ectopic transgene expression is induced in the activated *Hoxd* locus. The tail bud (E9.5) is a site in the embryo where both *Hoxb* and *Hoxd* are active and localise towards the edge or outside of their respective CTs (Chambeyron et al., 2005; Morey et al., 2007). The transgene adopts a similar nuclear organisation (Fig. 5A and Table 2). Conversely, in rhombomeres 1 and 2 (r1/r2) of the hindbrain, where endogenous *Hoxb1*, *Hoxb1-LacZ* and *Hoxd* genes are not expressed, the transgene was mainly located well inside (>0.4 μm) of the CT, similar to the organisation of endogenous *Hoxd* (P=0.41), and significantly more internal in position than endogenous *Hoxb1* (P<0.001). All regions were also condensed in r1/r2 of both wild-type and transgenic embryos and, by comparison, were decondensed in the tail bud (Fig. 5C and Table 2). The levels of decondensation around the transgene were similar to those at the endogenous *Hoxd* in wild-type embryos (P=0.131 for the 5′ side and P=0.076 to the 3′end).

An interesting situation arises in r4. The *Hoxb1-LacZ* transgene, when randomly inserted in the genome, is able to recapitulate endogenous *Hoxb1* expression in r4 (Marshall et al., 1994), but, when transposed into 5′ *Hoxd*, transgene expression in r4 is abolished (Kmita et al., 2000). We analysed the nuclear organisation, along the rostro-caudal axis of transgenic embryos, of the *Lnp* and *Hoxd* regions and a genomic region extending further 5′ of *Lnp* (5′ flank; RP23-288B11; Fig. 1A) that did not show any nuclear movement in any of the tissues analysed so far in wild-type embryos (Morey et al., 2007). As expected, no CT looping out was observed with any of the regions in r1/r2 (Fig. 5B), whereas there was a relocation of the *Lnp* and *Hoxd* regions, but not of the 5′ flanking region, to the outside of the CT in the tail bud (Fig. 5B and Table 2). However, in r4, where neither the *Hoxd* genes nor the transgene are expressed, a modest, but significant, relocalisation towards the edge of the CT of both *Lnp* and *Hoxd* regions was detected in comparison with r1/r2 (Fig. 5B and Table 2). The transgene itself also appears to have a position less internal to the CT than it does in r1/r2; however, this does not reach statistical significance (P=0.28; Table 2). The nuclear position of the transgene in r4 is quite different from the positional behaviour of endogenous *Hoxb1* (P<0.001), which extensively localises outside of its CT in this part of the hindbrain (Chambeyron et al., 2005) (Fig. 5A).

There was also some decondensation of the 3′ *Hoxb1-LacZ* flanking region, but not of the 5′ flanking region, in r4 compared with that in r1/r2 (Fig. 5C and Table 2). These results suggest that, even though not expressed, the *Hoxb1-LacZ* transgene exerts an influence on the nuclear organisation and chromatin decondensation of adjacent genomic regions of the *Hoxd* locus in r4, although this effect is much attenuated compared with the events seen at endogenous *Hoxb1*.

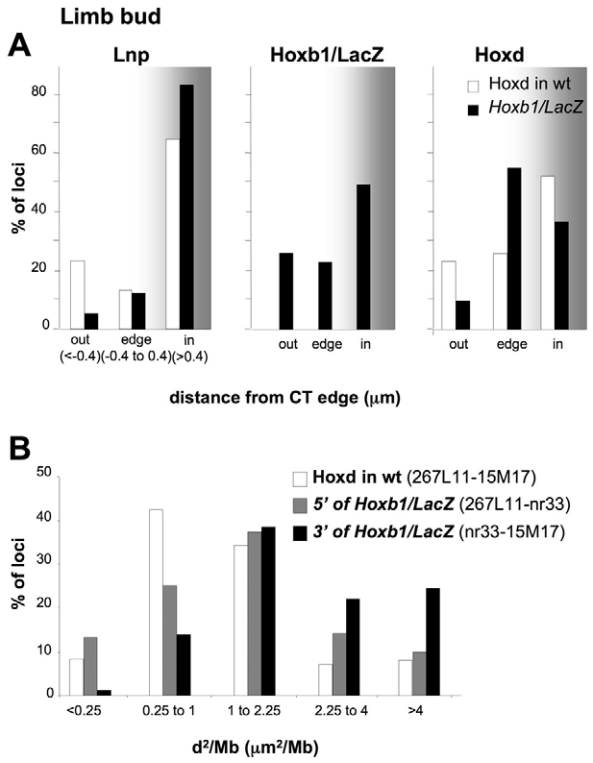

Fig. 4. Nuclear reorganisation and chromatin decondensation in the limb bud of E9.5 *Hoxb1-LacZ* embryos. (A) Histograms showing the percentage of *Lnp*, *Hoxb1-LacZ* or *Hoxd* hybridisation signals inside, at the edge or outside of the MMU2 CT in the forelimb bud of E9.5 *Hoxb1-LacZ* embryos (black bars) compared with the endogenous *Hoxd* (white bars). The cut-offs used for the edge category are 0.4 μm and –0.4 μm from CT edge. (B) Distributions of squared interphase distances (d^2 in μm^2) standardised to genomic separation (in Mb) measured between *Lnp* and *Hoxd* probes (white bars) at endogenous *Hoxd* in forelimb bud nuclei from E9.5 wild-type embryos or between the *Lnp* and *Hoxb1-LacZ* probes (grey bars) and between the *Hoxb1-LacZ* and *Hoxd* probes (black bars) in forelimb nuclei from E9.5 *Hoxb1-LacZ* embryos.

Discussion

Here, we have shown the contrasting behaviours of a *Hoxb1-lacZ* transgene, which has been transposed into the 5′ end of the *Hoxd* locus, at different embryonic stages and in different tissues. In E7.5, the transgene is able to recapitulate some of the nuclear reorganisation and chromatin condensation seen at the endogenous *Hoxb1* locus (Figs 1 and 3). This indicates that the transgene contains the *cis*-acting elements needed to initiate these changes in higher-order chromatin structure during gastrulation, even though it is embedded in a *Hoxd* locus that is normally still silent at this stage. Whether the elements in the transgene responsible for chromatin decondensation are the same as those involved in CT reorganisation remains to be determined. The observation that looping out from the CT is restricted to the vicinity of the transgene (Fig. 2), but that transgene-induced chromatin decondensation spreads to adjacent genomic regions (Fig. 3), further serves to illustrate the fact that these two facets of nuclear reorganisation can be independent of

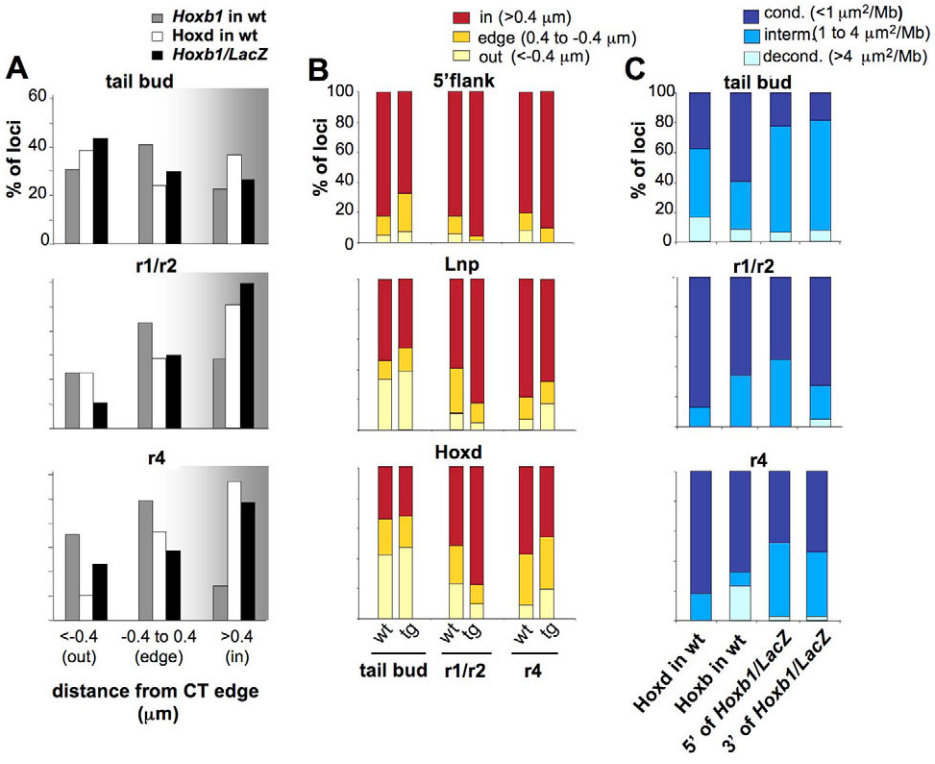

Fig. 5. Nuclear reorganisation of the *Hoxb1-LacZ* transgene and surrounding regions along the antero-posterior axis of E9.5 embryos. (A) Histograms showing the percentage of *Hoxb1-LacZ* hybridisation signals inside, at the edge or outside of the MMU2 CT in the tail bud, r1/r2 or r4 of E9.5 *Hoxb1-LacZ* embryos (black bars) compared with endogenous *Hoxd* (white bars) or endogenous *Hoxb1* on MMU11 in wild-type E9.5 embryos (grey bars). (B) Histograms showing the percentage of signals located either inside (red bars), at the edge (orange bars) or outside (yellow bars) of the CT for the 5′ flanking, the *Lnp* and the *Hoxd* probes in tail bud, r1/r2 or r4 of either wild-type (wt) or *Hoxb1-LacZ* transgenic (tg) embryos. (C) Percentages of nuclei showing condensed (dark blue), intermediate (blue) or decondensed (pale blue) chromatin structures for the regions between *Lnp* and *Hoxd* probe signals (*Hoxd* wt) and between *Hoxb1* and *Hoxb9* probe signals (*Hoxb* wt) in tail bud, r1/r2 or r4 of wild-type embryos or between *Lnp* and *Hoxb1-LacZ* probe signals (5′ *Hoxb1-LacZ*) and between *Hoxb1-LacZ* and *Hoxd* probe signals (3′ *Hoxb1-LacZ*) in *Hoxb1-LacZ* embryos.

one another (Morey et al., 2007). Interestingly, ectopic gene expression in the PS of the transgenic embryos is limited to the *Hoxd13* gene immediately adjacent to the transgene (Kmita et al., 2000), suggesting that this might be a consequence of the nuclear repositioning of *Hoxb1-LacZ*.

We have previously shown that the normal *Hoxd* region, when activated in the E9.5 limb bud, decondenses but does not loop out from its CT (Morey et al., 2007). Therefore it is surprising here to find that the *Hoxb1-LacZ* transgene does loop out from the CT in the limb bud. These results show that the transgene does not contain any DNA elements preventing its nuclear reorganisation and chromatin decondensation in tissues where *Hoxb1* is normally silent, and that the absence of *Hoxd* looping out from the CT in the limb bud is not, as we had previously suggested (Morey et al., 2007), just due to the pathway activating *Hoxd* expression in this tissue, but that it is also dependent on the sequence of the *Hoxd* locus itself.

So far, these experiments have shown that the nuclear behaviour of the transposed transgene cannot be dissociated from its pattern of gene expression. In the r4 hindbrain region of E9.5 embryos, although the extensive looping out and chromatin decondensation seen at endogenous *Hoxb1* are attenuated at the transgene, coincident with the suppression of its expression here, we noticed a discrete effect of the transgene on adjacent *Hoxd* regions, which are relocated significantly more towards the outside of the CT, and are more decondensed, in r4 of transgenic embryos than are these regions in wild-type embryos (Fig. 5 and Table 2). Interestingly, this effect was more pronounced towards the 3′ side of the transgene, where the early mesoderm enhancer and the *Hoxd* cluster are located (Fig. 1A). This result suggests that the transgene, which does contain the regulatory elements necessary for its autonomous expression in r4 when randomly integrated in the genome, including the r4 autoregulatory enhancer (Graaff et al., 2003; Marshall et al., 1994; Popperl et al., 1995; Studer et al., 1994), contains the elements that can initiate some degree of nuclear reorganisation at 5′ *Hoxd* in r4 even in the absence of expression and that it is likely that the chromatin environment of the rest of *Hoxd* in this tissue prevents subsequent events that are necessary for gene expression per se.

The nuclear reorganisation at the transposed *Hoxd* locus in r4 therefore suggests that chromatin decondensation, and looping out from the CT, can occur upstream of gene expression and so are unlikely to be just passive consequences of, for example, RNA polymerase II activity (although we cannot exclude that there might be noncoding transcription from the transgene construct). Similarly, a recent study has shown that decondensation and looping out from the CT of the human major histocompatibility complex, stimulated by interferon-γ through the JAK-STAT signalling pathway, also occurs upstream of transcriptional activity (Christova et al., 2007). Interestingly, given the presence of RAREs on the *Hox1-LacZ* transgene described here, this was correlated with the recruitment of the chromatin remodelling enzyme Brg1. Brg1 has been shown to interact with nuclear hormone receptors (Fryer and Archer, 1998). A better understanding of the molecular mechanisms driving nuclear reorganisation and chromatin decondensation of the *Hoxb* and *Hoxd* loci at different stages of development is now required.

Materials and Methods

Mouse embryo sectioning and staging

Analysis on E7.5 and E9.5 embryos was performed as described previously (Chambeyron et al., 2005). We used embryos from crosses between *Dct-LacZ* homozygous transgenic CD1×CD1 mice as wild type (Chambeyron et al., 2005; Morey et al., 2007). *Hoxb1-LacZ* homozygous embryos were collected from TgHb1×TgHb1 crosses (Kmita et al., 2000). The day on which the vaginal plug was detected was considered as 0.5 days of gestation (E0.5). *Dct-LacZ* and *Hoxb1-LacZ* E7.5 embryos in the deciduas and E9.5 embryos were fixed in 4% formaldehyde-PBS 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 DNA-FISH and haematoxylin-eosin (HE) staining. HE-stained sections from E7.5

embryos were used for embryo staging according to criteria established previously (Downs and Davies, 1993). A minimum of three independent embryos where used for the analyses.

3D DNA-FISH

Sagittal sections of E7.5 embryos that contained the PS, and sections from E9.5 embryos containing r1/r2, r4, forelimb buds and the tail bud were selected for FISH analysis. Sections laid on Superfrost slides (Menzel) were heated to 60°C for 20 minutes and washed four times in xylene for 10 minutes each before rehydration through an ethanol series. They were then microwaved for 20 minutes in 0.1 M citrate pH 6 buffer, washed in water and rinsed once in 2×SSC before use. For FISH, slides were incubated in 2×SSC for 5 minutes at 75°C, denatured for 3 minutes at 75°C in 70% formamide-2×SSC, plunged into ice-cold 70% ethanol for 3 minutes, dehydrated through an ethanol series and air dried. Hybridisation, washes and detection were performed as described previously (Chambeyron and Bickmore, 2004).

The BACs RP23-288B11 (5′ flank), RP24-267L11 (*Lnp*), RP23-15M17 (*Hoxd*) and the MMU2 chromosome paint used as probes in this study have been described previously (Morey et al., 2007). The nr33 (*Hoxb1-LacZ*) probe consisted of the vector used for homologous recombination and therefore includes the genomic region extending from the 3′ end of *Evx2* up to the 5′ end of *Hoxd13* and the *Hoxb1-LacZ* reporter construct (Kmita et al., 2000) (Fig. 1A). The *Hoxb1* and *Hoxb9* probes have been described previously (Chambeyron et al., 2005; Chambeyron and Bickmore, 2004). Probes were prepared and labelled as described previously (Morey et al., 2007). Approximately 200 ng of biotin-paint and 100 ng of digoxigenin- or biotin-labelled BAC or plasmid probes were used per slide together with 10 μg mouse *Cot*1 DNA (Invitrogen) and 5 μg sonicated salmon sperm DNA.

Image capture and analysis

For 3D FISH, slides were examined using a Zeiss Axioskop fluorescence microscope with Plan-neofluar (numerical aperture=1.3) or Plan apochromat ×100 objectives, a 50 W Hg source (Carl Zeiss, Welwyn Garden City, UK) and Chroma #83000 triple band-pass filter set (Chroma Technology, Rockingham, VT) with the excitation filters installed in a motorised filter wheel (Ludl Electronic Products, Hawthorne, NY). A piezoelectrically driven objective mount (PIFOCi model P-721, Physik Instrumente GmbH, Karlsruhe) and a Princeton Instruments Micromax CCD camera with Kodak 1400e sensor (Universal Imaging, Maldon, UK) were used to control movement in the z dimension and collect image stacks with a 0.2 μm step. Hardware control, image capture and analysis were performed using in-house scripts written for IPLab Spectrum (Scanalytics, Fairfax, VA). Images were deconvolved using a calculated point spread function (PSF) with the constrained iterative volume algorithm of Microtome (Scanalytics, Fairfax, VA). 3D distance measurements were as described previously (Chambeyron et al., 2005). A minimum of 50 nuclei were analysed for each tissue. Chromatin condensation/decondensation was then assessed from the relationship between mean-squared interphase distance (d^2 in μm^2) between probes of known genomic separation (in Mb) (Chambeyron et al., 2005; Morey et al., 2007; Yokota et al., 1997).

Statistical analysis

The statistical relevance of data (Table 2) was assessed using the non-parametric Kolmogorov-Smirnov (KS) test to examine the null hypothesis that two sets of data show the same distribution. Data sets consisted of at least 50 nuclei (100 territories/loci) for each embryonic tissue, and for each combination of probes. A P value of <0.05 was considered statistically significant.

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