

Nuclear reorganisation and chromatin decondensation are conserved, but distinct, mechanisms linked to Hox gene activation

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The relocalisation of some genes to positions outside chromosome territories, and the visible decondensation or unfolding of interphase chromatin, are two striking facets of nuclear reorganisation linked to gene activation that have been assumed to be related to each other. Here, in a study of nuclear reorganisation around the *Hoxd* cluster, we suggest that this may not be the case. Despite its very different genomic environment from *Hoxb*, *Hoxd* also loops out from its chromosome territory, and unfolds, upon activation in differentiating embryonic stem (ES) cells and in the tailbud of the embryo. However, looping out and decondensation are not simply two different manifestations of the same underlying change in chromatin structure. We show that, in the limb bud of the embryonic day 9.5 embryo, where *Hoxd* is also activated, there is visible decondensation of chromatin but no detectable movement of the region out from the chromosome territory. During ES cell differentiation, decondensed alleles can also be found inside of chromosome territories, and loci that have looped out of the territories can appear to still be condensed. We conclude that evolutionarily conserved chromosome remodelling mechanisms, predating the duplication of mammalian Hox loci, underlie Hox regulation along the rostrocaudal embryonic axis. However, we suggest that separate modes of regulation can modify *Hoxd* chromatin in different ways in different developmental contexts.

KEY WORDS: Chromatin condensation, Chromosome territory, Embryonic stem cells, Hox, Limb bud, Mouse

INTRODUCTION

Chromatin condensation and organisation in the nucleus are considered important facets of the regulation of gene activation and gene silencing. Nuclear reorganisation of genes relative to 'transcription factories' (Osborne et al., 2004) and chromosome territories (CTs) has been correlated with active transcription. In the latter case, fluorescence in situ hybridisation (FISH) detects a relocalisation of some active genes to positions beyond the visible limits of CTs (Volpi et al., 2000; Williams et al., 2002; Mahy et al., 2002). An 'opening', or decondensation, of higher-order chromatin structure is also thought to be linked to gene activation (Mohd-Sarip and Verrijzer, 2004). Indeed, it has been suggested that the 'looping out' of gene loci from CTs is a visual manifestation of a large-scale chromatin decondensation (Volpi et al., 2000; Gilbert et al., 2004).

To understand how changes in complex chromatin structure relate to the expression of genes, and to work towards an understanding of the mechanisms that initiate and regulate them, requires an inducible system of co-regulated gene expression. One system that affords this is Hox gene loci. Transgene studies and genetically induced rearrangements of Hox loci have led to a suggestion that there might be a progressive opening of chromatin structure through Hox loci from 3' to 5' that controls sequential gene activation colinear with the order of the genes on the chromosome (van der Hoeven et al., 1996; Kmita and Duboule, 2003). Support for this model has come from our previous analysis of the murine *Hoxb* locus both ex vivo, during the differentiation of embryonic stem (ES) cells, and in vivo in cells of the primitive streak and adjacent mesoderm during early

embryogenesis and also along the rostrocaudal (head to tail) axis of the embryo later in development. In both cases, there is visible chromatin decondensation and a looping out of *Hoxb* genes from their CTs as the expression of *Hoxb* is induced (Chambeyron and Bickmore, 2004; Chambeyron et al., 2005).

If these large-scale chromatin remodelling events are fundamental to the regulation of Hox loci, and possibly also their colinear expression, then they should be conserved between paralogous gene clusters. The four mammalian autosomal Hox gene loci are the result of an ancestral duplication that occurred at the origin of vertebrate evolution (Ferrier and Minguillon, 2003). Each cluster still exhibits temporal and spatial colinearity of gene expression along the rostrocaudal axis of the trunk. However, *Hoxd*, but not *Hoxb*, genes have been co-opted more recently in evolution into the regulation of developing limbs and external genitalia and this required the appearance of novel regulatory elements around *Hoxd*. An enhancer controlling expression of *Hoxd10-d13* and the adjacent *Lnp* gene in digits and in the distalmost part of the limb buds has been identified ~160 kb 5' of *Hoxd* (Spitz et al., 2003; Spitz et al., 2005). Similarly, control regions 3' of *Hoxd* have been postulated to control colinear *Hoxd* expression in the nascent limb bud (Fig. 1) (Zakany et al., 2004; Deschamps and van Nes, 2005) and gut (Spitz et al., 2005). Limb malformations in humans with chromosomal rearrangements 5' and 3' of *HOXD* also suggest that regulatory elements in the large-scale genomic context around the locus are important for correct gene expression or for protection from position effects (Spitz et al., 2002; Dlugaszewska et al., 2006).

No such regulatory elements flanking *Hoxb* have been described. Therefore, it was unclear whether chromatin decondensation and looping out from the CT, similar to that at *Hoxb*, would occur at *Hoxd*, nor whether the nuclear reorganisation accompanying *Hoxd* expression would be the same along the rostrocaudal embryonic axis and in the limb bud.

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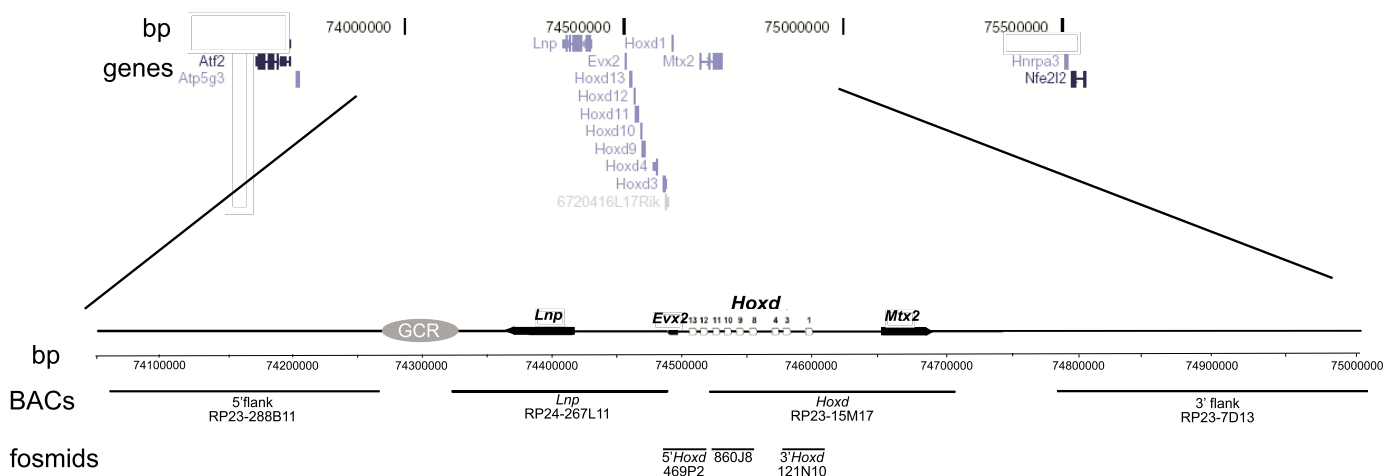


Fig. 1. Genomic structure of *Hoxd*. Map of the genomic region surrounding *Hoxd* on MMU2, showing the position of genes. Map position is in bp. Below this is a detailed view of the *Hoxd* locus itself. Genes are depicted as arrowed boxes indicating the orientation of transcription. White arrowheaded boxes correspond to the nine *Hoxd* genes (from 1 to 13) and black arrowheaded boxes represent the flanking genes. The grey oval locates a region of non-coding sequence conservation that includes the GCR. The location of BAC and fosmid clones used as FISH probes are shown (see also Table 2). Data and map positions are taken from NCBI Build 35 of the mouse genome (<http://genome.ucsc.edu/cgi-bin/hgGateway>) and from ENSEMBL v37, Feb 2006 (http://www.ensembl.org/Mus_musculus/index.html).

Here, we have analysed the nuclear organisation of a ~1 Mb region around the *Hoxd* cluster in both differentiating ES cells and the embryonic day (E) 9.5 embryo. As at *Hoxb*, we see chromatin decondensation and a looping out of the *Hoxd* region from its CT, suggesting that these are part of ancestral chromatin-based mechanisms for regulating Hox loci. However, contrary to expectations, we have dissociated these two facets of nuclear reorganisation from each other. During ES cell differentiation we show that movement of the *Hoxd* region towards the outside of the CT can occur before its apparent chromatin decondensation. In the embryo, both looping out and decondensation occur in the tailbud region, but in the limb bud there is decondensation of the *Hoxd* region in the absence of discernable looping out. This may reflect different modes of *Hoxd* regulation that occur in different developmental contexts.

MATERIALS AND METHODS

ES cell culture and differentiation

OS25 ES cells allow for the selection of undifferentiated (*Oct4*-expressing) cells using Hygromycin (hyg), and differentiated cells using Ganciclovir (selection against *Oct4* expressing cells) (Billon et al., 2002). They were maintained undifferentiated by growth on 0.1% gelatin-coated dishes in Glasgow's minimal Eagle's medium supplemented with 10% fetal calf serum, nonessential amino acids, 1 mmol/l sodium pyruvate, 0.3 mg/ml L-glutamine, 0.1 mmol/l 2-mercaptoethanol, 1000 U/ml human recombinant leukaemia inhibitory factor (LIF) and 100 µg/ml hyg (Chambeyron and Bickmore, 2004). Differentiation was induced by plating the cells at low density without LIF or hyg for 1 day. Retinoic acid (RA) 5×10^{-6} M was then added. On day 3 the cells were replated in RA-containing medium complemented with 2.5 µmol/l Ganciclovir. Samples for RNA extraction and nuclei preparation were collected every 2 days of differentiation. Similar results were obtained on samples from two independent differentiation experiments. Data presented in this paper all come from the same differentiation.

Mouse embryo sectioning and DNA FISH

Analysis of E9.5 embryos was as previously described (Chambeyron et al., 2005). Embryos from crosses between *Dct-LacZ* homozygous transgenic CD1XCD1 mice were collected at E9.5, fixed in 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 DNA FISH and Haematoxylin-Eosin staining. For FISH, sections laid on Superfrost slides 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 mol/l citrate pH 6 buffer, washed in water and rinsed once in $2 \times$ SSC before use. FISH was performed as described below, except for the denaturation step, which was for 3 minutes at 75°C in 70% formamide/ $2 \times$ SSC pH 7.5 followed by 3 minutes in ice-cold 70% ethanol.

RT-PCR

Total RNA was extracted using Tri-reagent (SIGMA) and assessed by electrophoresis. Random-primed reverse transcriptions were performed at 42°C for 1 hour on 5 µg total RNA by using 1 Unit Superscript II Reverse transcriptase (RT) (Invitrogen) and 4 µl random hexamers (50 mmol/l; Amersham Pharmacia) in a 50 µl reaction. A negative control reaction, lacking enzyme, was performed in parallel for each RNA sample. One microlitre of these reactions were amplified by PCR using 1 Unit Taq polymerase (Amplitaq, Roche), 2 mmol/l magnesium chloride and 1 µmol/l forward and reverse primers in a 20 µl reaction. All primer pairs used span exons and therefore only detected the spliced transcripts. PCR conditions and primer sequences are described in Table 1.

Multicolour fluorescence in situ hybridisation

Nuclei for 2D and 3D FISH were prepared as previously described (Chambeyron and Bickmore, 2004). FITC-labelled paint for MMU2 was purchased from CAMBIO. BAC and fosmid clones covering the *Hoxd* region were chosen from ENSEMBL v37, Feb 2006 (http://www.ensembl.org/Mus_musculus/index.html). BAC clones were purchased from BACPAC resources center and fosmid clones were kindly provided by the Sanger Institute (Cambridge, UK) (for coordinates and names, see Table 2). DNA from clones was prepared using standard alkaline lysis and labelled by nick-translation with digoxigenin-11-dUTP or biotin-16-dUTP (Roche). Approximately 200 ng FITC-paint, 100 ng biotin-labelled BAC or fosmid probe and 100 ng digoxigenin BAC or fosmid probe were used per slide, together with 15 µg mouse Cot1 DNA (GIBCO BRL) and 5 µg salmon sperm DNA. Digoxigenin-labelled probes were detected using Rhodamine anti-digoxigenin and Texas Red anti-sheep IgG (Vector Laboratories); biotin-labelled probes were detected using Cy5 streptavidin and biotinylated anti-avidin (Vector Laboratories); and the FITC signal from the MMU2 chromosome paint was amplified using F1 rabbit anti-FITC and

Table 1. RT-PCR primers and amplification conditions

Gene	Primer sequences (5'→3')	Product size (bp)	PCR conditions	Reference
<i>Mtx2</i>	CAGTGCCTGTCAAGCCCTTTC TGGCACACTTCTCAGTGTC	671	(95°C 30 sec; 55°C 1 min; 72°C 30 sec) ×40	
<i>Hoxd1</i>	CCACAGCACTTTCGAGTGGA TGGCTCACATAGCAGCGTTT	659	(95°C 30 sec; 62°C 1 min; 72°C 30 sec) ×35	
<i>Hoxd3</i>	GAAGTCCAGCAGAAGAAGCAG GCAGCTGGCCGGAGTAAGC	698	(95°C 30 sec; 62°C 1 sec; 72°C 30 sec) ×40	(Condie and Capecchi, 1993)
<i>Hoxd4</i>	TGAAAAAGGTGCACGTGAAT GAAGAAGACCTGCCCTTGGT	262	(95°C 15 sec; 56°C 1 sec; 72°C 15 sec) ×40	(Mizusawa et al., 2004)
<i>Hoxd8</i>	CTTAAATCAGAGCTCGTCTC TTGGGTCTCCATCCTTTGC	293	(95°C 15 sec; 56°C 1 sec; 72°C 15 sec) ×33	(Mizusawa et al., 2004)
<i>Hoxd9</i>	CTAAAGTCTCCCAAGTGGAG GCTGGTTGGAGTATCAGACT	142	(95°C 15 sec; 56°C 1 sec; 72°C 15 sec) ×45	(Mizusawa et al., 2004)
<i>Hoxd10</i>	GTGCAGGAGAAGGAAAGCAA GGTCAGTTCTCGGATTTCAT	276	(95°C 15 sec; 56°C 1 sec; 72°C 15 sec) ×40	(Mizusawa et al., 2004)
<i>Hoxd12</i>	GACCCGAAGACCAAGGTCAAG CCCGTGGGCGAGCTAATAGAG	571	(95°C 30 sec; 62°C 1 min; 72°C 30 sec) ×35	
<i>Hoxd13</i>	GCACGCTTCTCTCGGAGGTT CCGCCGCTGTCTCTTGT	457	(95°C 30 sec; 60°C 1 min; 72°C 30 sec) ×35	(Akasaka et al., 2001)
<i>Evx2</i>	GCGCCTGGAGAAAGAGTTC GACTGGCTGCTGTGACAACT	588	(95°C 30 sec; 58°C 1 min; 72°C 30 sec) ×35	
<i>Lnp</i>	GTGGAAGGCTCAAGCTCAAC TGCTGGGCAATCTGAATATG	455	(95°C 30 sec; 60°C 1 min; 72°C 30 sec) ×35	
<i>Oct4</i>	GCCGTTCTCTTTGGAAAGGTGTTT CTCGAACCACATCCTTCTCT	312	(95°C 30 sec; 55°C 1 min; 72°C 30 sec) ×35	(Chambeyron and Bickmore, 2004)
<i>Hprt</i>	CCTGCTGGATTACATTAAAGCACTG GTCAAGGCATATCCAACAACAAAC	333	(95°C 30 sec; 55°C 1 min; 72°C 30 sec) ×40	(Zeltser et al., 1996)

The sequences of forward and reverse primers used for the RT-PCR analysis in Fig. 3 are shown. The corresponding amplification conditions and the references to the article where the PCR assays were initially described are indicated. sec, seconds; min, minutes.

F2 FITC anti-rabbit (CAMBIO). Hybridisation, washes and detection were as described previously (Chambeyron and Bickmore, 2004). Slides were counterstained with 0.5 µg/ml DAPI.

Image capture and analysis

For 2D FISH, slides were examined using a Zeiss Axioplan II fluorescence microscope with Plan-neofluar objectives, a 100 W Hg source (Carl Zeiss, Welwyn Garden City, UK) and a Chroma #84000 quadruple band pass filter set (Chroma Technology Corp., Rockingham, VT) with the excitation filters installed in a motorised filter wheel (Ludl Electronic Products, Hawthorne, NY). Greyscale images were captured with a Hamamatsu Orca AG CCD camera [Hamamatsu Photonics (UK) Ltd, Welwyn Garden City, UK]. Image capture and analysis were performed using in-house

scripts written for IPLab Spectrum (Scanalytics Corp., Fairfax, VA). The distance between two probes, and probe position relative to the CT edge, were calculated as described previously (Chambeyron and Bickmore, 2004).

For 3D FISH, slides were examined using a Zeiss Axioskop fluorescence microscope with Plan-neofluar or Plan apochromat objectives, a 50 W Hg source (Carl Zeiss, Welwyn Garden City, UK) and Chroma #83000 triple band pass filter set (Chroma Technology Corp., 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 & Co., Karlsruhe) and a Princeton Instruments Micromax CCD camera with Kodak 1400e sensor (Universal Imaging, Maldon, UK), were used to control movement in the z

Table 2. BAC and fosmid probes used as DNA-FISH probes

Probe	Region	Whitehead (Sanger) name	Ensembl name	Coordinates		Size (bp)
				Start	Finish	
BACs	5' flanking <i>Hoxd</i> , upstream of the GCR (5' flank)	–	RP23-288B11	74055714	74278271	222558
	<i>Lnp</i> region (lnp)	–	RP24-267L11	74315270	74495825	180557
	<i>Hoxd</i> cluster (<i>Hoxd</i>)	–	RP23-15M17	74521323	74709231	187908
	3' flanking <i>Hoxd</i> cluster (3' flank)	–	RP23-7D13	74783115	75018064	234950
Fosmids	<i>Evx2-Hoxd13</i> (5' <i>Hoxd</i>)	W11-469P2	G135P67444A12	74479816	74518662	38847
	<i>Hoxd12-Hoxd8</i>	W11-860J8	G135P68612D9	74518434	74561498	43065
	<i>Hoxd4-Hoxd1</i> (3' <i>Hoxd</i>)	W11-121N10	G135P67844B8	74572642	74611097	38456

Names are from Ensembl v37, February 2006 (http://www.ensembl.org/Mus_musculus/index.html). For fosmid clones, the annotation in the Whitehead nomenclature to identify the corresponding clone in the library is also indicated. Genome coordinates are taken from NCBI Build 35 of the mouse genome (<http://genome.ucsc.edu/cgi-bin/hgGateway>).

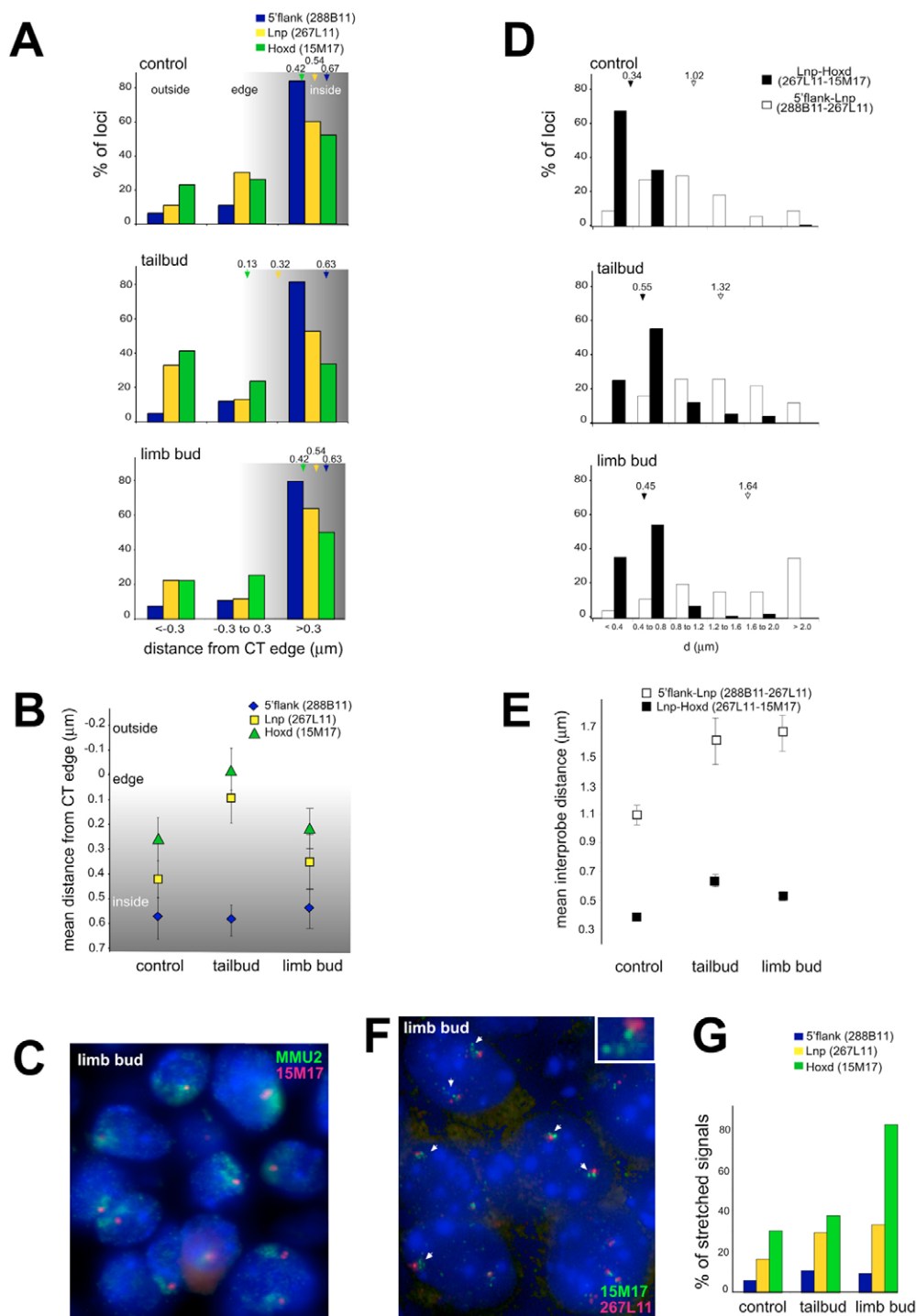


Fig. 2. Nuclear reorganisation of the *Hoxd* regulatory domain in the embryo. (A) Histograms showing the 3D position of hybridisation signals for a BAC lying within the 5' flanking region (RP23-288B11, blue), a BAC covering the *Lnp* gene (RP24-267L11, yellow), or a *Hoxd* BAC (RP23-15M17, green), relative to the MMU2 CT edge in nuclei from E9.5 control tissues, tailbud or limb bud. Coloured arrowheads indicate the median location for each respective probe. $n > 100$ loci, obtained from three embryos. (B) Mean position $\pm$ s.e.m. (μm), relative to the edge of the MMU2 CT for the 5' flank (blue), *Lnp* (yellow) and *Hoxd* (green) BACs. (C) Three-dimensional DNA FISH using the *Hoxd* probe (red) hybridised together with a MMU2 chromosome paint (green) on DAPI counterstained nuclei of a 4 μm limb bud section from E9.5 embryo. Raw image before deconvolution. (D) Distribution of 3D interphase distances (d) in μm , measured between the *Lnp* and *Hoxd* BACs (black bars), or between the 5' flank and the *Lnp* BACs (white bars) in control tissues, tailbud or limb bud from E9.5 embryos. Black or white arrowheads indicate the median separation between each probe pair. In total, $n > 100$ loci, obtained from three embryos. (E) Mean $\pm$ s.e.m. interphase separation (μm), measured between *Lnp* and *Hoxd* BACs (black square), or between the 5' flank and the *Lnp* BAC (white squares) in control tissues, tailbud or limb bud from E9.5 embryos. (F) Three-dimensional DNA FISH with the *Lnp* (red) and the *Hoxd* (green) BACs on DAPI counterstained nuclei from E9.5 limb bud, image after deconvolution. Arrowheads point to stretched signals. (G) Histogram showing the percentage of stretched 3D FISH signals detected with the *Hoxd*, *Lnp* and 5' flank BAC probes in the control tissues, tailbud and limb bud from E9.5 embryos.

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 Corp., Fairfax, VA). Images were deconvolved using a calculated PSF with the constrained iterative volume algorithm of Microtome (Scanalytics Corp., Fairfax, VA). Three-dimensional distance measurements were as described previously (Chambeyron et al., 2005). A minimum of 50 nuclei was analysed for each tissue.

Statistical analysis

The statistical relevance was assessed using the non-parametric Kolmogorov-Smirnov 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) for each differentiation time point or embryonic tissue, and for each combination of probes. A P -value <0.05 was considered statistically significant.

RESULTS

Hoxd is looped out from the chromosome territory in the tailbud but not the limb bud

We have previously shown that nuclear reorganisation of *Hoxb* occurs in expressing tissues during embryonic development (Chambeyron et al., 2005). In the E9.5 embryo, differential looping out of *Hoxb* from its CT occurs along the rostrocaudal axis of the neural tube. *Hoxb1*, at the 3' end of *Hoxb*, is extensively looped outside its CT in nuclei of rhombomere 4 (r4), where it is strongly expressed, but not more caudally in r5 or more posterior parts of the spinal cord, where it is not expressed. The tailbud is the most caudal part of the spinal cord, resulting from the regression of the primitive streak earlier in development. Both 3' and 5' *Hoxb* genes are widely expressed in the tailbud and, concomitantly, both 3' and 5' ends of the *Hoxb* locus locate just outside the CT in this embryonic region (Chambeyron et al., 2005). *Hoxd* genes are also widely expressed in the tailbud region (Tarchini and Duboule, 2006; Zakany et al., 2001). Therefore, we used 3D DNA FISH on E9.5 mouse embryo sections to determine whether the *Hoxd* region is looped out from its CT in the tailbud. Rhombomeres 1, 2 and 4, where *Hoxd* genes are not expressed (McKay et al., 1994; Zakany et al., 2001; Zhang et al., 1994), were used as control tissues. Nuclear distances between the probe signals and the edge of mouse chromosome 2 (MMU2) CT, where *Hoxd* is located, were determined using a previously described script (Mahy et al., 2002; Chambeyron and Bickmore, 2004; Chambeyron et al., 2005). In this analysis a probe signal is considered to be outside the CT when its distance to the CT edge is negative. Distances >0 correspond to probe signals inside the CT.

In control tissues (r1, 2 and 4), signals from a BAC probe covering the *Hoxd* cluster ('*Hoxd*' RP23-15M17 in Fig. 1 and Table 2) were mainly inside the MMU2 CT, but in the tailbud 40% of them were located outside the MMU2 CT (Fig. 2A). This looping out was seen in $>80\%$ of tailbud nuclei, but was usually restricted to only one of the two alleles. Therefore, in the tailbud, movement of the *Hoxd* locus to the edge and to the outside of the CT coincided with its expression ($P=0.001$). This nuclear reorganisation was not restricted to *Hoxd* itself. Signals from a BAC clone just 5' of *Hoxd* and overlapping the *Lnp* gene ('*Lnp*' RP24-267L11) were also relocalised towards the outside of the CT in 70% of tailbud nuclei ($P=0.003$ compared with control), even though there is no evidence that *Lnp* expression is induced there (Spitz et al., 2003). However, signals from a BAC located more 5' ('5'flank' RP23-288B11 in Fig. 1) remained inside the CT in both control and tailbud ($P=0.66$) (Fig. 2A). Even in the nonexpressing control tissues, *Hoxd* was located significantly ($P<0.001$) closer to the CT

edge than the two more 5' regions, suggesting that the CT may be organised such that *Hoxd* is poised before its activation (Fig. 2B).

Throughout the emerging limb bud of the E9.5 embryo, 3' *Hoxd* genes, up to and including *Hoxd9*, are also expressed (Tarchini and Duboule, 2006; Tarchini et al., 2006). However, we found no significant looping out of the region from the CT in the forelimb bud, compared with control tissues ($P>0.09$ for all three BACs) (Fig. 2A-C). Therefore activation of *Hoxd* in limb bud occurs in the absence of a relocalisation of the locus outside the CT, whereas in the tailbud *Hoxd* expression occurs in the context of a looping out of a large region from the CT. There appears to be a boundary to the extent of this looping out located between the BACs *Lnp* and 5' flank.

Decondensation of the *Hoxd* locus occurs in both the tailbud and limb bud

At *Hoxb*, movement away from the CT was also accompanied by a visible increase in the nuclear distance between the 3' and the 5' ends of the cluster, which is thought to represent a decondensation, or unfolding, of higher-order chromatin structure (Chambeyron and Bickmore, 2004; Sachs et al., 1995; Yokota et al., 1995). To establish whether this also occurs along the *Hoxd* region, we measured the interphase distances (d) between the 3D DNA FISH signals for the *Hoxd*, and *Lnp* BACs in nuclei from E9.5 embryos. There was significant ($P<10^{-3}$) movement to larger d values in both the tailbud and limb bud compared with the *Hoxd* nonexpressing control tissues. Interphase decondensation was also detected between the 5' flank and *Lnp* BACs ($P=0.01$ and $<10^{-3}$ in tailbud and limb bud, respectively) (Fig. 2D,E).

Another manifestation of chromatin decondensation lies in the shape of the BAC hybridisation signals. Whereas the signals detected with the 5' flank BAC were well-defined pinpoints, the *Lnp* and *Hoxd* BACs often gave stretched-out tracks of signals. This was especially prominent for the *Hoxd* probe in the limb bud (Fig. 2F,G).

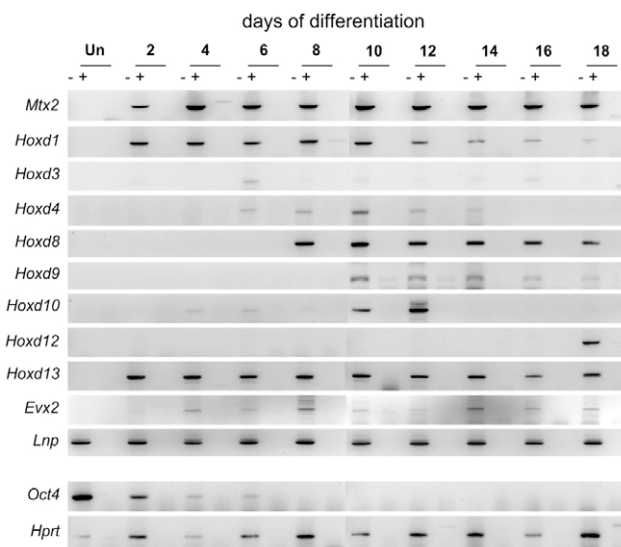


Fig. 3. Expression of *Hoxd* and flanking genes during ES cell differentiation. RT-PCR analysis of *Hoxd* and flanking genes (*Mtx2*, *Evx2* and *Lnp*) in undifferentiated (Un) OS25 ES cells and during 18 days of RA-induced differentiation. Loss of *Oct4* expression was used to monitor differentiation and *Hprt* serves as a constitutively expressed control. +, with RT; -, without RT.

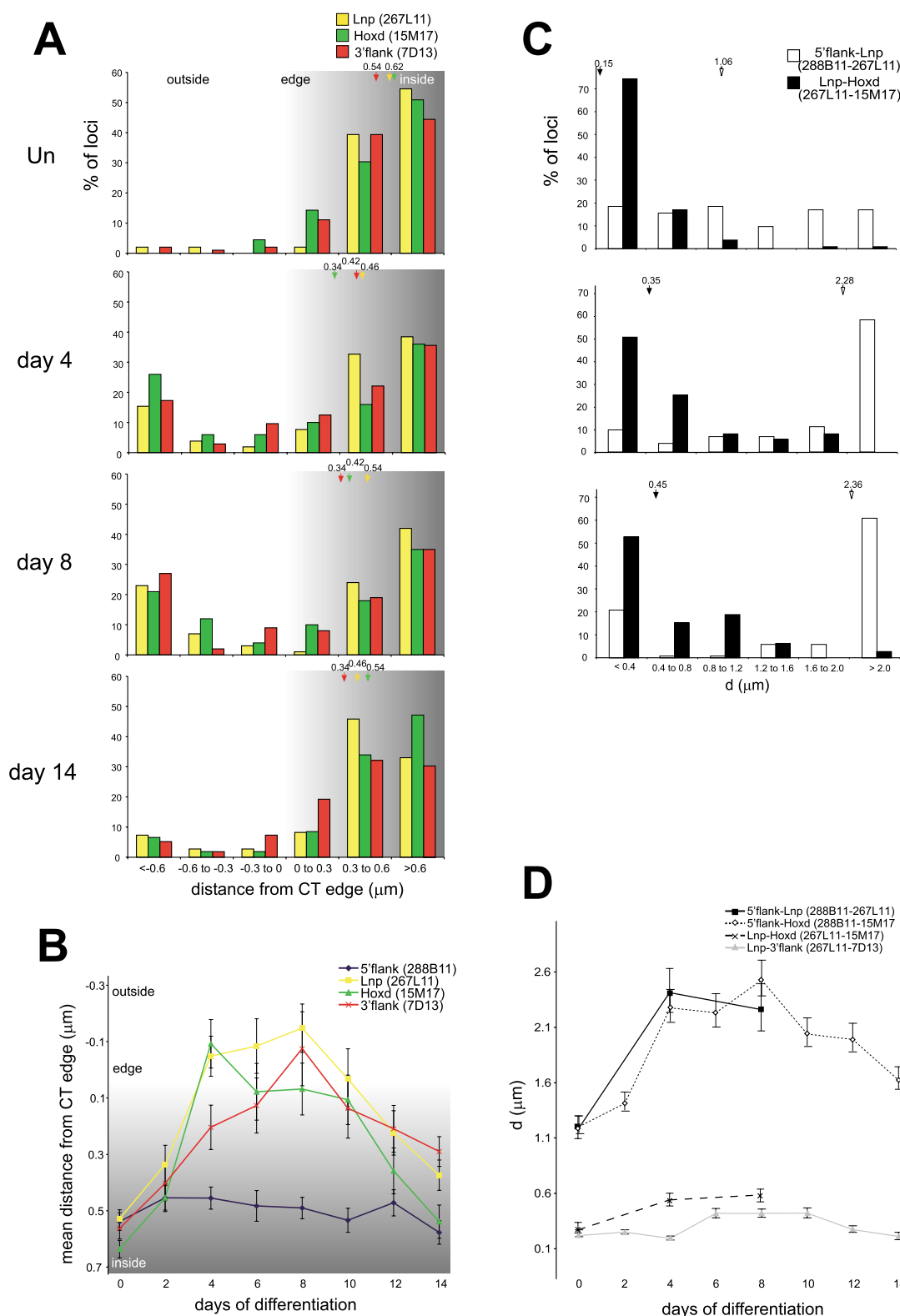


Fig. 4. Nuclear reorganisation at and around *Hoxd* during ES cell differentiation. (A) Histograms showing the distribution of 2D FISH hybridisation signals for the *Hoxd* (green), *Lnp* (yellow) and 3' flank (RP23-7D13, red) BACs relative to the edge of the MMU2 CT during differentiation. Arrowheads show the median positions for each probe. (B) Mean $\pm$ s.e.m. position (μm), relative to the edge of the MMU2 CT for the hybridisation signals from the *Hoxd*, *Lnp* and 5' and 3' flanking BACs during the timecourse of differentiation. (C) Distribution of interphase separation (d) in μm , measured between hybridisation signals for the *Lnp* and *Hoxd* BACs (black bars), or between the 5' flank and the *Lnp* BAC (white bars) during the first 8 days of differentiation. Black or white arrowheads indicate the median separation between each probe pair. (D) Mean $\pm$ s.e.m. interphase separation (d), in μm , measured between four pairs of probe signals (as indicated) during the timecourse of differentiation. For all experiments, $n > 100$ loci.

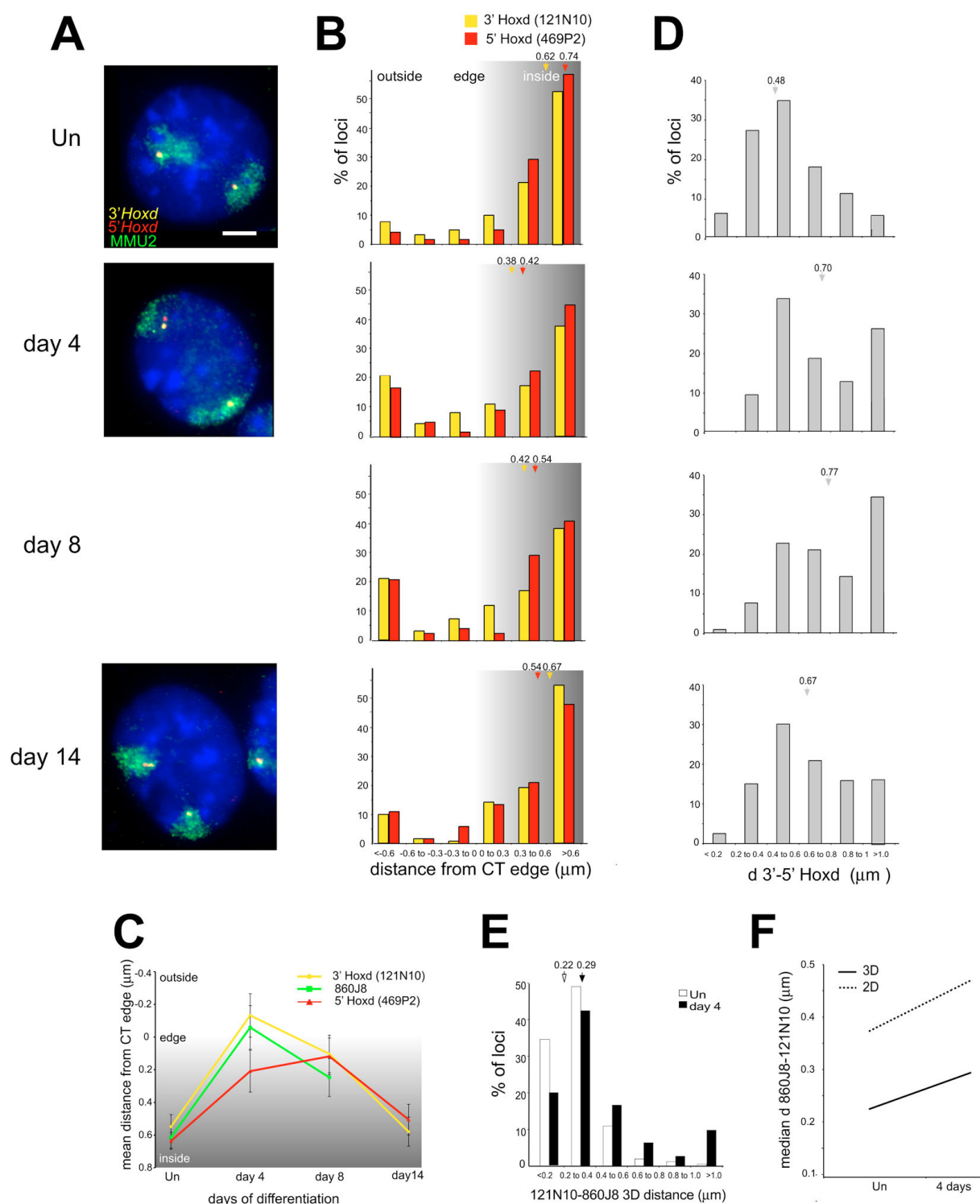


Fig. 5. Nuclear reorganisation within the *Hoxd* locus. (A) Four-colour DNA FISH using fosmid probes 3' *Hoxd* (121N10, yellow), 5' *Hoxd* (469P2, red) and an MMU2 chromosome paint (green), on DAPI (blue) counterstained nuclei from undifferentiated (Un) OS25 ES cells and cells differentiated for 4 and 14 days. Scale bar: 5 μm . (B) Histograms showing the distribution of 3' *Hoxd* (121N10, yellow) and 5' *Hoxd* (469P2, red) hybridisation signals relative to the edge of the MMU2 CT during differentiation. Arrowheads show the median values for each probe. (C) Mean position $\pm$ s.e.m. (μm), relative to the edge of the MMU2 CT, for all three *Hoxd* probes during the timecourse of differentiation. (D) Distribution of interphase separations (d) in μm , measured between 3' and 5' *Hoxd* (121N10-469P2) fosmid probe signals in 2D FISH, during the timecourse of differentiation. Arrowheads show the median values. In B, C and D, $n > 100$ territories. (E) Distribution of interphase separations in μm , measured between 121N10 and 860J8 3D FISH signals in undifferentiated ES cells (white bars) and cells differentiated for 4 days (black bars). Arrowheads show the median values. (F) Comparison of median interphase distance (d) between signals for probes 860J8 and 121N10 in undifferentiated cells and after 4 days of differentiation after 3D (solid line) or 2D (dotted line) FISH.

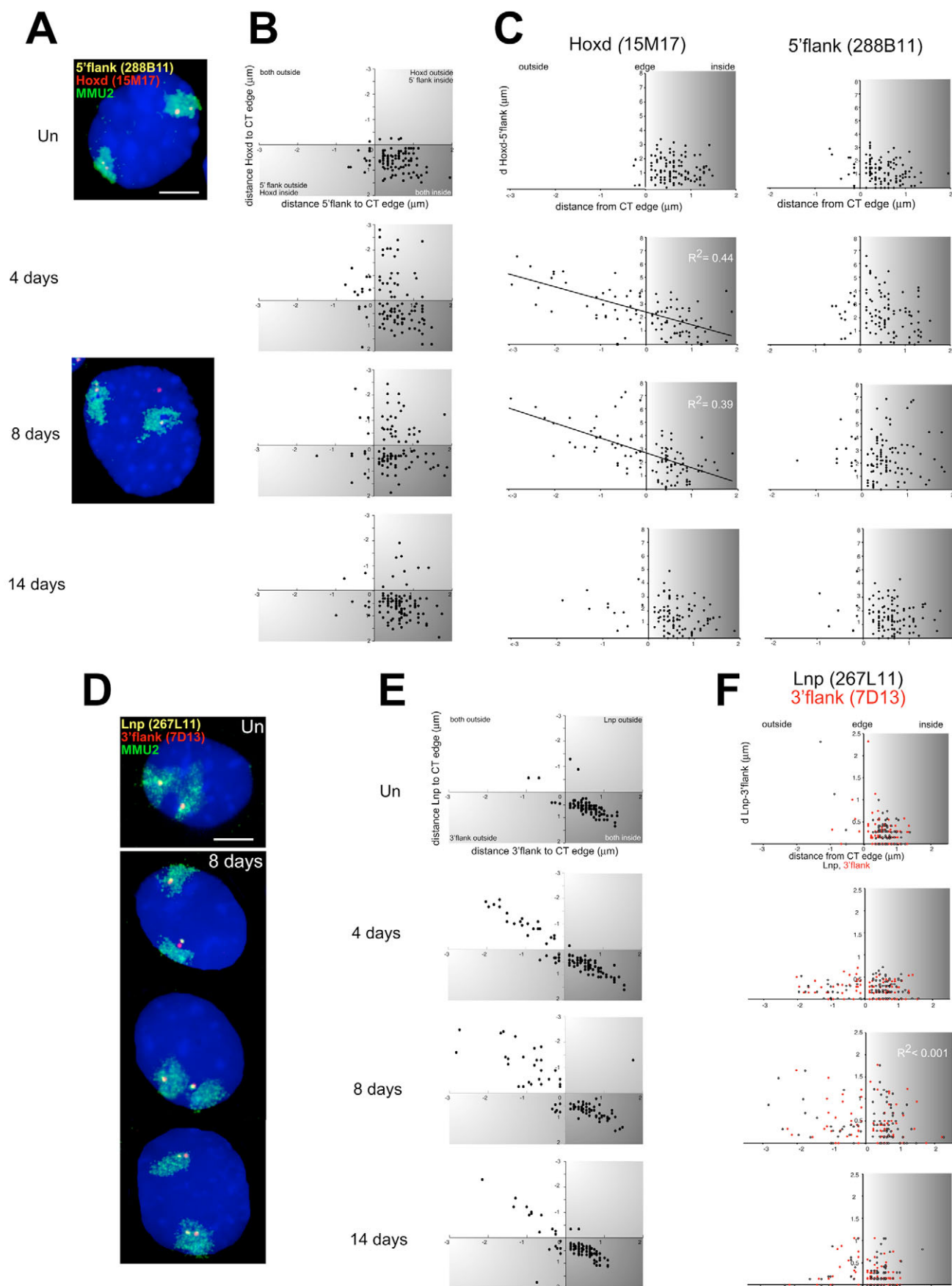


Fig. 6. See next page for legend.

Fig. 6. Coordination of looping and decondensation in the *Hoxd* region. (A) FISH using probes 5' flank (RP23-288B11, yellow), *Hoxd* (RP23-15M17, red) and MMU2 chromosome paint (green) on DAPI stained nuclei of undifferentiated ES cells or after 8 days of differentiation. (B) Scatter plots of the position of *Hoxd* (y-axes) and 5' flank (x-axes) BAC probe signals relative to the edge of the MMU2 CT during differentiation. The bottom right quadrant represents territories in which both probes are inside the CT; the top left quadrant represents situations in which both probes were located outside the CT. The other two quadrants represent discordant positions for the two BAC probe signals (one inside and one outside the CT); $n > 100$ territories. (C) Scatter plots of the interphase separation (d) in μm between *Hoxd* and the 5' flank probe signals (y-axes of both graphs) and the position of each probe relative to the edge of the MMU2 CT (μm) (x-axes) (*Hoxd* left panels; 5' flank on right). The diagonal lines indicate the best-fit linear regression lines and their corresponding R^2 values; $n > 100$ territories. (D) As in A, but with the probes *Lnp* (RP23-267L11, yellow) and 3' flank (RP23-7D13). At day 8 of differentiation, examples of decondensed (widely spaced probe signals) chromatin outside the CT (upper picture), of condensed chromatin outside the CT (middle picture) or of decondensed chromatin inside the CT (lower picture) are shown. (E) As in B but with BACs *Lnp* and 3' flank. (F) Scatter plots of the d (μm) between the *Lnp* (black dots) and 3' flank (red dots) signals (y-axes) and the position (μm) of each probe relative to the edge of the MMU2 CT (x-axes). Scale bars: 5 μm .

Therefore, long-range decondensation of the entire region extending from *Lnp* to the *Hoxd* cluster correlated with *Hoxd* expression in E9.5 embryos. In the tailbud, 20% of *Hoxd* loci appeared decondensed, which is similar to the percentage of loci localised outside the MMU2 CT. However, there was also decondensation of *Hoxd* in the limb bud, where there was no relocalisation of this region with respect to the CT. This suggests that 'looping out' and decondensation are not just different manifestations of the same event of nuclear reorganisation.

Sequential activation of *Hoxd* genes during ES cell differentiation

To dissect the events of nuclear reorganisation in more detail and in a more tractable system, we used an ES cell differentiation system. Gene expression and nuclear reorganisation could be induced at *Hoxb* by triggering the differentiation of murine ES cells with retinoic acid (RA) (Chambeyron and Bickmore, 2004). To determine whether similar activation occurs at *Hoxd* we used RT-PCR to analyse the expression of *Hoxd* genes (Fig. 3) in undifferentiated OS25 ES cells, and during 18 days after the withdrawal of LIF and the addition of RA. As for *Hoxb*, there was no detectable expression of *Hoxd* genes in undifferentiated cells. The extinction of *Oct4* expression upon differentiation was accompanied by the rapid induction (by day 2) of *Hoxd1* expression, but not of the more 5' genes *Hoxd3* through to *Hoxd12* (Fig. 3). Expression of *Hoxd3* and *Hoxd4* were detected by day 6, *Hoxd8* by day 8, *Hoxd9* and *Hoxd10* by day 10 and *Hoxd12* expression was not detected until day 18. *Hoxd1* expression declined at later stages of differentiation, but not as rapidly as seen for *Hoxb1* (Chambeyron and Bickmore, 2004).

Hoxd is flanked by structurally and functionally unrelated genes. Expression of *Mtx2*, located 3' of *Hoxd1* (Fig. 1), is induced by day 2 (Fig. 3), suggesting that this gene might also be subject to temporal colinearity. However, at the 5' end of *Hoxd*, the early detection of *Hoxd13* expression (by day 2) suggests a break in the temporal colinearity at this end of the cluster in this system. A large conserved

noncoding region 5' of *Hoxd* (Fig. 1B), termed a global control region (GCR), contains digit enhancers that act on *Hoxd13*, *Lnp* and *Evx2* (Tarchini and Duboule, 2006). Neural enhancers in the GCR also act on *Evx2* and *Lnp* (Spitz et al., 2003). As *Evx2* is also activated early in the timecourse of differentiation, this suggests that the GCR may have some activity in ES cells. *Lnp* expression in ES cells is constitutive (Fig. 3) (Spitz et al., 2003). This analysis shows that the colinear activation of the *Hoxd* cluster is mostly recapitulated upon RA-induced ES cell differentiation.

Nuclear reorganisation of *Hoxd* is induced upon ES cell differentiation

We used 2D interphase FISH to determine whether the events of nuclear reorganisation at *Hoxd*, seen in the embryo, also occur in differentiating ES cells. As in control embryonic tissues, hybridisation signals from the *Hoxd* and *Lnp* BAC probes were almost all located inside the CT in undifferentiated cells (Fig. 4A,B), even though *Lnp* was expressed in them (Fig. 3). These regions relocalise outside the MMU2 CT upon differentiation ($P < 0.005$), but whereas the highest proportion (32%) of *Hoxd* alleles located $> 0.3 \mu\text{m}$ outside the CT occurred by day 4 of differentiation, the maximum frequency of extra-territory *Lnp* alleles was not seen until day 8 (Fig. 4A). The nuclear behaviour of the region 3' of *Hoxd* and *Mtx2*, detected by the RP23-7D13 (3' flank) BAC clone (Fig. 1), was similar to that of *Lnp*, with a maximal frequency of looping out from the CT at day 8 of differentiation. After 14 days, signals from all three probes were relocated back inside the CT (Fig. 4A,B). As in the embryo, there was no movement detected with the 5' flank BAC at any time point during differentiation (Fig. 4B). Therefore, there is a remarkably similar domain of chromatin around *Hoxd* that is located outside its CT in the tailbud of the embryo, and that is induced to transiently loop out from the CT during ES cell differentiation.

There is also a significant ($P < 10^{-3}$) increase in the interphase distances separating the *Hoxd* cluster (RP23-15M17) from *Lnp* (RP24-267L11), and *Lnp* from the 5' flank (RP23-288B11) during differentiation (Fig. 4C). At day 14, and concomitant with the relocalisation of the region back inside the CT, there is recondensation of the whole region (Fig. 4D). As in the embryo, extended tracks of hybridisation signals were often detected in differentiating ES cells with the *Hoxd* and the *Lnp* BAC probes (data not shown).

The relative kinetics of the intra-CT movements detected with probes across the *Hoxd* region (Fig. 4A) suggests that these events initiate within the *Hoxd* cluster itself, rather than in flanking regions. As the temporal activation of *Hox* genes begins at the 3' ends of the clusters, we analysed nuclear behaviour within the *Hoxd* locus itself using three fosmid clones (121N10, 860J8 and 469P2; Fig. 1 and Table 2). In undifferentiated ES cells, almost all ($> 88\%$) 3' *Hoxd* signals (121N10 probe) were within the CT (Fig. 5A,B). By day 4 of differentiation, there was a significant ($P = 0.01$) shift of signals away from the CT, such that 25% of them were then located outside. This is the same time point at which 'looping out' of *Hoxb1* signals is seen from its CT (Chambeyron and Bickmore, 2004). By day 14, 3' *Hoxd* signals were back within the CT, similar to the situation before differentiation ($P = 0.95$) (Fig. 5A,B). Similar results were obtained with 860J8, encompassing the central *Hoxd* genes (data not shown). At the 5' end of *Hoxd* (469P2), there was also movement towards and outside the CT edge, but this did not reach maximal significance ($P = 0.004$) until day 8 (Fig. 5B). This suggests that nuclear reorganisation initiates at the 3' end of *Hoxd* and this is also supported by a comparison of the mean probe positions during the timecourse of differentiation (Fig. 5C).

Chromatin decondensation also occurs within *Hoxd*. The midpoints of the 3' (121N10) and 5' (469P2) *Hoxd* probes were 90 kb apart and their hybridisation signals were barely separable in undifferentiated cells (Fig. 5A). At days 4–8 of differentiation, there was a significant ($P < 10^{-3}$) increase in the interphase separation between them (Fig. 5D). There was some recondensation by day 14, but their separation was still greater than that before differentiation ($P = 0.006$), suggesting that the structure of the *Hoxd* cluster remains in an altered state. To confirm that decondensation over such short genomic distances was not an artefact of the 2D DNA FISH, we confirmed that there was also a significant increase in the interphase separations between 121N10 and 860J8 fosmids during differentiation, using 3D FISH (Fig. 5E). A similar relative degree of decondensation was detected by 2D and 3D FISH, even though the former exaggerated the absolute interphase distances (Fig. 5F).

Looping out and chromatin decondensation can be distinct modes of nuclear reorganisation at *Hoxd*

Superficially, the similar kinetics of looping out and chromatin decondensation of the *Hoxd* region during ES cell differentiation are consistent with the suggestion that looping out from CTs is a visual manifestation of chromatin decondensation that has been propagated over a long range (Volpi et al., 2000; Gilbert et al., 2004). However, our observation in the embryo proper, of extensive decondensation in the absence of looping out, led us to question this. To determine whether interphase decondensation and looping out are indeed synonymous events, we used four-colour DNA FISH to simultaneously measure the nuclear positions of different parts of *Hoxd* both relative to each other and relative to the CT during ES cell differentiation. When we looked between the 5' flanking region, which remained fixed inside the CT, and either the *Lnp* or *Hoxd* regions, which moved away to locations outside the CT during differentiation, the increase in inter-probe distances was indeed correlated with looping of *Hoxd* outside the MMU2 CT ($R^2 = 0.44$ at day 4) (Fig. 6A–C).

However, a very different pattern emerged when we examined probes within the region that loops out of the CT (from *Lnp* to the 3' flank, including *Hoxd* itself). Movement of *Lnp* and the 3' flank occurred together: it was rare to find one probe outside a CT while the other remained inside (Fig. 6D,E). However, this movement preceded interphase separation, as both probes found outside the CT at day 4 were still spatially close together ($< 0.5 \mu\text{m}$) (Fig. 6F). At day 8, when there was both looping out and apparent decondensation, there was no correlation between the two events (in linear regression analysis $R^2 < 0.001$) (Fig. 6E). *Lnp* and 3' flank signals located far outside the bounds of the visible CT could appear to still be quite closely juxtaposed, and widely separated signals could be seen within the CT (Fig. 6D). The same lack of correlation between movement and interphase separation was also observed between *Lnp* and the *Hoxd* cluster itself, and even between the 3' and 5' ends of *Hoxd* (data not shown).

DISCUSSION

Nuclear reorganisation of *Hox* clusters is an ancestral mechanism

We have shown previously that the murine *Hoxb* locus loops out from its CT (mouse chromosome 11) and decondenses when the locus is activated during ES cell differentiation (Chambeyron and Bickmore, 2004), or during embryonic development (Chambeyron et al., 2005). Here we show that similar events of nuclear reorganisation also occur at *Hoxd* in the tailbud region of the E9.5

embryo (Fig. 2) and during ES cell differentiation (Fig. 4). Nuclear reorganisation of *Hox* loci therefore reflects a basic mechanism underlying the specific activation of these gene clusters in differentiation, and in development along the rostrocaudal embryonic axis. Thus it seems likely that this mechanism predates the duplication and divergence of vertebrate *Hox* clusters more than 500 million years ago (Pollard and Holland, 2000; Ferrier and Minguillon, 2003).

Nuclear reorganisation initiates within *Hoxd*, but then spreads out to encompass unrelated genes

Although activation of *Hoxd* is accompanied by its nuclear reorganisation, a simple escape from the CT cannot be sufficient to explain the temporal activation of gene expression during ES cell differentiation. Whereas the kinetics of *Hoxd1* activation is contemporaneous with its movement away from the CT by day 4, expression of *Hoxd3* (on the same fosmid clone as *Hoxd1*) is not detected by RT-PCR until day 6 (Fig. 2). Conversely, maximal relocalisation of the 5' *Hoxd* fosmid is seen by day 8 of differentiation, but *Hoxd12* expression is not detected by RT-PCR until day 18, at which time the *Hoxd* region has been retracted back into the CT. This suggests that movement away from the CT is not sufficient to activate the expression of all *Hoxd* genes and that additional chromatin modifications must occur to allow for gene expression per se. Also, it seems unlikely that it is necessary for 5' *Hox* genes (*Hoxd12*) to be outside the CT when they are expressed, although we cannot exclude the possibility that *Hoxd12* expression is originating from that small proportion (10%) of alleles that remain outside the CT at the end of differentiation (Fig. 5B). Combined RNA and DNA FISH might resolve this, but we have been unable to detect *Hox* expression by RNA FISH. This may be due to the fact both that *Hox* genes have only one small intron, and that microarray analysis shows that levels of *Hoxd* gene expression are not very high (data not shown).

Looping out of chromatin away from the CT, initiated within *Hoxd*, then spreads out in both directions to encompass unrelated flanking genes (Figs 2, 4). The activation of *Mtx2* expression early in differentiation 3' of *Hoxd* (Fig. 3) might be a response to the spread of nuclear reorganisation. Similarly, the timecourse of *Evx2* expression immediately 5' of *Hoxd* appears to parallel the spread of chromatin looping and decondensation (Fig. 4). Interestingly the proximity of *Evx* genes to another *Hox* locus (*Hoxa*) suggests that *Evx2* was part of an ancestral *Hox* cluster (Pollard and Holland, 2000; Ferrier and Minguillon, 2003), so it may have evolved to respond to global chromosomal cues regulating *Hox* regulation. However, beyond *Evx2*, *Lnp* is a recent evolutionary recruit to this *Hox* locus. Its expression does not respond to *Hoxd* nuclear reorganisation during ES cell differentiation (Fig. 3), or in the tailbud of the E9.5 embryo (Spitz et al., 2003), even though it is relocalised towards the outside of the CT in these situations (Figs 2, 4).

Pathways leading to looping out and chromatin unfolding

Our previous analyses showing both interphase probe separation and looping out at *Hoxb*, in ES cell differentiation (Chambeyron and Bickmore, 2004), and in the embryo (Chambeyron et al., 2005), appeared to be consistent with the idea that looping out from CTs and chromatin decondensation are two sides of the same coin (Volpi et al., 2000; Gilbert et al., 2004). However, our analysis here of *Hoxd* has thrown up discordances between inter-locus interphase distances and movement of the loci to the outside

of CTs. In two different *Hoxd*-expressing regions of the E9.5 embryo, one – the tailbud – shows both looping out and decondensation at *Hoxd*, whereas the other – the limb bud at E9.5 – shows extensive chromatin decondensation but no evidence of any looping out of *Hoxd* (Fig. 2). We therefore assayed simultaneously, at each allele during ES cell differentiation, the localisation with respect to the CT, and the interphase probe separation. Whereas the movement of the *Hoxd* region to sites outside the CT correlates with its interphase separation from a 5' probe (288B11) that remains inside the CT, a more complex situation was seen within the *Hoxd* region itself – from Lnp to the 3' flank (Fig. 6). The first (day 4) nuclear events seen within this region are movement to the outside of the CT, without chromatin decondensation as assayed by interphase separation. When a movement to larger inter-probe distances is seen at day 8, this occurs regardless of position relative to the CT. One possibility is that long-range unfolding of chromatin structure allows the locus a greater degree of mobility so that it can move dynamically in and out of the CT. Thus, although both events are triggered upon ES differentiation, looping out does not depend on a prior decondensation of the region, and chromatin decondensation may not lead directly to a looping out from the CT.

Most published *in situ* hybridisations on embryos at around E9.5 suggest that levels of *Hoxd* expression in the tailbud and limb bud are similar (e.g. Tarchini and Duboule, 2006), but there is evidence for stronger expression of *Hoxd4* in the tailbud compared with the limb bud (Akasaka et al., 2001) (<http://genex.hgu.mrc.ac.uk/das/jsp/submission.jsp?id=EMAGE:48>). Therefore at this stage, we cannot exclude that the differential nuclear organisation of *Hoxd* in tailbud and limb bud is due to different levels of *Hoxd* transcription. Also, different regulatory pathways might be responsible for *Hoxd* activation along the rostrocaudal and the secondary axis of the embryo. The temporal activation of *Hoxd1-d9* in the early limb bud is thought to be under the control of an early limb bud activating element (ELCR) located 3' of the cluster (Tarchini and Duboule, 2006), but its role in the regulation of *Hoxd* along the rostrocaudal axis has not been fully explored. Understanding how different modes of regulation feed in to altered chromatin structures at Hox loci will be key to further elucidating how the expression of these developmental players is regulated so exquisitely in time and space.

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