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Citation for published version:
https://doi.org/10.1101/gad.292104

Digital Object Identifier (DOI):
10.1101/gad.292104

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Genes & Development

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Chromatin decondensation and nuclear reorganization of the HoxB locus upon induction of transcription

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The colinearity of genes in Hox clusters suggests a role for chromosome structure in gene regulation. We reveal programmed changes in chromatin structure and nuclear organization upon induction of Hoxb expression by retinoic acid. There is an early increase in the histone modifications that are marks of active chromatin at both the early expressed gene Hoxb1, and also at Hoxb9 that is not expressed until much later. There is also a visible decondensation of the chromatin between Hoxb1 and Hoxb9 at this early stage. However, a further change in higher-order chromatin structure, looping out of genes from the chromosome territory, occurs in synchrony with the execution of the gene expression program. We suggest that higher-order chromatin structure regulates the expression of the HoxB cluster at several levels. Locus-wide changes in chromatin structure (histone modification and chromatin decondensation) may establish a transcriptionally poised state but are not sufficient for the temporal program of gene expression. The choreographed looping out of decondensed chromatin from chromosome territories may then allow for activation of high levels of transcription from the sequence of genes along the cluster.

Keywords: Chromatin decondensation; chromosome territory; differentiation; gene expression; histone modification; nuclear structure

Received November 18, 2003; revised version accepted April 2, 2004.

To understand how chromatin regulates gene expression it is necessary to analyze not only nucleosome structure and modifications but also higher-order chromatin condensation and nuclear organization. Many correlations between transcription and nucleosome modifications have been found. For example, active β-globin loci are enriched in hyperacetylated histones H3 and H4, and H3 dimethylated on lysine 4 (met2H3-K4), whereas the silent domain flanking the chicken locus has hypoacetylated histones and H3 dimethylated on K9 (met2H3-K9; Litt et al. 2001; Bulger et al. 2003; Kim and Dean 2003). How histone modifications influence transcription is still debated. They can act as binding sites for specific regulatory proteins (Fischle et al. 2003) and may directly affect higher-order chromatin structure and condensation (Tse et al. 1998; Wolff and Hayes 1999; Carruthers and Hansen 2000; Wang et al. 2001).

Chromatin organization at and beyond the level of the 30-nm chromatin fiber is poorly understood. Cytologically, chromatin decondensation is seen at transcriptionally active regions in the polytene chromosomes of dipteran insects (Andersson et al. 1984) and when transcriptional regulators are artificially targeted to sites in the mammalian genome (Tumbar et al. 1999; Tsukamoto et al. 2000; Muller et al. 2001; Ye et al. 2001; Nye et al. 2002). It is not clear whether chromatin decondensation accompanies the induction of endogenous mammalian gene expression.

There is also a complex relationship between transcription and the position of genes within chromosome territories (CTs). Some studies show inactive genes located in the interior of CTs and active genes concentrated at the territory periphery (Kurz et al. 1996; Dietzel et al. 1999), but active genes can be transcribed from inside of CTs (Mahy et al. 2002b). Moreover, regions with a high density of coordinately expressed genes locate in loops that extend outside of CTs in expressing cells but not in nonexpressing cells (Volpi et al. 2000; Williams et al. 2002). Localization outside of CTs also occurs at genomic regions with a high-density of broadly expressed genes (Mahy et al. 2002a). Hence, it has been suggested that there is a correlation between high levels of transcriptional activity and localization outside of CTs (Mahy et al. 2002a).

It is not known whether chromatin decondensation and CT organization are just consequences of the changes in histone modifications accompanying gene activation, or whether they are independent levels of chromatin structure over and above the level of the nucleo-
some. At the mammalian β-globin locus, altered nuclear organization (movement away from heterochromatin) correlates with general histone acetylation and the LCR appears to be responsible for localizing the region outside of CTs prior to gene activation. These studies suggest that nuclear reorganization is upstream of histone modifications in a pathway to chromatin opening and that it is necessary to create a transcriptionally poised state that precedes gene expression (Schubeler et al. 2000; Ragoczy et al. 2003).

To understand the relationships between gene expression, nucleosome modification, chromatin decondensation, and nuclear organization, we have studied the developmentally regulated Hoxb gene cluster. Establishing correct spatial and temporal domains of Hox expression is important for anteroposterior (AP) patterning (Krumlauf 1994; Popperl et al. 1995; Studer et al. 1998). This is partly achieved through the colinearity of gene order along the chromosome, with the time and place of gene activation (Fig. 1A; for review, see Kmita and Duboule 2003). The mechanisms for vertebrate Hox gene regulation are largely unknown, but cis-acting regulatory elements (see Marshall et al. 1994; Popperl et al. 1995; Maconochie et al. 1997; Oosterveen et al. 2003), as well as higher-order silencing mechanisms (Kondo and Duboule 1999) are involved. A progressive transition from an inactive (closed) to an active (open) chromatin state, propagated through the cluster from 3' to 5', has been proposed (Van der Hoeven et al. 1996; Kondo and Duboule 1999; Roelen et al. 2002). These models are inferred from transgenic reporter approaches (see Marshall et al. 1994; Maconochie et al. 1997) and from rearrangements that reposition Hox genes within a cluster (Van der Hoeven et al. 1996, Kondo et al. 1998).

Given the interest in the control of Hox gene expression, it is surprising that little is known about their chromatin structure. By using retinoic acid (RA) to induce regulated expression of Hoxb genes in mouse embryonic stem (ES) cells, we have performed chromatin immunoprecipitation (ChIP) to track histone modifications, and fluorescence in situ hybridization (FISH) to analyze chromatin condensation and nuclear organization. Within 4 d of induction, there are increases in histone modifications associated with transcriptional activity [AcH3-K9 and met2H3-K4] at both the early expressed Hoxb1 and also at genes that will not be transcribed until later in the developmental program (Hoxb9). At this stage there is also a dramatic decondensation of the HoxB cluster, which is not a simple consequence of the increase in histone acetylation. There is then a choreographed extrusion of genes outside of CTs that is coincident with the temporal program of gene expression. We propose a two-step model for how chromatin structure regulates expression from HoxB. First a locus-wide change in chromatin structure (histone modification and chromatin decondensation) may create a transcriptionally poised

Figure 1. Organization and differential activation of the Hoxb locus in ES cells. [A] Chromosomal organization of genes (open boxes) in the murine Hoxb complex. 5' and 3' indicate the direction of the transcription. The distance between genes is indicated below the map, as is the position of FISH probes (gray bars) for Hoxb1 and Hoxb9. The arrow indicates the colinear properties of the Hoxb cluster with respect to spatial expression [anterior to posterior], time of expression [early to late], and sensitivity to RA. Modified from Hunt and Krumlauf (1992). [B] Differential expression of Hoxb genes in OS25 ES cells. RT–PCR analysis of Hoxb1, 9, and 13 in undifferentiated (UN) cells and in cells induced to differentiate with RA for 2 to 12 d. Differentiation was confirmed by the loss of Oct4 expression. Total RNA from E11.5 embryo (E) and analysis of Gapdh were used as positive controls.
state. Then a progressive 3’ to 5’ change in higher-order chromatin structure, which is manifest as the movement outside of CTs, is necessary to allow for the programmed expression of genes along the cluster.

Results

RA induces a temporal program of Hoxb gene expression in ES cells

In vivo the activation of Hox gene expression by retinoids is mediated by cis-acting RA response elements [RAREs; for review, see Gavalas and Krumlauf 2000]. Hox genes also show a colinear temporal response to RA ex vivo. In embryonal carcinoma cells, genes at the 3’ extremity of the clusters respond earlier, and at lower RA concentrations, than do more 5’ genes (Fig. 1A; Simmone et al. 1990, 1991; Papalopulu et al. 1991). RA also induces Hox expression in ES cells (Papalopulu et al. 1991). We induced expression of the HoxB cluster with RA in wild-type ES cells but obtained a nonhomogeneous response as revealed by the expression of SSEA-1 antigen in residual undifferentiated ES cells [Brown et al. 1993; data not shown]. Because studies of nuclear organization rely on analysis at the single cell level, it was important to have a more homogenous population of cells, before and after differentiation. Therefore, we chose to use OS25 ES cells that allow for G418 selection (Chen et al. 2002; data not shown). No expression of Hoxb genes was detected in these cells (Fig. 1B). Differentiation, induced by withdrawing LIF and adding RA, resulted in the complete loss of alkaline phosphatase and SSEA-1 markers, and Oct4 expression in the presence of LIF and G418 (Billon et al. 2002; data not shown).

Changes in histone H3 modification at Hoxb1 and Hoxb9

Transcriptionally active genes are generally characterized by high levels of histone acetylation and metH3-K4 [for review, see Zhang and Reinberg 2001; Lachner et al. 2003]. We used ChIP and quantitative real-time PCR to examine changes in H3 modification at Hoxb1 and Hoxb9 before differentiation (neither gene expressed) and at day 4 [Hoxb1 expressed] and day 10 [Hoxb9 expressed] of differentiation. We also examined the r4 enhancer upstream of Hoxb1, which is required for its rlongerene 4 restricted expression [Popperl et al. 1995], and the RARE (3’DR2) located 1.2 kb 3’ of Hoxb1 that mediates the early response to RA [Marshall et al. 1994, Fig. 2A]. The presence of nucleosomes in these regions was confirmed by ChIP with antibody that detects the C terminus of H3 [Verreault et al. 1996]. By day 4, increased H3 acetylated at K9 [H3K9] was detected at all sites in Hoxb1 and Hoxb9 that were examined, even though only Hoxb1 is expressed at this time. Levels of met3H3-K4 also increased at Hoxb1 by day 4 of differentiation, and surprisingly, constitutively high levels of this histone modification were found at exon 1 and the promoter of the silent Hoxb9 both before and after differentiation. ACH3-K9 and met3H3-K4 were lost from Hoxb1 by day 10, when the gene has been silenced, but they persist at Hoxb9 [Fig. 2B].

We conclude that RA rapidly induces histone modifications [ACH3-K9 and met3H3-K4], which are considered as marks of active chromatin, at not only the 3’ end of the cluster where gene expression is induced at an early stage but also at more 5’ regions [Hoxb9]. At these later sites, the changes in histone modifications preceed transcriptional activation by many days. The subsequent loss of these histone modifications from Hoxb1 parallels the cessation of Hoxb1 expression. However, at no stage did we detect substantial levels of met3H3-K9 [Fig. 2B], suggesting that this marker of gene repression is not important for the switch on or off of Hoxb expression in this system.

Chromatin decondensation at Hoxb

It has been suggested that an “opening” of chromatin structure may contribute to the regulation of Hox clusters [van der Hoeven et al. 1996; Kondo and Duboule 1999, Roelen et al. 2002]. Cytological chromatin decondensation accompanies transcriptional activation in some reporter systems [Tumbar et al. 1999; Tsukamoto et al. 2000, Muller et al. 2001]. To determine if there are similar levels of chromatin decondensation accompanying induction of Hoxb, we measured the distance [d] between Hoxb1 and Hoxb9 hybridization signals in nuclei before and after RA induction. At probe separations of <2 Mb, there is a linear relationship between the mean-square interphase distances between them [d<sup>2</sup>] and their separation in kilobases [van den Engh et al. 1992]. This analysis has been used to show that different parts of the human genome have different levels of chromatin compaction [Yokota et al. 1997].

Hoxb1 and Hoxb9 are 90 kb apart (Fig. 1A), and in nuclei of undifferentiated ES cells, they could barely be separated from one another. After day 2 of differentiation, they were readily separable in both MAA and pFa-fixed cells [Fig. 3A]. Some recondensation of this region was then seen by day 10. The distribution of d values after day 2 conforms to a Rayleigh distribution [Sachs et al. 1995] (SD/d) = 0.56 and median/(d) = 0.9) indicating
that the decondensed chromatin describes a random walk path.

Decondensation is not a general consequence of differentiation. The interphase separation between two control genes (Rcn and Pax6), the expression of which is not induced by RA and which are separated by 300 kb on MMU2, is not significantly different before and after 2 d of differentiation (P = 0.46; Fig. 3B).

The visual decondensation of Hoxb could represent a specific change in its higher-order chromatin structure or could just be a passive consequence of the increase in histone acetylation at the locus [Fig. 2] and its influence on folding of the chromatin fiber. To distinguish between these two possibilities, we experimentally increased histone acetylation in undifferentiated ES cells, by inhibiting the action of histone deacetylases [HDACs] with trichostatin A (TSA), in the absence of RA. Expression of Hoxb was determined by real-time RT–PCR. During the normal RA-induced differentiation program, there is an ~10-fold increase in the levels of Hoxb1 mRNA by day 4. In contrast, only a very small (less than twofold) increase in Hoxb1 mRNA was detected after exposure of undifferentiated ES cells to TSA, and there was no expression of Hoxb9 (data not shown). TSA pretreatment does not prevent the normal execution of the program of RA-induced Hoxb expression because the correct sequence of Hoxb1 and Hoxb9 expression was seen at appropriate times after exposure of TSA-treated undifferentiated cells to RA (data not shown).

ChIP and real-time PCR showed that, in undifferentiated cells, TSA increased AcH3-K9 levels at Hoxb1 and its regulatory elements to the same extent as that seen when untreated cells are differentiated with RA (data not shown).
not shown). However, as well as no induction of Hoxb expression, there was no visual decondensation of the locus in TSA-treated cells (Fig. 3A). Therefore, we conclude that there is an approximate ninefold transient decondensation of the Hoxb complex upon induction with RA, and that this decondensation is not just a simple consequence of increased levels of histone acetylation.

**Extrusion from the CT accompanies Hoxb gene expression**

Although we have detected changes in histone modification and chromatin decondensation upon induction of Hoxb, the timing of these events early in the induction program (by day 4) does not account for the temporal program of gene expression—in particular, why is Hoxb9 expression not seen until day 10? Domains of high transcriptional activity have been correlated with a distinctive chromatin structure at the cellular level, a looping out from CTs (Mahy et al. 2002a). Moreover, for some domains of coordinately regulated genes, the extrusion of these loops correlates with the up-regulation of gene expression (Volpi et al. 2000; Williams et al. 2002).

We therefore used FISH to examine how Hoxb is organized relative to its CT, before and after induction. Probes encompassing Hoxb1 or Hoxb9 [Fig. 1A] and a paint for mouse chromosome 11 (MMU11) were cohybridized to nuclei from undifferentiated and from cells at day 4 or 10 of differentiation [Fig. 4A]. We calculated the distance [in micrometers] between the Hoxb gene signals and the nearest CT edge as described previously [Fig. 4B; Table 1; Mahy et al. 2002a]. In undifferentiated ES cell nuclei, most hybridization signals for both Hoxb1 and Hoxb9 were within the CT. By day 4 of differentiation, when Hoxb1 is expressed, most of its hybridization signals are now located well (>0.2 µm) outside of the CT, but Hoxb9 remains inside (Table 1, Fig. 4B,D). This was also confirmed by simultaneous detection of Hoxb1, Hoxb9, and MMU11 CT [Fig. 4C]. A two-tailed distribution Student’s t test showed that the relocalization of Hoxb1 outside of the CT upon differentiation is statistically significant ($P < 0.000$). By day 10, Hoxb1 expression is switched off and the gene relocalizes back toward the CT ($P = 0.02$; Fig. 4B,D). However, its location is still more exterior to the CT than in undifferentiated cells ($P < 0.000$). At day 10, Hoxb9 moves to the CT surface [Fig. 4D], and a significantly ($P = 0.001$) increased frequency of Hoxb9 signals are now found outside of the CT, coincident with the expression of this gene [Fig. 4B; Table 1].

**Figure 3.** Decondensation of Hoxb. (A) FISH of Hoxb1 [red] and Hoxb9 [green] to MAA-fixed nuclei from undifferentiated (UN) cells, from cells at 2 or 10 d of differentiation, and from undifferentiated cells treated with TSA. Nuclei were counterstained with DAPI [blue]. Bar, 5µm. Below it, each image the mean-square interprobe distance—$d^2$ (µm$^2$) ± S.E.M.—is shown ($n = 50$ cells). The value for cells at 2 d, fixed with pFa, is also shown. (B) FISH of Pax6 [red] and Rcn [green] probes on cells before and after (2 d) differentiation.
Figure 4. Progressive looping of the HoxB cluster from 3' to 5'. (A) FISH with MMU11 chromosome paint (green) and gene-specific probes (red) for Hoxb1 (top) or Hoxb9 (bottom) on MAA-fixed nuclei from undifferentiated (UN) cells, and from cells at 4 or 10 d of differentiation. Nuclei were counterstained with DAPI (blue). Bars, 5 µm. (B) Histograms showing the distribution of hybridization signals for Hoxb1 (open bars) or Hoxb9 (filled bars) relative to the MMU11 territory edge (µm). Negative distances indicate signals located beyond the visible limits of the detectable CT. n = 100 territories. (C) FISH with MMU11 chromosome paint (red) and gene-specific probes for Hoxb1 (green/red, 1:1) or Hoxb9 (green) on nuclei from cells at 10 d of differentiation. Nuclei were counterstained with DAPI (blue). Bar, 5 µm. (D) Mean position (µm ± S.E.M.) of Hoxb1 and Hoxb9 relative to the MMU11 territory edge during 12 d of differentiation. The position of Rcn in the MMU2 territory is shown as control (Mahy et al. 2002a). n = 100 territories. Normalization of the data to the size of the nucleus showed that the Hoxb movements are not due to the increased size of the nucleus and CT upon differentiation [data not shown].
We conclude that in undifferentiated ES cells Hoxb1 resides within, but close to, the surface of its CT. Upon induction with RA, it loops out away from the CT with kinetics that parallel those of its transcription, so that when Hoxb1 expression is silenced after day 4, the frequency and extent of its looping decreases. However, it does not return to the same location as it was in before induction. Compared with Hoxb1, Hoxb9 is located further inside of the CT in undifferentiated cells and remains there immediately after induction. Looping out of Hoxb9 is not detected until day 10, and it never reaches the same extent as that seen for Hoxb1 at day 4. Therefore, unlike the coordinate looping of the MHC (Volpi et al. 2000), the induction of expression from the Hoxb complex results in a progressive remodeling and a looping out of the region from the CT, which parallels the temporal activation of genes in the cluster.

Table 1. Localization of Hoxb genes outside of CTs

<table>
<thead>
<tr>
<th>Gene</th>
<th>% signals outside of MMU11 CT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Undiff</td>
</tr>
<tr>
<td>Hoxb1 (MAA)</td>
<td>36</td>
</tr>
<tr>
<td>Hoxb1 (pFa)</td>
<td>15</td>
</tr>
<tr>
<td>Hoxb9 (MAA)</td>
<td>24</td>
</tr>
</tbody>
</table>

The percentage of Hoxb1 or Hoxb9 hybridization signals manually scored as being outside of the MMU11 CT by FISH to either MAA- or pFa-fixed nuclei from undifferentiated ES cells, and from cells after 4 and 10 d of differentiation. Undifferentiated cells that had been treated with TSA, and TSA-treated cells induced to differentiate with RA for 2 d, were also examined. N/D indicates not determined.

Hoxb1 moves toward the center of the nucleus upon induction

Looping could just be a passive consequence of chromatin decondensation, or it could represent the recruitment of the locus to a particular region of the nucleus or nuclear compartment. In the former case, there need be no directionality to the movement relative to the center or the periphery of the nucleus. In the latter case, there may be directional movement. To test this, we determined the distribution of Hoxb1 and Hoxb9 hybridization signals in images of nuclei eroded into concentric shells, from the edge (shells 1 and 2) to the center (shells 4 and 5) of the nucleus (Croft et al. 1999). A significant (P = 0.001) movement of Hoxb1 toward the nuclear center occurs at day 4 of differentiation, and by day 10, it has returned to its original nuclear location (Fig. 5). In contrast, there is a progressive movement of Hoxb9 toward the nuclear center between day 4 and 10 (P = 0.008; Fig. 5). There was no accompanying movement of the MMU11 CT in the same direction, suggesting that when HoxB loops out from the CT, it is specifically drawn toward the center of the nucleus.

Discussion

Changes in high-order chromatin structure and levels of gene expression

We have correlated the temporal pattern of gene expression from the Hoxb cluster with changes in nucleosome modification (Fig. 2), chromatin decondensation (Fig. 3), and an extrusion of HoxB genes out of the CT (Fig. 4). We detect an increase (by day 4) of histone H3 acetylation (at
K9, and methylation of H3 at K4, upon induction by RA. This is consistent with the known associations of these histone marks with states of gene activation (Lachner et al. 2003). However, the modifications are gained at both the transcriptionally activated \( \text{Hoxb}1 \) and at \( \text{Hoxb}9 \), which will remain silent for a further 6 d before its activation [Fig. 2]. Therefore, these histone marks seem to be associated with poising of the genes for expression, rather than expression per se. We detect little met\(_{\text{H3-K9}} \) at \( \text{Hoxb}1 \) or \( \text{Hoxb}9 \), no loss of met\(_{\text{H3-K9}} \) upon induction, and no acquisition of this histone modification when \( \text{Hoxb}1 \) expression ceases after day 4. This suggests that this mark of gene repression is not involved in the early regulation of \( \text{HoxB} \).

Although transcriptionally active chromatin is often considered to have an “open” chromatin structure, implying a decondensed chromatin structure, direct evidence for this has been lacking. Here we have shown for the first time that activation of an endogenous mammalian gene locus is accompanied by a visible approximation of histone acetylation at H3K9 (Roelen et al. 2002). The rapid response to RA in ES cells [Fig. 1B; Papalopulu et al. 1991], and the absence of met\(_{\text{H3-K9}} \) prior to induction, suggests that \( \text{Hoxb}1 \) is similarly poised for transcription in these cells. Because we have found that \( \text{Hoxb}1 \) is inside of its CT in undifferentiated ES cells [Fig. 4], we suggest that the looping out of decondensed chromatin from the territory represents the transcriptionally active state and not a poised state [Fig. 6].

The mechanisms that mediated chromatin decondensation and extrusion from CTs have yet to be determined. It has been suggested that histone modifications may directly influence higher-order chromatin structures, for example, by altering nucleosome–DNA or nucleosome–nucleosome interactions, and by neutralizing charge in the histone N-terminal tails (Tse et al. 1998; Wolffe and Hayes 1999; Carruthers and Hansen 2000, Wang et al. 2001). However, increasing the levels of histone acetylation at \( \text{HoxB} \) experimentally with TSA does not lead to the changes in high-order chromatin structure that normally occur at the locus upon differentiation. Therefore, we suggest that chromatin compaction and nuclear organization represent a level of chromatin structure that is not simply a reflection of underlying histone acetylation.

**Chromatin structure and the regulation of Hox gene expression**

In vivo, \( \text{Hoxb}1 \) expression is initiated in primitive streak mesoderm early in gastrulation [E7.2]; however, \( \text{Hoxb}1 \) and \( \text{Hoxb}2 \), but not more 5’ genes in the cluster, are poised to respond to exogenous RA before primitive streak formation (by E6.5; Roelen et al. 2002). In this regard, it is interesting to note that even in the nuclei of undifferentiated ES cells, the intrachromosomal organizations of \( \text{Hoxb}1 \) and \( \text{Hoxb}9 \) are different from each other. Whereas \( \text{Hoxb}1 \) is located at the CT surface, \( \text{Hoxb}9 \) is further inside the territory [Fig. 4D]. The proximity of \( \text{Hoxb}1 \) to the territory surface may contribute to its poised transcriptional state and may underlie its ability to respond rapidly and robustly to RA [Fig. 6]. It will therefore be interesting to analyze the nuclear organization of \( \text{Hoxb}1 \) when placed in inappropriate genomic locations (Kmita et al. 2000).
We propose a two-step model through which chromatin structure regulates Hoxb expression (Fig. 6). In response to RA, there appears to be a widespread alteration in histone modification and chromatin condensation that is not restricted to the most 3’ gene (Hoxb1). We suggest that this establishes a transcriptionally competent chromatin state. The progressive looping out of genes from the CT may then contribute to the temporal pattern of gene expression and be necessary to support high rates of transcription. This later step, but not the first one, has similarities to models that propose that a 3’ to 5’ opening of chromatin structure that leads to the sequential accessibility of Hox genes for transcription (Van der Hoeven et al. 1996, Kondo and Duboule 1999, Roelen et al. 2002).

Materials and methods

ES cells culture, differentiation, and TSA treatment

Undifferentiated OS25 ES cells were maintained on 0.1% gelatin-coated dishes in Glasgows’ minimal Eagle medium containing 10% fetal calf serum, nonessential amino acids, 1 mM sodium pyruvate, 0.3 mg/ml L-glutamine, 0.1 mM 2-mercaptoethanol, 1000 U/mL human recombinant LIF, and 100 µg/mL G418 (Billon et al. 2002).

To induce differentiation, 5 × 10^5 cells were plated in 25-cm² tissue culture flasks (Costar) without LIF or G418 for 1 d; 5 × 10^6 M RA was then added for 4 d. After 2 d (day 2 of differentiation) the cells were replated in 75-cm² flasks, and the RA-containing medium was supplemented with 2.5 µM Ganciclovir to select against undifferentiated cells for another 2 d (day 4). Subsequently, media was supplemented only with Ganciclovir (no RA) for eight more days until day 12. The medium was changed every 2 d during the differentiation protocol (Billon et al. 2002).

The 5 × 10^6 undifferentiated ES cells, maintained in medium supplemented with G418 and LIF, were treated with 160 nM TSA for 24 h.

Real-time RT-PCR

Twenty micrograms of total RNA, prepared by using Bio/RNA-X-cell [Bio/Gene Limited], were treated with 20 U Rnase-free DNase I [Promega]. After phenol/chloroform extraction, the RNA was ethanol precipitated in 1 v of 5 M ammonium acetate and dissolved in H₂O. Two micrograms of RNA were reverse-transcribed by using Superscript II Rnase H [Invitrogen] and 4 µL of Random Hexamers [50 µM, Amersham Pharmacia], and 1 µL was used as template for real-time PCR, using the DNA-intercalating SYBR Green reagent and Master mix [Bio-Gene], on a lightcycler [Roche Molecular Biochemicals]. Each reaction was performed in duplicate. PCR primers and conditions are described in Table 2. The 1:10, 1:100, and 1:1000 dilutions of β-actin PCR product of known concentration were used to produce a standard curve. The results were analyzed by Lightcycler software, version 3.5.

Chromatin immunoprecipitation

The ChIP protocol was modified from that of Christenson et al. (2001). ES cells were replated onto 10-cm tissue culture dishes. One day later, formaldehyde [Sigma], to a final concentration of 1%, was added to the medium for 10 min at room temperature. Cells were washed once in cold phosphate-buffered saline (PBS) before scraping in buffer A (85 mM KCl, 5.5% sucrose, 10 mM Tris at pH 7.5, 0.2 mM EDTA, 0.5 mM spermidine, 250 µM PMSF, and 1:100 protease inhibitor cocktail; Roche), containing

Table 2. PCR primers and amplification conditions

<table>
<thead>
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<th>Gene/locus</th>
<th>Primers</th>
<th>Product size (bp)</th>
<th>PCR conditions</th>
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<tbody>
<tr>
<td>RT–PCR</td>
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<tr>
<td>Oct4</td>
<td>GGCCTTCCTCTTPGAAGGCTGTC</td>
<td>312</td>
<td>15 min 95°C [30 sec 95°C, 30 sec 55°C, 30 sec 72°C] × 30</td>
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<tr>
<td></td>
<td>CTCGACAGATCCCTTTCTCT</td>
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<tr>
<td>Gapdh</td>
<td>CTTCTGCTGCTGAGCTCTCT</td>
<td>70</td>
<td>15 min 95°C [30 sec 95°C, 30 sec 60°C, 40 sec 72°C] × 25</td>
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<tr>
<td>Hoxb1</td>
<td>CCAGTCTCCGAGCAGCTTT</td>
<td>450</td>
<td>15 min 95°C [30 sec 95°C, 30 sec 62°C, 40 sec 72°C] × 35</td>
</tr>
<tr>
<td>Hoxb9</td>
<td>CCAGGAGGCTGCTGGCTTAT</td>
<td>176</td>
<td>15 min 95°C [30 sec 95°C, 30 sec 68°C, 40 sec 72°C] × 30</td>
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<tr>
<td>Hoxb13</td>
<td>CCTGACACAGCCTGCTTTT</td>
<td>445</td>
<td>15 min 95°C [30 sec 95°C, 30 sec 57°C, 40 sec 72°C] × 35</td>
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<tr>
<td>β-actin</td>
<td>GGTGAGAAGGGACTCTCTTGG</td>
<td>475</td>
<td></td>
</tr>
</tbody>
</table>

| ChIP       |         |                 |                |
| Hoxb1 r4 + promoter + initiation site | TCCCATCTCCCTGCTTACAG | 172 | 15 min 95°C [30 sec 95°C, 30 sec 64°C, 20 sec 72°C] × 25 |
| Hoxb1 3’DR2 | TAGAGGAGGCTTCTCGCTTT | 110 | 15 min 95°C [30 sec 95°C, 30 sec 61°C, 20 sec 72°C] × 25 |
| Hoxb1 exon 1 | CCATTATCCCGGAGCAGCAG | 104 |                |
| Hoxb1 promoter | TCCGCTCTGAGCTTCTGCTT | 80 |                |
| Hoxb9 promoter | TCCGAGAGCCAGGCTTCTCAG | 110 |                |
| Hoxb9 exon 1 | CCATCCCTGCTGCTGCTTCT | 112 |                |
For that. Coturniculus cayot. After centrifugation (2 min, times in buffer A, were added to the supernatant and incubated 1996), 5 µL anti-met2H3-K4 (Upstate Biotechnology, 07-212), antibody that recognizes the C terminus of H3 (Verreault et al. mM Tris at pH 8, 150 mM NaCl, and 0.5% Triton X-100) and active control (mock IP). was divided into aliquots of 150 µg DNA. One aliquot was kept aside (input), and one was incubated with no antibody as a negative control (mock IP).

Each test aliquot was diluted in 250 µL IP dilution buffer (100 mM Tris at pH 8, 150 mM NaCl, and 0.5% Triton X-100) and rotated overnight at 4°C, 2000 g. EDTA, 50 mM Tris at pH 8, and 250 µM PMSF and sonicated NaCl, wash buffer (10 mM Tris at pH 8.0, 500 mM LiCl, 1% NP40, and 1% deoxycholate), and TE (pH 8.0). One hundred microliters of TE (pH 8.0) with 500 µg/mL of RNaseA were added to the beads for 30 min at 37°C. Samples were eluted in 1% SDS and 0.1 M NaHCO3. NaCl was added to 200 mM and the mixture heated for 6 h at 65°C to reverse the formaldehyde cross-links. Samples were digested with 20 µg/mL proteinase K for 1 h at 55°C and the DNA purified by phenol/chloroform extraction and ethanol precipitation. The DNA pellet was re-suspended in 20 µL of sterile H2O, and 1 µL aliquots were used as templates in real-time PCR.

PCR on immunoprecipitated DNA

One microliter of immunoprecipitated DNA was quantified by real-time PCR. For PCR in the 3′DR2 region and exon 1, DNA-intercalating SYBR Green reagent and The Master Mix (Bio-Gene) was used. The QuantiTect SYBR Green PCR kit (Qiagen) was used for the Hoxb1 and Hoxb8 promoter. Each ChIP and each real-time PCR was done at least twice. Input sonicated DNA (150 µg) from ES cell lysate was diluted 1:10, 1:100, and 1:1000, and 1 µL amplified in parallel with the immunoprecipitated DNA (IP) to produce a standard curve. Each 10-fold dilution increased the Ct by about three cycles. For each amplification, the background corresponding to the mock IP was subtracted from the IP, and the specific enrichment was calculated from the standard curve.

FISH

Paint for mouse chromosome 2 [MMU2] was PCR-labeled with biotin-16-UTP as previously described (Mahr et al. 2002b). Biotin-16-UTP-labeled paint for MMU11 was purchased from Cambio. BACs 501s and 67L02 for Rcr and Pax6, respectively (Mahr et al. 2002b), were labeled with digoxigenin or biotin by nick translation. The 50 kb BAC MPP-4 [T. Jinks, M.T. Martinez-Pastor, and R. Krumlauf, unpublished] encompassing Hoxb1 (Fig. 1A) was labeled with digoxigenin-11-dUTP by nick translation. A plasmid containing Hoxb9 (P. Hunt and R. Krumlauf, unpublished) was labeled with digoxigenin-11-dUTP or biotin-16-UTP. Approximately 200 ng of paint and 150 ng BAC/plasmid were used per slide, together with 15 µg mouse Cori DNA (GIBCO BRL) and 5 µg ssDNA.

Two-dimensional analysis was as described previously (Croft et al. 1999; Mahy et al. 2002b). To preserve three-dimensional nuclear structure, ES cells grown on slides were washed three times in PBS before permeabilization in CSK buffer (100 mM NaCl, 300 mM sucrose, 3 mM MgCl2, 10 mM PIPES at pH 6.8, and 0.5% Triton X100) for 5 min on ice. After washing three times in PBS, slides were fixed with 4% paraformaldehyde in 20% glycerol/PBS and FISH as described previously (Kurz et al. 1996; Mahy et al. 2002a,b).

Image analysis

Two-dimensional samples were examined on a Zeiss Axioskop fluorescence microscope fitted with a triple band-pass filter (Chroma no. 83000). Gray-scale images were captured with a cooled CCD camera (Princeton Instruments Pentaxmax) and custom IPLab scripts. For three-dimensional analysis, a focus motor was used to collect image stacks at 0.5-µm intervals along the z-axis. Images were captured with a Xillig CCD camera.

Analysis of probe position relative to CTs in 2D was as previously described [Mahy et al. 2002a,b]. Because the size of the nucleus and the CTs increased upon differentiation, the locus to territory edge distance was also normalized by dividing it by the radius of the circle of equal area to that of the territory [Mahy et al. 2002b].

To calculate the d between two probes in two dimensions the hybridization signals were manually segmented and the xy coordinates of the centroid of each were determined. d was then calculated from d = [(x1 − x2)2 + (y1 − y2)2]. Calculation of d in stacks of three-dimensional images was similar. Briefly, thexyz coordinates of the centroid of each signal were determined. The distance between the two probes (or space diagonal) was the distance between two spots at opposite corner of a cuboid. dxyz = |a2 + b2 + c2|; a, b, and c are the length of the side of the cuboid.

Probe position relative to the nuclear periphery and the nuclear center was determined by erosion of the nuclear area into five concentric shells [1 through 5] of equal area from the periphery of the nucleus to the center [Croft et al. 1999]. The proportion of DAPI fluorescence and CT signal was calculated for each of the five shells, and the shell containing the gene signal was manually recorded. To do a t test for the gene probe, an arbitrary value was given to each shell [one at the periphery and five at the more center shells], and the number of probes located in each was multiplied by this value. To perform a t test for the CT position, the percentage of signal was multiplied by the mean radius for each shell. In a plane-projected sphere, the mean radius of random a point for each shell was calculated as 2/3(r12 + r22 - r32)/2[r12 - r32].

Acknowledgments

S.C. was supported by fellowships from La Ligue Contre le Cancer and the Wellcome Trust (GR071481). W.A.B. is a Centennial fellow of the James S. McDonnell foundation. We thank Austin Smith [Institute for Stem Cell Research, Edinburgh] for OS25 cells, and Robb Krumlauf [Stowers Institute, Kansas City] for Hoxb BACs and for stimulating our research in this area. The H3-C terminal antibody was a gift of Alain Verreault (Cancer Research UK, Clare Hall). We are grateful to Nick Hastic, Robin Allshire, and Richard Meechan for critical reading of the manuscript.
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References


