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Vertebrate *HoxB* gene expression requires DNA replication

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To study the relationship between DNA replication and transcription *in vivo*, we investigated *Hox* gene activation in two vertebrate systems: the embryogenesis of *Xenopus* and the retinoic acid-induced differentiation of pluripotent mouse P19 cells. We show that the first cell cycles following the mid-blastula transition in *Xenopus* are necessary and sufficient for *HoxB* activation, whereas later cell cycles are necessary for the correct expression pattern. In P19 cells, *HoxB* expression requires proliferation, and the entire locus is activated within one cell cycle. Using synchronous cultures, we found that activation of *HoxB* genes is colinear within a single cell cycle, occurs during S phase and requires S phase. The *HoxB* locus replicates early, whereas replication is still required for maximal expression later in S phase. Thus, induction of *HoxB* genes occurs in a DNA replication-dependent manner and requires only one cell cycle. We propose that S-phase remodelling licenses the locus for transcriptional regulation.

Keywords: chromatin domains/DNA replication/*Hox*/mouse/*Xenopus*

Introduction

It has long been thought that transcription and DNA replication might be mechanistically linked in eukaryotic cells to coordinate formation of chromatin with gene activity on replicating DNA and to avoid initiating DNA replication within transcribing genes (Wolffe, 1991). DNA replication is coupled to chromatin reformation and thus provides an opportunity for transcriptional reprogramming. Early evidence for a coupling of replication and transcription came from observations that transcriptionally active genes replicate early and vice versa (Goldman *et al*., 1984), and studies using genome-wide microarray analysis of transcription and timing of DNA replication in *Drosophila* and yeast have addressed the generality of this notion. Whereas, in yeast, no correlation was observed between replication timing and transcriptional activity (Raghuraman *et al*., 2001), the opposite result was obtained in *Drosophila* (Schübeler *et al*., 2002). DNA microinjected into human cells is also more likely to be transcribed in early rather than late S phase (Zhang *et al*., 2002). Thus, coregulation of DNA replication and transcription may have evolved in higher eukaryotes to coordinate cell proliferation and patterning during development.

In both invertebrates and vertebrates, cell differentiation can in some cases be uncoupled from proliferation (Harris and Hartenstein, 1991; de Nooij and Hariharan, 1995), whereas in other cases cell division is necessary to specify a change in cell fate (Ambros, 1999; Mennerich and Braun, 2001). DNA replication may provide a mechanism to link proliferation to such patterning decisions by activating or repressing transcription. Some *in vitro* experiments have suggested that this may be the case (Crowe *et al*., 2000). We have chosen to investigate this question *in vivo* in a developmentally regulated locus, the *HoxB* locus.

The *Hox* loci are contiguous sets of genes encoding homologous transcription factors involved in patterning of various tissues in all animals (Krumlauf, 1994). *Hox* gene transcriptional activation occurs in vertebrate embryos according to temporal and spatial colinearity, which dictate that activation timing and anterior boundaries of expression follow the order of the genes along the complex (Duboule and Morata, 1994; Krumlauf, 1994; Gaunt and Strachan, 1996). Many *cis* elements that regulate transcription of different *Hox* genes have been identified (for reviews, see Duboule, 1998; Gellon and McGinnis, 1998), but there is as yet no mechanistic explanation for colinearity. Although vertebrate *Hox* activation has been proposed to be controlled by proliferation, which would mediate progressive opening of the *Hox* locus (Duboule, 1994), this has not yet been tested. Nevertheless, cell division may be associated with *Hox* patterning (Ohsugi *et al*., 1997), and disrupting the somitic cell cycles of chick embryos produces homeotic defects (Primmett *et al*., 1989). Temporal colinearity may be an intrinsic feature of embryonic expression of *Hox* genes, since it also occurs, over the space of a week, on *HoxB* induction by retinoic acid (RA) treatment of pluripotent human NT2 cells (Simeone *et al*., 1990), although the relationship of this activation with cell proliferation is not known.

In this paper, we show that *Hox* gene activation is dependent on DNA replication in two different systems. We found that, in *Xenopus*, all *HoxB* genes studied are activated in a short time window at the mid-blastula transition (MBT). Inhibition of DNA replication before the MBT prevents *HoxB* activation, whereas inhibition one or two cell cycles after the MBT alters the *Hox* expression pattern. Using synchronous P19 cells induced by RA, we demonstrate that expression of the entire *HoxB* locus can occur within one cell-cycle period after stimulation and is sensitive to inhibition of DNA replication and that colinear activation of *Hox* expression occurs during S phase. We found that the *HoxB* locus replicates early, whereas colinear activation occurs later, implying that DNA replication licenses the locus for transcriptional control.

We propose that the domain structure and chromatin of the *HoxB* locus has evolved for coregulation of DNA replication and transcription, providing support that such coregulation, as implied from microarray analysis in *Drosophila*, also applies to vertebrates.

Results

HoxB genes are expressed between the MBT and gastrulation in a replication-dependent manner

In *Xenopus*, it has been observed that embryos exposed to aphidicolin at late blastula, mid-gastrula or neurula stages continue to differentiate and that the expression of various embryo-specific genes was unaffected by the arrest of DNA synthesis (Rollins and Andrews, 1991; Harris and Hartenstein, 1991). Although replication-inhibited embryos do not develop correctly, neural determination can still take place. We wished to precisely define the moment at which differentiation becomes independent of DNA replication, so we targeted the first cell cycles after the MBT (Figure 1A). *Xenopus* embryos undergo the first cleavage after 90 min and the following 11 synchronous divisions every 30 min (Newport and Kirschner, 1982a; Boterenbrood *et al*., 1983). Cleavage 12–13, 7 h after fertilization, is marked by an abrupt transition, the MBT, with an increase of the cell-cycle length and the onset of zygotic transcription (Newport and Kirschner, 1982a,b). Cell cycles 12, 13 and 14 last 40, 60 and 75–100 min, respectively, with increasing asynchrony (Satoh, 1977; Newport and Kirschner, 1982a; Boterenbrood *et al*., 1983; Masui and Wang, 1998). We used a combination of hydroxyurea and aphidicolin (HU-A) that rapidly and completely inhibits DNA replication in embryos (Harris and Hartenstein, 1991), which we added at short intervals around the MBT (Figure 1A). Figure 1B shows the increase in genomic DNA content due to DNA replication in embryos and shows that HU-A treatment inhibits DNA replication within 60–90 min. If HU-A is added 90 min before the MBT, embryos do not develop further and disaggregate a day later. However, morphological aspects of neurulation occurred normally if HU-A was added only 30 min before the MBT (Figure 1C), allowing the MBT cell cycle to take place (Figure 1B). Adding HU-A 30 min after the MBT allows development until the early tailbud stage, but the embryos are truncated and have less definition in the head, although they do have cement glands. Adding HU-A 2 h after the MBT permits tailbud progression, but head development is affected and embryos are curved (Figure 1C). Neural precursors can differentiate but not divide in such embryos, allowing generation of all types of neurones, although fewer in number (Harris and Hartenstein, 1991).

These data confirm and extend previous studies (Harris and Hartenstein, 1991; Rollins and Andrews, 1991) showing that extensive morphogenesis can occur in the absence of DNA replication in post-blastula embryos. We also show that HU-A added during early cleavages blocks the cell cycle and further development after the MBT and that very few cell cycles after the MBT, possibly only one, are sufficient to permit neural determination. Allowing DNA replication for a slightly longer time allows a dramatic increase in morphological development. Intervals of no more than one cell cycle are probably responsible for differences in patterning between groups of progressively later-inhibited embryos.

From these data, we expected that *HoxB* expression might occur if DNA replication is permitted for only one or two cell cycles after the MBT. Expression of the *Hoxb3–Hoxb9* domain in HU-A-treated and control embryos was measured by RT–PCR. Figure 1D shows that constitutively expressed ornithine decarboxylase mRNA did not significantly vary whether or not HU-A was present. In contrast, *HoxB* mRNA expression was induced from 12 h after fertilization (Figure 1D; data not shown), within the fourth cell cycle after the MBT. *HoxB* genes are partly (*Hoxb3* and *Hoxb5*) or normally (*Hoxb7–Hoxb9*) induced if DNA replication is inhibited after the MBT (HU-A added at 6 h 30 or 7 h 30), whereas all are repressed when HU-A is added during the early cleavage stage (5 h 30). Similar results were obtained from three experiments. We conclude that *HoxB* transcription occurs if the cell cycle corresponding to the MBT, or one cell cycle after, is permitted and that expression of *HoxB* genes occurs within a short window and does not require further rounds of replication.

We wondered whether this cell-cycle linkage and subsequent uncoupling is unique to *Hox* genes or whether other developmental genes are similarly regulated. We therefore looked at the expression of other genes activated around or shortly after the MBT. Figure 1D shows data for two of these: *DG70*, expressed at gastrulation with similar timing to *HoxB* genes; and *XSox17α*, involved in endoderm formation. The cell-cycle dependence of their expression represents the two situations we found (coupling or not). For *XSox17α*, addition of HU-A 90 min before the MBT does not prevent normal induction of gene expression, showing that some zygotic gene expression has no need for cell-cycle progression for its programming. However, *DG70* expression is greatly reduced if passage through the cell cycle at the MBT is blocked. Thus, in general, determination of gene expression affected by the cell cycle occurs very early in *Xenopus*: one or two cell cycles permits high level expression significantly later in development.

The experiment described above did not address whether *HoxB* expression was correctly localized. In normal tailbud embryos, *Hoxb1* is expressed in the hindbrain around the otic vesicle and in somites, and *Hoxb9* is expressed in the neural tube and in somites (Godsave *et al*., 1994). Spatial localization of *HoxB* expression in replication-inhibited embryos and controls is shown in Figure 2. Embryos in which DNA replication was inhibited by HU-A at 30 min (Figure 2B) or 90 min after the MBT (Figure 2C) express both *Hox* genes in the area where somites condense, whereas neither was detectable in the central nervous system (CNS), even though neural precursors are present (Harris and Hartenstein, 1991).

Induction of HoxB genes in embryonal carcinoma cells occurs in a period of time corresponding to one cell cycle and requires S phase

The induction of *HoxB* genes during early *Xenopus* development suggested a cell-cycle dependence of their expression. However, in embryos, due to asynchronous cell divisions among blastomeres after the MBT (Satoh,

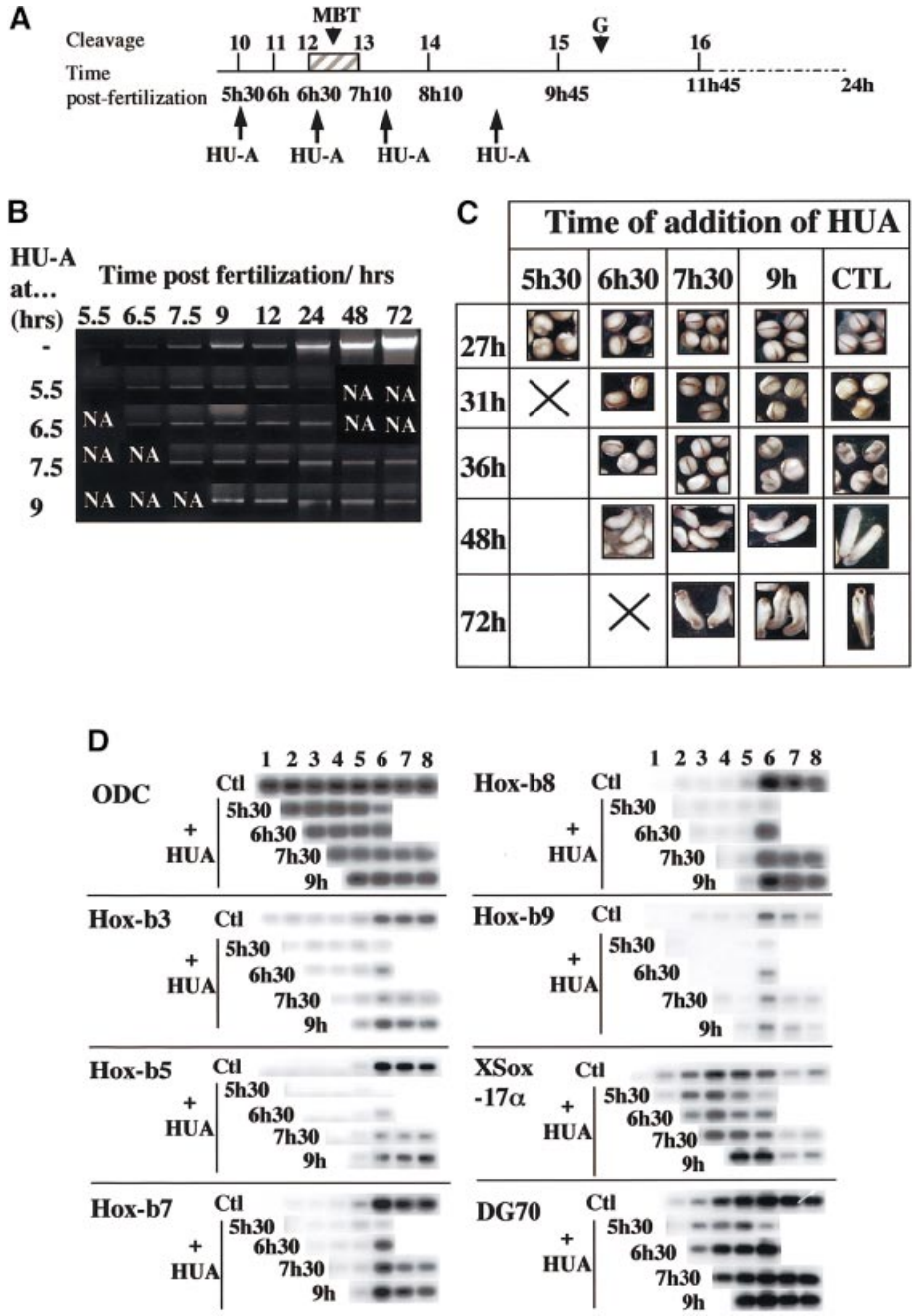

Fig. 1. Inhibition of DNA replication in *Xenopus* embryos and *Hox* expression. (**A**) Summary of this experiment: schema showing cell-cycle stages and timing during early *Xenopus* development and timing of hydroxyurea and aphidicolin (HU-A) addition. MBT, mid-blastula transition; G, onset of gastrulation. (**B**) HU-A was added to developing *Xenopus* embryos before or just after the MBT at the indicated times (left) and maintained throughout development. Genomic DNA was quantitatively prepared from embryos frozen during the time course at the indicated times (top), and DNAs from 0.5 embryo equivalents were loaded on a gel and analysed by gel electrophoresis and ethidium bromide staining. NA, not applicable, since these embryos (i) at these time points are from the control group and shown in the control lanes, thus no DNA was loaded in these lanes (left) or (ii) started to disaggregate by 24 h (right). (**C**) Phenotype of embryos photographed at the indicated times after fertilization. (**D**) Total *HoxB* expression in replication-inhibited embryos. At the indicated times from the five different time courses, five embryos were lysed for total RNA preparation, and semiquantitative RT–PCR and Southern blotting was performed for the indicated *Hox* genes, the ornithine decarboxylase gene (ODC) and the genes *XSox17*α and *DG70*. Time points were (1) 5 h 30, (2) 6 h 30, (3) 7 h 30, (4) 9 h, (5) 12 h, (6) 24 h, (7) 48 h and (8) 72 h.

1977), a strict relationship between cell-cycle progression and *Hox* expression cannot be established. We therefore used pluripotent embryonal carcinoma (EC) cells to further study this relationship.

Figure 3A shows the main characteristics of the mouse *HoxB* locus. RA can induce *Hox* expression in EC cells, and this activation occurs with temporal colinearity over the space of a week for *HoxB* genes in human NT2 EC cells (Simeone *et al*., 1990), suggesting that such cells may retain a similar *Hox* regulation to that of mouse embryos. We chose the mouse EC cell line P19, in which RA can stimulate differentiation from any point in the cell cycle

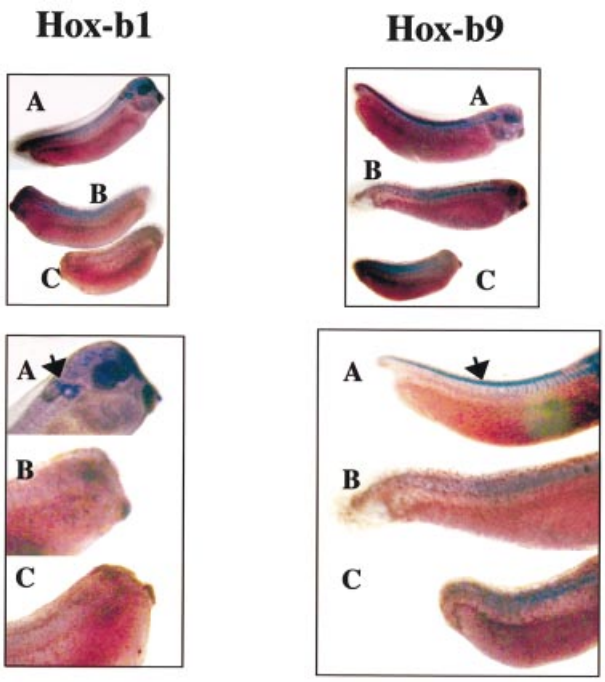

Fig. 2. *HoxB* expression in the CNS is disrupted in replication-inhibited *Xenopus* embryos. *In situ* hybridization (see Materials and methods) for *Hoxb1* (left) and *Hoxb9* mRNA (right) in normal (**A**) or replication-inhibited embryos to which hydroxyurea and aphidicolin was added at 9 h (**B**) or 7.5 h (**C**) after fertilization and maintained in the medium. Embryos were fixed at 72 h after fertilization. *HoxB* expression was fixed for all embryos after the same length of time of the staining reaction. The lower images are close-ups of the same embryos (left, A and C, and right B) or embryos treated in exactly the same way (left, B, and right, A and C) as those in the upper images. The arrows point to normal expression of the two *Hox* genes in the CNS (see text for details).

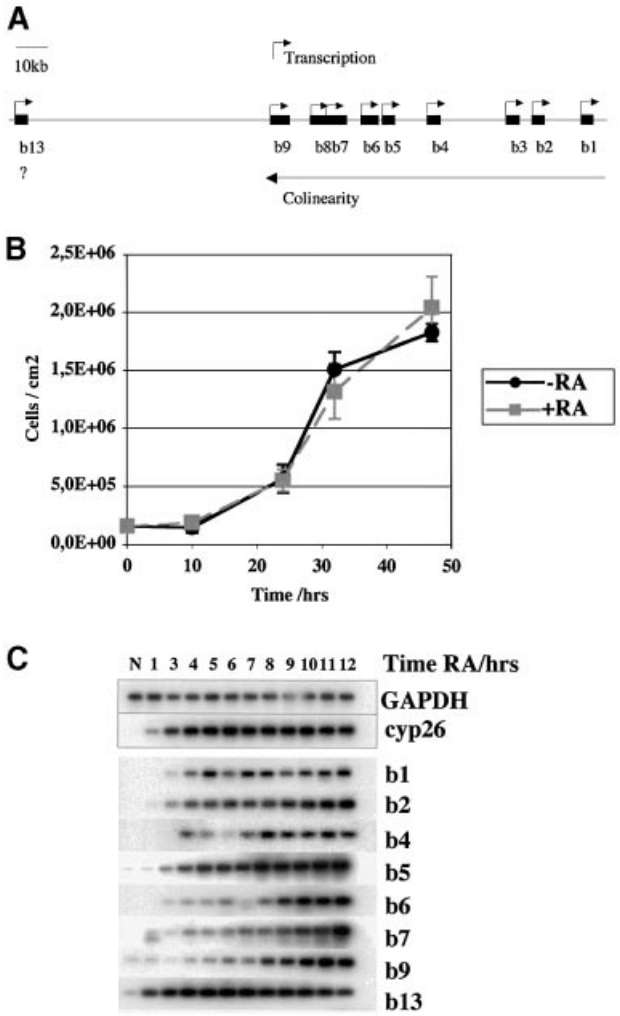

Fig. 3. Expression of all *HoxB* genes in P19 cells is activated during the first cell cycle after induction. (**A**) Schema showing the position, size, orientation and colinearity of gene transcription in the mouse *HoxB* locus. (**B**) Cell proliferation continues after retinoic acid (RA) stimulation. P19 cells were seeded at 10% confluence, incubated overnight and then either stimulated (+RA) or not (-RA) with 0.5 μM RA, and triplicate samples were trypsinized and counted. (**C**) *HoxB* activation occurs within one cell cycle. P19 cells were grown to early exponential phase, aggregated in suspension overnight, aggregates stimulated by RA, and extracts prepared during a 12 h time course for subsequent analysis by semiquantitative RT–PCR and Southern hybridization.

(Berg and McBurney, 1990) and induce *HoxB* expression. In P19 cells, *HoxB* genes were maximally induced at 0.5 μM RA (data not shown). The main questions we addressed were whether *HoxB* expression occurs in dividing cells and with what timing, whether it depends on cell division, whether P19 cells show temporal colinearity and, if so, whether this is related to the cell cycle.

Figure 3B shows that RA-treated P19 cells continue to proliferate for at least three cell divisions, at a similar rate to control cells (~10 h per cell cycle). Flow cytometry analysis of DNA content showed a similar cell-cycle distribution to controls (data not shown). Addition of RA rapidly induces the control *Cyp26* gene, encoding a cytochrome-p450 RA hydroxylase (Sonneveld *et al*., 1999), as expected (Figure 3C), whereas constitutively expressed *GAPDH* did not vary. Initiation of expression of the entire *HoxB* locus occurs within 4 h, and genes are well expressed within 9–10 h, thus in the time corresponding to less than one cell cycle (Figure 3C). Highest levels of expression were obtained after 24 h (data not shown; see also Figure 4A). We could not detect temporal colinearity under these conditions.

Since RA-activated *HoxB* expression requires less than one cell cycle, we asked whether it requires passage through the cell cycle by treating cells with 15 μM aphidicolin at the same time as RA. We verified by flow cytometry that aphidicolin blocks DNA replication in these cells (data not shown). A time course of activation of *HoxB* genes in the absence or presence of aphidicolin is shown in Figure 4. In untreated cells, *HoxB* mRNAs are not detectable, and after RA addition they become detectable after 1–3 h, increasing throughout the experiment to 48 h, apart from *Hoxb13*, which remains stable or decreases by 10 h (as in Figure 3B). In the presence of aphidicolin, *Hoxb1* and *Hoxb13* at the 3′ and 5′ ends of the domain (Figure 3A) are activated with normal timing, as is *Cyp26*. However, expression of the *Hoxb2–Hoxb9* genes was significantly reduced by aphidicolin treatment (Figure 4; see also Figures 6C, 9C and 10B). Quantification of these results is provided in Supplementary data

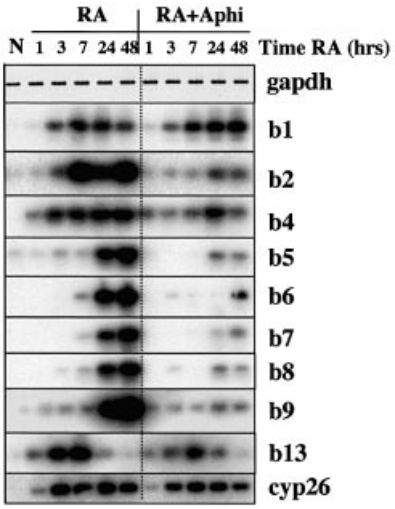

Fig. 4. Inhibition of DNA replication disrupts *HoxB* expression in P19 cells. P19 cell aggregates were treated with retinoic acid (RA) with or without aphidicolin (Aphi) and samples were lysed for semiquantitative RT–PCR at the indicated times and detected by Southern blotting using labelled probes for the relevant *HoxB* genes (abbreviated to b1, b2, etc.) and *Cyp26* (for *GAPDH*, staining of the gel is shown). No expression was detected without cDNA input in the PCR (data not shown). N, non-treated P19 cell aggregates.

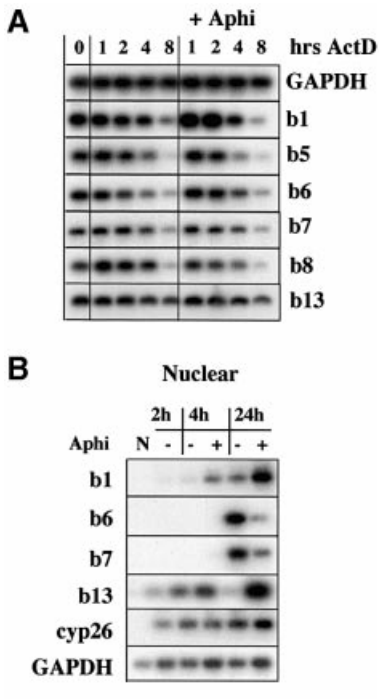

Fig. 5. Inhibition of DNA replication disrupts *HoxB* transcription in P19 cells. (**A**) Aphidicolin does not destabilize *HoxB* mRNA. P19 aggregates were stimulated by retinoic acid (RA) for 18 h and duplicate samples were incubated either with actinomycin D (ActD) alone (left) or actinomycin D and aphidicolin (+Aphi, right) for an 8 h time course. Samples were prepared for semiquantitative RT–PCR and Southern hybridization at the indicated times. (**B**) Experiment performed as in Figure 4, but cells were lysed for nuclear transcript purification, followed by semiquantitative RT–PCR and Southern blotting. N, non-treated P19 aggregates.

(available at *The EMBO Journal* Online). We also reproducibly observed a gradient of sensitivity to aphidicolin, with expression of the more internal domain *Hoxb6–Hoxb8* the most sensitive to inhibition of DNA replication, followed by *Hoxb4* and *Hoxb5*, then *Hoxb2* and *Hoxb9*, and finally *Hoxb1* and *Hoxb13* at opposite ends of the domain either weakly or not at all inhibited by aphidicolin (see also Figure 10B). The overall profile of this inhibition was similar in six separate experiments.

This inhibition of *HoxB* expression could be due to either mRNA destabilization in the cytoplasm or reduced nuclear transcription. To address this issue we performed two experiments. In the first, *HoxB* mRNA was induced by 18 h RA alone, then transcription was blocked by actinomycin D and the effect of aphidicolin on the stability of *HoxB* mRNA followed for 8 h. Figure 5A shows that aphidicolin had little or no effect on the stability of *HoxB* mRNA. We then analysed the effect of aphidicolin on accumulation of nuclear transcripts (Figure 5B; see also Materials and methods). Nuclear *HoxB* transcripts were absent in untreated P19 cells and detectable after RA treatment, as expected, whereas aphidicolin inhibits RA induction of the *Hoxb6–Hoxb7* domain (Figure 5B), but, interestingly, transcription of *Hoxb1* and *Hoxb13* are again not inhibited, but rather increased, by aphidicolin treatment in P19 cells.

The expression of central HoxB genes is induced in one cell cycle and depends on DNA replication

Aphidicolin blocks the DNA polymerase machinery (Sheaff *et al*., 1991) and thus blocks the cell cycle in S phase, consequently also preventing progression through the rest of the cell cycle in most of the cells of asynchronous populations. To see whether initiation of DNA replication is required for *HoxB* expression, a double block protocol was necessary. P19 cells were first pre-synchronized with 2 mM thymidine, released and then placed in the presence of RA with either aphidicolin, NG97, a cdk inhibitor (see Materials and methods) which we found blocks P19 cells at G_2–M (D.Fisher, unpublished data), or cycloheximide (CHX), which blocks protein synthesis and thus blocks the cell cycle in both G_1 and G_2 (Figure 6A). The three treatments block DNA synthesis by arresting the cells, respectively, at the G_1–S transition (+Aphi), or in G_1 and G_2 (+CHX) or at the G_2–M transition (+NG97) (Figure 6B), whereas aphidicolin does not block the G_2–M transition.

All three treatments significantly inhibited RA-induced expression of *Hoxb4*, *Hoxb5* and *Hoxb7* (Figure 6C). Thus, induction of *HoxB* requires initiation of S phase rather than mitosis, since cells can undergo mitosis in the presence of aphidicolin after release into G_2, yet *Hoxb4*, *Hoxb5* and *Hoxb7* expression is still inhibited. *Hoxb1* and *Hoxb13* are again less sensitive to inhibition of DNA synthesis, although they are sensitive to a block of the G_2–M transition (Figure 6C). We also reproducibly observed that *Hoxb13* was overexpressed in the presence of CHX. We conclude that RA induction of the more central *HoxB* cluster depends on DNA replication, whereas passage through mitosis is sufficient for induction of the two genes bordering this cluster (*Hoxb1* and *Hoxb13*).

Detection of temporal colinearity depends on synchronous DNA replication

In human NT2 cells, colinear *HoxB* activation occurred over the space of 1 week (Simeone *et al*., 1990). However,

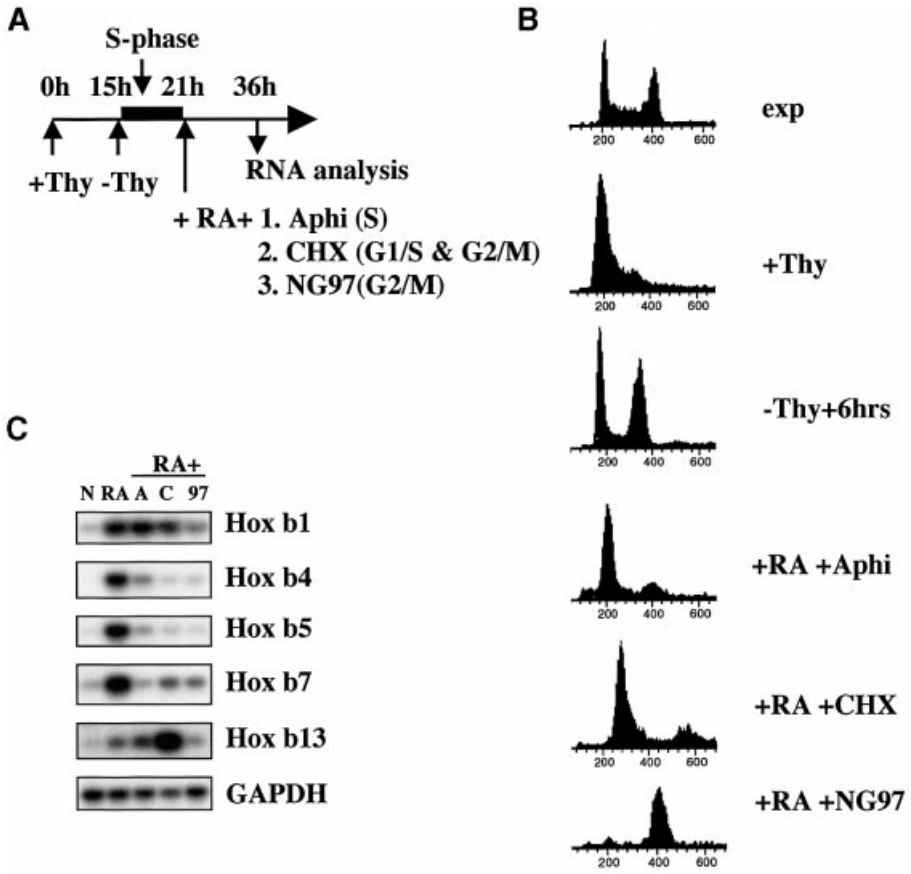

Fig. 6. Passage through DNA replication, rather than mitosis, is required for correct *HoxB* expression. (**A**) Exponentially growing (exp) P19 cell aggregates were pre-synchronized with 2 mM thymidine (+Thy) for 15 h and then released for 6 h (-Thy) before adding retinoic acid (RA) for a further 15 h, either alone or in combination with aphidicolin (Aphi), cycloheximide (CHX) or NG97. (**B**) Progression through the cell cycle was checked by flow cytometry. (**C**) Southern blots of semiquantitative RT–PCR products from the same experiment. N, non-treated aggregates; RA alone, or with aphidicolin (A), cycloheximide (C) or NG97 (97).

we failed to detect temporal colinearity in P19 cells, which activate the entire locus in a few hours, less than one cell cycle (Figure 3C). We found a dependence of *HoxB* expression on DNA replication. As such, timing of normal activation of *HoxB* genes could also be linked to DNA replication. If so, colinearity of expression might be masked in asynchronous populations.

To test this possibility, RA-induced *HoxB* expression was analysed in P19 cells synchronized by a double thymidine block (Figure 7A). Cells were released into G_2 and induced with RA, and progression through the cell cycle was followed by flow cytometry (Figure 7A). In these conditions, a striking colinearity of *HoxB* expression from *Hoxb1–Hoxb8* was unveiled (Figure 7B). *Hoxb1* is detectable 1 h after induction, and *Hoxb2–Hoxb8* is successively detectable 4–8 h after induction, corresponding to the time at which cells undergo S phase. *Hoxb9* may also follow temporal colinearity, but its constitutive basal level of expression partly masks it. *Hoxb13* did not follow colinearity. The colinearity of *Hoxb13*, although previously reported (Zeltser *et al*., 1996), has not been extensively studied. In mice, this gene is ~100 kb upstream of the main *HoxB* locus, which itself spans ~100 kb, and as such may belong to a different chromatin domain. This agrees with the fact that deletion of the entire locus from *Hoxb1* to *Hoxb9* does not affect transcription of *Hoxb13* (Medina-Martinez *et al*., 2000). We conclude that temporal colinearity of expression of the *Hoxb1–Hoxb9* locus does occur after RA induction. Furthermore, this temporal colinearity occurs within one single cell cycle. Finally, colinearity appears to be lost for *Hoxb13*.

Replication timing of the HoxB locus and timing of requirement for DNA replication

The observation that transcriptionally active genes replicate early in S phase and that non-expressed genes replicate later suggests a link between replication timing and transcriptional regulation in metazoans (Gilbert, 2002; Schübeler *et al*., 2002). We asked whether such a link could contribute to *HoxB* colinearity. The above results suggested that colinearity might be related to replication timing, in which case a progression of replication timing through the locus might be observed. Alternatively, colinearity may be revealed in synchronously dividing cells due to early DNA replication licensing the domain for transcriptional regulatory mechanisms, which determine colinearity independently.

This experiment was performed in exponentially growing monolayer P19 cells to analyse the undifferentiated state. Cells were labelled for 45 min with bromodeoxyuridine (BrdU), and after fixation they were sorted by FACS analysis into four different S-phase groups: early,

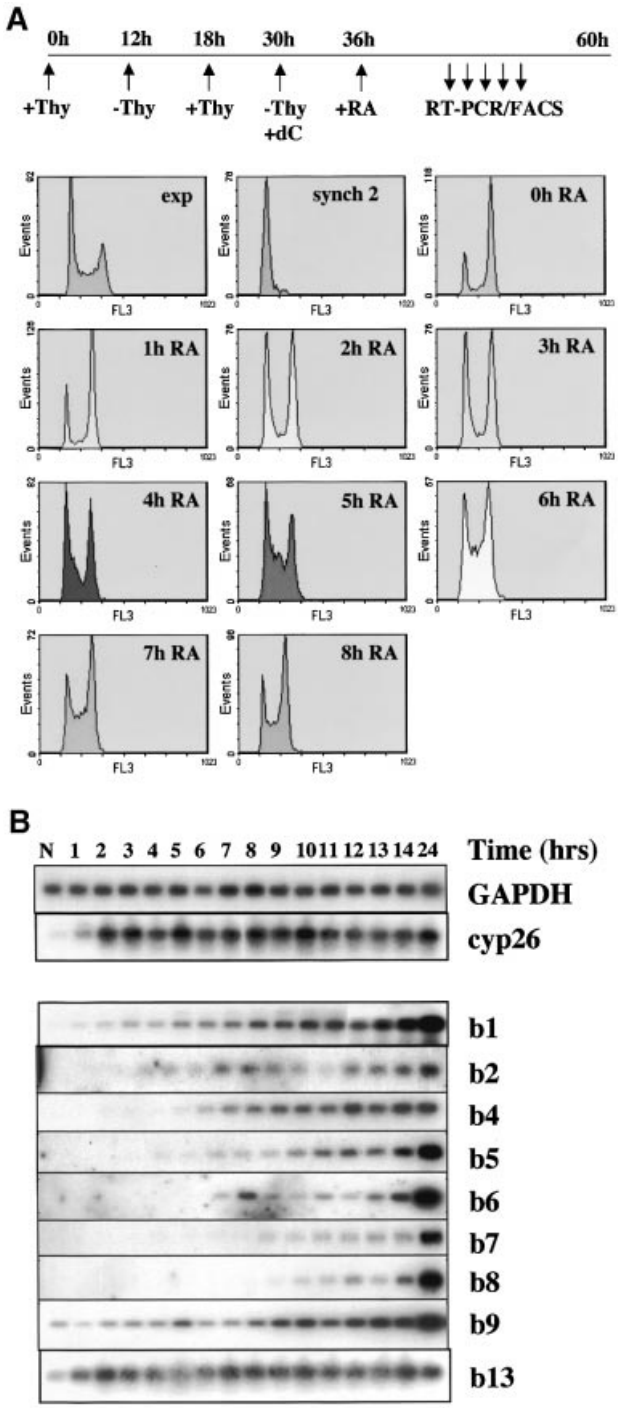

Fig. 7. Temporal colinearity of *HoxB* expression occurs within one cell cycle, and most *HoxB* genes are activated during S phase. Aggregates of P19 cells were synchronized by a double thymidine (Thy) block, as schematized in (**A**) and described in Materials and methods. Progression through the cell cycle was checked by flow cytometry. RA, retinoic acid. (**B**) Samples were taken at 1 h intervals for semi-quantitative RT–PCR and Southern blotting.

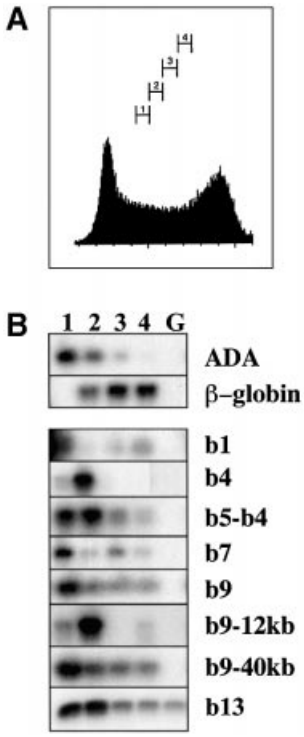

Fig. 8. The *HoxB* locus replicates early in untreated P19 cells. Exponentially growing monolayer P19.6 cells were treated with a 45 min bromodeoxyuridine (BrdU) pulse and fixed and sorted by flow cytometry (**A**) into four S-phase compartments (1–4). (**B**) Replicated DNA was recovered by anti-BrdU immunoprecipitation from these four samples (1–4) and the equivalent quantity of unlabelled P19 genomic DNA (G). Semiquantitative PCR was performed using primers corresponding to the *Hox* genes shown on the right (b1–b9), or a region of the *Hox* locus at a set distance from *Hoxb9* (–40 kb and –12 kb) or regions of the adenosine deaminase (ADA) and β-globin loci.

early–mid, mid–late and late (Figure 8A, pools 1–4). Genomic DNA was purified from sorted cells and control unlabelled cells, sonicated to 1 kb and used for immunoprecipitation with anti-BrdU antibodies to recover replicating DNA. As control loci, we used adenosine deaminase (ADA), expressed in all cells, and β-globin, expressed only in haematopoietic cells. Figure 8B shows that the entire *HoxB* locus replicates early: the strongest signal is obtained in either the early or early–mid compartments. There may be more than one replication origin, or perhaps replication forks move very slowly in this region, leading to differences in timing. The controls *ADA* and β-globin replicate early and late, respectively, as expected. We conclude that the *HoxB* locus is already organized as an early replicating chromatin domain in pluripotent exponentially growing P19 cells.

We wished to find out whether DNA replication of the *HoxB* genes themselves is necessary for their transcriptional upregulation, or whether the latter is tied to some other aspect of S phase. Because it is not possible to specifically abolish DNA replication of one locus, we wanted to see whether there is a correlation between replication timing and the moment at which DNA replication is dispensable for high levels of *HoxB* expression. If replication of the gene itself is required to stimulate gene expression, then, after it is replicated, inhibition of replication by aphidicolin should no longer affect the subsequent expression level. If, alternatively, some other aspect of S-phase biochemistry is required, there might be no such abrupt transition in aphidicolin sensitivity of gene expression. This could only be performed in synchronized

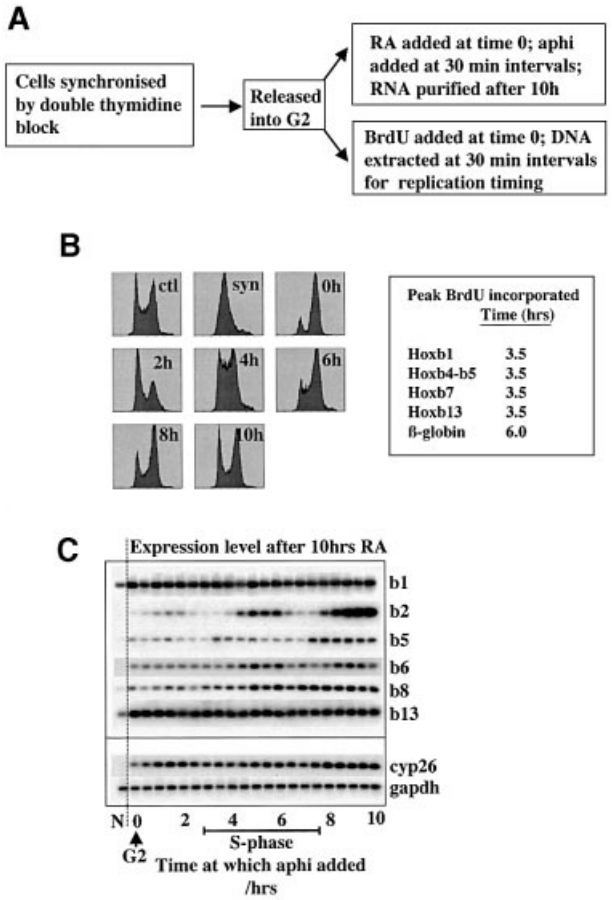

Fig. 9. S-phase transitions in dependence of *HoxB* expression on DNA replication. Parallel cultures of double thymidine synchronized P19 cell aggregates (syn) were released into G_2 for 6 h and retinoic acid (RA) was added, corresponding to time zero (0 h), to one population and bromodeoxyuridine (BrdU) to the other. The time course was then followed for 10 h. (**A**) Summary of experiment (aphi, aphidicolin). (**B**) Left: samples were also withdrawn and fixed for flow cytometry every 2 h to monitor cell-cycle progression (ctl, exponentially growing control cells). Right: summary of replication timing analysis for the loci shown, as determined by quantitative anti-BrdU chromatin immunoprecipitation and PCR, from parallel time course with samples every 30 min. (**C**) RA was added at 0 h to all samples; aphidicolin was then added to samples at half-hour intervals within the 10 h time course, and RNA was extracted from all samples at the end of the 10 h, for analysis of gene expression by semiquantitative RT–PCR and Southern blotting. N, non-RA-treated cells.

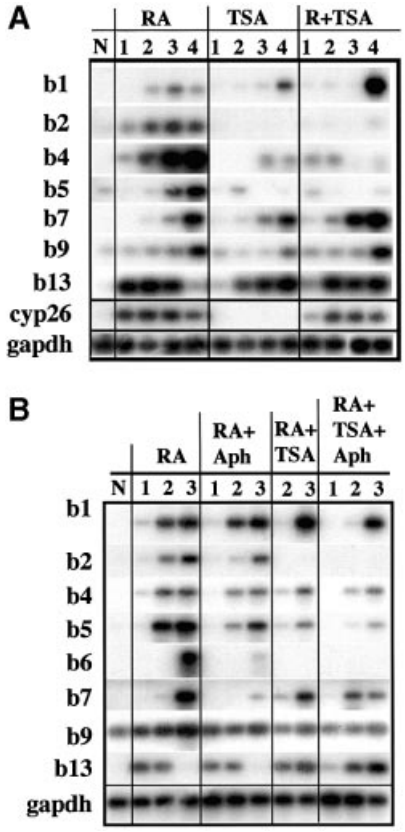

Fig. 10. Inhibition of histone deacetylation and methylation affects *HoxB* expression but does not mimic retinoic acid (RA) induction. (**A**) Southern blots of semiquantitative RT–PCR products from an experiment in which P19 aggregates were treated with RA alone, trichostatin (TSA) alone or both (R+TSA). N, non-treated cells. Time points were (1) 1 h, (2) 3 h, (3) 9 h and (4) 24 h. No products were detectable in the PCR blank (data not shown). (**B**) An experiment similar to that in (A) to test whether TSA could restore normal *HoxB* expression to P19 cells blocked by aphidicolin. Aggregates were treated with RA alone or in combination with aphidicolin (+Aph), TSA or both. Time points were (1) 2 h, (2) 8 h and (3) 24 h. N, non-treated cells.

cells, with replication timing analysed in parallel by BrdU labelling of parallel cultures. This experiment is presented in Figure 9A. RA was added to cells synchronized in G_2, and replication timing and the point at which aphidicolin is no longer effective at blocking subsequent *HoxB* expression was followed.

Figure 9B shows that S phase occurs from shortly after 2 h to between 6 and 8 h; this was confirmed by hybridizing genomic DNA from the parallel culture with anti-BrdU antibodies (data not shown). Replication timing analysis, by chromatin immunoprecipitation and quantitative PCR of BrdU-labelled DNA at half-hour intervals, confirmed that, as shown in Figure 8, the *HoxB* locus replicates early: peak values for all regions tested were from 3 to 3.5 h (Figure 9B, right; data not shown). In parallel samples, aphidicolin was added at half-hour intervals, and all samples were left until the end of the experiment, at 10 h after adding RA, before determining the final *Hoxb* expression level. For *Hoxb1*, *Hoxb13* and *Cyp26*, as expected, aphidicolin had no effect on final expression level, whereas for *Hoxb2*, *Hoxb5*, *Hoxb6* and *Hoxb8* there was a clear and abrupt transition, if not two transitions, in the level of *Hoxb* expression subsequently achieved (Figure 9C). This may correspond to sequential replication of the two alleles or, alternatively, to activation of transcriptional derepression at two specific points in S phase, one early and one late. Also, at either one or two points in S phase, if DNA replication is inhibited at this point the resulting *HoxB* expression is less than if replication is inhibited either earlier or later, which could be explained by an S-phase position-specific abolition of transcription coupled with a constant rate of mRNA degradation. Interestingly, this occurs just before the point at which DNA replication is required for the transition to a higher level of *HoxB* expression and is particularly intriguing in the light of the observed wave-like expression behaviour of *Hox* genes in embryos, which is dynamic,

covering one somite segmentation round of ~2 h (Zakany *et al*., 2001).

These experiments confirm that DNA replication plays an important part in *HoxB* expression, where polar repressor–enhancer interactions may be revealed by chromatin remodelling during DNA replication.

Inhibition of histone deacetylation and methylation affects HoxB expression but does not mimic RA induction

Histone acetylation is a general mechanism increasing chromatin accessibility that is regulated by the competing activity of histone acetylases and histone deacetylases. We analysed whether this regulation may contribute to *HoxB* expression. We observed that trichostatin (TSA), a histone deacetylase inhibitor, can partly induce the *HoxB* cluster, without RA addition (Figure 10A). Of this cluster, *Hoxb1*, *Hoxb7*, *Hoxb9* and *Hoxb13* are the most responsive to TSA, whereas *Hoxb2*, *Hoxb4* and *Hoxb5* are less sensitive to TSA induction. The control genes *GADPH*, a constitutively expressed gene, and the RA-responsive *Cyp26* are not affected by TSA (Figure 10A). We conclude that favouring histone acetylation with TSA is sufficient to induce the expression of some of the *HoxB* genes but does not reproduce RA induction. However, TSA probably does not mimic endogenous regulation of histone acetylation.

When coupled to RA induction (Figure 10), histone deacetylase inhibition increases the expression of the border genes of the cluster (*Hoxb1* and *Hoxb13*) but reproducibly inhibits the induction of the internal genes of the cluster (*Hoxb2* and *Hoxb4–Hoxb6*). This general effect is more pronounced if DNA synthesis is blocked by aphidicolin (Figure 10B). We conclude that inhibition of histone deacetylases, although it can act independently from RA induction, does not reverse the inhibition of *HoxB* expression by DNA synthesis inhibition. Nevertheless, this does not necessarily mean that RA-induced *HoxB* activation is dependent on DNA replication independently of changes in histone acetylation.

Another epigenetic modification that could influence *HoxB* expression is DNA methylation. We observed that 5′-azacytidine, a methylation inhibitor, alone does not activate any expression of *HoxB* genes, although it strongly enhances expression of the border genes of the cluster (*Hoxb1* and *Hoxb9*) in the presence of RA (data not shown). We analysed the global methylation status of five regions located between *Hoxb1* and *Hoxb13* using digestion by methylation sensitive (*Hpa*II) or insensitive (*Msp*I) enzymes. Although differences of methylation status of these regions were observed, this did not change on RA treatment (see Supplementary data). We conclude that RA-induced *HoxB* expression in P19 cells does not rely on global DNA demethylation of the *HoxB* locus.

Discussion

HoxB gene induction requires at least one cell cycle in both Xenopus development and mouse P19 cells

We report that inhibition of the cell cycle disrupts *HoxB* activation in two different embryonal model systems (*Xenopus* embryos and P19 EC cells) and provides evidence that *Hox* expression is linked to DNA replication.

In *Xenopus* embryos, we find significant differences in subsequent embryonic patterning using only small differences in timing of inhibition of DNA replication, which shows that patterning decisions in *Xenopus* are tied to the early cell cycles following the MBT. Arresting DNA replication at or before the MBT prevents activation of *Hox* genes, probably by blocking activation of the zygotic genome, whereas permission for DNA replication during MBT or the next cell cycle allows *HoxB* activation. However, *HoxB* expression in replication-inhibited embryos occurs only in somites and is not detectable in the CNS. This suggests that DNA replication may be required at a later stage for *HoxB* patterning of the CNS, even though neural precursors are present and can differentiate (Harris and Hartenstein, 1991).

Mouse P19 EC cells can act analogously as neural precursors (MacPherson *et al*., 1997), and we find that proliferation of these cells is also required for normal levels of *HoxB* activation. In aggregated P19 EC cells, RA stimulates neural differentiation, and cells continue to proliferate. All *HoxB* genes are activated in the first cell cycle after RA stimulation, and DNA replication is required for normal *HoxB* expression.

Hox gene expression and DNA replication: a license to express?

Temporal colinearity of *HoxB* expression in P19 cells is clear only when cells are synchronized within the cell cycle, suggesting that *HoxB* activation is linked to progression through the cell cycle. If *HoxB* genes are activated in a defined order during the cell cycle, each successive gene will be activated at the same time in all cells in synchronous cultures, which would explain why differences in timing of activation (i.e. temporal colinearity) are clearly visible.

The colinearity of expression of the *HoxB* domain within a single cell cycle has a striking relationship with its sensitivity to inhibition of DNA replication. Thus, the later the genes are expressed, the more they are sensitive to inhibition of DNA synthesis. The peak of sensitivity is for paralogues 6–8, whereas the border paralogues *Hoxb9* and *Hoxb13* at the 5′ end and *Hoxb1* at the 3′ end are less sensitive. All *HoxB* genes, other than *Hoxb13* (which does not obey temporal colinearity in these cells) and *Hoxb1*, are activated in S phase when stimulated in G_2. *Hoxb1* and *Hoxb13* are activated before S phase and thus not surprisingly do not require S phase for correct activation.

G_1–S progression is not sufficient for *Hoxb2–Hoxb9* expression, since aphidicolin, which specifically blocks DNA polymerase function, prevents *HoxB* expression in G_2-synchronized cells. This is not due to the requirement for RA itself to act at a particular cell-cycle stage, since a transient pulse of RA can induce neural differentiation from any phase of the cell cycle (Berg and McBurney, 1990), and in our experiments the control gene *Cyp26*, as well as *Hoxb1* and *Hoxb13*, are activated normally in replication-inhibited cells.

These results are compatible with current models for temporal colinearity. Colinearity of *HoxD* genes in the mouse embryo appears to be due in part to a polar release from transcriptional repression by a distal enhancer (Kondo and Duboule, 1999; Kmita *et al*., 2002).

Furthermore, *HoxB* activation along the anterior–posterior axis in chick embryos suggests that progressive opening of the *HoxB* locus occurs in all regions of the neural tube but genes are only expressed if *cis*-acting factors are present (Bel-Vialar *et al*., 2002). Thus, colinearity might be achieved by two component steps: regulated derepression of the locus by DNA replication, making it permissive to regulated expression of specific transcription factors.

Two recent papers demonstrate a differential regulation of anterior and posterior *HoxB* genes in the response to RA and FGF signalling (Bel-Vialar *et al*., 2002; Osterveen *et al*., 2003). FGF can stimulate expression of *Hoxb6–Hoxb9* partly through effects on *cdx* expression and partly by rendering this part of the locus more accessible to Cdx activation. Furthermore, in general, anterior *HoxB* transgenes integrated at ectopic locations recapitulate endogenous expression patterns, whereas posterior *HoxB* transgenes do not, suggesting that higher-order organization of the *HoxB* locus is required for posterior *HoxB* expression. Our experiments suggest a similar difference in accessibility of the chromatin to transcriptional activation, since posterior *HoxB* genes are more dependent on DNA replication than anterior genes. It would be interesting to know whether the synergy between FGF and *cdx* expression requires proliferation, and whether expression from ectopically integrated *HoxB* transgenes requires DNA replication.

We propose that the gradient of sensitivity to aphidicolin observed according to the position of *HoxB* paralogues on the chromatin domain is related to polar competition between transcriptional repressor and activator elements, in which DNA replication strongly favours expression of repressed chromatin by creating nascent DNA upon which new chromatin is formed or by restructuring the domain during S phase. In P19 cells the *HoxB* locus may be in a predetermined state, since it replicates early and is mainly unmethylated and activation of the entire locus is rapid, yet DNA replication is still required to allow normal *Hox* gene expression. Possibly, the *HoxB* locus in P19 cells is organized into chromatin in such a way that passage through a single cell cycle allows relief of repression over the entire locus in much the same way that a single DNA replication derepresses chromatin to allow long-range enhancer function in mouse embryos (Forlani *et al*., 1998).

In some vertebrates, cell-cycle lengths show an anterior–posterior gradient (long to short) (Sanders *et al*., 1993), and there is a caudal–rostral wave of initiation of *HoxB* expression in the neural tube in these embryos (Gaunt and Strachan, 1996). Furthermore, cell-cycle synchrony occurs in developing somites (Primmett *et al*., 1989), which also show waves of *HoxB* expression (Zakany *et al*., 2001). As such, the spatial and temporal organization of proliferation could provide a template for developmental gene expression in which staggered DNA replication along a developing axis relieves gene expression from transcriptional silencing.

Materials and methods

Xenopus embryos

Xenopus eggs were fertilized *in vitro*, dejellied in 2% cysteine and maintained in 0.2× MBSH at 23°C. A mixture of aphidicolin (150 μM, from 20 mg/ml stock in DMSO) and hydroxyurea (20 mM) was applied to sibling embryos at the times indicated. Whole-mount *in situ* hybridizations were performed according to Harland (1991), with modifications (Islam and Moss, 1996). The *Hoxb1* and *Hoxb9* clones used for preparing the probes were gifts from Jonathon Slack (Bath, UK). Genomic DNA was prepared by homogenizing four embryos frozen in liquid nitrogen at the time points indicated and, after thawing, in 0.3 ml of SETS (150 mM NaCl, 10 mM EDTA, 50 mM Tris pH 7.5, 0.5% SDS) and subsequent proteinase K/RNase/phenol treatment as described previously (Sambrook *et al*., 1989), carefully ensuring at each step that the maximum DNA was retained. DNAs from each sample were resuspended in 50 μl, and 6 μl were loaded on an agarose gel.

Cell culture and manipulation of P19 cells

P19.6 cells were a gift from Pierre Chambon (Strasbourg, France). Manipulation and induction of differentation were performed according to Rudnicki and McBurney (1987). RA was added to the culture medium at 0.5 μM. We verified RA dose dependence for *HoxB* expression and found no difference between monolayers and aggregates of P19 cells. Monolayers were used for the experiment in Figure 3B, in which quantitative recovery and counting of cells was essential, and in Figure 8, in which we wished to study cells as undifferentiated as possible, but aggregates were used in experiments involving *HoxB* expression analysis (all other experiments), since this is the standard procedure for neural differentiation of P19 cells. Aphidicolin was used at 15 μM, and cells were either pretreated for 1 h before RA or treated simultaneously with RA, as indicated. NG97 was a gift from Laurent Meijer (Roscoff, France) and was used at 10 μM. CHX was used at 1 μg/ml. Synchronous culture was performed by treating with 2 mM thymidine for 15 h, washing twice for 5 min with PBS and twice for 10 min with culture medium and releasing for 6 h into G_2. For the double thymidine block, after release into G_2, 2 mM thymidine was re-applied and the cells incubated for 15 h, before washing as above and release into thymidine-free medium containing 25 μM deoxycytidine. Cells were fixed for flow cytometry by trypsinizing ~10^6 cells, washing once with culture medium and twice with PBS, resuspending in 90% ethanol/PBS and storing at –20°C. Flow cytometry was performed by rehydrating the cells in PBS, washing three times in PBS/1%BSA, resuspending in 1 ml PBS/BSA with 100 μg/ml RNase A and 50 μg/ml propidium iodide and analysing using an EPICS or FACscan cytometer.

DNA replication timing

By cell sorting: 2×10^8 exponentially growing cells to which 50 μM BrdU was added for 45 min were fixed for flow cytometry; 60 000 cells were sorted from each of four S-phase compartments. By synchronous culture: 50 μM BrdU was added to synchronized P19 cell aggregates, and DNA was prepared from 10^6 cells at 30 min intervals using DNAzol (Invitrogen). DNA replication timing was analysed by semiquantitative PCR from substrates prepared by anti-BrdU (Becton Dickinson) immunoprecipitation exactly as described for the β-globin locus (Cimbora *et al*., 2000). A control sample contained an equal quantity of genomic DNA from P19 cells grown without BrdU.

RT–PCR analysis of gene expression

RNA was prepared from ~10^6 cells or five embryos using the SV RNA isolation kit (Promega). RT was performed using 2 μg of total RNA with Superscript II according to the manufacturer's instructions (Life Technologies). First-strand cDNAs were diluted to 100 μl, and 2 μl were used as substrates for PCR. Semiquantitative PCR was performed as follows. For each primer set, a mixture of all first-strand cDNAs for each experiment were subjected to 15–40 cycles of PCR to determine the optimum number of cycles for mid-exponential amplification (1 ng of product per 20 μl of reaction). This number of cycles was then performed on individual samples. Sequences of PCR primers are available on request. Products were separated by gel electrophoresis, Southern blotted with radioactive probes and quantified by PhosphorImager, using standard procedures.

Analysis of nuclear transcript levels

Aggregates of 10^6 P19 cells after different treatments were rinsed twice in cold PBS, and nuclei were isolated by osmotic lysis on ice in 2 ml of 10 mM Tris pH 7.5, 3 mM $MgCl_2$, 10 mM NaCl and 0.3% NP-40. After 5 min, cells were lysed by eight strokes in a Dounce homogenizer, centrifuged at 4°C for 10 min, washed twice in 20 mM Tris, pH 8.0, 140 mM KCl, 10 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM DTT and 20% glycerol and snap frozen in liquid nitrogen in 100 μl of 50 mM Tris pH 8.3, 5 mM $MgCl_2$, 0.1 mM EDTA and 40% glycerol. *In vitro* transcript extension

was performed on thawed samples by the addition of 125 µl of 2× reaction buffer (300 mM KCl, 5 mM $MgCl_2$, 10 mM Tris pH 7.9) and 25 µl of rNTPs (2.5 mM), with aphidicolin (15 µM) or the equivalent dilution of DMSO. After incubation at 30°C for 20 min, 10 µl of DNase was added and incubation continued at 37°C for 10 min. RNAs were prepared by stopping the reactions with 1.5 ml of Tri-reagent (Molecular Research Centre) and following the manufacturer's protocol. One-tenth of each RNA was used for RT, as above, and PCRs were normalized with respect to GAPDH.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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