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Long-range regulation by shared retinoic acid response elements modulates dynamic expression of posterior *Hoxb* genes in CNS development

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ABSTRACT

Retinoic acid (RA) signaling plays an important role in determining the anterior boundary of *Hox* gene expression in the neural tube during embryogenesis. In particular, RA signaling is implicated in a rostral expansion of the neural expression domain of 5' *Hoxb* genes (*Hoxb9–Hoxb5*) in mice. However, underlying mechanisms for this gene regulation have remained elusive due to the lack of RA responsive element (RARE) in the 5' half of the *HoxB* cluster. To identify *cis*-regulatory elements required for the rostral expansion, we developed a recombineering technology to serially label multiple genes with different reporters in a single bacterial artificial chromosome (BAC) vector containing the mouse *HoxB* cluster. This allowed us to simultaneously monitor the expression of multiple genes. In contrast to plasmid-based reporters, transgenic BAC reporters faithfully recapitulated endogenous gene expression patterns of the *Hoxb* genes including the rostral expansion. Combined inactivation of two RAREs, DE-RARE and ENE-RARE, in the BAC completely abolished the rostral expansion of the 5' *Hoxb* genes. Knock-out of endogenous DE-RARE lead to significantly reduced expression of multiple *Hoxb* genes and attenuated *Hox* gene response to exogenous RA treatment *in utero*. Regulatory potential of DE-RARE was further demonstrated by its ability to anteriorize 5' *Hoxa* gene expression in the neural tube when inserted into a *HoxA* BAC reporter. Our data demonstrate that multiple RAREs cooperate to remotely regulate 5' *Hoxb* genes during CNS development, providing a new insight into the mechanisms for gene regulation within the *Hox* clusters.

Introduction

Hox genes play a fundamental role in determining positional identity along the anterior–posterior body axis during embryogenesis (Krumlauf, 1994; Mallo et al., 2010). Precise control of the spatio-temporal domain of expression is crucial for the function of these genes. In mammals, 39 *Hox* genes are present in four genomic clusters, and within each cluster the expression pattern of Hox genes generally follows colinearity whereby the position of genes in the cluster is correlated with their temporal and spatial expression pattern along the body axis (Kmita and Duboule, 2003). Generally, *Hox* genes in 3' regions of clusters are transcriptionally activated earlier and expressed in more anterior regions of the developing embryo than their 5' counterparts. In this study, we define posterior or 5' *Hox* genes as those of paralog groups 5–13 and anterior or 3' *Hox* genes as those of paralog groups 1–4. Regulatory constraints have been postulated to be a contributing factor in maintaining the organization of *Hox* clusters during vertebrate evolution. It has been demonstrated that some aspects of *Hox* gene regulation depend on this clustered organization to act on multiple genes through the use of enhancer sharing (Gould et al., 1997; Sharpe et al., 1998; van der Hoeven et al., 1996) or long-range regulation (Duboule, 2007; Spitz et al., 2003). For example, in the *HoxD* cluster, distal enhancers residing far away from the cluster control multiple *Hoxd* genes during development of limbs, genitals and other organs (Andrey et al., 2013; Tschopp and Duboule, 2011). Clustered organization is also important for proper *Hox* gene regulation along the main body axis as some neural and somites enhancers are shared by neighboring *Hoxb* genes and elements can work over a long range (Sharpe et al., 1998; Valarché et al., 1997).

During gastrulation, *Hox* genes are sequentially activated in the primitive streak and their expression domain expands rostrally to

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establish anterior expression boundary in the neural tube and mesoderm (Alexander et al., 2009; Deschamps and van Nes, 2005). Establishment and maintenance of *Hox* gene expression is controlled by major signaling cascades, such as FGFs, Wnts and retinoic acid (RA) in combination with transcriptional and epigenetic regulatory mechanisms (Alexander et al., 2009; Young et al., 2009). In the nervous system, RA signaling plays an important role in setting the anterior boundaries of *Hox* gene expression during hindbrain segmentation and in the spinal cord during embryogenesis (Hernandez et al., 2007; Linville et al., 2004; Rhinn and Dolle, 2012; Sirbu et al., 2005; Tümpel et al., 2009). RA signaling is mediated by RA receptors (RARs), which form heterodimers with RXRs and bind to consensus DNA sequences called RA response elements (RAREs) in the genome to modulate transcription of target genes (Duester, 2008; Rhinn and Dolle, 2012). Multiple RAREs have been identified within the vertebrate *Hox* clusters and regulatory studies have demonstrated that they play diverse roles in regulation of immediately adjacent *Hox* genes (Dupé et al., 1997; Gould et al., 1998; Mainguy et al., 2003; Nolte et al., 2003, 2013; Studer et al., 1998, 1994). In addition, several RAREs have been found in the 3' flanking regions of the *HoxA* and *HoxB* clusters within enhancers with the potential to regulate multiple *Hox* genes during cardiac and endoderm development (Bertrand et al., 2011; Nolte et al., 2013; Soshnikova et al., 2013).

While initial patterns of expression and anterior boundaries are often maintained during later development for some *Hox* genes, others undergo dynamic changes in levels of expression or shifts in expression boundaries (Alexander et al., 2009; Deschamps and van Nes, 2005). For example, three mouse 5' *Hoxb* genes (*Hoxb5*, *Hoxb6* and *Hoxb8*) display similar dynamic shifts in their anterior boundaries of neural expression between at E9.5 and 11.5 (Oosterveen et al., 2004). This rostral shift is dependent upon RA signaling, as it is not observed in mouse embryos lacking the retinoic acid-synthesizing enzyme retinaldehyde dehydrogenase 2 (Oosterveen et al., 2003a). However, no RARE, which could account for this dynamic shift, has been identified in the 5' half of the *HoxB* cluster (Mainguy et al., 2003). This raises the possibility that RA could control these rostral shifts in domains of expression either over a long range via remote RAREs or indirectly through intermediates, such as the *Cdx* genes or Wnt signaling as observed in axial elongation (Young et al., 2009).

RAREs have been found 3' of both the *Hoxb4* and *Hoxb5* genes. The RARE 3' of *Hoxb4* functions as an early neural enhancer (ENE) to initiate *Hoxb4* expression, which is maintained in later stages through auto- and cross-regulatory inputs (Gould et al., 1998, 1997). This RARE-dependent enhancer is also shared with the adjacent *Hoxb3* gene (Gould et al., 1997). An RARE was identified 3' of *Hoxb5* in the DE neural enhancer which regulates expression of both *Hoxb5* and *Hoxb4* (Oosterveen et al., 2003a, 2003b; Sharpe et al., 1998). Regulatory analysis of the *Hoxb8* gene suggested that elements important for proper domains of expression maybe located in more distal regions, adjacent to the *Hoxb5* gene (Oosterveen et al., 2003a; Valarche et al., 1997). The presence of these shared RARE-dependent enhancers suggests a hypothesis that the DE- and ENE-RAREs may work together over a long range to modulate the expression of multiple 5' *Hoxb* genes in the developing nervous system.

To test the possibility that the DE- and ENE-RAREs may act on several genes through enhancer sharing or long-range regulation, we developed a recombineering technology to serially label multiple genes with different reporters in a single bacterial artificial chromosome (BAC) vector containing the mouse *HoxB* cluster. Mutations of the RAREs in the BAC reporter lines and the endogenous locus altered the expression of multiple 5' *Hoxb* genes. Our results identify the position of highly conserved *cis*-elements that mediate the responses of the *Hox* clusters to retinoids and demonstrate that enhancers embedded in the cluster can work over a long-range on multiple genes. This shared regulatory mechanism is likely to be a contributing factor in maintaining the clustered organization.

Materials and methods

Mouse strains and RA gavage

Hoxb1-b9$^{\Delta}$ and *Zp3-cre* mice have been described previously (de Vries et al., 2000; Ramirez-Solis et al., 1995). To generate the targeting construct for DE-RARE, a 1.6 kb left homology arm (LHA) and a 2.1 kb right homology arm (RHA) were amplified with the following primers using a mouse BAC clone (#RP23-196F5) as a template: 5'-GACGTCTGCTCTTTGACCTGTAG (LHA forward primer), 5'-GCGGCCGCTTGATGAGATATGAGCGTTGA (LHA reverse primer), 5'-TGCGAGCGCCGGCCTATGTG (RHA forward primer) and 5'-CATATGTCTAGGAACTAGGGGTCTG (RHA reverse primer). LHA and RHA were sequentially subcloned upstream and downstream, respectively, of a *NeoR* cassette flanked by LoxP sites in pGEM-T Easy vector (Promega). The targeting construct was linearized by *Aat*II/*Nde*I digestion, ethanol precipitated and electroporated into W4 (129S6/SVEvTac) ES cells. G814-resistant clones were screened by genomic PCR in which the forward primer (5'-TCTTGGAGAAACAAGCCCTAGT) anchors upstream of LHA and the reverse primer (5'-AAGGAACCCGCGCTATGAC) within the *NeoR* cassette. Correct targeting was further confirmed by amplifying and sequencing a PCR product spanning across RHA with the following primer pair: 5-TTTCCGCCTCAGGACTCTTC (forward) and 5'-TCTAGCACTGACCCAAAGTC (reverse). One correctly targeted clone was injected into C57BL/6 blastocysts to generate chimeric males which were subsequently crossed with C57BL/6 females for germline transmission. In order to remove the *NeoR* cassette from the targeted allele, heterozygous *DE-RARE*^{neo} mice were crossed with *Zp3-cre* mice to obtain *DE-RARE*^{neo}*;Zp3-cre* females, which were then crossed with C57BL/6 males resulting in mice heterozygous for the *DE-RARE*$^{\Delta}$ (*DE*$^{\Delta}$) allele in which DE-RARE is replaced with a LoxP site.

For *in utero* RA treatment, a 25 mg/ml stock of all-trans retinoic acid (Sigma) dissolved in dimethyl sulfoxide (Sigma) was mixed with vegetable oil and administered to pregnant mice by oral gavage at a dose of 20 μg/g body weight and embryos were harvested 8 h after the gavage.

For transgenic reporter analysis, plasmid or BAC DNA constructs were linearized and injected into C57Bl/10JxCBA-F1 one-cell embryos to generate either transgenic founder lines or transient transgenic embryos. Established transgenic lines were maintained in C57Bl/10JxCBA background.

All experiments involving mice were approved by the Institutional Animal Care and Use Committee of the Stowers Institute for Medical Research (Protocol 2013-0114).

HoxB BAC recombineering

The mouse *HoxB* BAC clone RP23-196F5 was modified by the bacterial recombination technology (Copeland et al., 2001; Lee et al., 2001) to trim off its ends, introduce an insulator and label multiple *Hoxb* genes with different reporters. The LoxP variant sites, which become inactivated after single Cre-mediated recombination, were utilized for sequential modifications of the BAC (Branda and Dymecki, 2004; Lee and Saito, 1998; Parrish et al., 2011). The sequences of homology arms used for each recombination step are listed (Table S1).

The 1.2 kb chicken β-globin insulator named *cHS4* (Pikaart et al., 1998) was amplified by PCR using chicken genomic DNA as template with the following primers: 5'-GGTAC CACGGGGAGAGCCCCCCCCCAAAG and 5'-GGATCCAATATTCCCCCATCCTCACTGACTC. After sequencing, *cHS4* was cloned downstream of the *NeoR* cassette flanked by a

LoxP variant site to make *NeoR-cHS4*. Subsequently, SceI homology arm (HA) and *Hoxb9* upstream HA were cloned upstream and downstream of *NeoR-cHS4*, respectively. Recombineering with the resulting construct and subsequent removal of *NeoR* after *Cre* induction replaced a LoxP511 site of the pBACe3.6 backbone vector with *cHS4*. In addition, 43 kb at the 5' end of the initial *HoxB* genomic region was removed leaving the 24.6 kb right upstream of *Hoxb9*.

The second round of recombineering was performed with a construct in which a *TetR* cassette is followed by a LoxP and *Hoxb1* downstream HA. Recombination with this construct and following removal of the *TetR* cassette with *Cre* induction lead to elimination of the partial *TetR* and the *sacBII* gene from pBACe3.6. The resulting BAC with a 136.9 kb region covering the *HoxB* cluster and a copy of *cHS4* at its 5' end was named as B137.

Different reporter genes were sequentially inserted in-frame into the first coding exon of *Hoxb9*, *Hoxb7*, *Hoxb5* and *Hoxb4* by recombineering. Among the reporters, Cerulean is a variant of CFP and its coding sequences are 97.8% identical to those of eGFP potentially causing unwanted recombination events during multiple rounds of recombineering. Therefore, 31 silent mutations were introduced into *Cerulean* so that at least one mismatch is present in every 30 bases when compared to *eGFP*. In addition to the reporters, sequences encoding FLAG and myc were added to the 3' end of *Hoxb8* and *Hoxb6*, respectively. To generate plasmid constructs, a 17 kb *Sbf*I fragment and a 15 kb *Eco*RV fragment containing *Hoxb7-LacZ* and *Hoxb9-mCherry*, respectively, were isolated from the labeled B137 and cloned into pGEM-T Easy.

Serial deletions of B137 were performed using recombineering constructs which contains *Hoxb1* downstream HA at 3' end and the following HAs at 5' end: b4u for B72, b4d for B84, b3u for B95 and b3u2 for B102.

For seamless mutagenesis of DE- and ENE-RARE, wild-type sites were first replaced with a *sacB-NeoR* cassette (Muyrers et al., 2000). The second recombineering was performed with constructs identical to the wild-type sequence except the point mutations located in the middle.

HoxA BAC recombineering

Both ends of the mouse *HoxA* BAC clone RP23-329P3 were modified by similar recombineering strategies similar to those used for the *HoxB* BAC. First, using the *NeoR-cHS4* cassette flanked by SceI and *Hoxa1* downstream HA, the LoxP511 site and the 3' 20 kb of the *HoxA* region in the original BAC was replaced with *cHS4* leaving the 23.2 kb downstream of *Hox1a*. The second round of recombineering was performed with a construct in which a *TetR* cassette is followed by a LoxP and *Hoxa13* upstream HA to eliminate the partial *TetR*, the *sacBII* gene and 5' 29.1 kb of the *HoxA* region leaving the 23.4 kb upstream of *Hoxa13*. The resulting BAC with a 152 kb region covering the *HoxA* cluster and a copy of *cHS4* at its 3' end was named as A152. Subsequently, *LacZ* with a polyA signal from the bovine growth hormone gene, *eGFP*, *H2A:mCherry* and *H2B:Cerulean* were sequentially inserted in-frame into the first coding exon of *Hoxa2*, *Hoxa1*, *Hoxa7* and *Hoxa9*, respectively, by recombineering (Table S1). The 137 bp fragment containing DE-RARE (underlined) and adjacent sequences (5'-ACCAAAGCTATTCTC—CGGGATCACGCAGAGGTCAGCAGACGGGGGT) was inserted into the *Hoxa5-Hoxa4* intergenic region using a54 left HA and a54 right HA together with a Lox variant site 5' to the element.

β-Gal staining, in situ hybridization and imaging

To detect β-galactosidase activity, embryos were fixed in either 0.1% paraformaldehyde/0.2% glutaraldehyde for 10–60 min on ice. After several washes in phosphate buffered saline (PBS), samples were stained in X-Gal for 6–18 h at 4 °C or at room temperature. Whole-mount *in situ* hybridization was performed with embryos fixed in 4% PFA overnight according to standard protocols using DIG-labeled anti-sense riboprobes. Fluorescent images were obtained by the Leica M205 Stereomicroscope (Leica Microsystems).

Embryo dissection and real-time PCR

The anterior neural tubes from the midbrain–hindbrain boundary to the level of the third dorsal root ganglion (C3) were dissected out from wild-type and mutant embryos in PBS. For gene expression analysis, total RNA was isolated from 3 to 5 dissected neural tubes were pooled for total RNA extraction using Trizol (Life Technologies). Real-time PCR was performed using High Capacity RNA-to-cDNA, TaqMan Gene Expression Master Mix and FAM-labeled probes according to the manufacturer's protocol (Life Technologies). PCR results were analyzed with the SDS 2.3 and DataAssist softwares (Life Technologies) using *ActinB* and *GAPDH* as controls.

Results

5′ Hoxb genes undergo rostral expansion in their neural expression domain

We performed *in situ* hybridization with mouse embryos to carefully monitor and verify dynamic temporal changes in the neural expression domain of 5' *Hoxb* genes (Fig. 1A). As previously reported (Oosterveen et al., 2004), our *in situ* data indicated that *Hoxb5*, *Hoxb6* and *Hoxb8* undergo dramatic rostral expansion of their expression domains in the developing neural tube between E10.5 and E11.5 (Fig. 1A). In addition, *Hoxb7* displayed a rostral expansion with an anterior boundary similar to that of *Hoxb8*. While *Hoxb9* also showed a rostral expansion the degree of the shift was much less than observed for the other 5' *Hoxb* genes. There is a colinear order in the respective anterior boundaries of expression of these 5' *Hoxb* genes at E10.5 which is maintained after the rostral shift (Fig. 1C). The fact that all 5 of the *Hoxb* genes display relatively similar dynamic shifts in expression suggests that this may be mediated by a common *cis*-regulatory mechanism.

The *HoxA* cluster is closely related to the *HoxB* cluster and contains a similar set of *Hox* paralogs. Therefore, we also examined the expression pattern of 5' *Hoxa* genes for comparative analysis (Fig. 1B). *Hoxa5* and *Hoxa6* also displayed a rostral expansion between E10.5 and E11.5 and the anterior boundaries of expression of these genes were similar to their *Hoxb* paralogs at both stages (Fig. 1C). In contrast, *Hoxa7* and *Hoxa9*, did not show any significant change in their neural expression boundary over this time period. Consequently, *Hoxa7* and *Hoxa9* have anterior expression boundaries more posterior than those of *Hoxb7*, *Hoxb8* and *Hoxb9* at E11.5 indicating a difference in the regulatory landscape between the two clusters.

Multiplexed reporters in *HoxB* BAC transgenic mice recapitulate endogenous expression patterns during the CNS development.

As a first step to identify *cis*-elements responsible for establishing the correct expression boundaries of 5' *Hoxb* genes and their dynamic rostral shifts in the neural tube, we utilized a BAC clone, B137, which contains all the *Hoxb* genes except *Hoxb13*, plus 24.6 kb 5' to *Hoxb9* and 16.2 kb 3' to *Hoxb1* (Fig. 2A). To simultaneously monitor the expression of multiple *Hoxb* genes in transgenic embryos carrying this BAC, it was sequentially modified through recombineering (Parrish et al., 2011) so that *mCherry*, *LacZ*, *eGFP* and *H2B:Cerulean* reporters were inserted in-frame into the first coding exon of *Hoxb9*, *Hoxb7*, *Hoxb5* and *Hoxb4*, respectively (Fig. 2A). Sequences encoding FLAG and myc epitopes were

also added to the 3' end of *Hoxb8* and *Hoxb6*, respectively, to permit detection of these proteins by antibodies. In addition, to minimize the positional effect due to random integration of transgenes into the genome and the potential cross regulation among concatemerized transgenes, the 1.2 kb insulator from the chicken *β-globin* locus (Pikaart et al., 1998) was introduced into the 5' end of the B137 multiplexed reporter construct.

Analyses of independent transgenic mouse lines harboring the modified B137 ($N=6$) indicated that *LacZ* and fluorescent reporter expression mimics the endogenous expression patterns of *Hox* genes in the neural tube (Fig. 2B). These B137 transgenic mice were crossed with a line harboring a deletion of the *HoxB* cluster (*Hoxb1-b9*$^{\Delta}$) which enabled us to monitor the expression of all of the *Hoxb* genes on B137 by *in situ* hybridization. *Hoxb8* and *Hoxb6* transcripts from B137 were detected in their normal expression domains (Fig. 2B) as were more 3' genes such as *Hoxb1* (data not shown). This suggests that B137 contains most, if not all, of the *cis*-elements necessary to control expression of 5' *Hoxb* genes in the neural tube during early embryogenesis.

To search for *cis*-elements capable of mediating the dynamic CNS expression we first examined the potential of local regions surrounding individual genes. Transgenic reporter lines were generated with smaller fragments containing either *Hoxb7-LacZ* (17 kb) or *Hoxb9-mCherry* (15 kb) isolated from B137 (Fig. 2A). Unlike the full BAC reporter, these smaller constructs generated neural and mesodermal boundaries that were more posterior than those of endogenous genes at E10.5 (Fig. 1A and Fig. 2C). At E11.5 and later stages, the smaller reporters failed to display a rostral expansion in the neural tube (Fig. 2C and data not shown). These results imply that 5' *Hoxb* genes require remote *cis*-elements to establish their early expression boundaries and later to drive the rostral expansion in the neural tube.

The region between *Hoxb5* and *Hoxb3* is required to establish and maintain proper neural expression domains of 5' *Hoxb* gene reporters.

We hypothesized that the remote *cis*-elements responsible for the rostral expansion of 5' *Hoxb* genes may reside in the 3' region of the cluster as 3' *Hoxb* genes are expressed in the more anterior region of the neural tube. To test this idea, we generated transgenic lines with serial 3' deletions of B137 (Fig. 3A). We discovered that the pattern of reporter expression from BACs of 102 kb or larger is comparable to that of B137 (Fig. 3B and data not shown). In contrast, BACs truncated at 94 kb and 84 kb (B94 and B84) showed very weak and patchy reporter expression along the spinal cord (Fig. 3B). This is surprising because these constructs contain the same *Hoxb7* (17 kb) and *Hoxb9* (15 kb) regions that we demonstrated are capable of directing expression in the small reporter assays (Fig. 2C). A further 3' deletion to 72 kb (B72) restored some neural expression, but it was weak and did not undergo a rostral expansion. This suggests the presence of a repressive element in this region (region I, 72–84 kb). Although patchy, the presence of *LacZ*-expression cells in the anterior region of the spinal cord in both B94 and B84 suggests that *cis*-elements required for the rostral expansion are also present in region I. However, additional *cis*-elements upstream of *Hoxb3* (region II, 94–102 kb) are required for maintaining normal expression levels of 5' *Hoxb* genes in the neural tube.

This deletion analysis suggests that the concerted actions of multiple *cis*-elements present in the region between *Hoxb5* and *Hoxb3* are necessary to establish and maintain normal expression

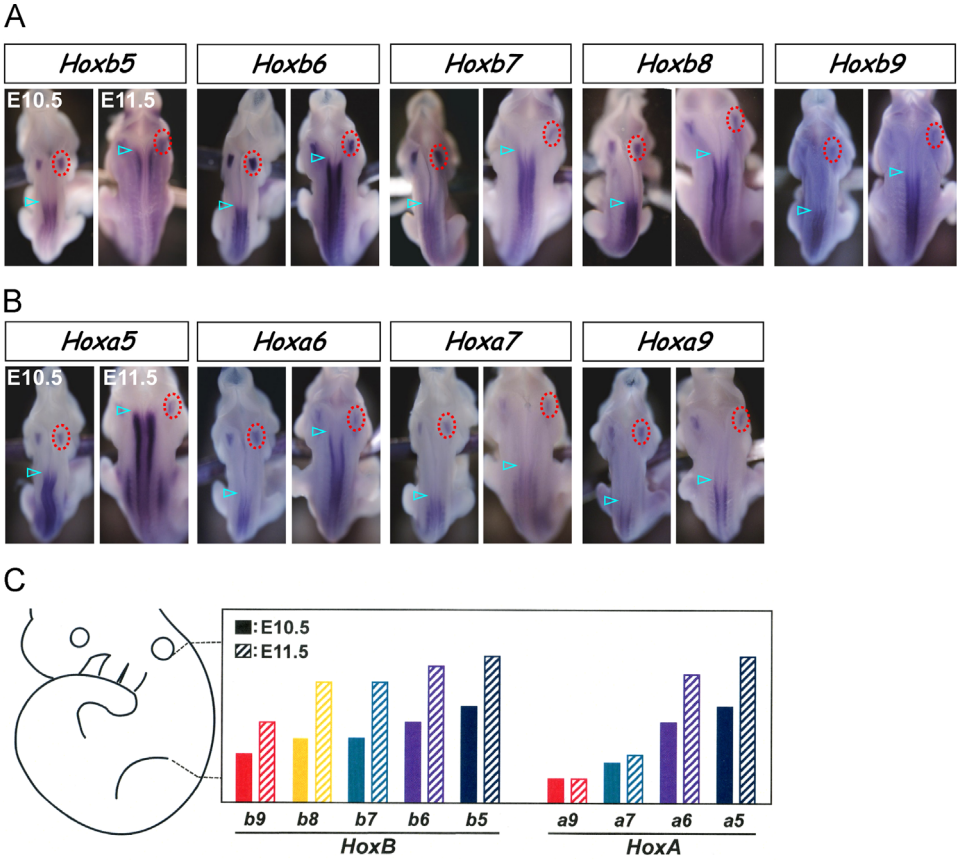

Fig. 1. Dynamic expression patterns of 5' *Hoxb* and 5' *Hoxa* genes in the neural tube. (A) Whole mount *in situ* hybridization of mouse embryos shows that 5' *Hoxb* genes undergo the rostral expansion of their expression domain in the neural tube between embryonic day 10.5 (E10.5) and E11.5. (B) In the *HoxA* cluster, *Hoxa5* and *Hoxa6* display the rostral expansion while *Hoxa7* and *Hoxa9* do not. The otic vesicles are marked by a dashed circle as a reference and the arrowheads marked the anterior boundary of expression in the neural tube. (C) A summary of the relative neural expression domain of the 5' *Hoxb* and 5' *Hoxa* genes at E10.5 (solid bar) and E11.5 (striped bar).

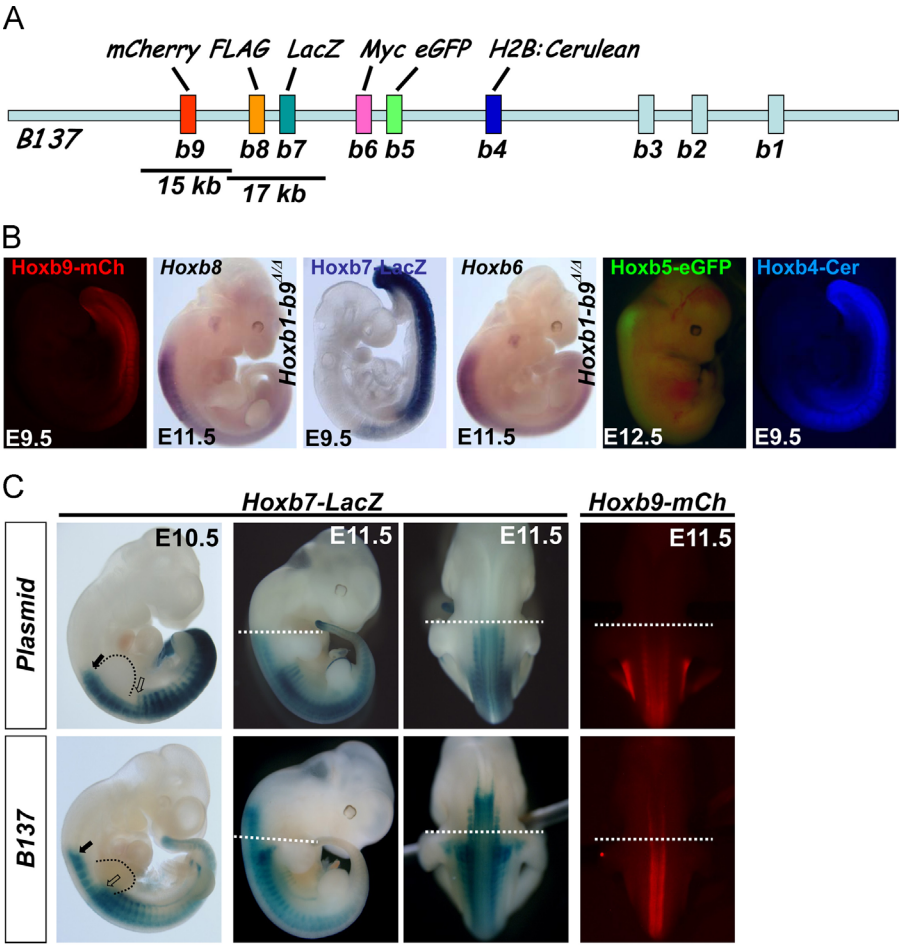

Fig. 2. Multiplexed *HoxB* BAC reporters recapitulate endogenous gene expression patterns in transgenic mice. (A) Schematic diagram showing labeling of multiple *Hoxb* genes in a 137 kb *HoxB* BAC (B137). (B) Expression pattern of the reporters in B137 mimic that of endogenous *Hox* genes. *Hoxb8* and *Hoxb6* transcripts from B137 were detected by *in situ* hybridization with mice lacking endogenous *Hoxb9-Hoxb1*. (C) Plasmid-based transgenic reporters containing either *Hoxb7-LacZ* (17 kb) or *Hoxb9-mCherry* (15 kb) display anterior expression boundaries more posterior than those of B137. The solid and open arrows indicate the anterior boundary in the neural tube and the mesoderm, respectively. The dashed lines outline the edge of the forelimb bud (E10.5) or mark the level of the anterior limit of the forelimb bud along the body axis (E11.5).

of 5' *Hoxb* genes including the rostral expansion. The regulatory activities of individual enhancer and repressor elements in the cluster display context-dependent properties, indicating that they cooperate to mediate appropriate endogenous expression. Consistent with this concept, our deletion analysis indicated that the 72 kb construct alone is not sufficient for mediating the rostral expansion of reporter expression even though it contains a small DE fragment 3' to *Hoxb5* that has been shown to be capable of anteriorizing the expression domain of small *Hoxb8-LacZ* and *Hoxb5-LacZ* reporters with their proximal *cis*-elements (Oosterveen et al., 2003a; Valarche et al., 1997).

Two RAREs are redundantly required for the rostral expansion of 5' Hoxb gene reporters

While RA signaling has been implicated in the rostral expansion of *Hox* genes in the spinal cord, no RAREs have been found in the region 5' to *Hoxb5*. However, the regions between *Hoxb5* and *Hoxb3*, required for the rostral expansion based on the deletion analyses above, contain two previously characterized RAREs, DE and ENE (Fig. 3A). These two RAREs are good candidates for involvement in remote regulation of multiple 5' *Hoxb* genes because they have been shown to participate in regulation of neural expression and have the potential to be shared between the immediately adjacent genes (Gould et al., 1998, 1997; Oosterveen et al., 2003a, 2003b; Sharpe et al., 1998). Therefore, we tested their ability to work over long range on the expression of multiple 5' *Hoxb* genes by inactivating them individually or in combination in context of the full B137 multiplexed reporter (Fig. 4A).

Mutations in both halves of the consensus binding sites of the direct repeats in ENE-RARE ($B137^{mENE}$) lead to reduced expression of *Hoxb4-Cerulean* in the neural tube at E9.5 (Fig. 4C), consistent with its role in initiating the early phase of *Hoxb4* expression (Gould et al., 1998). However, this mutation had little effect on the expression of the 5' *Hoxb* gene reporters at E12.5 (Fig. 4B and data not shown). In contrast, mutation of DE-RARE ($B137^{mDE}$) lead to a reduction or loss of the 5' *Hoxb* gene reporter expression in their anterior neural domain in 5 out of 8 transgenic lines (Fig. 4B and data not shown). When both of these RAREs were mutated ($B137^{mDE/ENE}$), the rostral neural expansion was no longer observed for any of the reporters (Fig. 4B and C; $N=4$). In addition, *in situ* analysis revealed that *Hoxb6* and *Hoxb8* also failed to display a rostral expansion in expression (Fig. 4D). Together, these results demonstrate that both DE- and ENE-RAREs contribute to regulation of the rostral expansion in neural expression of 5' *Hoxb* genes, with the DE-RARE element playing a more prominent role.

DE-RARE regulates multiple Hoxb genes in the endogenous cluster

We further investigated the role for DE-RARE by targeted mutation of the element in the endogenous *HoxB* cluster (Fig. 5).

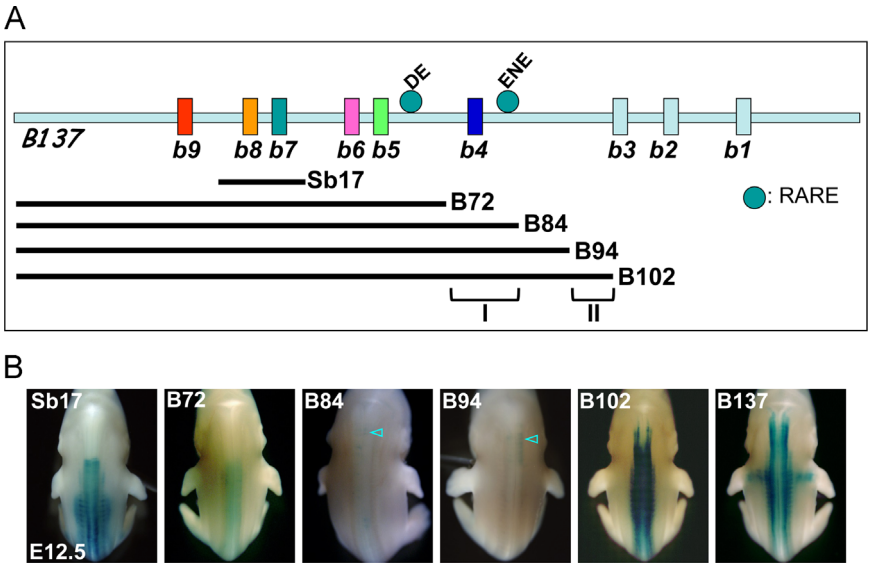

Fig. 3. Serial deletion analyses of the *HoxB* BAC reporters. (A) Schematic diagram showing 3' serial deletions of B137 and the positions of the retinoic acid response elements (RAREs). (B) Expression of *Hoxb7-LacZ* from the deletion constructs at E12.5. The arrowheads indicate weak and patchy *LacZ*-expression domains from the B84 and B94 constructs in the anterior spinal cord.

Initially, DE-RARE was replaced by a floxed *NeoR* cassette through homologous recombination in embryonic stem cells. The *NeoR* cassette was then removed by Cre-mediated recombination leaving a LoxP site, resulting in a deleted allele (*DE-RARE*$^{\Delta}$). *DE-RARE*$^{\Delta/\Delta}$ mice were viable and fertile and displayed no skeletal defects (data not shown). While *in situ* hybridization analysis with E11.5 embryos detected no obvious change in the expression patterns, real-time RT-PCR analysis with dissected hindbrain and anterior spinal cord indicated that *Hoxb1*, *Hoxb5* and *Hoxb6* are significantly down-regulated in *DE-RARE*$^{\Delta/\Delta}$ mice (Fig. 5B and data not shown). This confirms that the DE-RARE does participate in the endogenous regulation of multiple *Hoxb* genes. The relatively modest effect of DE-RARE knock-out is consistent with the redundancy between DE- and ENE-RARE observed with the BAC reporter analyses. Together this data supports the idea that ENE-RARE and possibly other elements in the *Hoxb5-Hoxb3* region partially compensate for the loss of DE-RARE in the endogenous *HoxB* cluster. The BAC regulatory analyses and targeted mutagenesis results are consistent with a model whereby the DE- and ENE-RAREs work over a long range to regulate the anterior shifts of multiple 5' *Hoxb* genes (Fig. 5C).

DE-RARE is required for robust response of Hoxb genes to exogenous RA

It has been known that exposure to RA during pregnancy results in developmental abnormalities accompanied by mis-regulation of *Hox* genes (Conlon and Rossant, 1992; Kessel, 1992; Marshall et al., 1992; Oosterveen et al., 2003a). We investigated whether the DE-RARE also plays a role in the response of *Hoxb* genes to exogenous RA. RA treatment of pregnant mice at E10.5 led to premature rostral expansion and up-regulation of expression of *Hoxb4*, *Hoxb5*, *Hoxb6* and *Hoxb8* in the neural tube of wild-type embryos (Fig. 6A–D). For comparison we also examined several 5' *Hoxa* genes. Interestingly, *Hoxa6* showed no significant change in the pattern of expression after RA treatment, while *Hoxa5* showed a robust response (Fig. 6E–F).

In *DE-RARE*$^{\Delta/\Delta}$ mice, the RA response was significantly attenuated for *Hoxb5*, *Hoxb6* and *Hoxb8*, but not for *Hoxb4* (Fig. 6A–D). Real-time RT-PCR analysis with dissected hindbrain and anterior spinal cords further demonstrated reduced induction of *Hoxb5* and *Hoxb6* to ectopic RA in the mutant embryos (Fig. 6G). In addition, *Hoxb1* expression was also reduced in *DE-RARE*$^{\Delta/\Delta}$ embryos after RA treatment (Fig. 6G). These results further support the role for the DE-RARE in directly mediating regulation of multiple *Hox* genes by RA signaling during embryogenesis.

DE-RARE can anteriorize the neural expression boundary of 5' Hoxa reporters

Based on our regulatory analysis in mice, we next investigated whether the RAREs implicated in regulation of 5' *Hoxb* genes are a conserved feature of the *HoxB* cluster among vertebrates and whether the other *Hox* clusters contain similar elements. Phylogenetic sequence analyses using VISTA Browser (http://pipeline.lbl.gov/cgi-bin/gateway2?selector=vista) indicates that the DE- and ENE-RAREs are highly conserved among vertebrates and can be found in the *HoxB* cluster of teleosts, *Xenopus* and other mammals, consistent with their significant roles in regulation of 5' *Hoxb* genes (Fig. 7A and data not shown). In general there is very little sequence conservation in the intergenic region between *Hoxb5* and *Hoxb4*. However, in addition to DE there is another conserved region which we note also contains an RARE, b4u (Fig. 7A). In extending this analysis to other *Hox* clusters, we find an equivalent b4u RARE 5' to all *Hox4* paralogs, namely a4u-, c4u- and d4u-RAREs, which has been conserved across the species (Fig. 7A). Interestingly, a stretch of sequences adjacent to the consensus RAR-RXR binding site is highly conserved implying potential interplay between RA signaling and other transacting factors (Fig. 7B). In addition all Hox clusters in other vertebrate species also contain an RARE 3' to *Hox4* paralogs equivalent to ENE-RARE in the *HoxB* cluster (data not shown). This suggests that these two RAREs flanking *Hox4* paralogs were present in an ancestral *Hox* cluster prior to the genome wide duplications associated with vertebrate evolution (Meyer, 1998) and may have an ancient role in gene regulation. However, a conserved RARE equivalent to DE is not present in other *Hox* clusters (Fig. 7A), hence DE appears to be distinct from the RAREs positioned closer to *Hox4* paralogs.

These comparative sequence analyses raise the possibility that the DE-RARE gives the *HoxB* cluster its distinct regulatory properties with respect to the more dynamic rostral shift in boundaries of

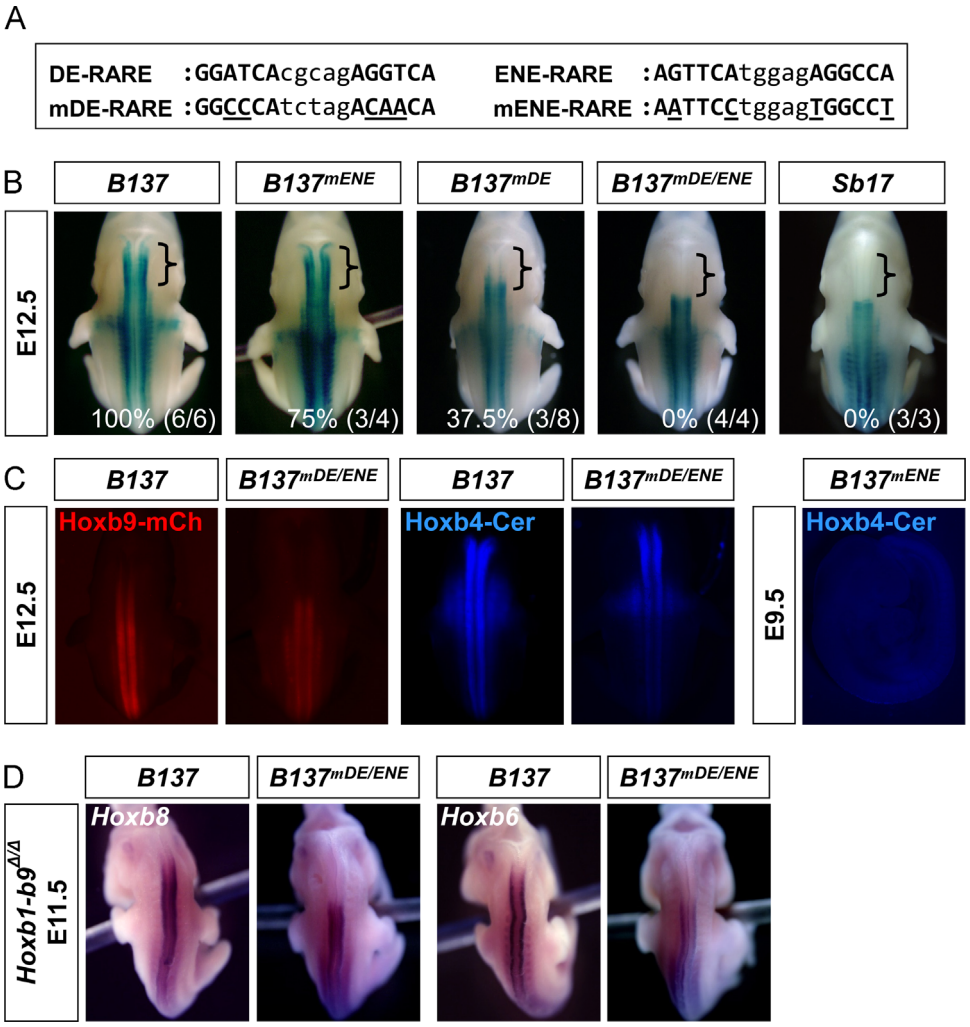

Fig. 4. Mutational analyses of DE- and ENE-RAREs in the *HoxB* BAC reporters. (A) Sequences of the wild-type and mutated DE- and ENE-RAREs. Mutations introduced into the consensus binding sites are underlined. (B) Comparison of *Hoxb7-LacZ* expression at E12.5 from the wild-type B137 BAC and BACs with single and double RARE mutations. Percentage of independent transgenic lines with the rostral expansion of *Hoxb7-LacZ* expression is shown at the bottom of each panel. (C) The rostral expansion of *Hoxb9-mCherry* is abolished in the double RARE mutant BAC, B137$^{mDE/ENE}$, while *Hoxb4-H2B:Cerulean* is not significantly affected at E12.5. At E9.5, the mutations in ENE-RARE lead to reduced expression of *Hoxb4-H2B:Cerulean* in the neural tube (see Fig. 2B for comparison to the wild-type BAC). (D) *Hoxb8* and *Hoxb6* transcripts from B137$^{mDE/ENE}$ do not show the rostral expansion as seen *in situ* hybridization with mice lacking endogenous *Hoxb9-Hoxb1*.

neural expression of 5' *Hoxb* genes compared to 5' *Hox* genes in the other clusters (Fig. 1) (Akin and Nazarali, 2005) and the robust RA response of these genes (Fig. 6).

To test this hypothesis, we investigated whether DE is capable of imparting these regulatory properties to other *Hox* clusters. Towards this goal, a 137 bp fragment containing DE-RARE and surrounding sequences was inserted into the corresponding position between *Hoxa5* and *Hoxa4* of a 152 kb *HoxA* BAC, A152, in which *Hoxa9* and *Hoxa7* were labeled with fluorescent reporters (Fig. 7C). In transgenic embryos carrying the control A152 BAC, *Hoxa9-H2B:Cerulean* and *Hoxa7-H2A:mCherry* were expressed in neural domains similar to the endogenous genes (Fig. 1 and Fig. 7C). Strikingly, these expression domains were shifted anteriorly in a version of this BAC containing the inserted *HoxB* DE-RARE fragment, A152^{DE} (Fig. 7C). These data demonstrate that an ectopic DE-RARE in *HoxA* has the ability to control multiple 5' *Hoxa* genes and anteriorize their expression domains in the neural tube, in a manner analogous to its normal role in the *HoxB* cluster. This supports a model (Fig. 5C) whereby a newly acquired DE-RARE accounts at least in part for the differences between *HoxB* and the other clusters in regard to the dynamic neural expression patterns and RA response of 5' *Hox* genes.

Discussion

In this study, by utilizing multiplexed BAC reporter constructs in transgenic mice we generated evidence that enhancer sharing and long-range regulation provide a mechanistic basis to explain the coordinated anterior shifts in neural expression of 5' *Hoxb* genes. We demonstrated that the combined activities of multiple RAREs (DE and ENE) embedded in the cluster are required for proper rostral expansion of reporter expression of multiple 5' *Hoxb* genes. Furthermore, a targeted deletion of the DE element in the *HoxB* cluster also impacts the endogenous expression of multiple *Hoxb* genes. These findings highlight the key role that retinoids play in establishing and maintaining domains of *Hox* gene expression. They provide further support that mechanisms involving shared *cis*-regulatory elements are likely to be a contributing factor in conservation of the cluster organization of *Hox* genes during vertebrate evolution.

Cooperative interactions between regulatory regions

In combination with reporters that permit the simultaneous detection of multiple *Hox* genes in the same embryos, our BAC

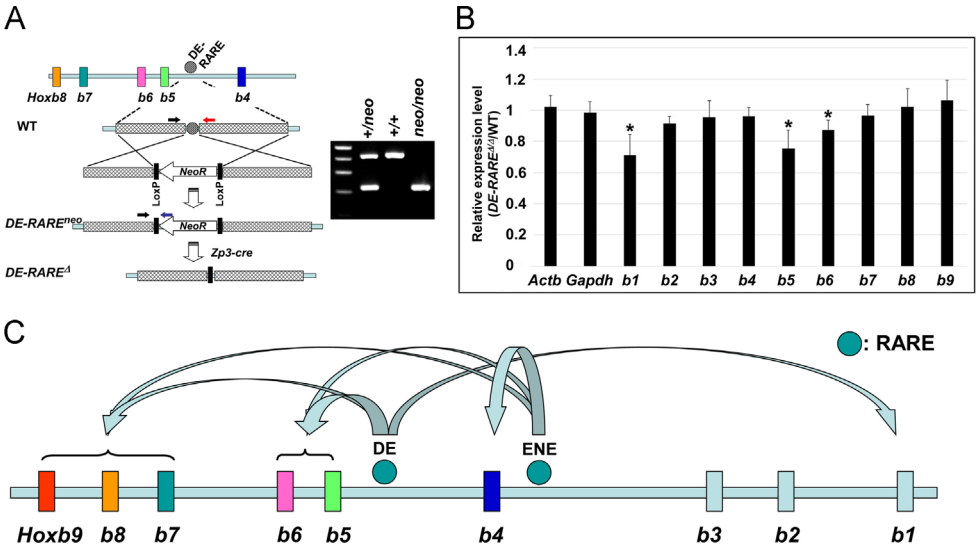

Fig. 5. DE-RARE regulates multiple *Hoxb* genes in the endogenous cluster. (A) Schematic diagram showing the targeting strategy for DE-RARE and an example of PCR-genotyping assay of mice with the targeted allele. (B) Real-time RT-PCR analysis with dissected hindbrain and anterior spinal cord indicates significant down-regulation (∗*p*-value < 0.02) of *Hoxb1*, *Hoxb5* and *Hoxb6* in *DE-RARE*$^{\Delta/\Delta}$ embryos at E11.5. Average fold change in the mutant in comparison to WT mice are shown (4 biological replicates for each). (C) A model showing regulation of multiple *Hoxb* genes by DE- and ENE-RARE in the neural tube. See the text for details.

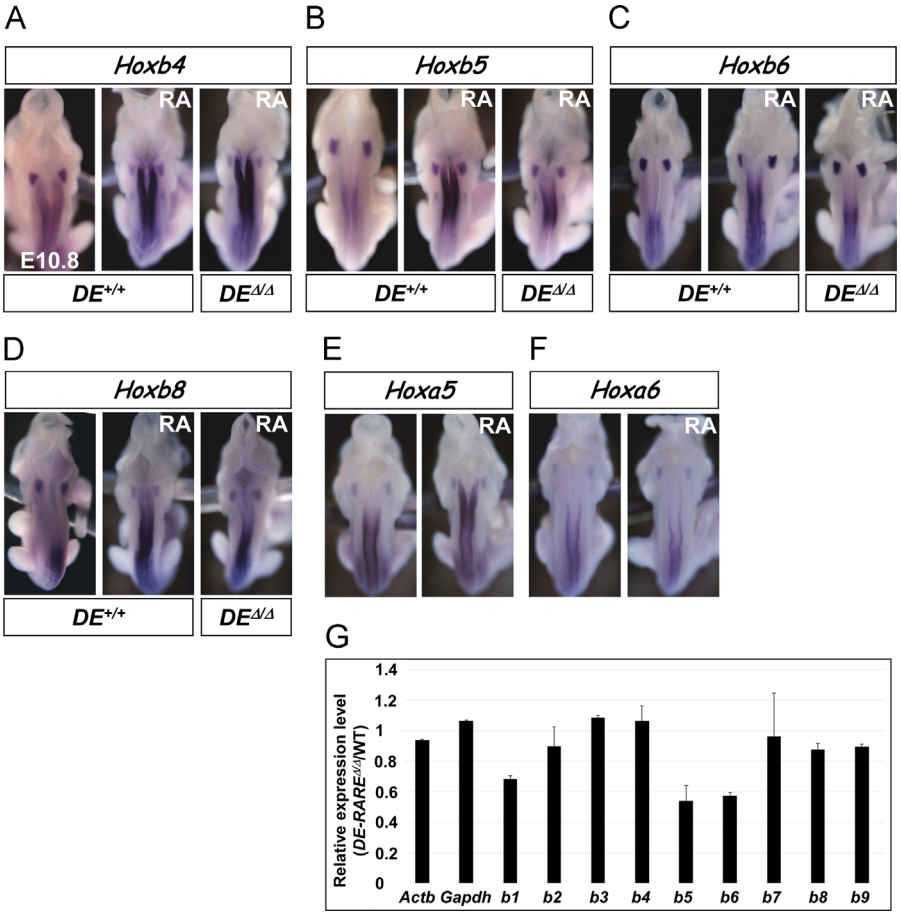

Fig. 6. DE-RARE is required for the RA response of the 5' *Hoxb* genes. ((A)–(D)) The *in utero* RA treatment at E10.5 induces elevation and rostral expansion of *Hoxb* gene expression in the neural tube as shown by *in situ* hybridization. This RA response is significantly attenuated for *Hoxb5*, *Hoxb6* and *Hoxb8* in *DE-RARE*$^{\Delta/\Delta}$ embryos while the response of *Hoxb4* remains the same. ((E)–(F)) In wild-type embryos, *Hoxa5* responds to the RA treatment but *Hoxa6* does not. (G) Real-time RT-PCR analysis with dissected hindbrain and anterior spinal cord shows reduced induction of *Hoxb1*, *Hoxb5* and *Hoxb6* to ectopic RA in *DE-RARE*$^{\Delta/\Delta}$ mutants. Embryos were harvested for dissection at E11.8 8 h after RA gavage. Average fold change in the mutant in comparison to wild-type mice are shown (2 biological replicates for each).

reporter approach, which preserve more of the regulatory landscape of the *Hox* clusters than small plasmid constructs, provided us with a great opportunity to explore more complex regulatory interactions involving multiple *cis*-elements within the clusters *in vivo*. Our regulatory analyses clearly demonstrated that DE- and ENE-RAREs need to interact with other regulatory components in the *HoxB*

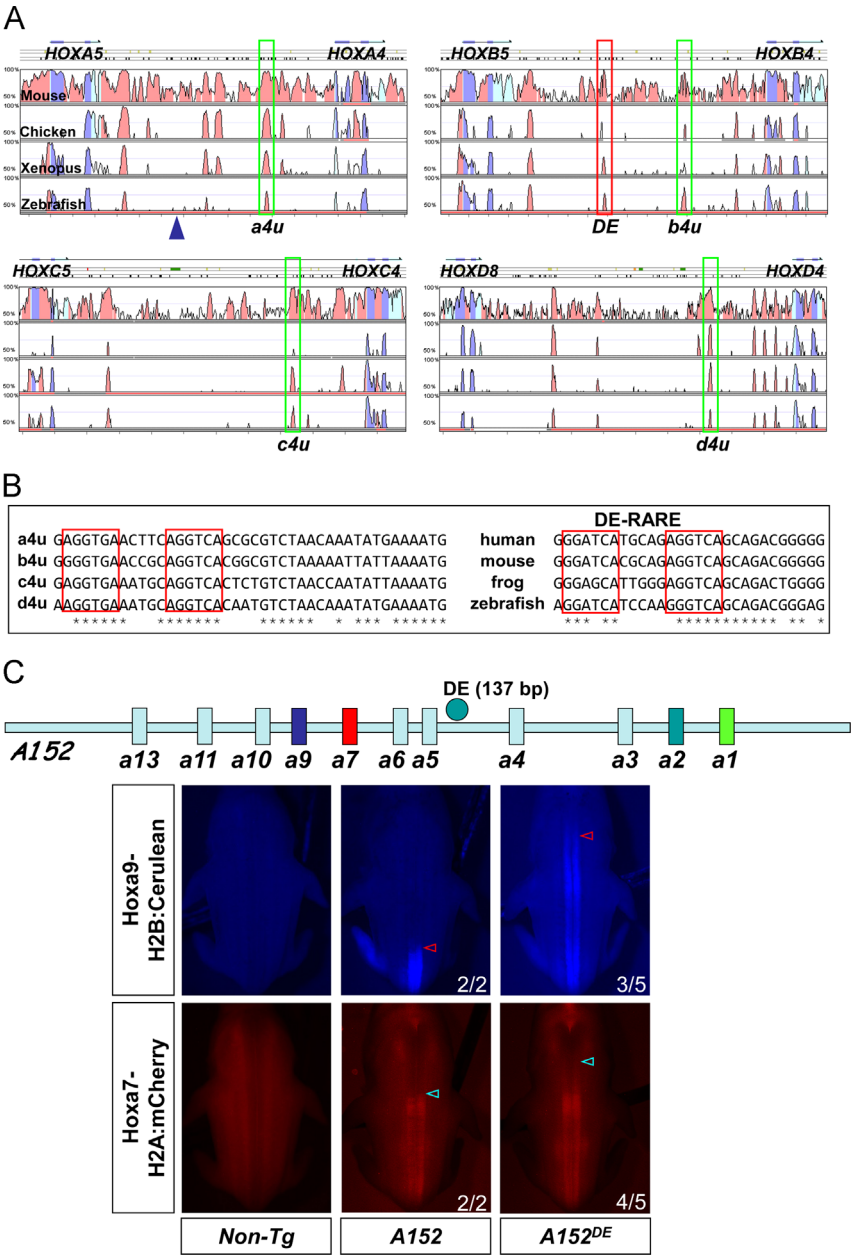

Fig. 7. DE-RARE anteriorizes the neural expression boundary of 5' *Hoxa* gene reporters. (A) Phylogenetic sequence analysis of the *Hox5-Hox4* intergenic region of the *Hox* clusters uncovers conserved regions containing RAREs. A conserve element equivalent to DE-RARE (red rectangle) is found only in the *HoxB* cluster while all the *Hox* clusters contain an RARE (a4u, b4u, c4u and d4u, green rectangles) 5' to *Hox4* paralogs. (B) (Left) The sequences of the RAREs (a4u, b4u, c4u and d4u) 5' to *Hox4* paralogs are very similar not only in the consensus binding sites (red rectangles), but also in the adjacent sequences. (Right) DE-RARE is conserved among vertebrate species. (C) A 137 bp fragment containing DE-RARE was inserted into the corresponding position (also shown in (A) by an arrowhead) of a *HoxA* BAC, A152, in which *Hoxa9*, *Hoxa7*, *Hoxa2* and *Hoxa1* were labeled with *LacZ* and fluorescent reporters. The insertion of the DE-RARE element results in more anteriorized neural expression domains of *Hoxa9-H2B:Cerulean* and *Hoxa7-H2A:mCherry* in the majority of independent transgenic lines, 3/5 and 4/5, respectively. Arrowheads mark the anterior boundary of expression in the neural tube.

cluster to exert their functional roles in controlling neural expression of multiple 5' *Hoxb* genes. For example, deletion analysis of *HoxB* BACs revealed that sequences between 94 and 102 kb (region II) are required to potentiate high levels of reporter expression even when DE and ENE are present (Fig. 3). Furthermore, a region between 72 and 84 kb (region I) appeared to contain elements that can repress reporter expression dependent upon DE- and ENE-RARE. It is interesting that the transposition of DE-RARE into the context of the *HoxA* cluster is sufficient to mediate the rostral expansion of *Hoxa7* and *Hoxa9* in a manner similar to its regulatory influences on 5' *Hoxb genes* (Fig. 7). This suggests that the *HoxA* cluster may also have elements similar to those in regions I and II which work together with DE-RARE to modulate regulatory activity.

Enhancer sharing and long-range regulation in the HoxB cluster

The long-range regulation by remote *cis*-elements has been well studied in the *HoxD* cluster, where global enhancer elements residing at either ends of the cluster control *Hoxd* gene expression in the limb bud, genitalia and intestinal hernia (Andrey et al., 2013; Tschopp and Duboule, 2011). The collinear expression of *Hox* genes along the main body axis in several tissues including the neural tube is in large part controlled by local *cis*-regulatory elements, often shared by neighboring genes (Alexander et al., 2009; Gould et al., 1997; Kmita and Duboule, 2003; Sharpe et al., 1998; van der Hoeven et al., 1996). Nonetheless, the precise control of the spatio-temporal domain of expression of some *Hox* genes in the neural

tube can only be achieved in the context of the whole *Hox* cluster, as shown from the previous promoter analyses of *Hoxb7* and *Hoxb8* (Charite et al., 1995; Vogels et al., 1993). In these studies, the proximal *cis*-elements around each gene failed to recapitulate the endogenous expression boundaries in the neural tube implying the presence of distal *cis*-elements.

Here we have demonstrated that the DE and ENE-RAREs play roles in long-range regulation of *Hoxb* genes. The primary effect of mutating the ENE-RARE alone was seen on the adjacent *Hoxb4* gene (Fig. 4B and C). In contrast, based on the BAC reporter analysis, mutation of DE-RARE has a major impact on the expression of *Hoxb5–Hoxb9* (Fig. 4 and data not shown). Mutation of both of the RAREs completely abolished the anterior shift in neural expression of 5' *Hoxb* genes, implying that they cooperate and have some degree of overlapping function on a common set of *Hoxb* genes. The b4u-RARE might also contribute to regulation of these *Hoxb* genes alone or in combination with these other RAREs, but we did not score its regulatory potential.

Targeted mutation of DE-RARE indicated that it is indeed required for proper regulation in the endogenous *HoxB* cluster. However, not all *Hox* genes are affected in the same manner by the mutation. *Hoxb5* and *Hoxb6* are more strongly effected than *Hoxb7–Hoxb9* (Fig. 5B), which may reflect their relative proximity to this *cis*-element. An unexpected finding is that *Hoxb1* is also down-regulated in the neural tube suggesting that DE-RARE is a part of a shared enhancer that can exert long-range influences in both 5' and 3' directions. While we did not observe significant changes in expression level of *Hoxb2–Hoxb4* upon mutation of DE-RARE, it may still have input on these genes which is masked by the regulatory activities of other *cis*-elements in the 3' part of the cluster. For example, an auto-regulatory enhancer for *Hoxb4* maintains the neural expression of the gene once triggered by ENE-RARE (Gould et al., 1997). In addition, there are other RAREs and enhancers which are known to modulate neural expression of *Hoxb2* and *Hoxb3* (Kwan et al., 2001; Maconochie et al., 1999). Alternatively, it is possible that there is an unknown mechanism that directs the activity of DE-RARE toward 5' *Hoxb* genes. The relative selectivity for 5' *Hoxb* genes may be related to looping or other topological constraints in the cluster.

Promoter selectivity could be another means by which DE-RARE preferentially acts on 5' *Hoxb* genes. In a previous study, we showed that the neural enhancer containing DE-RARE is able to activate the *Hoxb5* promoter, but not the *Hoxb4* promoter as a result of competition between the two promoters in a double labeled reporter mimicking the endogenous genomic context (Sharpe et al., 1998). Conversely, the ENE-RARE enhancer primarily modulates *Hoxb4* and *Hoxb3* (Fig. 4 and Gould et al., 1997). However, the DE-RARE containing enhancer was able to activate both the *Hoxb5* and *Hoxb4* promoters when tested individually (Sharpe et al., 1998). Furthermore, we were unable to identify any obvious feature that distinguish the promoters of 5' *Hoxb* genes from those of 3' *Hoxb* genes. Therefore, we favor the idea that genomic context of the *Hoxb* genes with respect to DE-RARE determines the relative degree of influence the DE-RARE has on those genes. It would be interesting to know whether those genes which shift anteriorly in the neural tube share any other features in their global expression specificities which might suggest common regulatory mechanisms.

Differences in timing of RARE activity

There are interesting differences in the stage when ENE-RARE and DE-RARE have an impact on expression of *Hoxb* genes. In general, the activation dynamics progresses from 3' to 5' so DE-RARE and ENE-RARE, positioned 5' and 3' of *Hoxb4*, respectively, would be expected to be functional and activate expression of *Hoxb4* and *Hoxb5/6* at the similar stage in the anterior spinal cord. However, ENE-RARE exerts its regulatory activity on *Hoxb4* at ~E9.0, whereas DE-RARE functions on 5' *Hoxb* genes at E10.5–11.5 (Figs. 2 and 4). This suggests that additional elements or factors are involved in controlling the timing of the RA regulation mediated by these two elements. This is consistent with findings from the deletion experiments (Fig. 3) which revealed that DE-RARE alone is not sufficient to mediate activation and maintenance of 5' *Hoxb* gene expression in the anterior spinal cord. It has also been found that the activity of an RARE located 3' of *Hoxd4* is modulated by flanking *cis*-elements (Nolte et al., 2006).

It has been shown that RA treatment triggers rapid clearance of repressive histone H3K27me3 of *Hox* clusters followed by acquisition of H3K79me2 (Kashyap et al., 2011; Mazzoni et al., 2013). These chromatin modifications are associated with induction of *Hox* genes suggesting that RA signaling can mediate changes in the chromatin modifications and hence facilitate gene expression. The later activity of DE-RARE might be due to a delay in the chromatin modifications in the 3' part of the cluster in the absence of additional factors. It is also possible that DE-RARE works to modify the epigenetic state of the cluster at early stages to allow activation 5' *Hoxb* genes through additional regulatory inputs at E10.5–11.5. Alternatively, once the chromatin state initially becomes open, the promoters of 5' *Hoxb* genes may be repressed and DE-RARE need to work together with other *cis*-elements through a mechanism of de-repression to activate these genes.

Differences in regulation of HoxA and HoxB

Consistent with the lack of a DE equivalent RARE in the *HoxA* cluster, *Hoxa7* and *Hoxa9* displayed no significant rostral shift in neural expression between E10.5–E11.5 and *Hoxa6*, *Hoxa7* and *Hoxa9* did not respond to RA treatment (Figs. 1 and 6). In contrast, *Hoxa5* and *Hoxa6* underwent a rostral shift and *Hoxa5* strongly responded to exogenous RA. We speculate that the two conserved RAREs, one 5' to *Hoxa4* and the other 3' to *Hoxa4*, may account for the anterior shift and ectopic RA response of some of *Hoxa* genes with their close proximity. There are no obvious differences in the consensus binding sites among DE, ENE or b4u-RAREs and related RAREs in other *Hox* clusters, yet they displayed significant differences in their ability to modulate genes over a long-range. This suggests that flanking or additional sequences may potentiate their activities possibly by recruiting other trans-acting factors.

In conclusion, the concerted action of multiple RAREs working on a group of 5' *Hoxb* genes at a distance helps to explain their similar domains and ordered rostral shifts in neural expression. This study has underscored the fact that enhancer sharing is a common and important feature in the regulation of multiple *Hox* genes in diverse tissues. This added degree of complexity in the regulation of clustered *Hox* genes and may represent one of the selective biases which have served to maintain the clustered organization of *Hox* genes in vertebrate evolution.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2014.01.027.

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