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Selectivity, sharing and competitive interactions in the regulation of *Hoxb* genes

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The clustered organisation of *Hox* complexes is highly conserved in vertebrates and the reasons for this are believed to be linked with the regulatory mechanisms governing their expression. In analysis of the *Hoxb4–Hoxb6* region of the *HoxB* complex we identified enhancers which lie in the intergenic region between *Hoxb4* and *Hoxb5*, and which are capable of mediating the correct boundaries of neural and mesodermal expression for *Hoxb5*. We examined their regulatory properties in the context of the local genomic region spanning the two genes by transgenic analysis, in which each promoter was independently marked with a different reporter, to monitor simultaneously the relative transcriptional read-outs from each gene. Our analysis revealed that within this intergenic region: (i) a limb and a neural enhancer selectively activate *Hoxb4* as opposed to *Hoxb5*; (ii) a separate neural enhancer is able to activate both genes, but expression is dependent upon competition between the two promoters for the enhancer and is influenced by the local genomic context; (iii) mesodermal enhancer activities can be shared between the genes. We found similar types of regulatory interactions between *Hoxb5* and *Hoxb6*. Together these results provide evidence for three separate general mechanisms: selectivity, competition and sharing, that control the balance of *cis*-regulatory interactions necessary for generating the proper spatial and temporal patterns of *Hox* gene expression. We suggest that these mechanisms are part of a regulatory basis for maintenance of *Hox* organisation.

Keywords: enhancer sharing/gene regulation/*Hoxb* complex/promoter competition/transgenic mice

Introduction

Hox genes are involved in specifying positional information along the anterior–posterior axis of most animals (Duboule, 1992; Krumlauf, 1994; Carroll, 1995). This is achieved by regulatory mechanisms which establish and maintain their spatially-restricted domains of expression during embryogenesis. In vertebrates they are organised into four complexes, each containing up to 11 genes, and the position of each gene within the complex correlates in a colinear manner with its anterior limit of expression along the axis (Duboule and Dolle, 1989; Graham *et al.*, 1989; Boncinelli *et al.*, 1991; McGinnis and Krumlauf, 1992; Godsave *et al.*, 1994). This suggests that the highly conserved and clustered organisation of *Hox* gene complexes is necessary for the co-ordination of their nested and ordered spatial expression. To explain colinearity and the precise spatially-restricted domains of expression, a number of mechanisms have been proposed which would operate in a complex-wide 'global' manner, such as a progressive de-heterochromatinisation process (Peifer *et al.*, 1987; Dolle *et al.*, 1989), a maintenance or imprinting system (Gaunt and Singh, 1990; Orlando and Paro, 1995; Pirrotta, 1997), or by analogy to the β-globin cluster, a locus control region (Dillon and Grosveld, 1993).

To date, transgenic regulatory analysis of *Hox* genes has primarily identified locally acting enhancers, capable of mediating specific subsets of the endogenous expression patterns on reporter genes outside of the normal clustered context. In the *Hoxb* complex, for example, the four genes at the 3′ end of the complex (*Hoxb1–Hoxb4*) have *cis*-acting elements, either within or immediately flanking their loci, responsible for directing the correct anterior expression boundaries in a variety of tissues (Whiting *et al.*, 1991; Sham *et al.*, 1992, 1993; Marshall *et al.*, 1994; Studer *et al.*, 1994). Furthermore, in the hindbrain specific *trans*-acting factors have also been identified which are involved in mediating the segmental expression of these genes (Sham *et al.*, 1993; Pöpperl *et al.*, 1995; Gould *et al.*, 1997; Maconochie *et al.*, 1997; Manzanares *et al.*, 1997). In contrast, it has not been possible to reconstruct the proper anterior boundaries of expression for more 5′ genes (*Hoxb6–Hoxb8*) using similar approaches, although enhancers directing *Hox*-like axial expression have been found (Schughart *et al.*, 1991; Eid *et al.*, 1993; Vogels *et al.*, 1993; Charite *et al.*, 1995). In these cases, appropriate regulation may require interaction between many different components (Charite *et al.*, 1995; Valarché *et al*., 1997), some of which might exert their influences over a long range, as suggested for regulation of *Hoxd* gene expression in the limb (van der Hoeven *et al.*, 1996). Hence, there may be differences in the mechanisms regulating 3′ versus 5′ genes in the *Hoxb* complex, which might be correlated with differences in patterning the head versus the trunk.

Recently it has emerged that, unlike the *Drosophila Hox* cluster, some positive and negative control regions can be shared between adjacent murine *Hox* genes (Sham *et al.*, 1992; Gérard *et al.*, 1996; Gould *et al.*, 1997; Zakany *et al.*, 1997). This suggests that there are unlikely to be absolute boundary or insulator elements between all adjacent *Hox* genes in the vertebrate complexes restricting the influence of local enhancers only to a single gene. Therefore, if the sharing of control regions is a general feature of the vertebrate *Hox* complexes, then locally

important elements might also have a global impact on the regulation of other genes. However, the ability of *Hox* genes to share *cis*-elements raises the question as to how their distinctly different spatial and temporal patterns of gene expression are generated and maintained. Other global or local influences must be operating to restrict their specific patterns of expression.

In this regard, *Hoxb5* is interesting because it is positioned in the middle of the complex between the 5′ and 3′ groups of genes, and the mechanisms involved in regulating its proper spatially-restricted expression patterns are unknown. It was previously noted that an enhancer within the intron of *Hoxb4* directed expression boundaries similar to those of *Hoxb5*, suggesting that this region might be shared between the two genes and be responsible for *Hoxb5* regulation (Whiting *et al.*, 1991). In this study we have used transgenic approaches to locate and characterise the properties of *cis*-control elements in the region between *Hoxb4* and *Hoxb7*, with a particular focus on the basis of *Hoxb5* regulation. We identified enhancers capable of directing the proper boundaries of *Hoxb5* expression that are positioned near *Hoxb4*. Using a double reporter system that allowed us to simultaneously monitor expression from both *Hoxb4* and *Hoxb5* promoters, we investigated the relative ability of these enhancers to function on either gene. Our results reveal that both selectivity and competition between promoters, in addition to the sharing of enhancer elements, are important for generating the restricted domains of *Hox* expression. Therefore, the interplay between locally acting elements could be as important as global mechanisms in providing an evolutionary constraint for maintaining the clustered organisation.

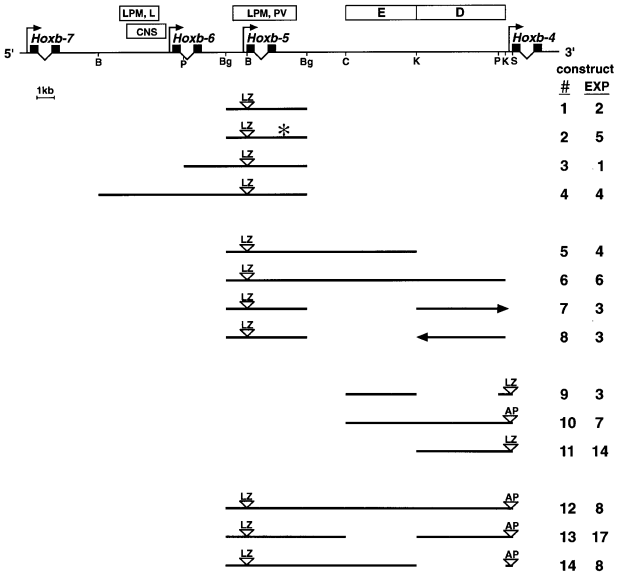

Fig. 1. Transgenic constructs. The structure of the *Hoxb* complex between *Hoxb7* and *Hoxb4* is shown on the top line. The boxes labelled D, E and LPM,PV denote enhancer domains defined in this study, and the enhancers marked CNS and LPM,L were previously identified. The line below this diagram represents regions of genomic DNA included in each of the transgenic constructs, and to the right the construct numbers (#) and number of independent embryos or lines generated which express the constructs are indicated. Triangles show the insertion sites for either the *lacZ* (*LZ*) or alkaline phosphatase (*AP*) reporter genes. The asterisk above construct 2 indicates the removal of a 200 bp *Pvu*II fragment from the 3′ untranslated region of *Hoxb5*. The arrowheads in constructs 7 and 8 display the orientation of region D. LPM, lateral plate mesoderm; PV, prevertebrae; L, limb; CNS, central nervous system; B, *Bam*HI; P, *Pvu*II; Bg, *Bgl*II; C, *Cla*I; K, *Kpn*I; S, *Sal*I.

Results

Identification of Hoxb5 control regions

Initially we examined the genomic region between *Hoxb4* and *Hoxb7* to search for *cis*-acting regulatory elements important for controlling the *Hoxb5* gene, and attempted to determine the regulatory relationship of these elements with adjacent genes in the complex. As a basis for transgenic analysis, a fusion gene was created in which a *lacZ* reporter was inserted in-frame at the *Bam*HI site within the first exon of *Hoxb5* (Figure 1). This construct (1) contained the *Hoxb5* transcription unit and 1 kb of both 5′ and 3′ flanking sequences; larger variants (constructs 3–8 and 12–14) were created by the addition of 5′ and 3′ regions to test the regulatory activity of flanking sequences.

Construct 1 contained regulatory regions that produced very weak reporter expression in the mesonephros, metanephros and other derivatives of the lateral plate mesoderm at 12.5 days post coitum (d.p.c.) (Figure 2a; Table I). We also deleted a 200 bp *Pvu*II fragment from the 3′ untranslated region, which contained an AU-rich motif found to reduce the stability of the *Hoxb5* mRNA in F9 teratocarcinoma cells (R.Krumlauf, unpublished). Embryos transgenic for this construct (2) reproducibly had stronger transgene expression in the lateral mesoderm derivatives, but we also detected weak staining in posterior prevertebrae (Figure 2b; Table I). The increased efficiency and levels of expression are consistent with the idea that this motif also affected RNA stability *in vivo*, and indicates that construct 1 has elements able to direct expression in a number of the endogenous *Hoxb5* domains. However, expression in the neural tube was still absent, and in both constructs 1 and 2 the most anterior extent of expression in the lateral plate mesoderm and somitic derivatives was more posterior than in the normal *Hoxb5* pattern.

To expand the search for regions capable of directing the missing domains of *Hoxb5* expression, two 5′ extensions were generated that terminated either in the intron of the adjacent *Hoxb6* gene (construct 3) or 4 kb 5′ of *Hoxb6* (construct 4), which includes half of the intergenic region between *Hoxb6* and *Hoxb7* (Figure 1). Even in the largest construct (4) there was no difference in expression compared with constructs 1 and 2, except for a new domain of expression in the forelimb bud (Figure 2c and d; Table I). This limb expression correlates with the previous characterisation of a limb enhancer just 5′ of *Hoxb6* (Schughart *et al.*, 1991; Eid *et al.*, 1993; Becker *et al.*, 1996). These results show that this limb enhancer is capable of working on both the *Hoxb5* and *Hoxb6* genes, since construct 4 contains both promoters.

Previous analysis of other upstream regions around *Hoxb7* and *Hoxb8* failed to detect any regulatory regions capable of directing anterior *Hoxb5*-like expression patterns (Eid *et al.*, 1993; Vogels *et al.*, 1993; Charite *et al.*, 1995). Therefore, we analyzed the regulatory potential of the 3′ intergenic region between *Hoxb5* and *Hoxb4* by making a series of 3′ extensions to construct 1. A 6.1 kb 3′ extension (construct 5) provided strong staining in the

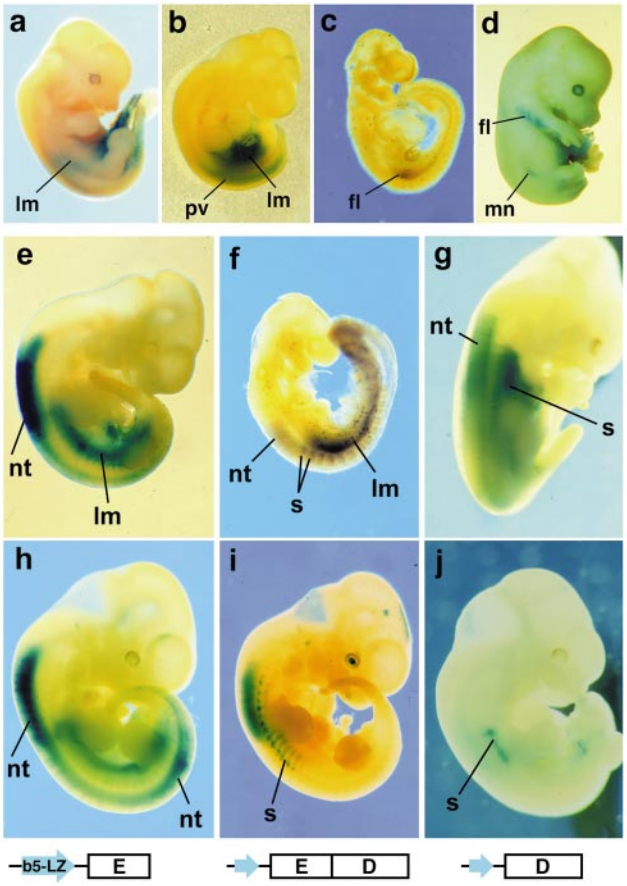

Fig. 2. Mapping *Hoxb5* control regions. (a–d) Lateral views of transgenic embryos showing expression from constructs 1 (a), 2 (b) and 4 (c and d). Stages are (**a**) 12.0 d.p.c., (**b**) 11.0 d.p.c., (**c**) 9.5 d.p.c. and (**d**) 13.5 d.p.c. These embryos all display expression in lateral mesoderm but (c) and (d) also show staining in the forelimbs. (e and h) Lateral views of *lacZ* expression from construct 5 containing the region E neural enhancer at (**e**) 10.5 d.p.c. and (**h**) 12.0 d.p.c. (f and i) Lateral views of reporter staining in (**f**) 9.5 d.p.c. and (**i**) 12.5 d.p.c. embryos with construct 6 containing enhancers D and E. (**g**) Dorsal view of staining in a 11.5 d.p.c. embryo with region D alone in the positive orientation (construct 7). (**j**) Lateral view of embryonic expression at 11.5 d.p.c. from region D in negative orientation (construct 8). Constructs are noted below the figure. Lateral plate mesoderm, lm; prevertebrae, pv; forelimb, fl; metanephros, mn; neural tube, nt; somites, s.

neural tube up to the correct anterior limit for endogenous *Hoxb5* for the first time (Figure 2e and h), in addition to those sites seen with construct 2 (Table I). Neural expression was initially detected at 9.5 d.p.c. in a small domain adjacent to the forelimb bud, and by 10.5 d.p.c. had expanded to reach the normal *Hoxb5* boundary at the spinal cord–hindbrain junction (Figure 2e). At 12.0 d.p.c. a second, more posterior, neural domain was also detected adjacent to the hindlimb bud (Figure 2h).

A further 3′ extension of 4.5 kb (construct 6), to within 300 bp of the *Hoxb4* P1 promoter (Gutman *et al.*, 1994), was now capable of mediating reporter expression in anterior somites/paraxial mesoderm in addition to the pattern seen with construct 5 (Figure 2f and i). The general expression from this construct (Figure 2f and i) closely resembled that of endogenous *Hoxb5* (see Figure 4a and e), and from 9.5–12.5 d.p.c. the transgene was expressed in the somites and neural tube with the correct *Hoxb5* anterior limits. Since the addition of the 4.5 kb 3′ fragment (termed region D) generated expression in the somites, we tested whether the somitic activity could be attributed specifically to this fragment by linking it to the basic *Hoxb5–lacZ* fusion construct (1) in either orientation (constructs 7 and 8). In both orientations reporter expression was found in paraxial mesoderm in a manner identical to that seen in construct 6, indicating that region D could function as a somite enhancer (Figure 2g and j). Furthermore, the transgene (construct 7) displayed expression in the neural tube, indicating that region D also contains a neural control region capable of activating the *Hoxb5* promoter separate from that found in the adjacent region (Figure 2g; Table I). This analysis of the genomic region between *Hoxb4* and *Hoxb7* has identified new enhancers in the complex and shows that regulatory elements important for directing restricted expression of the endogenous *Hoxb5* gene lie on its 3′ side, close to *Hoxb4*.

The intergenic regions also regulate Hoxb4

Conserved *cis*-acting regulatory elements which can direct subsets of the *Hoxb4* pattern (regions A–C) have previously been found within the intron and 3′ flanking sequences (Whiting *et al.*, 1991; Aparicio *et al.*, 1995; Morrison *et al.*, 1995). However, in the case of the group 4 paralogs, *Hoxa4* and *Hoxd4*, important regulatory elements have also been found on the 5′ side of the genes (Behringer *et al.*, 1993; Pöpperl and Featherstone, 1993; Morrison *et al.*, 1996). The proximity of the *Hoxb5* control regions identified above to the *Hoxb4* gene raises the possibility that they may be equally important for controlling its expression; we therefore tested the ability of these elements to activate the *Hoxb4* promoter. The regulatory regions were linked to a *Hoxb4* promoter/reporter vector (construct 8; Whiting *et al.*, 1991) which on its own is unable to direct expression in any tissue except the superior colliculus. We first examined the fragment used for the 3′ extension in construct 5 and found that a 3.9 kb sub-fragment from its 3′ end, termed region E, produced the same pattern of neural expression from both the *Hoxb4* (construct 9; Figure 3a and d) and the *Hoxb5* (Figure 2e and h) promoters. At 10.5 d.p.c. expression was concentrated in an anterior domain, reaching the normal *Hoxb5* boundary, and later was also found in a second posterior domain, adjacent to the hindlimb bud. This demonstrates that region E, which functions in both orientations (data not shown), is a neural enhancer capable of directing expression with the same anterior–posterior limits from either the *Hoxb5* or *Hoxb4* promoter.

In contrast, when regions D and E were tested on the *Hoxb4* promoter we observed several major differences in the expression patterns compared with those from the same elements on *Hoxb5* (compare Figures 3b and e, and 2f and i). In particular, on the *Hoxb4* promoter (construct 10; Figure 3b and e), the human placental alkaline phosphatase (*AP*) reporter staining in the neural tube, lateral plate mesoderm and somites had the same anterior boundaries as those seen on the *Hoxb5* promoter (Figure 2f and i). The major difference is that *Hoxb4* expression extended more posteriorly, and both the fore- and hind-limb buds were strongly positive, which is never observed from *Hoxb5*. Similar differences in promoter read-outs were also observed with region D alone, as reporter expression was detected in the limbs and posterior domains on *Hoxb4* (construct 11; Figure

Table I. Summary of the sites of transgene expression from the *Hoxb4* and *Hoxb5* promoters

Construct		*Hoxb5* promoter					*Hoxb4* promoter				
No	enhancer	neural	somites	lpmeso	limb	mes/met	neural	somites	lpmeso	limb	mes/met
1	–	–	–	+	–	+			NA		
2	–	–	±	+	–	+			NA		
3	–	–	±	+	±	+			NA		
4	–	–	±	+	+	+			NA		
5	E	+	±	+	–	+			NA		
6	D+E	+	+	+	–	+			NA		
7	D	±	+	+	–	+			NA		
8	D	±	+	±	–	+			NA		
9	E			NA			+	–	–	–	+
10	D+E			NA			+	+	+	+	+
11	D			NA			±	+	+	+	+
12	D+E	+	+	+	-	+	+	+	+	+	+
13	D	–	+	±	–	–	±	+	+	+	+
14	E	–	–	–	–	–	+	–	+	–	+

(+) expression at proper boundaries; (±) weak expression or only a subset of proper spatial domain; (–) absence of expression; and (NA) not applicable. lpmeso, lateral plate mesoderm; mes/met, mesonephros and/or metanephros. In the column under enhancers, D, D+E and E denote which of the region D and E enhancers are included in the respective construct.

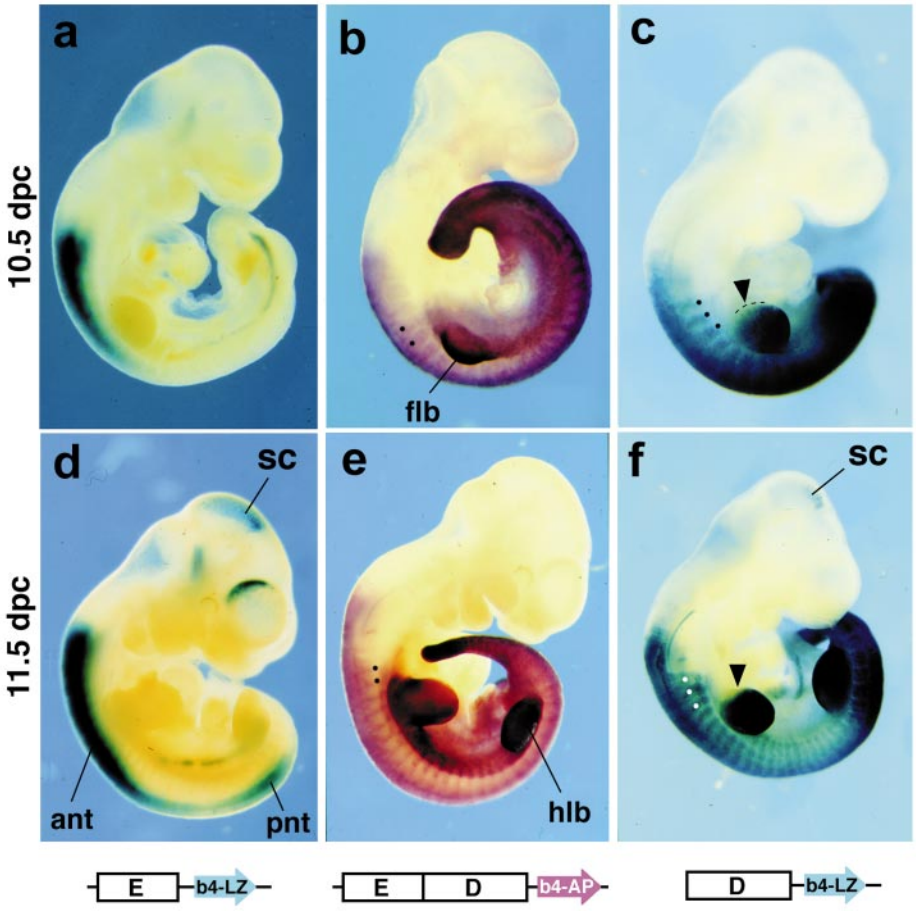

Fig. 3. Expression patterns of regions D and E on the *Hoxb4* promoter. Lateral views of transgenic embryos at 10.5 d.p.c. (**a**–**c**) and 11.5 d.p.c. (**d**–**f**). (a, d) *lacZ* reporter staining of embryos carrying the neural enhancer region E on the *Hoxb4* promoter. (b, e) *AP* reporter staining of embryos carrying regions D and E linked to *Hoxb4*. Note extensive staining in somites, limb and posterior regions compared with expression from the *Hoxb5* promoter (Figure 2f and i). (c and f) *lacZ* reporter expression in embryos carrying the region D enhancer on *Hoxb4*. Relevant constructs are noted at the bottom. Superior coliculus, sc; forelimb bud, flb; hindlimb bud, hlb; anterior neural tube, ant; posterior neural tube, pnt. In b, c, e and f the solid dots indicate number of expressing somites anterior to the limb bud. The arrowheads in c and f indicate loss of expression in the anterior part of the forelimb bud.

3c and f) but not on *Hoxb5* (Figure 2j). Hence region D is able to mediate similar prevertebral expression on *Hoxb4* and *Hoxb5*, but it directs distinctly different patterns from each promoter with respect to the limb, lateral mesoderm and posterior neural tube.

This implies that with respect to limb expression the

region D enhancer has an incompatibility with the *Hoxb5* promoter and selectively works on *Hoxb4* in this assay. It is possible that the lack of limb expression mediated by region D is related to its distance from the *Hoxb5* promoter, and not due to an incompatibility. However, when region D is positioned directly upstream of the *Hoxb5* promoter, instead of downstream, the same selectivity is observed (data not shown). Furthermore, the somite regulatory activity in region D is able to activate this promoter over the same 3′ distance (construct 6; Table I), supporting the idea that different promoter–enhancer interactions are generated by the various enhancer activities within region D. Therefore, our results show that while these intergenic enhancers are indeed capable of working on both *Hoxb4* and *Hoxb5*, there can be marked differences in the patterns generated from each promoter.

Double-marked constructs with the Hoxb4 and Hoxb5 promoters

The above assays evaluated the ability of the region D and E enhancers to function separately on the two adjacent promoters, but their properties may be altered if both promoters are available to compete for enhancer activity. Therefore, we generated the transgenic construct 12, which spans the full genomic region encompassing the two promoters, and used the *lacZ* reporter to mark *Hoxb5* and the *AP* reporter to mark *Hoxb4*. In this way it was possible to monitor the activities of the two promoters independently within the same embryo and make deletion variants to investigate the roles of the specific enhancers. In general, we find evidence for: (i) sharing of enhancers; (ii) selectivity or incompatibility; and (iii) competition. The results from all of these constructs are summarised in Table I and Figure 6, and illustrate the three different types of interactions which take place in the *Hoxb* complex.

In the double-marked constructs we wanted first to examine how the patterns of reporter expression for each gene corresponded with those of the single promoter constructs, and how they related to the endogenous patterns of expression. Assaying the *Hoxb5* promoter in construct 12, reporter staining at 9.5 d.p.c. and 10.5 d.p.c. displayed a sharp boundary in the neural tube and somites (Figure 4b and f), identical to that seen when only the *Hoxb5* promoter was present (construct 6; Figure 2f and i). To verify that the boundaries of reporter staining reflected those of the endogenous *Hoxb5* gene, we made a direct comparison using an anti-HOXB5 antibody (Wall *et al.*, 1992). At 9.5 d.p.c. the reporter staining is weak, but identical to the HOXB5 protein distribution (Figure 4a and b). At 10.5 d.p.c. the transgene and protein expression patterns have resolved into more restricted axial domains in the neural tube and somites, and are still identical (Figure 4e, f, i and j). In the somites(s), the anterior boundary of both the reporter expression and endogenous HOXB5 protein map to the s7/8 junction, which is two somites anterior of the forelimb bud (Figure 4k and l). In the neural tube, using the expression boundary within the adjacent somites as a marker, the anterior limits of both reporter and protein map to the same position (Figure 4i and j). Therefore, the *Hoxb5* reporter expression (construct 12) accurately reflects the endogenous HOXB5 distribution. Next, we assayed expression from the *Hoxb4* promoter (construct 12) and found that it generated an identical pattern (Figure 4c) compared with the single *Hoxb4* promoter construct (construct 10; Figure 3b).

Roles for regions D and E on both Hoxb4 and Hoxb5 promoters

Using the double staining method we were able to compare directly expression from *Hoxb4* and *Hoxb5* in the same embryo. The anterior boundaries of reporter expression in the somites and neural tube for *Hoxb4* were identical to those for *Hoxb5*, and corresponded to the endogenous *Hoxb5* pattern (Figure 4g, k and l). Furthermore, in the anterior domains of these tissues nearly all cells were doubly positive for both markers (Figure 7). This argues strongly that the two promoters are being regulated by the same enhancer(s), and that control regions are shared by both genes (Figure 6c).

As observed with the single-promoter constructs (6 and 10), the double-marking experiments (construct 12) demonstrated a selectivity/incompatibility with respect to expression in the limb buds and posterior neural tube. Staining for *Hoxb4* was generally stronger and more extensive than for *Hoxb5*. At 10.5 d.p.c. *Hoxb4* expression was detected in the fore- and hind-limb buds, and remained strong in posterior neural and mesodermal domains (Figure 4g and l). This contrasted with expression from the *Hoxb5* promoter, which was much more restricted at this stage (compare Figure 4f, g and l). This demonstrates that there is a genuine selectivity or preferential utilisation of the *Hoxb4* promoter in the limb and posterior neural tube by these enhancers (Figure 6i). Furthermore, a similar difference exists in the normal expression of these HOX proteins (Figure 4a, d, e and h), suggesting that the selectivity observed in the transgenic reporters is important for regulating normal expression of these genes.

Individual roles of D and E

We assessed the individual roles of the D and E enhancers by generating two variants of the double-marked construct in which either region E (construct 13) or region D (construct 14) were deleted. In the absence of the region E neural enhancer (13), the *Hoxb4* transgene expression pattern at 10.5 d.p.c. and 11.5 d.p.c. in limbs, mesoderm and the neural tube is identical to that of the unmodified construct (Figures 5b and e, and 6b and c). This is consistent with our findings that region D is able to direct the major aspects of this pattern from the *Hoxb4* promoter.

In contrast, monitoring expression of *Hoxb5* at 10.5 d.p.c. in this same embryo reveals that staining was specifically abolished in the neural tube, demonstrating that the neural activity in region E is essential for *Hoxb5* expression (Figures 5a, and 6b and c). Despite this change, *Hoxb5* reporter expression was unaltered in the somites, and both the *Hoxb4* and *Hoxb5* reporters still displayed staining with anterior boundaries identical to each other, and to the endogenous HOXB5 protein (Figures 5a and b, and 6b). This strengthens the idea that they are being controlled by the same shared somite element(s) in region D. Limb expression remains selectively associated with the *Hoxb4* promoter.

Interestingly, while at 10.5 d.p.c. the neural enhancer in region D is unable to direct expression from the *Hoxb5* promoter (Figure 5a), at 11.5 d.p.c. it does mediate *Hoxb5* reporter staining in the CNS, without loss of neural

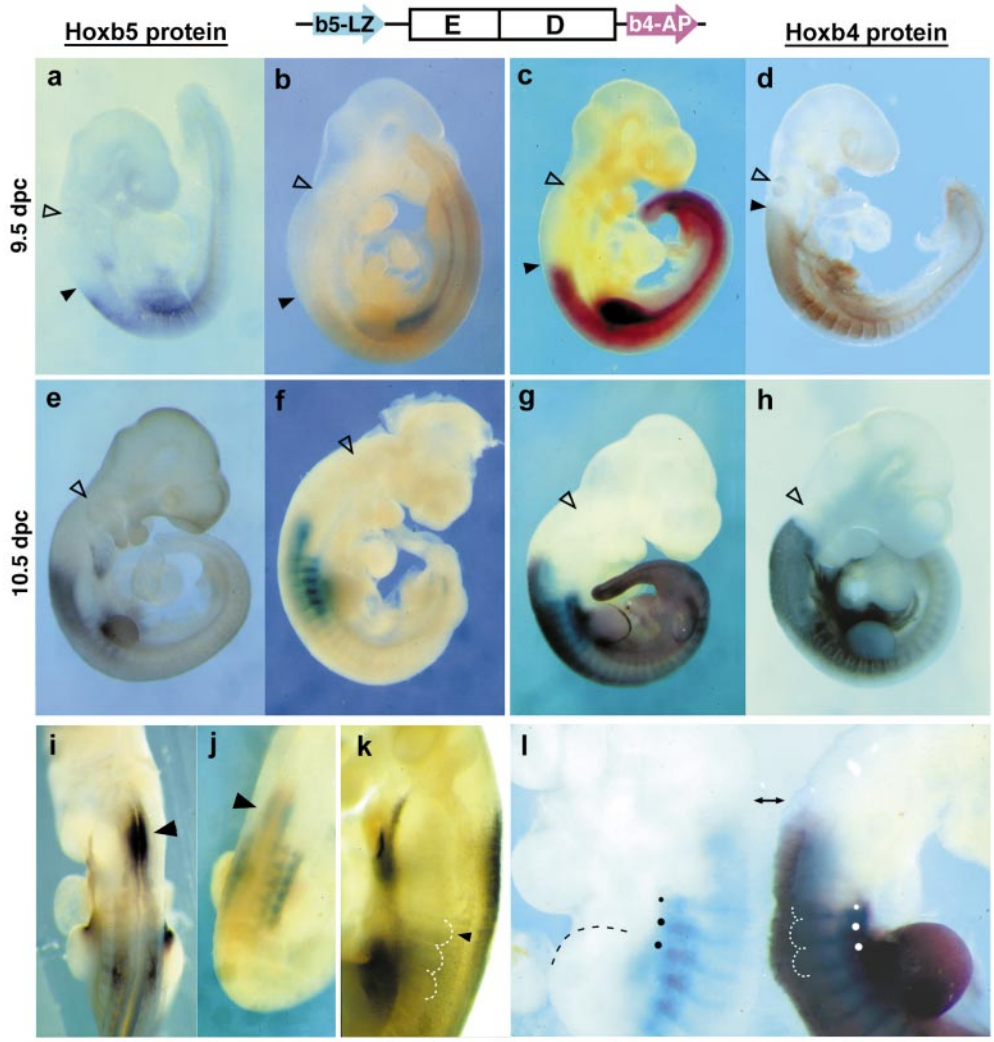

Fig. 4. Transgenic analysis of *cis*-interactions between *Hoxb4* and *Hoxb5* in a wild-type double-marked construct and comparisons with HOXB5 and HOXB4 protein distribution. (**a**, **e**, **i** and **k**) Lateral views (a and e) and dorsal views (i and k) of embryos stained with an anti-HOXB5 antibody. (**d** and **h**) Lateral views of embryos stained with an anti-HOXB4 antibody. (**b**, **c**, **f** and **g**) Lateral views of embryos with construct 12 stained for either a *lacZ* reporter marking the *Hoxb5* promoter (b and f) or an *AP* reporter marking the *Hoxb4* promoter (c and g). In (g) there is simultaneous staining for both reporters. (i and j) Dorsal views comparing HOXB5 protein, (i) with *lacZ* reporter staining and (**j**) for construct 12, indicating similarity in somite and neural (black arrowheads) expression. (**l**) Lateral view of a single embryo carrying construct 12, where one half was stained for *lacZ* (left) and the other half was stained for both *lacZ* and *AP* (right). Note that the neural boundaries (double arrowhead) and the somite boundaries (solid dots) are identical in both cases. The somite limit is the same as the endogenous *Hoxb5* boundary, denoted by the arrowhead in k. In (a–h) the open arrowheads indicate the position of the otic vesicle and the solid arrowheads the boundaries of reporter or protein expression in the neural tube. The respective proteins assayed and the transgenic construct are indicated at the top. Stages are (a–d) 9.5 d.p.c. and (e–l) 10.5 d.p.c.

expression from the *Hoxb4* promoter (Figure 5d and e). Therefore, the selective preference that region D displays for the *Hoxb4* promoter in the neural tube is time-dependent, whereas the selectivity of the limb regulatory element is absolute.

Competition for the region E neural enhancer

We demonstrated that the neural enhancer in region E was capable of working on either the *Hoxb5* or *Hoxb4* promoter separately (Figures 2e and h, and 3a and d), and on the *Hoxb5* promoter in the wild-type double-marked construct (Figure 4f). However, the removal of region D (construct 14) from the dual promoter construct altered the ability of region E to mediate neural expression. We observed a complete absence of neural expression from the *Hoxb5* promoter (Figure 6e), and at the same time neural expression was detected from the *Hoxb4* promoter. These results showed that region E now works preferentially on *Hoxb4*, although there were lower levels of expression and a more posterior boundary than when tested on *Hoxb4* or *Hoxb5* alone (Figures 5c, and 6d and e). The removal of region D also caused the loss of somitic expression from both promoters, consistent with the idea that it is shared between the genes.

Region E on *Hoxb5* alone (construct 5) also directs expression in lateral plate mesoderm (Figure 2e and h), and in construct 14 this domain of *Hoxb5* expression is absent. In fact, we detect no expression from the *Hoxb5* promoter in any tissue with this construct (Figure 6e). However, since the *Hoxb4* reporter expression persists in the lateral plate mesoderm (Figure 5c and f), it appears that the lateral plate mesoderm regulatory activity has also

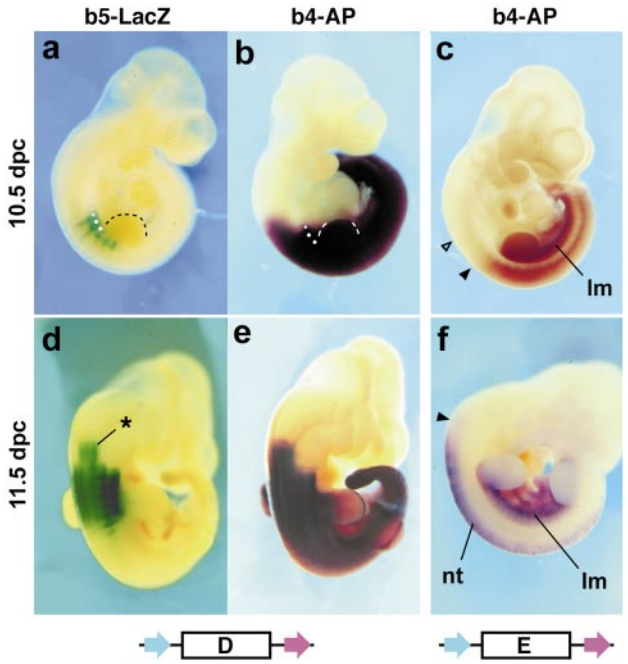

Fig. 5. Analysis of promoter selectivity in double-marked constructs deleting region D or E. (**a**, **b**, **d** and **e**) Lateral views of embryos carrying construct 13 with a deletion of region E stained for either a *lacZ* reporter marking the *Hoxb5* promoter (a and d), or an *AP* reporter marking the *Hoxb4* promoter (b and e). Note the difference in neural *lacZ* staining between the two stages (a and d). The solid dots in a and b indicate the number of positive somites anterior of the limb bud (see also Figure 6b). (**c** and **f**) *AP* reporter staining from the *Hoxb4* promoter in embryos carrying a deletion of region D (construct 14). Note the loss of anterior expression in the neural tube (nt) as shown by arrowheads and the persistence of staining in lateral plate mesoderm (lm). Constructs are indicated below the panels and the promoter/reporter combination above the panels.

switched its preference to the *Hoxb4* promoter (Figure 5c and f). These results indicate that interactions between region E and the two promoters are subject to competition, and that removal of region D changes the competitive balance between them, favouring the recruitment of region E to the *Hoxb4* promoter.

Discussion

In this study on the middle of the *Hoxb* cluster, the ability to simultaneously monitor gene expression from adjacent genes has revealed a series of complex regulatory interactions not apparent from an analysis of each locus individually. Our results demonstrate that there are three distinct mechanisms underlying the *cis*-regulation of *Hoxb4–Hoxb6* which help to generate their spatially restricted patterns of expression, and which are relevant to the conserved organisation of vertebrate *Hox* complexes.

One mechanism is enhancer sharing, whereby control regions mediate the same anterior boundaries of expression on both *Hoxb4* and *Hoxb5*. The second mechanism concerns selective interactions and incompatibility between promoters and enhancers. In this case, a limb and CNS enhancer will not interact with the *Hoxb5* promoter, and selectively mediate their regulatory activity only through the *Hoxb4* promoter. A third mechanism involves competition between the promoters for a control region. In this case, an anterior neural tube enhancer (region E) is capable of activating both promoters, but its relative preference for either is dependent upon the local genomic context.

These mechanisms lead to significant differences in the transcriptional read-out from the *Hoxb4* and *Hoxb5* promoters in the same constructs, which importantly parallel a similar difference in the endogenous distribution of these Hox proteins. We also find that these mechanisms are involved in regulation of other genes in the *Hoxb* complex. Therefore, the correct co-ordination of spatial and temporal expression, integral to *Hox* function, depends on a balanced network of local *cis*-regulatory interactions between genes which could affect expression more globally in the complex. This would make it difficult to disrupt the *Hox* complexes in vertebrates while maintaining their proper regulation, suggesting these interactions are part of the molecular basis for preserving the clustered organisation during evolution.

Sharing

Auto- and cross-regulation are important mechanisms *in trans* for cross-talk between *Hox* genes, but sharing of control elements provides an alternative mechanism operating *in cis* for co-ordinating expression. In this study we identified an intergenic somite enhancer (region D) that directs expression up to an s7/8 anterior boundary when tested on both the *Hoxb4* and *Hoxb5* promoters, individually or in combination (Figure 6a–c). The ability to monitor both reporter patterns within the same embryo is particularly useful in this case and has allowed us to determine that the somite boundaries are identical (Figure 4l), strengthening the idea that common components in region D are being shared between the *Hoxb4* and *Hoxb5* promoters. Hence, despite being immediately adjacent to, and operating on the *Hoxb4* promoter, this region generates an anterior boundary which corresponds precisely to that of endogenous *Hoxb5*. We observed another example of sharing in construct 4 containing *Hoxb5* and *Hoxb6*, where a limb enhancer originally identified upstream of *Hoxb6* (Schughart *et al.*, 1991; Eid *et al.*, 1993) was also able to activate the *Hoxb5* promoter (Figures 2c and d, and 6j). In combination with our finding that *Hoxb3* and *Hoxb4* share an r6/7 neural enhancer (Sham *et al.*, 1992; Gould *et al.*, 1997), these results suggest that sharing *cis*-control elements between adjacent genes is a common feature of the *Hoxb* complex, as summarised by the arrows above the genes in Figure 6j.

Mutagenesis and transposition experiments have shown that elements influencing expression in the limb and vertebrae are also shared in the *Hoxd* complex (Gérard *et al.*, 1996; van der Hoeven *et al.*, 1996; Zakany *et al.*, 1997). Furthermore, in recent transgenic analysis we found that the relative position and tissue-specificity of many control regions in group 4 genes (*Hoxa4*, *Hoxb4* and *Hoxd4*) are conserved (Morrison *et al.*, 1997). Some of these conserved regions correspond to enhancers from the *Hoxb4* locus that we have shown are shared with adjacent *Hoxb* genes, and hence they may be shared with adjacent genes in their respective complexes. At present we do not know how many different genes can share the same elements, but it appears that sharing is a regulatory mechanism common to all vertebrate *Hox* clusters.

To address whether sharing is at work within the same cell or in a mutually exclusive randomised fashion between

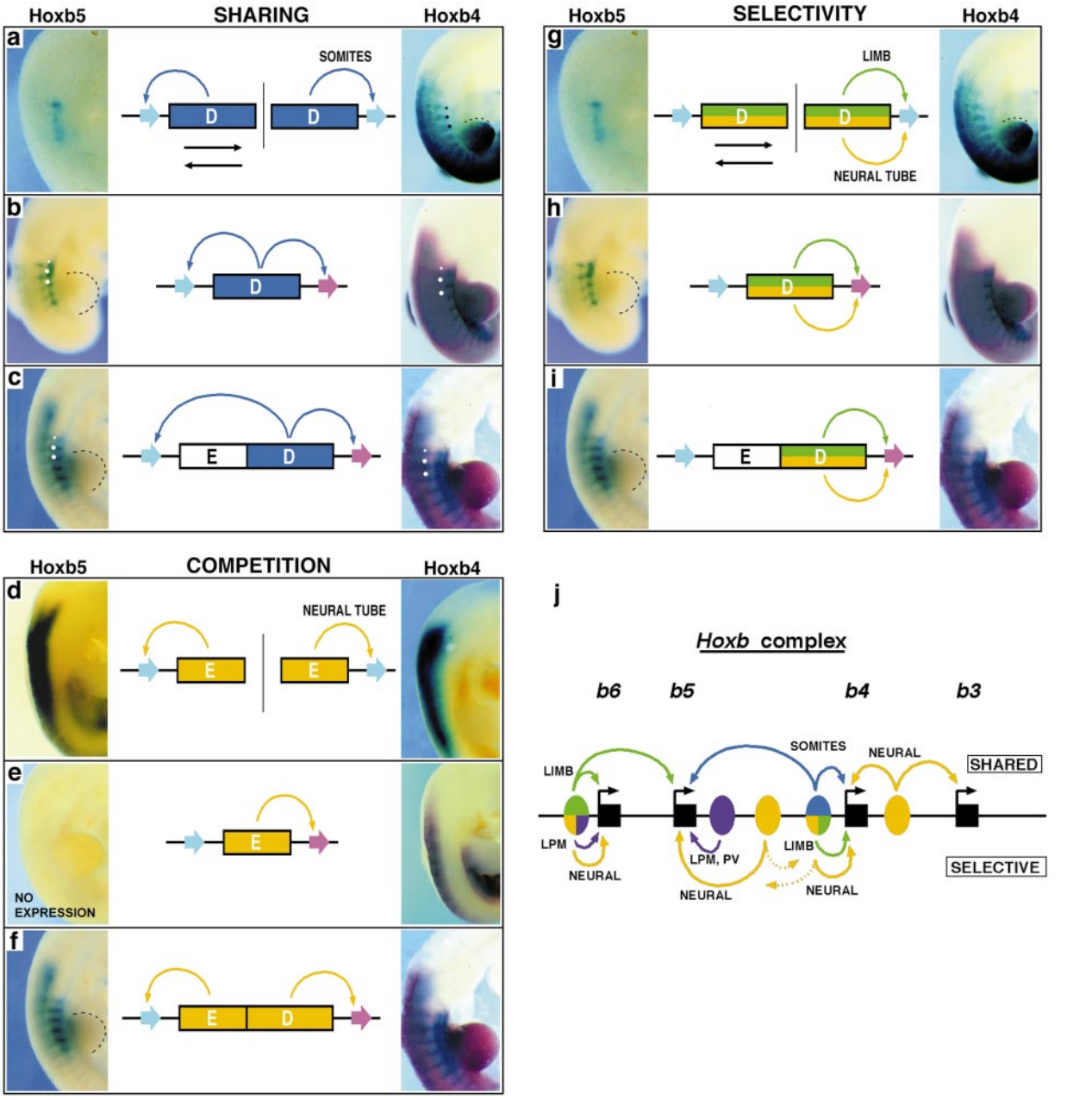

Fig. 6. Summary of the different activities and interactions displayed by regions D and E on the *Hoxb4* and *Hoxb5* promoters. (a–c) Somite regulatory activity from region D is shared equally by the two promoters. Reporter expression is detected up to the s7/8 boundary when region D is tested individually on either the *Hoxb5* (**a**, left) or the *Hoxb4* (a, right) promoters, and when tested on both promoters simultaneously, in the absence (**b**) or presence (**c**) of region E. (d–f) Competition for the neural activity of region E depends upon the context of the intergenic region. Region E directs neural expression independently from either the *Hoxb5* (**d**, left) or *Hoxb4* (d, right) promoters, but is unable to activate the *Hoxb5* promoter in a double-marked construct (**e**) unless region D is also present (**f**). (g–i) At 10.5 d.p.c. the neural and limb regulatory activities in region D display a complete selective preference for the *Hoxb4* promoter. Region D stimulates expression in the limb and neural tube from the *Hoxb4* promoter individually (**g**, right) or in combination with the *Hoxb5* promoter in double marked constructs in the absence (**h**) or presence (**i**) of region E, but fails to mediate similar expression from the *Hoxb5* promoter when tested individually (g, left) or in combination with *Hoxb4* (h and i). At 11.5 d.p.c. for construct 13, region D does stimulate *lacZ* staining in the neural tube (Figure 5a and d), indicating that some preferences can change over time. (**j**) Model summarizing the different regulatory interactions between Hoxb genes. Colours indicate tissue-specific enhancer activities: somites blue, limb green, lateral plate mesoderm purple and neural orange. The ovals mark the position of the enhancers and coloured looped arrows above the complex note shared interactions of these enhancers between promoters, while those below the complex mark enhancer/promoter interactions that are selective. Dashed arrows indicate interactions altered by context.

different cells, we examined expression of the double-marked constructs in sections at high power. Figure 7b shows that within the anterior neural tube both the *AP* and *lacZ* reporters are co-expressed at high levels in a large proportion of the same cells. This is also seen for expression in the somites (data not shown). However, we observe regions in which *AP* is either exclusively or much more strongly expressed than *lacZ* (Figure 7a). Similarly, there are regions where *lacZ* is either the predominant or only reporter expressed (Figure 7c). This indicates that the same regulatory element can indeed be shared within the same cell, but we do not know whether sharing

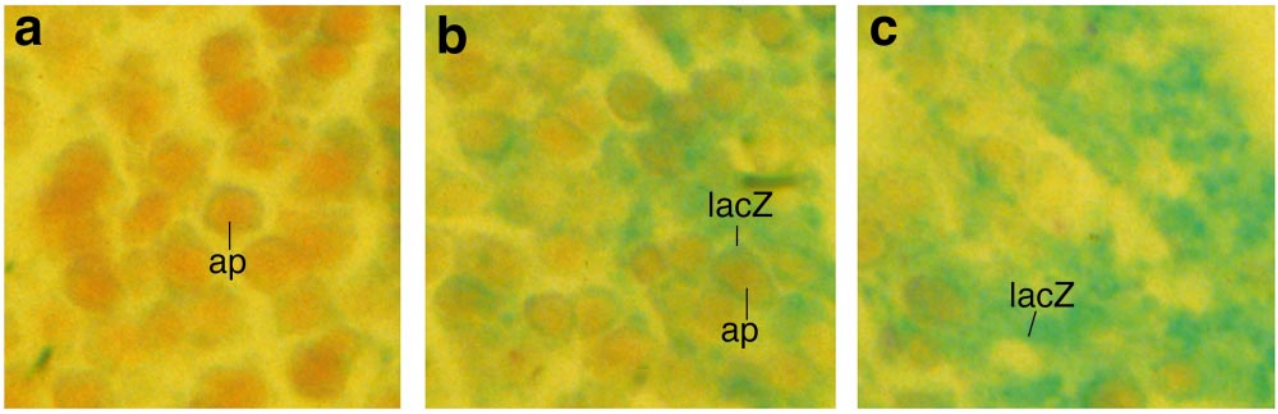

Fig. 7. Analysis of cellular distribution of *AP* and *lacZ* reporter staining in the neural tube. (a–c) Saggital sections through the rostral neural tube of a 10.5 d.p.c. transgenic embryo carrying construct 12, doubly stained for alkaline phosphatase (brownish yellow) and β-galactosidase (light blue) reporter activities. (**a**) A section through a region where the cells are predominantly expressing alkaline phosphatase but some cells also co-express a low level of β-galactosidase, as evidenced by the faint blue haloes around the nuclei. (**b**) A section through an area where the majority of the cells co-express both markers to a similar extent. (**c**) A section showing a region where most of the cells are expressing β-galactosidase at a high level and only a few cells co-express the *AP* reporter. ap, indicates alkaline phosphatase staining and *lacZ*, β-galactosidase activity.

between promoters occurs simultaneously or by a 'flip-flop' mechanism, as proposed for the β-globin gene cluster (Wijgerde *et al.*, 1995).

Selectivity

The widespread occurrence of sharing suggests that absolute insulators or boundary elements which completely isolate adjacent genes are not a common strategy for controlling vertebrate *Hox* genes. If boundary elements are fundamental to the regulatory process they must be exerting their influences to varying degrees on different elements even in the same tissue, or at different developmental stages. Therefore, alternative mechanisms must also be involved in mediating the specificity and directionality of regulatory elements.

One of these mechanisms is selectivity, as illustrated by the limb and neural enhancer activities in region D. Whether tested on the individual promoters or in the double-promoter constructs, limb expression is only found from *Hoxb4* and never from *Hoxb5* (Figure 6g–i). This occurs if region D is placed in either orientation or closer to the *Hoxb5* promoter, suggesting that it is not a distance effect (Table I; data not shown). Thus the limb enhancer displays an incompatibility with the *Hoxb5* promoter and selectively interacts with the *Hoxb4* promoter. The neural activity from region D also displays a similar selectivity for *Hoxb4* over *Hoxb5*, although interestingly, in this case it is time-dependent. Region D mediates no neural expression from the *Hoxb5* promoter at 10.5 d.p.c., but weak expression is detected at 11.5 d.p.c. (Figures 5a and d, and 6g–i). This temporal alteration in neural selectivity is distinct from the limb activity, which is restricted to *Hoxb4* at all stages examined.

Hoxb6 regulatory activities directing expression in the limb, CNS and lateral plate mesoderm are all contained within a small 2.0 kb region 5′ flanking region (Schughart *et al.*, 1991; Eid *et al.*, 1993; Becker *et al.*, 1996), and we have also found evidence for selectivity in the action of this enhancer. While the limb activity is shared between the *Hoxb6* and *Hoxb5* promoters (as noted above; Figure 2c and d), the CNS and mesodermal activities selectively activate only *Hoxb6* (Figure 6j). Therefore, promoter selectivity in the *Hoxb* complex is one of the important mechanisms by which directionality and specificity are regulated and it can be modulated in a temporal and tissue-specific manner. A summary of the selective interactions in the *Hoxb* complex is indicated by the coloured arrows below the genes in Figure 6j.

It is possible that this selectivity could be due to differences in the general class of the two promoters, in light of experiments by Ohtsuki *et al.* (1998), which show that promoters may be arranged into a hierarchy of enhancer-recruiting 'strengths'. The *Hoxb4* (Gutman *et al.*, 1994) and *Hoxb5* promoters do not contain TATA sequences, but putative DPE motifs were found (Burke and Kadonaga, 1996, 1997) located 36 and 38 bp downstream of their respective transcription start sites. Therefore, in our experiments the selectivity of the limb enhancer cannot be attributed to general differences between promoter classes. The promoter specificity we observed might instead be analogous to the types of promoter/enhancer incompatibility previously observed in the *Drosophila gooseberry* and *gooseberry neuro* loci (Li and Noll, 1994), and the loci around *dpp* (Merli *et al.*, 1996). In these cases, enhancers closely positioned to multiple genes are constrained by the properties of the promoters, so they operate exclusively on one gene. However, in our experiments some of the enhancers are able to work in both directions, and the selectivity can vary with time. This indicates that the properties of the *Hoxb* promoters themselves may contribute to, but are not sufficient for, mediating all aspects of the restricted regulation.

Competition

Competition between promoters is the third mechanism involved in controlling the regulatory interactions between adjacent genes. Region E directs an identical pattern of neural expression from the *Hoxb4* or *Hoxb5* promoter when tested separately, and shows no preference for one promoter over the other (Figure 6d). However, when region E alone is challenged with both promoters in the same construct (14) there is a dramatic preference for *Hoxb4* (Figure 6e). In fact, there is no detectable expression from the *Hoxb5 lacZ* reporter, suggesting that the *Hoxb4*

promoter completely out-competes *Hoxb5* for enhancer activity.

In the double-marked construct containing the full intergenic region (12), the competitive balance between the promoters for region E is altered through the inclusion of region D (Figure 6f). In this case, region E is now able to interact with the *Hoxb5* promoter, which demonstrates that the competitive balance between the two promoters and the neural enhancer is context-dependent. There are three ways in which region D could influence this competition: by creating a greater distance between E and *Hoxb4*; by containing an insulator element which specifically prevents *cis*-interactions; or by containing another neural enhancer which competes for the *Hoxb4* promoter. Regardless of the mechanism involved, our results illustrate that competition is likely to be important for restricting the interactions between vertebrate *Hox* enhancers and promoters.

Maintaining the Hox clusters

Figure 6j illustrates the complex network of shared and selective regulatory interactions spread over the core of the *Hoxb* complex. Together these have important implications, not only for generating the proper restricted patterns of expression, but also for maintaining the *Hox* clusters. The sharing of an enhancer between genes may make it difficult to disrupt the *Hox* clusters. However, if such enhancers exert their influence on these genes in a promoter-independent manner, then breaking up the cluster would not prevent appropriate regulation if the enhancer itself was duplicated and carried along with the dispersed gene. The interdigitation of promoters in, or upstream of, adjacent genes (Simeone *et al.*, 1988; Sham *et al.*, 1992) would put an added constraint on the system. However, the demonstration that a balance in promoter competition plays an important role in directing the appropriate spatial and temporal expression of *Hox* genes provides an even stronger reason for maintaining their clustered organisation. The loss or alteration of a promoter could affect not only its own regulation, but also the regulation of its neighbours, 'at a distance', simply by changing their relative competitive balance for a particular enhancer. Therefore, to correctly regulate a *Hox* gene outside of the complex it might be necessary to include not only the relevant enhancers, but also additional promoters to reconstruct the proper competitive balance.

In conclusion, we have demonstrated that within the central core of the *Hoxb* complex at least three different mechanisms operate to control enhancer–promoter interactions important for restricted expression. They do not in themselves explain colinear anterior–posterior expression, but could be an integral part of the process. While these enhancers and promoters influence adjacent genes they could also have longer range influences throughout the cluster.

Materials and methods

Plasmid constructions

Constructs 1–8 and 12–14 all contain a 5.0 kb *Bgl*ll genomic DNA fragment encompassing the *Hoxb5* locus, with the bacterial β-galactosidase reporter gene cloned into the *Bam*HI site of the first exon (Krumlauf *et al.*, 1987) to create a fusion protein. In constructs 1 and 2 this *Bgl*II fragment was cloned into pSal (Graham *et al.*, 1988); in construct 2 a 200 bp *Pvu*II fragment in the 3′-UTR of the *Hoxb5* gene was deleted from construct 1. In construct 3 a 2.4 kb *Pvu*II fragment from the intergenic region between *Hoxb5* and *Hoxb6* was first inserted into the *Pvu*II site of the vector pPolyIII, and then the 5.0 kb genomic fragment with the reporter (construct 1, described above) was cloned into the *Sal*I site of the same vector. To create construct 4, a 7.0 kb *Bam*HI–*Bgl*II fragment including the entire genomic region of *Hoxb6* was cloned into the *Bam*HI site of the pPolyIII, and construct 1 was inserted into the *Sal*I site. Construct 5 was generated by a 3′-extension of construct 1, using a 10.0 kb *Bgl*II–*Kpn*I genomic fragment. Construct 6 inserted a 4.5 kb *Kpn*I fragment to make a 3′-extension of construct 5. In constructs 7 and 8 the same 4.5 kb *Kpn*I fragment was inserted in both a positive and negative orientation in the *Kpn*I site of construct 1.

Constructs 9 and 11 were generated by inserting either the 3.9 kb *Cla*I–*Kpn*I genomic fragment (region E) or the 4.5 kb *Kpn*I fragment (region D) into the *Pst*I site of the *Hoxb4* minimal promoter vector (construct 8 from Whiting *et al.*, 1991). To create construct 10, a 17 kb fragment encompassing *Hoxb4* (construct 1 in Whiting *et al.*, 1991) was cloned into the vector pGP1f, then both the most 5′ of the two *Nco*I sites and the most 3′ of the two *Xho*I sites were each filled in. The *Sal*I–*Nco*I fragment was then replaced with an *AP/SV40pA* cassette. To convert this into the full double-marked construct 12, construct 5 was shortened to a *Bgl*II–*Cla*I fragment (by digesting with *Cla*I and religating the 8.5 and 5.1 kb fragments), and this was inserted into the *Xho*I site in the polylinker of construct 10. Constructs 13 and 14 were created by digesting construct 12 with *Kpn*I and religating either region D or E back into the *Kpn*I site. All constructs were linearised and purified away from vector DNA by gel electrophoresis.

Generation and analysis of transgenic and wild-type mice

Purification of DNA and mouse strains, generation of transgenic mice by pro-nuclear injection and *lacZ* reporter analysis were as previously described (Whiting *et al.*, 1991). Double staining of *lacZ* and *AP* was done with embryos fixed in 4% paraformaldehyde as described (Halliday and Cepko, 1992; Itasaki *et al.*, 1996). *lacZ* staining was performed first as the high temperature required for the *AP* staining procedure destroys the β-gal activity. For *AP* staining it was very important that the X-gal staining solution was thoroughly removed from the embryos, and five 15 min PBS washes with shaking were used. The embryos were then heated to 65°C for 30 min to inactivate the endogenous alkaline phosphatases, allowed to cool for 20 min before being transferred to staining buffer containing 240 mg/ml of levamisol and incubated for 1 h at room temperature. The BCIP and NBT stock solutions were then added (to a concentration of 100 mg/ml and 1 mg/ml respectively) and the embryos further incubated in the dark, at room temperature. The colour reaction was usually complete within 1–2 h, at which point it was stopped by adding a solution of 50 mM EDTA, pH 5.0, and then refixing the embryos in 4% paraformaldehyde. Immunostaining for HOXB5 and HOXB4 proteins was performed as described (Wall *et al.*, 1992; Gould *et al.*, 1997). Somite boundaries of expression were counted using the anterior margin of the forelimb bud as a landmark for absolute somite number, as previously described (Burke *et al.*, 1995; Morrison *et al.*, 1997).

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