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Independent regulation of initiation and maintenance phases of *Hoxa3* expression in the vertebrate hindbrain involve auto- and cross-regulatory mechanisms

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SUMMARY

During development of the vertebrate hindbrain, Hox genes play multiples roles in the segmental processes that regulate anteroposterior (AP) patterning. Paralogous Hox genes, such as *Hoxa3, Hoxb3* and *Hoxd3,* generally have very similar patterns of expression, and gene targeting experiments have shown that members of paralogy group 3 can functionally compensate for each other. Hence, distinct functions for individual members of this family may primarily depend upon differences in their expression domains. The earliest domains of expression of the *Hoxa3* and *Hoxb3* genes in hindbrain rhombomeric (r) segments are transiently regulated by *kreisler*, a conserved Maf b-Zip protein, but the mechanisms that maintain expression in later stages are unknown. In this study, we have compared the segmental expression and regulation of *Hoxa3* and *Hoxb3* in mouse and chick embryos to investigate how they are controlled after initial activation. We found that the patterns of *Hoxa3* and *Hoxb3* expression in r5 and r6 in later stages during mouse and chick hindbrain development were differentially regulated. *Hoxa3* expression was maintained in r5 and r6, while *Hoxb3* was downregulated. Regulatory comparisons of *cis*-elements from the chick and mouse *Hoxa3* locus in both transgenic mouse and chick embryos have identified a conserved enhancer that mediates the late phase of *Hoxa3* expression through a conserved auto/cross-regulatory loop. This block of similarity is also present in the human and horn shark loci, and contains two bipartite Hox/Pbx-binding sites that are necessary for its in vivo activity in the hindbrain. These HOX/PBC sites are positioned near a conserved *kreisler*-binding site (KrA) that is involved in activating early expression in r5 and r6, but their activity is independent of *kreisler*. This work demonstrates that separate elements are involved in initiating and maintaining *Hoxa3* expression during hindbrain segmentation, and that it is regulated in a manner different from *Hoxb3* in later stages. Together, these findings add further strength to the emerging importance of positive auto- and cross-regulatory interactions between Hox genes as a general mechanism for maintaining their correct spatial patterns in the vertebrate nervous system.

Key words: Hox genes, Hindbrain, Segmentation, Transgenic mice, Chick embryos, Auto/cross-regulation, Pbx, Meis

INTRODUCTION

The Hox transcription factors play important roles in regulating anteroposterior (AP) patterning in vertebrate embryos. In the developing nervous system, the subdivision of the hindbrain into a series transient metameric units, termed rhombomeres (r), is a fundamental mechanism used for generating and coordinating regional specification during vertebrate craniofacial development. In the hindbrain, both the expression patterns and function of Hox genes are linked with the process of segmentation (Lumsden and Krumlauf, 1996; Rijli et al., 1998; Trainor et al., 2000). Regulatory and mutational analyses in mice and other species have shown that Hox genes are involved in multiple steps of the segmental process, including specification of the AP identity of rhombomeric segments. For example, *Hoxa1* is required for the formation, growth and/or maintenance of r5 (Carpenter et al., 1993; Dollé et al., 1993; Mark et al., 1993); however, *Hoxa1* also synergizes with *Hoxb1* in establishing the initial segmental identity of r4 (Barrow et al., 2000; Gavalas et al., 1998; Studer et al., 1998) and in regulating the ability of r4 to generate cranial neural crest (Gavalas et al., 2001). Furthermore, through cross-regulatory

interactions *Hoxa1* directly participates in regulating the r4-restricted domain of *Hoxb1* expression, which is necessary for the maintenance of segmental identity (Alexandre et al., 1996; Bell et al., 1999; Goddard et al., 1996; Pöpperl et al., 1995; Studer et al., 1998; Studer et al., 1996; Zhang et al., 1994). There is also synergy between *Hoxa1* and *Krox20* in controlling patterning in r3 (Helmbacher et al., 1998).

The importance of the Hox cascade in hindbrain segmentation raises the question of how the rhombomere-restricted domains of Hox expression are established and maintained. Transgenic regulatory analyses of *cis*-elements, combined with mutant phenotype studies have shown that two transcription factors, *Krox20* and *kreisler*, play crucial roles in directly regulating the rhombomeric expression of multiple Hox genes. *Krox20* regulates the expression of *Hoxa2* and *Hoxb2* in r3 and r5 through the presence of conserved binding sites in enhancers located upstream of these genes (Nonchev et al., 1996a; Nonchev et al., 1996b; Sham et al., 1993; Vesque et al., 1996). However, there is a surprising degree of complexity in the number of *cis*-elements and regulatory components that contribute to the *Krox20*-dependent activity and segmental expression mediated by these enhancers (Maconochie et al., 2001). Similarly, *kreisler* is a conserved *Maf b-Zip* gene required for the formation of r5 and it regulates expression of *Hoxb3* and *Hoxa3* in r5-r6 by directly binding to sequences in their regulatory regions (Cordes and Barsh, 1994; Manzanares et al., 1999a; Manzanares et al., 1997; Manzanares et al., 1999b; Moens et al., 1998; Moens et al., 1996). However, there are distinct differences in the way that *kreisler* activates the segmental expression of these two Hox genes (Manzanares et al., 1999a).

The expression of both *kreisler* and *Krox20* in pre-rhombomeric territories is transient (Cordes and Barsh, 1994; Manzanares et al., 1999a; Moens et al., 1996; Nieto et al., 1991; Wilkinson et al., 1989a), but segmental expression of 3′ Hox genes persists for a longer period. Two different types of mechanisms have been suggested to play general roles in maintaining Hox expression patterns during embryogenesis. One is a chromatin-based or epigenetic model, which, by analogy to *Drosophila*, suggests that vertebrate members of the Polycomb and Trithorax groups serve to maintain a pre-established state of gene activity mediated by other factors (Gould, 1997; Pirrotta, 1997; Schumacher and Magnuson, 1997). The second involves the use of positive auto- and cross-regulatory interactions between Hox genes themselves to reinforce expression triggered by an independent process. In the hindbrain there is evidence that both mechanisms may be important. Targeted disruption of *rae28*, a homolog of the *Drosophila polyhomeotic* gene, results in a shift of *Hoxb3* and *Hoxb4* expression one rhombomere more anterior than their normal segmental boundaries (Takihara et al., 1997). Transgenic analysis has also shown that auto-, cross- and para-regulatory loops, which involve interactions between Hox proteins and their Pbx and Meis/Prep co-factors, are important for maintaining segmental expression of *Hoxb1*, *Hoxb2* and *Hoxb4* (Ferretti et al., 2000; Gould et al., 1998; Gould et al., 1997; Jacobs et al., 1999; Maconochie et al., 1997; Pöpperl et al., 1995; Studer et al., 1998).

Studies on targeted gene replacements and mutations (Condie and Capecchi, 1993; Greer et al., 2000; Manley and Capecchi, 1995; Manley and Capecchi, 1997; Manley and Capecchi, 1998) have shown that members of paralogy group 3 can functionally compensate for each other. The paralogous *Hoxa3, Hoxb3* and *Hoxd3* genes display many similarities in their expression and regulation but there are differences (Hunt et al., 1991a; Manzanares et al., 1999a; Manzanares et al., 1997). Hence, unique roles for individual members of this family may arise owing to differences in their expression domains, rather than differential activity (Greer et al., 2000). In this study we have compared the segmental expression and regulation of *Hoxa3* and *Hoxb3* to investigate how they are controlled after initial activation and if there are regulatory differences between these genes. We find that *Hoxb3* is downregulated after initial activation by *kreisler* and that *Hoxa3* expression in maintained through an auto/cross-regulatory mechanism. This illustrates differences in the temporal regulatory mechanisms of group 3 genes during hindbrain segmentation and underscores the importance of positive auto/cross-regulation in the *Hox* complexes.

MATERIALS AND METHODS

DNA constructs

A 5.5 kb *Kpn*I fragment in the vicinity of the chicken *Hoxa3* locus was isolated from a cosmid encompassing the 3′ region of the *HoxA* complex (a kind gift from Atsushi Kuroiwa). In order to identify conserved regions with the mouse *Hoxa3* r5/r6 enhancer, two four-cutter digest libraries (*Hae*III and *Mbo*I) from this fragment were generated in M13mp18, and randomly sequenced. In this way, a 500 bp *Hae*III fragment with high similarity to the mouse 600 bp *Eco*RV-*Sma*I r5/r6 element (constructs 3.4; Manzanares et al., 1999a) was found. Sequencing outwards on the original 5.5 kb *Kpn*I fragment confirmed that the similarity did not extend further. The relative location of the 500 bp fragment was mapped by PCR.

A 500 bp *Ava*I fragment from the mouse *Hoxa3* locus was isolated and used in the transgenic analysis, as the evolutionarily conserved region of the hindbrain enhancer is completely contained within this region. The 600 bp *Eco*RV-*Sma*I fragment previously used in regulatory analysis (Fig. 4; constructs 3.4; Manzanares et al., 1999a) only contains a part of the conserved domain with the KrA site and one of the HOX/PBC sites. The specific mutations in the MEIS/PREP, Pbx site A and Pbx site B sequences indicated in Fig. 6 were introduced by site-directed mutagenesis in M13 (Sculptor IVM System, Amersham). Multimerized oligonucleotides spanning the two HOX/PBC-binding sites were generated as described (Manzanares et al., 1997). The oligonucleotides used were HOX/PBC-A, 5′-GCGGGTTGATTATTGACCCAC-3′; HOX/PBC-B, 5′-AGCC-GAGTCATAAATCTTGCCC-3′; HOX/PBC-B+PREP/MEIS, 5′-CCGAGTCATAAATCTTGCCCAGCCATAAATGACAAAA-3′; and their complements. All constructs were checked by sequencing on an Applied Biosystems 373A sequencer.

Constructs c1, c2 and 6-11 were generated in a modified version of pBGZ40 (Yee and Rigby, 1993), that contains a basal human *β-globin* promoter, linked to the bacterial *lacZ* gene and an SV40 polyadenylation signal, while constructs 1-5 were generated in a reporter vector containing a basal mouse *Hoxb4* promoter (construct 8; Whiting et al., 1991). Constructs were separated from vector sequences before microinjection by electrophoresis and purified using Gelase (Epicentre Technologies).

Mouse and chicken transgenic analysis and in situ hybridization

The generation of mouse transgenic embryos by pronuclear injection and detection of *lacZ* reporter activity was as described (Whiting et al., 1991). Generation of transgenic chick embryos by in ovo

electroporation was as described (Itasaki et al., 1999), using the same *lacZ* reporter constructs tested in mice. Most constructs were assayed in founder (F_0) transgenic embryos; however, stable lines of mice were generated with several critical constructs, (construct number 1, 3 lines; construct 5, 4 lines) to detail and verify the temporal differences in segmental patterns. The reproducibility and criteria for positive (+) or negative (–) cases of transgene expression, as detailed in the Figures and Table 1, was determined as follows: we scored constructs 1-11 as (+) for r5, r6 or posterior domains (p) only if every transgenic embryo that expressed the relevant reporter construct was positive in those domains. (–) Indicates cases where all embryos expressing the reporter in other sites specifically lack a particular segmental domain. (+/–) notes cases (constructs 8, 9) where expression in the r5 was either very weak or absent in all embryos, even though these embryos had strong positive expression in the posterior domain. It is important to note that in all constructs, except 3 and 4, expression in the posterior neural tube is generated by virtue of independent regulatory elements contained in the enhancer fragments that are separate from those that regulate segmental expression (Manzanares et al., 1999a). Such elements serve as an internal control for the ability and reproducibility of transgene expression, and influences of integration site effects. Hence, in all these cases (–) refers to a specific loss of only rhombomeric expression. Constructs 3 and 4 lack the posterior enhancer elements and do not express in this domain. To confirm that the lack of expression of construct 4 in the r5, r6 and P domains, is meaningful, we generated a large number of transgenic embryos and scored for at least two examples with ectopic expression. This shows the transgene is capable of expressing in some tissues, just not in r5 or r6.

Whole-mount in situ hybridization on mouse and chick embryos was as described (Wilkinson, 1992). The probes used were mouse *Hoxb3*, a 700 bp *Bam*HI-*Hin*dIII genomic fragment containing 3′ coding and untranslated regions (Sham et al., 1992); mouse *Hoxa3*, a 650 bp *Hin*dIII-*Eco*RI *Hoxa3* genomic fragment (Gaunt et al., 1986); chick *Hoxb3*, a 400 bp *Eco*RI-*Sph*I genomic fragment from the 3′ coding and untranslated regions; and chick *Hoxa3*, a 900 bp *KpnI*-*Eco*RI genomic fragment spanning the second exon and 3′ untranslated region (Saldivar et al., 1996).

Electrophoretic mobility shifts assays

All pSG5-derived expression vectors containing *Pbx1a*, *Hoxb1*, *Hoxb3*, *Hoxd3*, *Hoxa3* or *Prep1* coding sequences were translated in vitro using the coupled TNT transcription/translation system (Promega), in the presence of ^{35}S-methionine (Amersham). Prep1 and Pbx were co-translated (plasmids in equimolar amounts). Proteins were visualized by SDS-PAGE followed by autoradiography to ensure they were the correct lengths. For EMSA, 2 μl of reticulocyte lysate containing the desired combinations of in vitro co-translated proteins, were mixed with binding buffer (10 mM Tris-Cl pH 7.5, 75 mM NaCl, 1 mM EDTA, 6% glycerol, 3 mM spermidine, 1 mM DT, 0.5 mM PMSF, 1 mg poly-dIdC, 40000 cpm ^{32}P-labeled oligonucleotide and, when used, unlabeled competitor double-stranded oligonucleotides) to a total volume of 20 μl. After 30 minutes of incubation on ice, the reactions were separated by 5% PAGE in 0.5× TBE. The sequences of the double stranded oligonucleotides A3-PP2, A3-PH1 and A3-PHP1 used in the EMSA of this study are shown in Fig. 3. The MUTA and MUTB oligonucleotides used as competitors contained the same sequences as wild type, except that the A and B Hoxa3/Pbx sites were mutated in the same bases in the same manner as those used for transgenic analysis (Fig. 6A).

Target sites and control elements

The references on the identification/analysis of the HOX/PBC target sites used in Fig. 6 with respect to HOX/PBC are HOX/PBC con (Mann and Chan, 1996); *labial* (Grieder et al., 1997); *Hoxb1* r1-r3 (Pöpperl et al., 1995); *Hoxb2* (Maconochie et al., 1997); *EphA2* (Chen and Ruley, 1998); *Hoxb4* (Chan et al., 1997; Gould et al., 1997); *Dfd EAE1*, *EAE2*, *NAE1* and *NAE2* (Bergson and McGinnis, 1990; Chan et al., 1997; Lou et al., 1995; Regulski et al., 1991; Zeng et al., 1994); *fkh* (Ryoo and Mann, 1999); and *Hoxb6 con*, *Hoxb7 con* and *Hoxa10 con* (Knoepfler et al., 1996). With respect to MEIS/PREP sites, target sites are MEIS/PREP con (Berthelsen et al., 1998b; Chang et al., 1997; Knoepfler et al., 1997; Rieckhof et al., 1997); and *Hoxb1* and *Hoxb2* (Berthelsen et al., 1998a; Ferretti et al., 2000; Jacobs et al., 1999; Maconochie et al., 1997; Pöpperl et al., 1995).

In Fig. 8 the references used for drawing the in vivo roles of factors or sites are, for initiation, *RARE Hoxb1* (Marshall et al., 1994; Studer et al., 1998); *RARE Hoxa1*, (Dupé et al., 1997; Frasch et al., 1995); *RARE Hoxb2*, M. M. and R. K., unpublished; *RARE Hoxa2* (Frasch et al., 1995); *RARE Hoxb4* (Gould et al., 1998; Gould et al., 1997); *RARE Hoxd4* (Zhang et al., 2000); and Kr, Ets and Krox sites in *Hoxa3* and *Hoxb3*, (Manzanares et al., 1999a; Manzanares et al., 1997; M. M. and R. K., unpublished data). With respect to the sites referred to for maintenance auto- and cross-regulatory activity, references are *Hoxb1* (Berthelsen et al., 1998a; Ferretti et al., 2000; Jacobs et al., 1999; Pöpperl et al., 1995); *Hoxb2* (Ferretti et al., 2000; Jacobs et al., 1999; Maconochie et al., 1997); and *Hoxb4* (Chan et al., 1997; Gould et al., 1997).

RESULTS

The paralogous *Hoxa3* and *Hoxb3* genes are differentially expressed in late phases of vertebrate hindbrain segmentation

At 8.25-8.5 days post coitus (dpc) the group 3 Hox genes all start to display sharp anterior limits of expression that map to the r4/r5 boundary. In subsequent stages, the relative levels of expression in the anterior rhombomeres varies for each member (Hunt et al., 1991a; Hunt et al., 1991b; Manzanares et al., 1999a; Wilkinson et al., 1989b). While *Hoxa3* expression is upregulated in both r5 and r6, *Hoxb3* is only upregulated in r5; *Hoxd3* is weakly expressed in these rhombomeres. The initial expression of *Hoxb3* and *Hoxa3* in these segments results from the fact that they are direct targets of *kreisler*.

As a first step in investigating the how segmental domains of *Hoxb3* and *Hoxa3* expression are maintained when *kreisler* is no longer expressed, we examined their expression patterns in mouse and chick embryos in the stages after their initial *kreisler*-dependent activation (Fig. 1). Whole-mount in situ hybridization was performed at 10.5 dpc in mouse embryos and at HH stage 16 in chick embryos. Surprisingly, we detected significant differences between the expression patterns at these stages in both species (Fig. 1). In mouse, *Hoxa3* expression was maintained in r5 and r6 (Fig. 1B,F); however, expression of *Hoxb3* was absent in these segments and a new anterior limit of expression appeared at the r6/r7 junction (Fig. 1A,E). An analogous change in the spatial expression patterns of these genes was detected in chick embryos at HH stage 16. *Hoxa3* was clearly expressed at high levels in r5-r6 (Fig. 1D,H), while *Hoxb3* was downregulated in all of r5 and most of r6, although there were two dorsal stripes of expression in r6 that extended to the r5 boundary (Fig. 1C,G). Interestingly, there was a weak domain of *Hoxb3* expression that extended up to the r3/r4 interface in chick embryos (Fig. 1C,G). This lower level of expression was similar to that seen in the mouse, where it had been previously shown that different *Hoxb3* promoters generate spatially distinct subsets of transcripts (Sham et al., 1992). These data demonstrate that the downregulation of *Hoxb3* in r5 and r6 is a conserved aspect of its regulation and

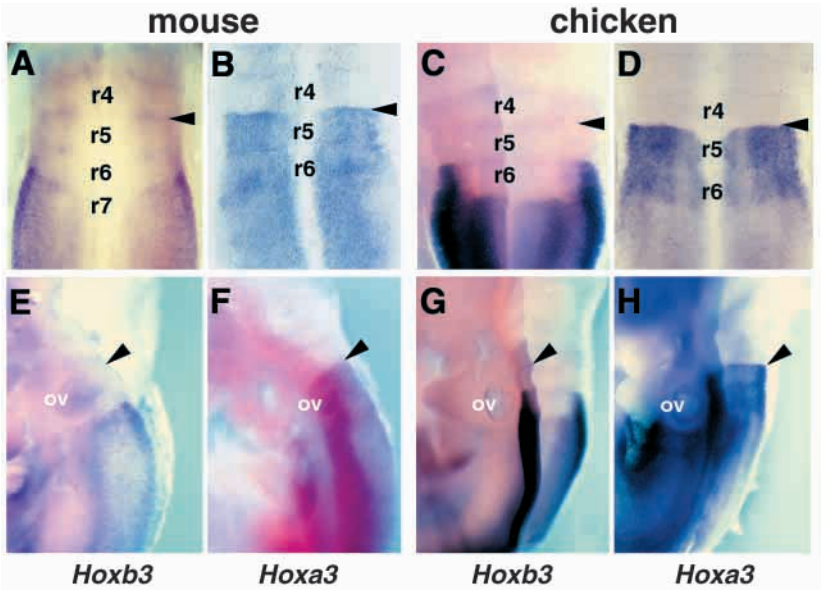

Fig. 1. Differential maintenance of *Hoxa3* and *Hoxb3* expression is conserved in mouse and chick embryos. (A-D) Dorsal views of flat-mounted mouse hindbrains (A,B) and chick hindbrains (C,D) hybridized with *Hoxa3* (B,D) or *Hoxb3* (A,C). (E-F) Lateral views of mouse (E,F) and chick embryos probed with *Hoxa3* (F,H) or *Hoxb3* (E,G). All mouse embryos and tissue were at stage 10.5 dpc, and chick embryos and flat-mounts at HH stage 16. The black arrowheads indicate the r4/r5 boundary. Note that in both species, *Hoxb3* is downregulated in r5 and r6, while *Hoxa3* is maintained in the anterior segments.

distinct from that of *Hoxa3*. Because the initiation of segmental *Hoxa3* and *Hoxb3* expression is regulated by *kreisler* (Manzanares et al., 1999a; Manzanares et al., 1997), which is no longer expressed during the phase of *Hoxb3* downregulation, these changes in late expression reflect differences in the maintenance mechanisms of these genes.

Characterization of a conserved r5/r6 enhancer from the chicken *Hoxa3* locus

To examine the basis of the *kreisler*-independent r5/r6 expression of *Hoxa3* in later stages we decided to first isolate and characterize the related *cis*-regulatory elements required for chick *Hoxa3* expression, so that we could compare them with those of the mouse and try to identify evolutionarily conserved sequence elements. To this end, 5′ genomic fragments upstream of the chick *Hoxa3*-coding region were inserted into a *lacZ* reporter vector (BGZ40) and electroporated in ovo into the chick neural tube to assay for their regulatory potential. In embryos (n=12) assayed at HH stage 14-16, we found that a 5.5 kb *Kpn*I fragment (construct c1; Fig. 2A), reproducibly mediated reporter staining in the neural tube up to an anterior limit at the r4/r5 boundary (Fig. 2B,E). This corresponds to the same segmental limit as that of the endogenous *Hoxa3* gene (Fig. 1D,H). To test if the regulatory activity of this chick fragment was conserved, we generated transgenic mouse embryos with the same c1 construct, and in all cases (n=3; Table 1) observed *lacZ* expression at 10.0 dpc in the neural tube, with a sharp anterior boundary at the level of r4/r5 (Fig. 2C,F). This pattern of reporter expression is virtually identical to that found in the transgenic chick embryos and also resembles that obtained with fragments from the mouse *Hoxa3* locus we previously characterized in isolating the *kreisler*-dependent r5/r6 enhancer (constructs 3 and 3.3; Manzanares et al., 1999a).

Based on the similar regulatory activities of the chick and mouse enhancers and the fact the chick elements will function in the mouse, we scanned the chick 5.5 *Kpn*I fragment for regions of sequence similarity to the mouse enhancer.

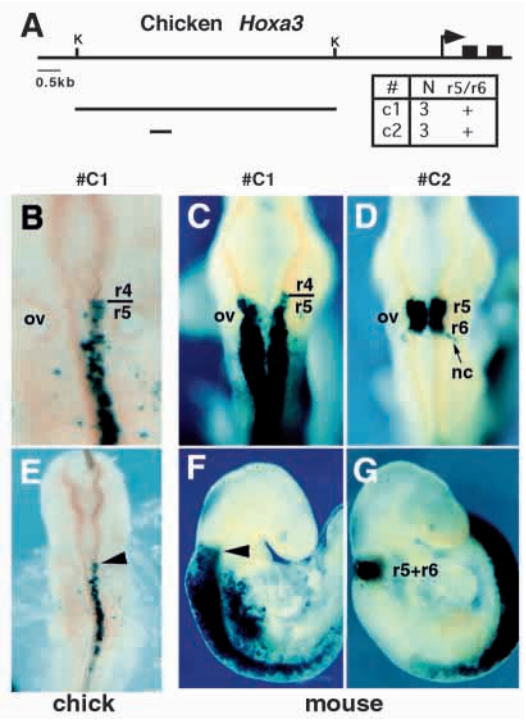

Fig. 2. Identification and activity of a chick *Hoxa3* r5/r6 enhancer in mouse and chick embryos. (A) Diagram of the upstream region of the chick *Hoxa3* locus and fragments used to make constructs for chick and mouse transgenic analysis. On the right is indicated the construct number (#) and also the number (N) of transgenic embryos, all reproducibly expressing the constructs in r5/r6 in the mouse analysis. (B,E) Reporter expression at HH16 in the chick neural tube electroporated on the right side with construct c1. Anterior expression reproducibly (n=12) maps to r4/r5. (C,F) Dorsal (C) and lateral (F) views of transgene expression in 10.0 dpc mouse embryos carrying construct c1. Note that the pattern and anterior boundary is the same as that detected in chick embryos (see B,E). (D,G) Dorsal (D) and lateral (G) views of reporter expression in r5/r6 directed by a region in construct c1 conserved with the mouse *Hoxa3* gene (see Fig. 3). nc, neural crest; ov, otic vesicle. The black arrowheads indicate the r4/r5 boundary. All mouse embryos shown are at 10.0 dpc.

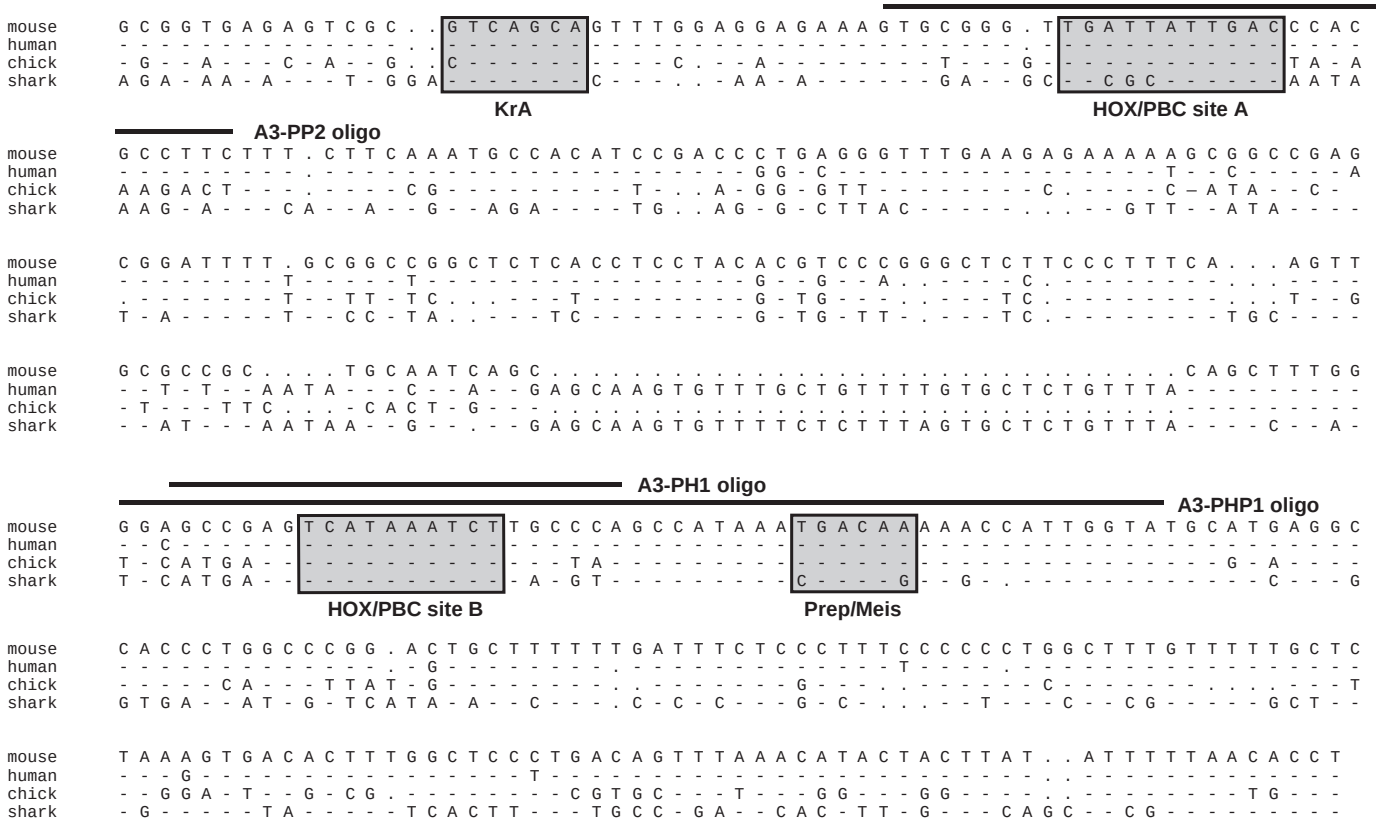

Fig. 3. Sequence alignment of a region conserved between the chick, mouse, human and horn shark *Hoxa3* 5′ flanking regions. The kreisler-binding site KrA previously identified in the mouse enhancer (Manzanares et al., 1999a) is boxed, as are the putative bipartite HOX/PBC sites A and B, and the Prep/Meis consensus motif. The unbroken black lines above the sequence indicate the double-stranded oligonucleotides A3-PP2, A3-PH1 and A3-PHP1 used in binding and competition assays (Fig. 6). Dashes represent an identity and dots a missing nucleotide.

Sequencing and comparisons identified a relatively large block of approximately 400 bp that had >70% similarity with the mouse r5/r6 element (Fig. 2A; Fig. 3). Outside of this region, similarity dropped to non-significant levels. This situation is very different from that of the *Hoxb3* r5 enhancer, where only two blocks of 19 and 45 bp are conserved between mouse (1 kb) and chick (800 bp) regulatory fragments (Manzanares et al., 1997). Furthermore, in comparing the conserved block in *Hoxa3* with database sequences of the complete human and horn shark (*Heterodontus francisci*) *HoxA* clusters, we identified a region of high similarity located in a similar position (5-7 kb) upstream of the *Hoxa3* ATG in both species (Fig. 3; GenBank Accession Number, AC004079; Kim et al., 2000).

We have previously demonstrated that *kreisler* regulates early segmental expression in r5 and r6 mediated by the *Hoxa3* enhancer through interacting directly with a Maf b-Zip DNA-binding motif, termed the KrA site (Manzanares et al., 1999a). This KrA site is also present in the conserved *Hoxa3* block of other species identified above (Fig. 3). The main difference in this conserved domain between species is that a 31 bp insertion occurs at the same position and with an identical sequence in both the shark and human blocks, when compared with mouse and chick (Fig. 3).

To examine its functional activity in stages following initial activation by *kreisler*, a 500 bp chick *Hae*III fragment

Table 1. Transgenic analysis on the ability of Hoxa3 genomic regions to direct segmental expression in r5 and r6

	Early Kr-dependent phase (8.25-9.0 dpc)			Late Kr-independent maintenance phase (9.5-10.5 dpc)			
Construct*	*n*	r5/r6	p	*n*	r5	r6	p
1	38*	+	+	65‡	+	+	+
2	1	–	+	3	–	–	+
3	2	+	–	1	+	+	–
4	n.d.	n.d.	n.d.	2§	–	–	–
5	17‡	–	+	32‡	+	–	+
6	2	+	+	3	+	+	+
7	3	–	+	3	+	–	+
8	n.d.	n.d.	n.d.	4	+/–	–	+
9	n.d.	n.d.	n.d.	4	+/–	–	+
10	n.d.	n.d.	n.d.	5	–	–	+
11	n.d.	n.d.	n.d.	5	+	–	+
C1	n.d.	n.d.	n.d.	3	+	+	+
C2	1	+	+	2	+	+	+

*Construct numbers tested in transgenic analysis using regions indicated in Fig. 4.

‡Numbers include transgenic founder embryos, permanent lines and their progeny.

§Ectopic expression domains where reporter is capable of expression, but only does so under the influence of elements in flanking integration sites.

n, total number of embryos expressing the transgene with an identical pattern; p, expression in posterior region of embryo; n.d., not tested.

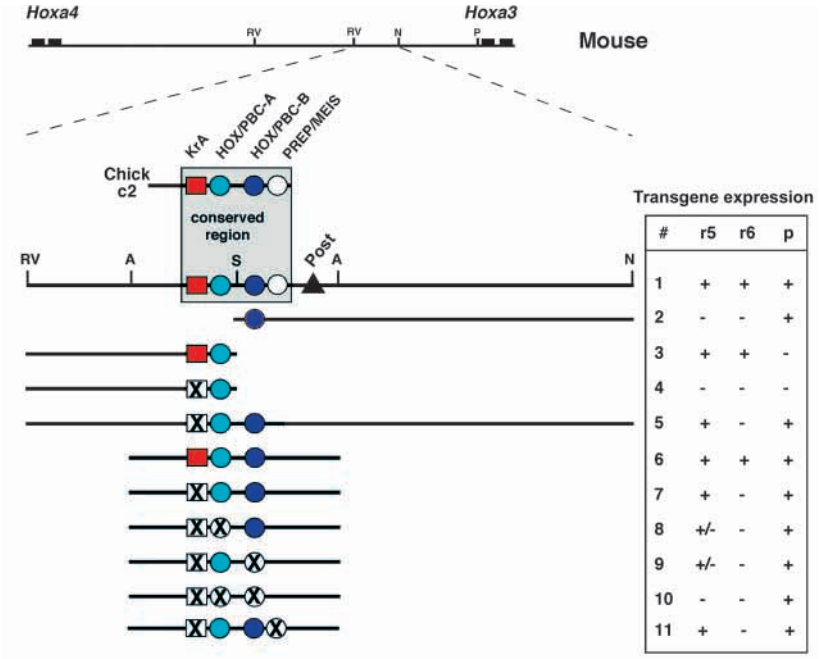

Fig. 4. The mouse *Hoxa3* locus: transgenic constructs tested for regulatory activity. At the top is a diagram of the genomic region and below an expanded view illustrating the wild-type and mutated genomic fragments linked to a *lacZ* vector used for stimulating reporter activity in transgenic analysis. The gray box indicates the conserved region whose sequence is shown in Fig. 3. The KrA-binding site is marked by a red box, the HOX/PBC-A site by a blue circle, the HOX/PBC-B site by a purple circle and the Prep/Meis site by a white circle. The black triangle (Post) represents elements that direct posterior expression in mesoderm and neural tissue; they function independently of the segmental elements. Black X in white squares or circles mark mutated sites. The construct numbers (#) for each fragment and a summary of their domains of expression in r5, r6 and posterior (p) regions is indicated at the right of each fragment. Details of the timing and numbers of expressing embryos are provided in Table 1. Restriction sites are RV, *Eco*RV; A, *Ava*I; S, *Sma*I; P, *Pst*I; and N, *Not*I.

spanning the conserved domain was tested in transgenic embryos. In 10.0 dpc mice, strong *lacZ* expression was detected in r5 and r6 of all (n=3; Table 1) transgenic embryos (construct c2; Fig. 2D,G). In a similar manner, construct c2 also directed robust staining in r5/r6 of all (n=8) in ovo electroporated chick embryos assayed at HH14-16 (Fig. 5D). These experiments demonstrate that this region of similarity contains *cis*-elements important for regulating conserved aspects of segmental expression of *Hoxa3* in later stages in vertebrates.

kreisler-independent control elements direct late segmental expression

We have previously shown (Manzanares et al., 1999a) that a 1.8 kb *Eco*RV-*Nco*I fragment from the mouse *Hoxa3* locus, which includes the conserved block (construct 1; Fig. 4), is capable of mediating both the early and late segmental expression patterns in r5-r6 (Table 1) that are characteristic of endogenous *Hoxa3*. In six founder embryos and progeny from three stable lines carrying this construct (Table 1), strong *lacZ* staining was detected in the future r5/6 territory at 8.25-9.0 dpc and in later stages (9.5-11.5 dpc) this expression was maintained at high levels in r5 and r6 (Fig. 5A,B; data not shown). In addition to the rhombomeric expression, this enhancer also mediated expression in posterior neural and mesodermal domains through separate regulatory regions (see triangle in Fig. 4).

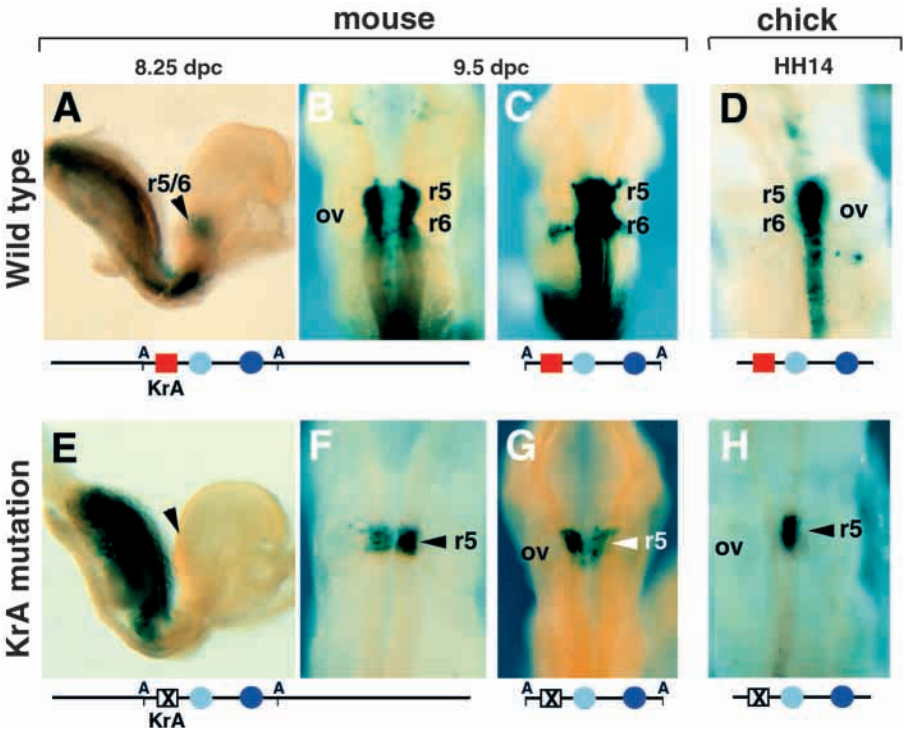

Fig. 5. A *kreisler*-independent activity in the conserved block regulates late segmental expression. (A-C) A large (A,B) and a minimal (C) fragment containing the conserved block from mouse mediates both early (8.25 dpc) and late (9.5 dpc) reporter expression in r5/r6. (E-G) Mutation of the KrA sites eliminates the early but not the late phase of r5 expression in transgenic mouse embryos. (D) A fragment containing the conserved block from chick reproducibly (n=8) directs reporter expression in r5/r6 at late stages (HH14) when electroporated in ovo into the right side of the neural tube. (H) Similar to the result in the mouse, mutation of the KrA site in the chick enhancer (n=11) does not abolish its ability to mediate later stage expression. The relevant constructs used in each case are indicated below each panel with the stages assayed listed above.

To begin to see if we could separate the regulatory regions involved in early and late segmental expression, we tested two subregions of the *Eco*RV-*Not*I fragment contained in construct 1 by taking advantage of a *Sma*I site that cuts in the middle of the conserved block (Fig. 4). When a 1.2 kb *Sma*I-*Nco*I subfragment (construct 2), which contains a 3′ part of the conserved block, was assayed in transgenic embryos, it was unable to direct reporter activity in hindbrain segments at any stage (Table 1; Fig. 4), although there was expression in posterior domains of the embryos (data not shown; Manzanares et al., 1999a). Next we tested a 600 bp *Eco*RV-*Sma*I genomic fragment from the 5′ part of the *Eco*RV-*Nco*I region. This construct (3) contains the KrA site necessary for early r5/r6 expression and part of the conserved block (Fig. 4). As expected, this construct directed reporter expression in r5 and r6 in transgenic mouse embryos, and mutation of the KrA site (4) abolished all regulatory activity (Table 1; Fig. 4).

Next, we tested whether the conserved region itself was sufficient to mediate early and late r5/r6 expression. Using a 500 bp *Ava*I fragment, containing the full mouse conserved region (construct 6; Fig. 4), we found that strong reporter staining was detected in both early and late stages (Table 1; Fig. 5C). Furthermore, when the conserved domain from chick (construct c2; Fig. 4) was tested by in ovo electroporation, it also mediated expression at later stages (n=8; Fig. 5D). To determine if the KrA site and, hence, *kreisler* is required in this context, we generated specific mutations in the KrA sites of the mouse (constructs 5, 7; Fig. 4) and chick fragments that were capable of directing the late patterns. These mutations abolish the early domains (8.25-9.0 dpc) of expression in r5 and r6 (Table 1), confirming the role of *kreisler* in initial activation. However, the KrA mutation does not affect the ability of these enhancers which span the conserved block, to direct late segmental expression in r5 and partially in r6 (Fig. 5E-H; data not shown). This demonstrates that sequences within both the mouse and chick conserved blocks can also function in a *kreisler*-independent manner to maintain *Hoxa3* expression at later stages.

Functional HOX/PBC binding sites in the conserved *Hoxa3* hindbrain enhancer

To identify the basis of this separate activity, we examined the sequences in more detail. While no obvious motifs arose from a database search, we found two putative sites (HOX/PBC-A and HOX/PBC-B; Fig. 3) related to consensus bipartite binding sites for Hox and Pbx proteins (Fig. 6A; Chan and Mann, 1996; Chan et al., 1997; Mann and Chan, 1996; Mann, 1995). In addition, we also found a putative binding site for the Prep/Meis family of homeodomain proteins (Figs 3, 6A), which are able to complex with Hox and Pbx proteins to facilitate the binding and the in vivo activity of Hox-responsive elements (Berthelsen et al., 1998a; Ferretti et al., 2000; Jacobs et al., 1999; Rieckhof et al., 1997; Ryoo et al., 1999). In light of the evidence suggesting that auto- and cross-regulatory loops are important in hindbrain patterning (Ferretti et al., 2000; Gould et al., 1997; Jacobs et al., 1999; Maconochie et al., 1997; Pöpperl et al., 1995; Studer et al., 1998), we examined these motifs in more detail.

The bipartite HOX/PBC sites A and B in the *Hoxa3* enhancer differ from the well characterized *labial/Hoxb1/Hoxa1* or group 1 target sites previously identified. They contain TA or TT in the center of the core instead of GG, and more closely resemble sites for paralogy groups 4-10 (Fig. 6A). We have previously demonstrated that multimerized versions of HOX/PBC sites from in vivo target genes can direct highly restricted patterns of expression in mouse and fly embryos, and that the central two base pairs in the core site are important in modulating the specificity of Hox proteins that act through these sites (Chan et al., 1997; Gould et al., 1997; Maconochie et al., 1997; Pöpperl et al., 1995). To test the potential of these sites in *Hoxa3*, we generated five copies of HOX/PBC-A linked to a *lacZ* reporter. Interestingly, this synthetic combination mediated staining in r5-r6 and/or posterior regions of the neural tube (Fig. 6B,C). This pattern is similar to the *Hoxa3* enhancer and distinct from the r6/7 and r4 patterns seen with multimerized HOX/PBC sites from *Hoxb4* (Chan et al., 1997) and *Hoxb1/Hoxb2*, respectively (Maconochie et al., 1997; Pöpperl et al., 1995). These results illustrate that subtle differences in the bipartite sites can result in dramatically different readouts of in vivo activity.

To confirm that these HOX/PBC sites are able to interact with Hox, Pbx and Prep proteins, we first used a previously established electrophoretic mobility shift competition assay based on the ability of combinations of these proteins to bind to HOX/PBC and Prep/Meis sites from the *Hoxb2* gene (Berthelsen et al., 1998a; Ferretti et al., 2000). Using double-stranded oligonucleotides spanning HOX/PBC site A (A3-PP2) or site B (A3-PHP1) as cold competitors, the binding of Pbx1a/Hoxb1 and Prep1/Pbx1a heterodimeric complexes, and of ternary Prep1/Pbx1a/Hoxb1 complexes to the *Hoxb2* motifs (B2-PP2) were all inhibited by wild-type sequences, but not by mutant forms (MUTB) of the HOX/PBC sites (Fig. 6A,D,E).

To test for direct binding of Hox group 3 and Pbx proteins, we used labeled double-stranded oligonucleotides spanning the *Hoxa3* HOX/PBC site B (A3-PH1; Fig. 3) as a substrate for complex formation. In the presence of Pbx1a and Hoxa3, Hoxb3 or Hoxd3 slower migrating complexes are formed by comparison with the addition of Pbx1a alone (Fig. 6F). Under these conditions, Hoxb3/Pbx1a heterodimers are more efficient at forming complexes on the HOX/PBC-B site that those with Hoxd3 or Hoxa3 and the relative efficiency appears to be Hoxb3>Hoxd3>Hoxa3. However, complex formation with each of these combinations of Hox group 3 and Pbx1a heterodimers is inhibited by an excess of cold competitor for the wild-type site (A3PH1) or anti-Pbxa antibodies (α-Pbxa) but not by an excess of competitor carrying the same mutations in the Hox/PBC-B site (MUTB), as tested in transgenic analysis (Fig. 6A,F). Together, these transgenic and in vitro results suggest that the sites in *Hoxa3* may be important for enhancer activity, by mediating interactions with Hox/Pbx/Meis-Prep transcriptional complexes.

Both HOX/PBC sites are required in vivo for the late phase of enhancer activity

Next we wanted to evaluate the relative in vivo roles of the HOX/PBC and Prep/Meis motifs found in the conserved regulatory region. Our previous analysis (Manzanares et al., 1999a) had suggested that the *kreisler* KrA site itself was sufficient for r5/r6 activity; in this study, construct 3, which contains a wild-type KrA site and only one of the HOX/PBC sites, properly initiated r5/r6 expression in transgenic mice. Furthermore, electroporation in chick embryos suggests that

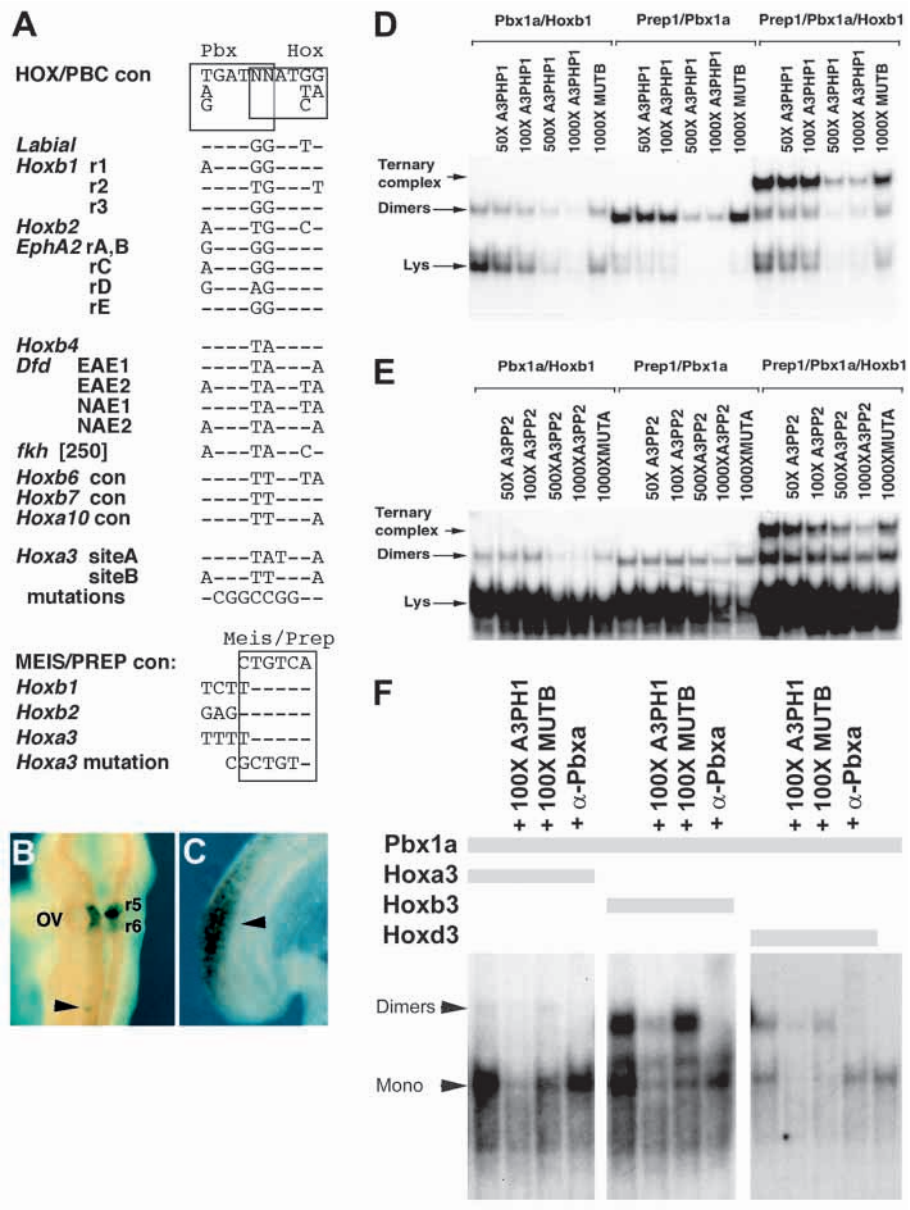

Fig. 6. Consensus bipartite HOX/PBC sites in the enhancer and their properties. (A) List of characterized HOX/PBC sites and Meis/Prep sites found in target genes aligned with those detected in *Hoxa3*. Mutated sequences used in binding and transgenic assays are indicated. (B,C) Reporter expression in 10.0 dpc mouse embryos carrying a construct with five copies of the HOX/PBC-A site linked to *lacZ*. Note strong expression in r5/r6; arrowhead marks posterior neural expression. (D,E) Electrophoretic mobility shift assays where a labeled double-stranded oligonucleotide containing the *Hoxb2* HOX/PBC site and its associated Prep/Meis-binding site (B2-PP2; Ferretti et al., 2000) has been mixed with the combinations of Pbx1a, Prep1 and Hoxb1 proteins (noted above the panels) in the absence or presence of varying amounts of cold competitor oligonucleotides spanning the HOX/PBC-A site (A3-PP2; E) or the HOX/PBC-B site (A3-PHP1; D). MUTA and MUTB are mutant forms of the competitors carrying the changes noted in A. Arrows at the sides indicate shifted complexes interacted with dimeric and trimeric combinations. (F) Gel shift assay where a labeled double-stranded oligonucleotide containing the *Hoxa3* HOX/PBC-B site (A3-PH1; Fig. 3) has been mixed with the combinations of Pbx1a, Hoxb3, Hoxa3 or Hoxd3 proteins (noted above the panels) in the absence or presence of a 100 times excess of cold competitor oligonucleotides containing the wild-type (A3-PH1) or mutated form of the HOX/PBC-B site (MUTB). The addition of anti-Pbxa antibodies (α-Pbxa) inhibits complex formation.

initial activation does not require the Hoxa3 HOX/PBC sites. Therefore, we generated a series of variant constructs where each HOX/PBC or PREP/Meis motif was separately mutated (constructs 8-11; Fig. 4), in order to assay for the roles of these elements in maintaining later patterns of segmental expression. These mutations were made in the context of the 500 bp *Ava*I fragment which spans the conserved block. These variants also contained a mutated KrA site (construct 7, Fig. 4) to ensure that only the *kreisler*-independent activity of the control region was being tested. In the version with the KrA mutation and the two wild-type HOX/PBC sites (construct 7), expression was not activated early in r5/r6, but came on later in r5 (Figs 5G, 7A,F; Table 1). Mutation of site A or site B dramatically reduced the levels of reporter expression in late stage embryos (Fig. 7B,C,G,H) and the combined mutations of both sites completely abolished enhancer activity (Fig. 7D,I). By contrast, a mutation in the Prep/Meis site in the same context of a mutated KrA site had no effect on reporter expression (Fig. 7E,J). Together, these experiments indicate that both of the HOX/PBC sites contribute to the late phase of segmental expression in r5/r6 and suggest that after the initial activation by *kreisler,* the maintenance of *Hoxa3*, as opposed to *Hoxb3*, is controlled by a conserved auto/cross-regulatory loop during hindbrain development.

DISCUSSION

In this study, we have shown that the segmental patterns of *Hoxa3* and *Hoxb3* expression in r5 and r6 initially activated by *kreisler* are differentially regulated and maintained in later stages of mouse and chick hindbrain development. We found that *Hoxb3* was downregulated in the anterior segments, while *Hoxa3* expression was maintained. Functional comparisons of chick and mouse *cis*-elements in the *Hoxa3* locus have allowed us to identify a conserved enhancer that mediates the late phase

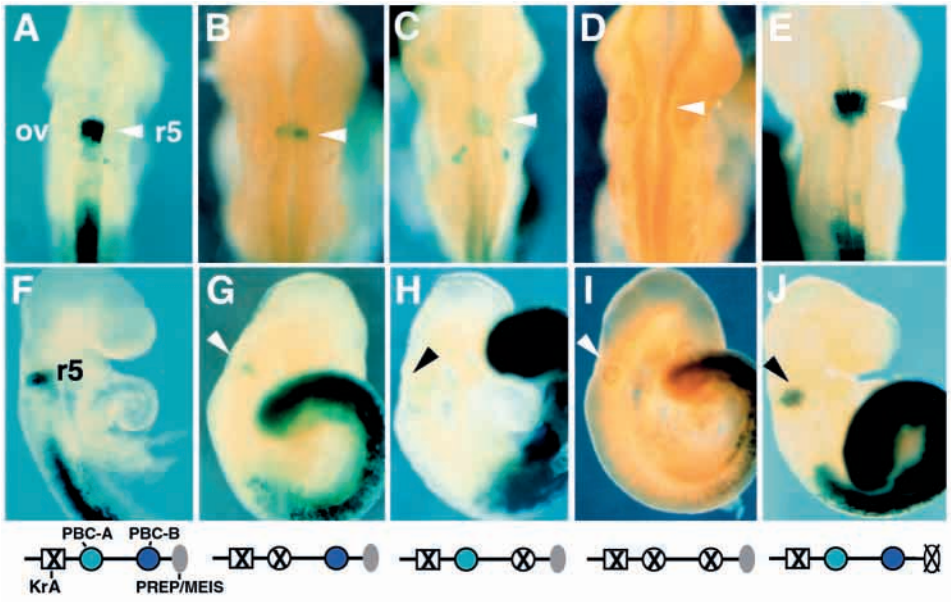

Fig. 7. Transgenic assay indicates HOX/PBC sites are necessary for enhancer activity in the hindbrain. (A,F) Dorsal (A) and lateral (F) views of reporter expression in 10.0 dpc embryos directed by a fragment that spans the conserved block from the mouse *Hoxa3* locus in which the KrA site has been mutated (construct 7, Fig. 4). The activity of this fragment is dependent upon the late control elements. (B,C,G,H) When the HOX/PBC-A (B,G) or the HOX/PBC-B (C,H) sites are mutated (constructs 8 and 9, respectively), expression in r5/r6 is reduced. (D,I) Reporter staining is completely abolished when both HOX/PBC sites (construct 10) are mutated. (E,J) Mutations in the Prep/Meis motif (construct 11) have no effect on reporter activity. All embryos are at 10.0 dpc. The respective constructs and mutated sites are noted at the bottom.

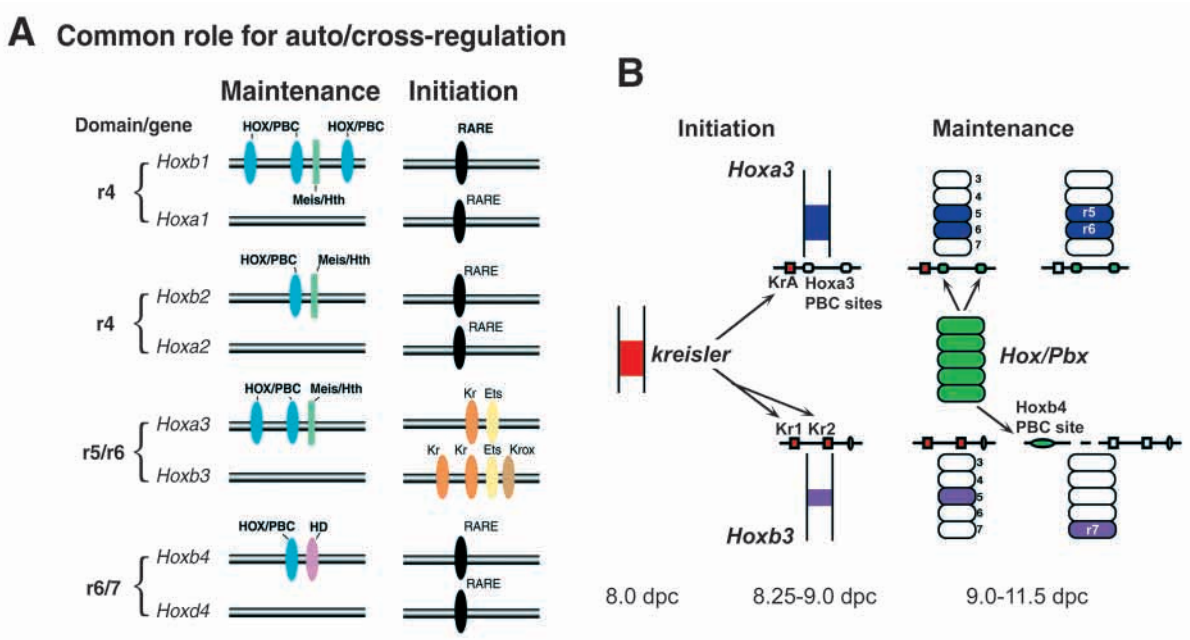

Fig. 8. Model for regulation of *Hoxa3* and *Hoxb3* and common role for auto/cross-regulatory mechanisms in maintaining of Hox gene expression in the developing hindbrain. (A) Hox genes are grouped in paralogous relationships and listed under the column 'Domain/gene'. The specific rhombomeric domain(s) of expression for each set of Hox genes is listed on the left. Under the column 'Initiation', is shown the types of factors or sites that are involved in triggering the initiation of segmental expression of the respective Hox genes in the nervous system. The ovals indicate binding sites for retinoic acid receptor elements (RARE, black); kreisler (Kr, orange); Ets factors (yellow); and Krox20 (Krox, brown). Under 'Maintenance' is illustrated the distribution of bipartite Hox/Pbx (HOX/PBC, blue ovals) and Meis/Prep/Hth (Meis/Hth, green box) -binding motifs that form the Hox auto or cross-regulatory elements. The purple oval for *Hoxb4* indicates a homeodomain (HD) binding site necessary for auto-regulatory activity in which the factor binding has not been identified. Note that, to date, only one paralog from each group has a *Hox* auto/cross-regulatory response element, while they often share common types of initiation elements. For references on the identification and roles of the sites see Materials and Methods. (B) Model comparing the regulatory interactions leading to similar, yet distinct patterns in the initiation and maintenance of the segmental expression of the paralogous *Hoxa3* and *Hoxb3* genes. Early expression of *kreisler* (red) at 8.0-8.25 dpc triggers segmental expression of *Hoxa3* (blue) and *Hoxb3* (purple) from 8.25-9.0 dpc, via the presence of kreisler-binding sites (KrA, Kr1 and Kr1) in the genes. Unlike the r5/r6 domain of *Hoxa3*, *Hoxb3* expression is restricted to r5, by cooperation of kreisler with Krox20 and Ets proteins. In later stages (9.0-11.5 dpc), when *kreisler* is downregulated, segmental expression of *Hoxa3* is maintained in r5 and r6 by Hox and Pbx factors through two HOX/PBC sites that are not present in *Hoxb3* control regions. *Hoxb3* is expressed more posteriorly in r7 of the hindbrain via the action of a shared enhancer, with Hoxb4 itself also dependent upon Hox /Pbx interactions.

of *Hoxa3* expression. This control region contains two bipartite HOX/PBC-binding motifs that will inhibit ternary complex formation by Hox, Pbx and Prep/Meis proteins on other well-characterized HOX/PBC sites (Pöpperl et al., 1995; Ferreti et al., 2000) and themselves bind heterodimeric complexes containing Pbx1a and Hoxb3, Hoxa3 or Hoxd3. Both of the Hoxa3 HOX/PBC sites contribute to and are necessary for full in vivo activity of the conserved enhancer in the hindbrain. These sites are positioned near conserved *kreisler*-binding sites involved in activating early expression in r5 and r6, but their activity is independent of *kreisler* and they form part of a conserved auto/cross-regulatory loop for feeding back on the regulation of *Hoxa3* at later stages. These results demonstrate that separate elements are involved in initiating and maintaining *Hoxa3* expression during hindbrain segmentation, and raise a number of interesting issues with respect to regulation of *Hox* genes and hindbrain patterning.

Auto/cross-regulation and maintenance of group 3 expression

The data in this study and our previous studies suggest a model for initiation and maintenance of *Hoxa3* and *Hoxb3* during hindbrain segmentation (Fig. 8B). The early transient expression of *kreisler* in the future r5 and r6 territories activates these Hox genes by binding to conserved *kreisler/Krml1* sites in their upstream enhancers. As *kreisler* expression decreases, *Hoxa3* is maintained in later stages through input from other Hox genes via an auto/cross-regulatory loop involving the conserved HOX/PBC sites (Fig. 8B). By contrast, expression of *Hoxb3* is downregulated in r5 and r6 as it lacks similar HOX/PBC sites associated with the *kreisler* response elements. However, *Hoxb3* expression is not lost in other regions of the hindbrain as a new anterior boundary is formed at the level of r6/r7 (Fig. 1A,E). This domain of expression coincides with that of *Hoxb4* (Wilkinson et al., 1989b) and is generated by an enhancer (region A) shared between *Hoxb3* and *Hoxb4*, whose late activity is also controlled by a *Hox/Pbx*-dependent mechanism (Fig. 8B; Gould et al., 1997; Sham et al., 1992; Whiting et al., 1991). Therefore, while the anterior boundaries of *Hoxb3* and *Hoxa3* are different from each other in later stages, both could be dependent upon *Hox* auto- and cross-regulatory loops.

The HOX/PBC site from the *Hoxb4* gene is capable of mediating a Hox response from members of multiple paralogous groups (Gould et al., 1997) and the same may be true for the *Hoxa3* HOX/PBC sites. In addition to the group 3 members, the *Hoxa2* and *Hoxb2* genes are also expressed in r5 at the relevant stages of hindbrain development when the enhancer is active. In this regard it is interesting to note that we found that the *Hoxa3* HOX/PBC sites are only capable of directing high levels of expression in r5, compared with r6 in later stages. This might be a consequence of the combined action of multiple inputs from the group 3 and group 2 genes in r5 on the HOX/PBC sites. Furthermore, when the wild-type *Hoxa3* enhancer carrying both the early and late elements was tested in a *kreisler* mutant background, r5/r6 expression was lost, but ectopic activity of the reporter was detected in r3 (Manzanares et al., 1999b). This could reflect a cryptic response to group 2 Hox products in a situation where the normal *kreisler* input is lost. In addition, multimers of the HOX/PBC-A site direct reporter expression in posterior neural domains outside the hindbrain, consistent with the idea they could be under the control of more 3′ or posterior Hox genes.

In the initiation phase, a variety of different factors and pathways (RA signaling, *Krox20*, *kreisler*, *Ets*) are involved in triggering expression of anterior Hox genes (groups 1-4; Gavalas and Krumlauf, 2000; Maconochie et al., 1996; Manzanares et al., 1999a; Trainor et al., 2000). By contrast, the identification of HOX/PBC sites in the late *Hoxa3* segmental enhancer, in combination with those found in rhombomere-restricted enhancers from the *Hoxb1*, *Hoxb2* and *Hoxb4* genes, suggest that auto- and cross-regulation of Hox genes during hindbrain segmentation is a common theme required for maintenance or diversity of expression in later stages (Fig. 8A). The organization and interplay between the *cis*-elements in these Hox maintenance control regions can exhibit considerable variation. HOX/PBC sites are presented in all of these regulatory elements associated with maintenance, but the number of sites varies from one to three (Fig. 8A). We also detected a possible binding site for the Prep/Meis family of Hox/Pbx co-factors in the *Hoxa3* enhancer, indicating that they are frequently found in close association with the HOX/PBC sites.

All of individual HOX/PBC sites have been shown to be important and contribute to enhancer activity in each respective gene (Fig. 7; (Chan et al., 1997; Gould et al., 1997; Maconochie et al., 1997; Pöpperl et al., 1995). However, the relative contribution made by the different Prep/Meis sites associated with these HOX/PBC motifs is more variable. In the case of *Hoxb2*, the Prep/Meis motif is essential for enhancer activity (Ferretti et al., 2000; Jacobs et al., 1999), but in *Hoxb1* and *Hoxa3* this site is not absolutely required for (Fig. 7; Ferretti et al., 2000). Furthermore, in the *Drosophila labial* gene, an HTH/Meis site has been shown to be essential for the activity of a *labial* response element in a minimal enhancer, but not if additional flanking regions are added (Ryoo et al., 1999). One reason for such variation might be that multiple HOX/PBC sites in *Hoxa3* and *Hoxb1* that contribute to expression compensate for loss of the Meis/Prep motif, while the single HOX/PBC site in *Hoxb2* cannot overcome this change.

Another important difference relates to the distinct regulatory abilities of the HOX/PBC sites present in the maintenance enhancers. Multimers of the *Hoxa3* HOX/PBC A site direct reporter expression in r5 and r6, mimicking part of the endogenous expression pattern. This pattern is different from those seen when multimerizing HOX/PBC sites from *Hoxb1* (r4), *Hoxb2* (r4) or *Hoxb4* (r6/r7; Chan et al., 1997; Gould et al., 1997; Maconochie et al., 1997; Pöpperl et al., 1995). Hence, despite the apparent similarity in these bipartite Hox/Pbx-binding motifs, this demonstrates that small variations in sequence can have dramatic effects on their in vivo activities (Fig. 6). This is in agreement with experiments that show changes in the central core can swap the specificity of these sites (Chan et al., 1997; Ryoo and Mann, 1999). While these sites and enhancers function as *Hox*-responsive elements, the mechanisms whereby their activity is restricted in a spatial and tissue-specific manner is unknown. Recent experiments have show that adjacent sequence motifs can also control the specificity of HOX/PBC response elements (Li et al., 1999). However, in the case of the multimerized motifs, the in vivo distribution of the Hox, Pbx and Meis protein is the same, so

their differential activities may reflect selectivity in the binding of different members of these co-factor families and/or differences in the ability of these motifs to integrate the interactions between commonly bound co-factors to form a transcriptionally active complex. These similarities and differences in *Hox*-responsive elements known to be important for normal in vivo function and regulation of the genes provides valuable information that will be useful in helping to identify downstream target genes in the Hox cascade.

Conservation and diversity of Hox group 3 regulation

It has been shown that paralogous Hox genes can functionally compensate for each other, indicating that they are expressed in highly similar domains. However, our analysis has demonstrated that while many aspects of *Hoxb3* and *Hoxa3* expression are similar, in the hindbrain there are differences in both their spatial and temporal patterns of expression. Our regulatory analyses have indicated that this arises owing to differences in the nature of *cis*-elements that control both initiation and maintenance (Fig. 8B). These genes arose by duplication from a common ancestor, and the shared role for *kreisler* in regulating the initiation of segmental expression reflects this process (Manzanares et al., 1999a). However, differences in the activity and organization of the *kreisler*-dependent initiation elements, and the presence of an r5-r6 maintenance loop in *Hoxa3* but not *Hoxb3* (Fig. 8B), shows that the *cis*-regulatory components of these genes have independently evolved. This provides further support for the idea that distinct roles for individual Hox genes can arise through subtle changes in regulation, which alter the dynamics of their expression. The large block of similarity in the chick and mouse *Hoxa3* control regions (400 bp) is presumably a consequence of the nested organization of the early and late elements for segmental expression. This may reflect the ancestral state, which has been lost in *Hoxb3*, leading to the relatively short (19 and 45 bp) conserved blocks in the mouse and chick control regions. It is interesting that the only other large block of sequence conservation outside of the coding regions we have found in comparing mouse and chick Hox genes is region A from *Hoxb4*, which also combines early and late neural elements in close proximity (Aparicio et al., 1995; Gould et al., 1998; Gould et al., 1997; Morrison et al., 1995). Hence, extended blocks of sequence similarity may tend to represent compound or complex arrays of *cis*-elements that control multiple parts of endogenous expression patterns. By contrast, the simple or short blocks of identity, such as those seen for the *Hoxb3 kreisler* and *Hoxa2* or *Hoxb2 Krox20*-dependent elements (Nonchev et al., 1996a), are likely to be characteristic of modules that regulate more restricted subsets of the endogenous pattern that is dependent upon a small set of common core components.

In conclusion, these findings add further strength to the emerging importance of positive auto and cross regulatory interactions between Hox genes as a general mechanism for maintaining the correct spatial patterns in the vertebrate nervous system. The finding that auto- and cross-regulation are important for maintaining *Hoxa3* expression and that of other *Hox* genes, does not negate roles for epigenetic or chromatin-mediated mechanisms. Such mechanisms could work together with or independent of crosstalk between the Hox genes in ensuring that the proper patterns of Hox expression are maintained through out development.

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REFERENCES

Alexandre, D., Clarke, J., Oxtoby, E., Yan, Y.-L., Jowett, T. and Holder, N. (1996). Ectopic expression of *Hoxa-1* in the zebrafish alters the fate of the mandibular arch neural crest and phenocopies a retinoic acid-induced phenotype. *Development* **122**, 735-746.

Aparicio, S., Morrison, A., Gould, A., Gilthorpe, J., Chaudhuri, C., Rigby, P. W. J., Krumlauf, R. and Brenner, S. (1995). Detecting conserved regulatory elements with the model genome of the Japanese puffer fish *Fugu rubripes*. *Proc. Natl. Acad. Sci. USA* **92**, 1684-1688.

Barrow, J. R., Stadler, H. S. and Capecchi, M. R. (2000). Roles of *Hoxa1* and *Hoxa2* in patterning the early hindbrain of the mouse. *Development* **127**, 933-944.

Bell, E., Wingate, R. and Lumsden, A. (1999). Homeotic transformation of rhombomere identity after localized *Hoxb1* misexpression. *Science* **284**, 2168-2171.

Bergson, C. and McGinnis, W. (1990). An auto-regulatory enhancer element of the *Drosophila* homeotic gene *Deformed*. *EMBO J.* **9**, 4287-4297.

Berthelsen, J., Zappavigna, V., Ferretti, E., Mavilio, F. and Blasi, F. (1998a). The novel homeoprotein Prep1 modulates Pbx-Hox protein cooperativity. *EMBO J* **17**, 1434-1445.

Berthelsen, J., Zappavigna, V., Mavilio, F. and Blasi, F. (1998b). Prep1, a novel functional partner of Pbx proteins. *EMBO J.* **17**, 1423-33.

Carpenter, E. M., Goddard, J. M., Chisaka, O., Manley, N. R. and Capecchi, M. R. (1993). Loss of *Hoxa-1 (Hox-1.6)* function results in the reorganization of the murine hindbrain. *Development* **118**, 1063-1075.

Chan, S.-K. and Mann, R. (1996). A structural model for a homeotic protein-extradenticle-DNA complex accounts for the choice of HOX protein in the heterodimer. *Proc. Natl. Acad. Sci. USA* **93**, 5223-5228.

Chan, S.-K., Ryoo, H.-D., Gould, A., Krumlauf, R. and Mann, R. (1997). Switching the *in vivo* specificity of a minimal *HOX*-responsive element. *Development* **124**, 2007-2014.

Chang, C. P., Jacobs, Y., Nakamura, T., Jenkins, N. A., Copeland, N. G. and Cleary, M. L. (1997). Meis proteins are major in vivo DNA binding partners for wild-type but not chimeric Pbx proteins. *Mol. Cell. Biol.* **17**, 5679-5687.

Chen, J. and Ruley, H. (1998). An enhancer element in the *EphA2 (Eck)* gene sufficient for rhombomere-specific expression is activated by *Hoxa1* and *Hoxb1*. *J. Biol. Chem.* **273**, 24670-24675.

Condie, B. and Capecchi, M. (1993). Mice homozygous for a targeted disruption of *Hoxd-3(Hox-4.1)* exhibit anterior transformations of the first and second cervical vertebrae, the atlas and axis. *Development* **119**, 579-595.

Cordes, S. P. and Barsh, G. S. (1994). The mouse segmentation gene *kr* encodes a novel basic domain-leucine zipper transcription factor. *Cell* **79**, 1025-1034.

Dollé, P., Lufkin, T., Krumlauf, R., Mark, M., Duboule, D. and Chambon, P. (1993). Local alterations of *Krox-20* and *Hox* gene expression in the hindbrain suggest lack of rhombomeres 4 and 5 in homozygote null *Hoxa-1 (Hox-1.6)* mutant embryos. *Proc. Natl. Acad. Sci. USA* **90**, 7666-7670.

Dupé, V., Davenne, M., Brocard, J., Dollé, P., Mark, M., Dierich, A., Chambon, P. and Rijli, F. (1997). *In vivo* functional analysis of the *Hoxa1* 3′ retinoid response element (3′ RARE). *Development* **124**, 399-410.

Ferretti, E., Marshall, H., Pöpperl, H., Maconochie, M., Krumlauf, R. and Blasi, F. (2000). Segmental expression of *Hoxb2* in r4 requires two distinct

sites that facilitate co-operative interactions and ternary complex formation between Prep, Pbx and Hox proteins. *Development* **127**, 155-166.

Frasch, M., Chen, X. and Lufkin, T. (1995). Evolutionary-conserved enhancers direct region-specific expression of the murine *Hoxa-1* and *Hoxa-2* loci in both mice and *Drosophila*. *Development* **121**, 957-974.

Gaunt, S., Miller, J., Powell, D. and Duboule, D. (1986). Homeobox gene expression in mouse embryos varies with position by the primitive streak stage. *Nature* **324**, 662-664.

Gavalas, A. and Krumlauf, R. (2000). Retinoid signalling and hindbrain patterning. *Curr. Opin. Genet. Dev.* **10**, 380-386.

Gavalas, A., Studer, M., Lumsden, A., Rijli, F., Krumlauf, R. and Chambon, P. (1998). *Hoxa1* and *Hoxb1* synergize in patterning the hindbrain, cranial nerves and second pharyngeal arch. *Development* **125**, 1123-1136.

Gavalas, A., Trainor, P., Ariza-McNaughton, L. and Krumlauf, R. (2001). Synergy between *Hoxa1* and *Hoxb1*: the relationship between arch patterning and the generation of cranial neural crest. *Development* **128**, 3017-3027.

Goddard, J., Rossel, M., Manley, N. and Capecchi, M. (1996). Mice with targeted disruption of *Hoxb1* fail to form the motor nucleus of the VIIth nerve. *Development* **122**, 3217-3228.

Gould, A. (1997). Functions of mammalian *Polycomb*-group and *trithorax*-group related genes. *Curr. Opin. Genet. Dev.* **7**, 488-494.

Gould, A., Itasaki, N. and Krumlauf, R. (1998). Initiation of rhombomeric *Hoxb4* expression requires induction by somites and a retinoid pathway. *Neuron* **21**, 39-51.

Gould, A., Morrison, A., Sproat, G., White, R. and Krumlauf, R. (1997). Positive cross-regulation and enhancer sharing: two mechanisms for specifying overlapping *Hox* expression patterns. *Genes Dev.* **11**, 900-913.

Greer, J. M., Puetz, J., Thomas, K. R. and Capecchi, M. R. (2000). Maintenance of functional equivalence during paralogous Hox gene evolution [see comments]. *Nature* **403**, 661-665.

Grieder, N. C., Marty, T., Ryoo, H. D., Mann, R. S. and Affolter, M. (1997). Synergistic activation of a Drosophila enhancer by HOM/EXD and DPP signalling. *EMBO J.* **16**, 7402-7410.

Helmbacher, F., Pujades, C., Desmarquet, C., Frain, M., Rijli, F., Chambon, P. and Charnay, P. (1998). *Hoxa1* and *Krox20* synergize to control the development of rhombomere 3. *Development* **125**, 4739-4748.

Hunt, P., Gulisano, M., Cook, M., Sham, M., Faiella, A., Wilkinson, D., Boncinelli, E. and Krumlauf, R. (1991a). A distinct *Hox* code for the branchial region of the head. *Nature* **353**, 861-864.

Hunt, P., Wilkinson, D. and Krumlauf, R. (1991b). Patterning the vertebrate head: murine Hox 2 genes mark distinct subpopulations of premigratory and migrating neural crest. *Development* **112**, 43-51.

Itasaki, N., Bel-Vialar, S. and Krumlauf, R. (1999). 'Shocking' developments in chick embryology: electroporation and *in ovo* gene expression. *Nat. Cell Biol.* **1**, E203-E207.

Jacobs, Y., Schnabel, C. and Cleary, M. (1999). Trimeric association of Hox and TALE homeodomain proteins mediates *Hoxb2* hindbrain enhancer activity. *Mol. Cell. Biol.* **19**, 5134-5142.

Kim, C. B., Amemiya, C., Bailey, W., Kawasaki, K., Mezey, J., Miller, W., Minoshima, S., Shimizu, N., Wagner, G. and Ruddle, F. (2000). Hox cluster genomics in the horn shark, Heterodontus francisci. *Proc. Natl. Acad. Sci. USA* **97**, 1655-1660.

Knoepfler, P. S., Lu, Q. and Kamps, M. P. (1996). Pbx-1 Hox heterodimers bind DNA on inseparable half-sites that permit intrinsic DNA binding specificity of the Hox partner at nucleotides 3′ to a TAAT motif. *Nucleic Acids Res.* **24**, 2288-2294.

Knoepfler, P. S., Calvo, K. R., Chen, H., Antonarakis, S. E. and Kamps, M. P. (1997). Meis1 and pKnox1 bind DNA cooperatively with Pbx1 utilizing an interaction surface disrupted in oncoprotein E2a-Pbx1. *Proc. Natl. Acad. Sci. USA* **94**, 14553-14558.

Li, X., Veraksa, A. and McGinnis, W. (1999). A sequence motif distinct from Hox binding sites controls the specificity of a Hox response element. *Development* **126**, 5581-5589.

Lou, L., Bergson, C. and McGinnis, W. (1995). *Deformed* expression in the *Drosophila* central nervous system is controlled by an autoactivated intronic enhancer. *Nucleic Acids Res.* **23**, 3481-3487.

Lumsden, A. and Krumlauf, R. (1996). Patterning the vertebrate neuraxis. *Science* **274**, 1109-1115.

Maconochie, M. K., Nonchev, S., Morrison, A. and Krumlauf, R. (1996). Paralogous *Hox* Genes: Function and Regulation. *Annu. Rev. Genet.* **30**, 529-556.

Maconochie, M., Nonchev, S., Studer, M., Chan, S.-K., Pöpperl, H., Sham, M.-H., Mann, R. and Krumlauf, R. (1997). Cross-regulation in the mouse *HoxB* complex: the expression of *Hoxb2* in rhombomere 4 is regulated by *Hoxb1*. *Genes Dev.* **11**, 1885-1896.

Maconochie, M. K., Nonchev, S., Manzanares, M., Marshall, H. and Krumlauf, R. (2001). Differences in krox20-dependent regulation of hoxa2 and hoxb2 during hindbrain development. *Dev. Biol.* **233**, 468-481.

Manley, N. and Capecchi, M. (1995). The role of Hoxa-3 in mouse thymus and thyroid development. *Development* **121**, 1989-2003.

Manley, N. and Capecchi, M. (1997). *Hox* group 3 paralogous genes act synergistically in the formation of somitic and neural crest-derived structures. *Dev. Biol.* **192**, 274-288.

Manley, N. and Capecchi, M. (1998). Hox group 3 paralogs regulate the development and migration of the thymus, thyroid and parathyroid glands. *Dev. Biol.* **195**, 1-15.

Mann, R. S. (1995). The specificity of homeotic gene function. *BioEssays* **17**, 855-863.

Mann, R. and Chan, S.-K. (1996). Extra specificity from extradenticle: the partnership between HOX and PBX/EXD homeodomain proteins. *Trends Genet.* **12**, 258-262.

Manzanares, M., Cordes, S., Kwan, C.-T., Sham, M.-H., Barsh, G. and Krumlauf, R. (1997). Segmental regulation of *Hoxb3* by *kreisler*. *Nature* **387**, 191-195.

Manzanares, M., Cordes, S., Ariza-McNaughton, L., Sadl, V., Maruthainar, K., Barsh, G. and Krumlauf, R. (1999a). Conserved and distinct roles of *kreisler* in regulation of the paralogous *Hoxa3* and *Hoxb3* genes. *Development* **126**, 759-769.

Manzanares, M., Trainor, P., Nonchev, S., Ariza-McNaughton, L., Brodie, J., Gould, A., Marshall, H., Morrison, A., Kwan, C.-T., Sham, M.-H. et al. (1999b). The role of *kreisler* in segmentation during hindbrain development. *Dev. Biol.* **211**, 220-237.

Mark, M., Lufkin, T., Vonesch, J.-L., Ruberte, E., Olivo, J.-C., Dollé, P., Gorry, P., Lumsden, A. and Chambon, P. (1993). Two rhombomeres are altered in *Hoxa-1* mutant mice. *Development* **119**, 319-338.

Marshall, H., Studer, M., Pöpperl, H., Aparicio, S., Kuroiwa, A., Brenner, S. and Krumlauf, R. (1994). A conserved retinoic acid response element required for early expression of the homeobox gene *Hoxb-1*. *Nature* **370**, 567-571.

Moens, C. B., Yan, Y.-L., Appel, B., Force, A. G. and Kimmel, C. B. (1996). *valentino*: a zebrafish gene required for normal hindbrain segmentation. *Development* **122**, 3981-3990.

Moens, C. B., Cordes, S. P., Giorgianni, M. W., Barsh, G. S. and Kimmel, C. B. (1998). Equivalence in the genetic control of hindbrain segmentation in fish and mouse. *Development* **125**, 381-391.

Morrison, A., Chaudhuri, C., Ariza-McNaughton, L., Muchamore, I., Kuroiwa, A. and Krumlauf, R. (1995). Comparative analysis of chicken *Hoxb-4* regulation in transgenic mice. *Mech. Dev.* **53**, 47-59.

Nieto, M. A., Bradley, L. C. and Wilkinson, D. G. (1991). Conserved segmental expression of *Krox-20* in the vertebrate hindbrain and its relationship to lineage restriction. *Development* **112 Suppl. 2**, 59-62.

Nonchev, S., Maconochie, M., Vesque, C., Aparicio, S., Ariza-McNaughton, L., Manzanares, M., Maruthainar, K., Kuroiwa, A., Brenner, S., Charnay, P. et al. (1996a). The conserved role of *Krox-20* in directing *Hox* gene expression during vertebrate hindbrain segmentation. *Proc. Natl. Acad. Sci. USA* **93**, 9339-9345.

Nonchev, S., Vesque, C., Maconochie, M., Seitanidou, T., Ariza-McNaughton, L., Frain, M., Marshall, H., Sham, M. H., Krumlauf, R. and Charnay, P. (1996b). Segmental expression of Hoxa-2 in the hindbrain is directly regulated by Krox-20. *Development* **122**, 543-554.

Pirrotta, V. (1997). *PcG* complexes and chromatin silencing. *Curr. Opin. Genet. Dev.* **7**, 249-258.

Pöpperl, H., Bienz, M., Studer, M., Chan, S., Aparicio, S., Brenner, S., Mann, R. and Krumlauf, R. (1995). Segmental expression of *Hoxb1* is controlled by a highly conserved auto-regulatory loop dependent upon *exd/Pbx*. *Cell* **81**, 1031-1042.

Regulski, M., Dessain, S., McGinnis, N. and McGinnis, W. (1991). High-affinity binding sites for the Deformed protein are required for the function of an auto-regulatory enhancer of the Deformed gene. *Genes Dev.* **5**, 278-286.

Rieckhof, G. E., Casares, F., Ryoo, H. D., Abu-Shaar, M. and Mann, R. S. (1997). Nuclear translocation of extradenticle requires *homothorax*, which encodes an extradenticle-related homeodomain protein. *Cell* **91**, 171-183.

Rijli, F., Gavalas, A. and Chambon, P. (1998). Segmentation and specification in the branchial region of the head: The role of *Hox* selector genes. *Int. J. Dev. Biol.* **42**, 393-401.

Ryoo, H. D. and Mann, R. S. (1999). The control of trunk Hox specificity and activity by Extradenticle. *Genes Dev* **13**, 1704-1716.

Ryoo, H. D., Marty, T., Casares, F., Affolter, M. and Mann, R. S. (1999). Regulation of Hox target genes by a DNA bound Homothorax/Hox/Extradenticle complex. *Development* **126**, 5137-5148.

Saldivar, J., Krull, C., Krumlauf, R., Ariza-McNaughton, L. and Bronner-Fraser, M. (1996). Rhombomere of origin determines autonomous versus environmentally regulated expression of *Hoxa3* in the avian embryo. *Development* **122**, 895-904.

Schumacher, A. and Magnuson, T. (1997). Murine *Polycomb*- and *trithorax*-group genes regulate homeotic pathways and beyond. *Trends Genet.* **13**, 167-170.

Sham, M.-H., Hunt, P., Nonchev, S., Papalopulu, N., Graham, A., Boncinelli, E. and Krumlauf, R. (1992). Analysis of the murine *Hox-2.7* gene: conserved alternative transcripts with differential distributions in the nervous system and the potential for shared regulatory regions. *EMBO J.* **11**, 1825-1836.

Sham, M. H., Vesque, C., Nonchev, S., Marshall, H., Frain, M., Das Gupta, R., Whiting, J., Wilkinson, D., Charnay, P. and Krumlauf, R. (1993). The zinc finger gene *Krox-20* regulates *Hoxb-2 (Hox2.8)* during hindbrain segmentation. *Cell* **72**, 183-196.

Studer, M., Lumsden, A., Ariza-McNaughton, L., Bradley, A. and Krumlauf, R. (1996). Altered segmental identity and abnormal migration of motor neurons in mice lacking *Hoxb-1*. *Nature* **384**, 630-635.

Studer, M., Gavalas, A., Marshall, H., Ariza-McNaughton, L., Rijli, F., Chambon, P. and Krumlauf, R. (1998). Genetic interaction between *Hoxa1* and *Hoxb1* reveal new roles in regulation of early hindbrain patterning. *Development* **125**, 1025-1036.

Takihara, Y., Tomotsune, D., Shirai, M., Katoh-Fukui, Y., Nishii, K., Motaleb, M., Nomura, M., Tsuchiya, R., Fujita, Y., Shibata, Y. et al. (1997). Targeted disruption of the mouse homologue of the *Drosophila polyhomeotic* gene leads to altered anteroposterior patterning and neural crest defects. *Development* **124**, 3673-3682.

Trainor, P., Manzanares, M. and Krumlauf, R. (2000). Genetic interactions during hindbrain segmentation in the mouse embryo. In *Mouse Brain Development*: *Results and Problems in Cell Differentiation*. Vol. 30 (ed. A. Goffinet and P. Rackic), pp. 51-89. Berlin-Heidelberg: Springer-Verlag.

Vesque, C., Maconochie, M., Nonchev, S., Ariza-McNaughton, L., Kuroiwa, A., Charnay, P. and Krumlauf, R. (1996). *Hoxb-2* transcriptional activation by *Krox-20* in vertebrate hindbrain requires an evolutionary conserved *cis*-acting element in addition to the Krox-20 site. *EMBO J.* **15**, 5383-5896.

Whiting, J., Marshall, H., Cook, M., Krumlauf, R., Rigby, P. W. J., Stott, D. and Allemann, R. K. (1991). Multiple spatially specific enhancers are required to reconstruct the pattern of *Hox-2.6* gene expression. *Genes Dev.* **5**, 2048-2059.

Wilkinson, D. G. (1992). Whole mount in situ hybridisation of vertebrate embryos. In *In Situ Hybridisation, A Practical Approach* (ed. D. G. Wilkinson), pp. 75-83. Oxford: IRL Press.

Wilkinson, D. G., Bhatt, S., Chavrier, P., Bravo, R. and Charnay, P. (1989a). Segment-specific expression of a zinc-finger gene in the developing nervous system of the mouse. *Nature* **337**, 461-465.

Wilkinson, D. G., Bhatt, S., Cook, M., Boncinelli, E. and Krumlauf, R. (1989b). Segmental expression of Hox-2 homeobox-containing genes in the developing mouse hindbrain. *Nature* **341**, 405-409.

Yee, S.-P. and Rigby, P., W. J. (1993). The regulation of *myogenin* gene expression during the embryonic development of the mouse. *Genes Dev.* **7**, 1277-1289.

Zeng, C., Pinsonneault, J., Gellon, G., McGinnis, N. and McGinnis, W. (1994). Deformed protein binding sites and cofactor binding sites are required for the function of a small segment-specific regulatory element in Drosophila embryos. *EMBO J.* **13**, 2362-2377.

Zhang, M., Kim, H.-J., Marshall, H., Gendron-Maguire, M., Lucas, A. D., Baron, A., Gudas, L. J., Gridley, T., Krumlauf, R. and Grippo, J. F. (1994). Ectopic *Hoxa-1* induces rhombomere transformation in mouse hindbrain. *Development* **120**, 2431-2442.

Zhang, F., Nagy Kovacs, E. and Featherstone, M. S. (2000). Murine hoxd4 expression in the CNS requires multiple elements including a retinoic acid response element. *Mech. Dev.* **96**, 79-89.