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

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Accepted 22 June 2001

SUMMARY

During development of the vertebrate hindbrain, Hox genes play multiple 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 kreasler, 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 kreasler-binding site (KrA) that is involved in activating early expression in r5 and r6, but their activity is independent of kreasler. 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 (Helmacher 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 kreasler, 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, kreasler 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 kreasler activates the segmental expression of these two Hox genes (Manzanares et al., 1999a).

The expression of both kreasler 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 on the other side of their normal segmental boundaries (Takahara 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 Capechi, 1993; Greer et al., 2000; Manley and Capechi, 1995; Manley and Capechi, 1997; Manley and Capechi, 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 kreasler and that Hoxa3 expression is 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 KpnI 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 (HaeIII and MboI) from this fragment were generated in M13mp18, and randomly sequenced. In this way, a 500 bp HaelIII fragment with high similarity to the mouse 600 bp EcoRV-Smal r5/r6 element (constructs 3.4; Manzanares et al., 1999a) was found. Sequencing outwards on the original 5.5 kb KpnI fragment confirmed that the similarity did not extend further. The relative location of the 500 bp fragment was mapped by PCR.

A 500 bp Aval 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 EcoRV-Smal 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'-GCCGGTTGAATTATGACCAC-3'; HOX/PBC-B, 5'-AGCGGATCTACTAATCTTTGCC-3'; HOX/PBC-B+PREP/MEIS, 5'-CCGACGTCATAAATCTTTGCCAGCCATAATGACAAA-3'; and their complements. All constructs were checked by sequencing on an Applied Biosystems 373A sequencer.

Constructs c1, c2 and c6-11 were generated in a modified version of pBGZ40 (Yee and Rigby, 1993), that contains a basal human lamp promoter, linked to the bacterial lacZ promoter (construct 8; Whiting et al., 1991). Constructs were separated from vector sequences before microinjection by electrophoresis and purified using Gelase (Epiconentre 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 (F0) 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 BamHI-HindIII genomic fragment containing 3’ coding and untranslated regions (Sham et al., 1992); mouse Hoxa3, a 650 bp HindIII-EcoRI Hoxa3 genomic fragment (Gaunt et al., 1986); chick Hoxb3, a 400 bp EcoRI-SphI genomic fragment from the 3’ coding and untranslated regions; and chick Hoxa3, a 900 bp KpnI-EcoRI genomic fragment spanning the second exon and 3’ untranslated region (Saldivar et al., 1996).

Electrophoretic mobility shifts assays
All pSG5-derived expression vectors containing Pbx3a, Hoxb1, Hoxb3, Hoxd3, Hoxa3 or Pbx1 coding sequences were translated in vitro using the coupled TNT transcription/translation system (Promega), in the presence of [35S]-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 32P-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 0.5x 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 (Pöppler et al., 1995); Hoxb2 (Manochoite et al., 1997); EphA2 (Chen and Ruley, 1998); Hoxb4 (Chan et al., 1997; Gould et al., 1997); Dfd

**RESULTS**

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

At 8.25-8.5 days post coitum (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...
distinct from that of \textit{Hoxa3}. Because the initiation of segmental \textit{Hoxa3} and \textit{Hoxb3} expression is regulated by \textit{kreisler} (Manzanares et al., 1999a; Manzanares et al., 1997), which is no longer expressed during the phase of \textit{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 \textit{Hoxa3} locus**

To examine the basis of the \textit{kreisler}-independent r5/r6 expression of \textit{Hoxa3} in later stages we decided to first isolate and characterize the related \textit{cis}-regulatory elements required for chick \textit{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 \textit{Hoxa3}-coding region were inserted into a \textit{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 \textit{KpnI} 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 \textit{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 \textit{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 \textit{Hoxa3} locus we previously characterized in isolating the \textit{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 \textit{KpnI} fragment for regions of sequence similarity to the mouse enhancer.
Fig. 3. Sequence alignment of a region conserved between the chick, mouse, human and horn shark Hoxa3 5' flanking regions. The krésis-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 krésis 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 krésis, a 500 bp chick HaeIII fragment

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

<table>
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<tr>
<th>Construct*</th>
<th>n</th>
<th>r5/r6</th>
<th>p</th>
<th>n</th>
<th>r5/r6</th>
<th>p</th>
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<tr>
<td>Early Kr-dependent phase (8.25-9.0 pc)</td>
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<td>Late Kr-independent maintenance phase (9.5-10.5 pc)</td>
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<td>Construct</td>
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<td>r5/r6</td>
<td>p</td>
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<td>r5/r6</td>
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<td>1</td>
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<td>65*</td>
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<tr>
<td>C1</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>3</td>
<td>n.d.</td>
<td>n.d.</td>
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<tr>
<td>C2</td>
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<td>+</td>
<td>+</td>
<td>2</td>
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</table>

*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.
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 EcoRV-NcoI 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).
To begin to see if we could separate the regulatory regions involved in early and late segmental expression, we tested two subregions of the EcoRV-NcoI fragment contained in construct 1 by taking advantage of a Smal site that cuts in the middle of the conserved block (Fig. 4). When a 1.2 kb Smal-NcoI 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 EcoRV-Smal genomic fragment from the 5’ part of the EcoRV-NcoI 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 AvaI 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., 1997; Maconomich et al., 1997; Pöpperl et al., 1995), 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; Maconomich 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 Hoxbl/Hoxb2, respectively (Maconomich 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-PHIP1) 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-PHIP1; Fig. 3) as a substrate for complex formation. In the presence of Pbx1a and Hoxa3, Hoxb3 or Hoxd3 slower migrating complexes are formed by competition 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-Pbxα) 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
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 AvaI 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. 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; Ferretti 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/Krm11 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 the hindbrain as a new anterior boundary is formed at the level of r5/r6 (Fig. 1A,E). This domain of expression coincides with the hindbrain as a new anterior boundary is formed at the level of r5/r6 (Fig. 1A,E). This domain of expression coincides with
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 kreasler in regulating the initiation of segmental expression reflects this process (Manzanares et al., 1999a). However, differences in the activity and organization of the kreasler-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 kreasler 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.

We thank Atsushi Kuroiwa for the gift of the chick Hoxa cosmids, Nobue Itasaka for advice and initial electroporation of the chick c2 construct, Kamala Maruthairar for sequencing, Dominic Farr for help in generating and sequencing the four-cutter libraries, Amanda Hewett, Peter Mealyer and Rosemary Murphy for animal husbandry, and members of the Krumlauf groups for advice on the paper and help in testing some mutant combinations in chick embryos. M. M. was supported by EU Marie Curie and HFSP Postdoctoral Fellowships; S. B. V. by fellowships from the French Cancer Research Association (ARC) and EMBO; and M. K. M. by the MRC. This work was funded by Core MRC Programme support and EEC Biotechnology Network grant (#BIO4 CT-960378) to R. K., and by a grant from TELETHON to F. B.

REFERENCES


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


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