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Conserved and distinct roles of *kreisler* in regulation of the paralogous *Hoxa3* and *Hoxb3* genes

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SUMMARY

During anteroposterior patterning of the developing hindbrain, the anterior expression of 3′ *Hox* genes maps to distinct rhombomeric boundaries and, in many cases, is upregulated in specific segments. Paralogous genes frequently have similar anterior boundaries of expression but it is not known if these are controlled by common mechanisms. The expression of the paralogous *Hoxa3* and *Hoxb3* genes extends from the posterior spinal cord up to the rhombomere (r) 4/5 boundary and both genes are upregulated specifically in r5. However, in this study, we have found that *Hoxa3* expression is also upregulated in r6, showing that there are differences in segmental expression between paralogues. We have used transgenic analysis to investigate the mechanisms underlying the pattern of segmental expression of *Hoxa3*. We found that the intergenic region between *Hoxa3* and *Hoxa4* contains several enhancers, which summed together mediate a pattern of expression closely resembling that of the endogenous *Hoxa3* gene. One enhancer specifically directs expression in r5 and r6, in a manner that reflects the upregulation of the endogenous gene in these segments. Deletion analysis localized this activity to a 600 bp fragment that was found to contain a single high-affinity binding site for the Maf bZIP protein Kreml1, encoded by the *kreisler* gene. This site is necessary for enhancer activity and when multimerized it is sufficient to direct a *kreisler*-like pattern in transgenic embryos. Furthermore the r5/r6 enhancer activity is dependent upon endogenous *kreisler* and is activated by ectopic *kreisler* expression. This demonstrates that *Hoxa3*, along with its paralog *Hoxb3*, is a direct target of *kreisler* in the mouse hindbrain. Comparisons between the Krml1-binding sites in the *Hoxa3* and *Hoxb3* enhancers reveal that there are differences in both the number of binding sites and way that *kreisler* activity is integrated and restricted by these two control regions. Analysis of the individual sites revealed that they have different requirements for mediating r5/r6 and dorsal roof plate expression. Therefore, the restriction of *Hoxb3* to r5 and *Hoxa3* to r5 and r6, together with expression patterns of *Hoxb3* in other vertebrate species suggests that these regulatory elements have a common origin but have later diverged during vertebrate evolution.

Key words: *Hox* gene, Hindbrain, Segmentation, *kreisler*, Transgenic mice, Transcriptional regulation, Mouse

INTRODUCTION

The vertebrate hindbrain is a segmentally organised structure, whereby a series of reiterated bulges, termed rhombomeres (r), are formed during anteroposterior patterning of the neural plate (Lumsden and Krumlauf, 1996). These metameric units behave as lineage-restricted cellular compartments that go on to adopt different identities (Fraser et al., 1990) and *Hox* genes are believed to be involved in the regulation of segmental identity (Keynes and Krumlauf, 1994; Krumlauf, 1994). Support for this comes from the ectopic expression of group 1 *Hox* genes in fish and mouse embryos, which leads to a posterior transformation in the hindbrain, whereby r2 adopts an r4-like identity (Alexandre et al., 1996; Zhang et al., 1994). Furthermore, analysis of mouse loss-of-function mutations in *Hoxb1* has revealed that it has a role in maintaining r4 identity (Goddard et al., 1996; Studer et al., 1996), while analysis of *Hoxa1* mutants suggests it has a role in segmentation (Carpenter et al., 1993; Dollé et al., 1993; Mark et al., 1993). Double mutant analysis has uncovered additional roles for these genes showing that they work synergistically in initiating r4 identity (Gavalas et al., 1998; Studer et al., 1998). This demonstrates that *Hox* genes can function in multiple steps of the segmental process of hindbrain patterning, and also shows

how paralogous *Hox* genes can have distinct and non-overlapping roles during development. This could be due to subtle differences in patterns and timing of expression, perhaps reflecting small variations in regulatory inputs.

With respect to regulation of the spatially restricted patterns of Hox expression, transgenic analysis has revealed that autoregulatory and cross-regulatory mechanisms between the *Hox* genes are important for maintaining segmental expression and identity (Gould et al., 1997; Maconochie et al., 1997; Pöpperl et al., 1995; Studer et al., 1998). In addition to this cross-talk between *Hox* genes, the retinoid pathway appears to play a direct role in initiating segmental expression of *Hoxa1*, *Hoxb1* and *Hoxb4* in the neuroectoderm (Dupé et al., 1997; Gould et al., 1998; Marshall et al., 1994; Studer et al., 1998). Furthermore, the zinc-finger-containing gene *Krox20* and the large Maf bZIP gene *kreisler* are upstream transcriptional activators of segmental *Hox* expression. *Krox20* directly controls the upregulation of *Hoxb2* and *Hoxa2* in r3 and r5 in the vertebrate hindbrain, through the presence of evolutionarily conserved high-affinity binding sites in enhancer regions from these genes (Nonchev et al., 1996a,b; Sham et al., 1993; Vesque et al., 1996). In support of this role, the segmental expression of these genes is changed in *Krox20* mutants and there is a failure to maintain r3 and r5, which are eventually lost (Schneider-Maunoury et al., 1993, 1997; Swiatek and Gridley, 1993). Very little is known about the evolution of regulatory elements, so the comparison of regulatory circuits between paralogous genes from the *Hox* clusters will be extremely informative in this respect.

In vertebrates, *kreisler/Krml1* and its zebrafish homologue *valentino* function in the specification of hindbrain segments (Cordes and Barsh, 1994; Manzanares et al., 1997; Moens et al., 1996, 1998). Previously, we have shown that *kreisler* also directly regulates segmental expression of *Hoxb3* in r5, through two conserved Krml1-binding sites present in both a mouse and chick enhancer (Manzanares et al., 1997). The activity of these enhancers is also dependent upon an equally conserved adjacent motif, the Ets-related activation site (ERAS). The ERAS is involved in both potentiating the enhancer activity and restricting it exclusively to r5 (Manzanares et al., 1997). This suggests that *kreisler* may have a later role in regulating segmental identity. Further support for this has come from ectopic expression of *kreisler* in the mouse hindbrain, indicating that it is sufficient to transform r3 to an r5-like identity (Theil et al., 1999).

Hoxa3 is another group 3 paralog that is upregulated in a segmental manner in the hindbrain (Hunt et al., 1991; Lumsden and Krumlauf, 1996). In this study, we used transgenic analysis to identify *cis*-acting control regions involved in mediating segmental expression of mouse *Hoxa3* and to determine what upstream factors and mechanisms are involved in potentiating the activity of these regions. Our analysis has shown that *Hoxa3* is segmentally expressed in r5 and r6, which is different from *Hoxb3* which is upregulated only in r5. We have identified an r5/r6 enhancer responsible for this activity and used in vivo and in vitro analyses to show that this enhancer functions as a readout of *kreisler* activity. This illustrates that *kreisler* regulates multiple *Hox* genes during hindbrain segmentation and comparison between the *Hoxa3* and *Hoxb3* enhancers has revealed significant differences in the way *kreisler* activity is coupled to the regulation of segmental expression.

MATERIALS AND METHODS

Transgenic mice and in situ hybridization

Transgenic embryos were generated by pronuclear injection into fertilized mouse eggs from an intercross of F_1 hybrids (CBA × C57Bl6) and stained for *lacZ* reporter activity as described (Whiting et al., 1991). In situ hybridization on whole-mount mouse embryos (Xu and Wilkinson, 1998) was carried out with a 650 bp *Hin*dIII-*Eco*RI *Hoxa3* genomic fragment (Gaunt et al., 1986) and a full-length *kreisler* cDNA probe (Cordes and Barsh, 1994). Flat mounts were prepared by removing the midbrain and anterior regions and rostral spinal cord and posterior regions. Embryos were then cut along the dorsal midline and opened like a book, with a glass coverslip on top. This presents dorsal regions laterally and ventral region medially. The *Hoxb4/lacZ* r6/7 line used for mating into the *Hoxa3* r5/r6 enhancer background was JL64 and carries construct #1 from Whiting et al. (1991).

Transgenic DNA constructs

Genomic DNA fragments from the *Hoxa3-Hoxa4* intergenic region were isolated from cosmid cos2 (Baron et al., 1987; Duboule et al., 1986). Most subfragments were cloned into an expression construct containing a basal *Hoxb4* promoter, the bacterial *β-galactosidase* gene and an SV40 polyadenylation signal (construct #8 in Whiting et al., 1991). However, constructs #1.4, #3.3 and those with the multimerised Krml1-binding site oligonucleotides where made using pBGZ40 which is an expression construct using human β-globin promoter linked to *lacZ* (Yee and Rigby, 1993). For microinjection inserts were separated from vector DNA by electrophoresis and purified using a gelase method provided by suppliers (Epicentre Technologies). Specific mutations in the enhancers were generated by site-directed mutagenesis in m13 (Sculptor IVM System, Amersham). Oligomerized versions of binding sites were generated as described (Manzanares et al., 1997). The oligonucleotides used to generate the double-stranded KrA site were 5′-CGCACTTTCTCCTCCAAAC-TGCTGACGCGA-3′ and its complement. The multimerized *Hoxb3 kreisler* sites (Kr1 and Kr2) were generated as previously described (Manzanares et al., 1997). The mutant variants and the copy numbers in multimerised constructs were all verified by sequencing.

Electrophoretic mobility shift assays and DNAseI footprinting

Electrophoretic mobility shift assays (EMSA) were carried out on the 1.8 kb *Eco*RV-*Not*I genomic fragment contained in construct #3.3, or on a 253 bp PCR fragment containing the putative *kreisler*-binding site/s generated by primers 5′-CTGAATTCTTTGCTCCAACG-CTCTC and 5′-CTGGATCCACGTGTAGGAGGTGAGAG. Both fragments were radioactively end-labelled with Klenow, and EMSA and DNAseI footprinting conditions were as described (Manzanares et al., 1997). To determine binding specificity, competitor T-MARE 5′-AGCTCGGAATTGCTGACGCATTACTC or random 5′-GAGTAATGAGGACTCCTCAATTCCGAG oligonucleo-tides were added in 10-fold or 100-fold molar excess of the radiolabelled probe at the start of the binding reaction. Oligonucleotides for the *Hoxb3 kreisler* sites were as described (Manzanares et al., 1997). The mutated version of the *Hoxa3* KrA site was 5′-TCCAAA-CcaagGACGCGACTCTCACCGC.

RESULTS

Mapping *Hoxa3* regulatory regions

To screen for regions involved in segmental regulation of *Hoxa3*, we linked 14.5 kb of genomic DNA covering the gene and its 5′ flanking regions to a *lacZ* reporter gene and assayed for activity in transgenic mice (Fig. 1A). A construct (#1)

containing a 3.8 kb *Hinc*II fragment that includes the two coding exons and 5′ upstream regions, was strongly expressed in lateral mesoderm and forelimb buds (Fig. 1B). In addition, there was weak reporter staining in a small population of dorsal cells in r5 and the posterior spinal cord (Fig. 1B,F). An adjacent upstream region (#2) mediated *lacZ* expression in a subset of the vagal neural crest, lateral and paraxial mesoderm and the posterior hindbrain and spinal cord (Fig. 1C,G). Low levels of staining were detected in the hindbrain, with an anterior boundary which roughly mapped to the r7-r8 territory, and higher levels of expression were found in the thoracic spinal cord. Finally, the next 9.5 kb 5′ flanking fragment (#3) directed high levels of staining in the hindbrain and spinal cord, third arch neural crest, somitic mesoderm, forelimb buds and the tailbud (Fig. 1D,H). Thus, all three fragments possess a diverse range of regulatory activities including some aspect of hindbrain expression.

The sum of the regulatory activities of these regions appears to account for most of the major domains and patterns of endogenous *Hoxa3* expression, as seen by in situ hybridisation (Fig. 1E,I; and Gaunt, 1987; Hunt et al., 1991; Manley and Capecchi, 1995). For example, the anterior limit of transgene expression (#3) in paraxial mesoderm maps to the same somite 4/5 boundary, as the endogenous gene (Figs 1D,E, 3C,E). In the hindbrain, construct #3 also directs expression to the correct anterior boundary at r4/5, and we noted high levels of reporter expression in r5 and r6 (Fig. 1D,H). This was surprising because on the basis of our previous in situ analysis, we expected *Hoxa3* to be similar to *Hoxb3* and to display upregulation only in r5 (Hunt et al., 1991). This led us to re-examine endogenous *Hoxa3* expression by whole-mount in situ analysis, and we observed that it is also upregulated in r6 (Fig. 1I). Therefore, the r6 expression from construct #3 represents a true difference between *Hoxa3* and *Hoxb3* and this construct closely mirrors the endogenous pattern of segmental *Hoxa3* expression in the hindbrain.

Analysis of the dorsal r5 enhancer

We investigated the ability of construct #1 to generate dorsal r5 expression by deletion analysis (Fig. 2A). This fragment contains the proximal *Hoxa3* promoter and we first wanted to determine whether the reporter expression was a result of promoter or enhancer activities. A 2.1 kb *Xba*I subfragment directed an identical pattern of expression to construct #1 in both orientations (#1.1, #1.2) on a heterologous promoter (Fig. 2B and data not shown). This suggests that the dorsal r5, limb bud and lateral mesoderm expression are controlled by an enhancer(s). Two copies of this subfragment (#1.3) stimulate reporter expression in a larger proportion of r5 cells, but have no effect on the other domains of expression (Fig. 2C). This indicates that the r5 activity of this enhancer is very weak and/or it may require additional elements to fully potentiate its activity.

Additional deletions (#1.4-#1.6), narrowed the enhancer activity to a 460 bp *Sty*I fragment (#1.5), which directs reporter staining in a manner similar to the entire fragment (Fig. 2D,E). Based on the role of *kreisler* in directly regulating *Hoxb3* in r5, we performed in vitro electrophoretic mobility shift assays (EMSA) and DNAseI footprint experiments to

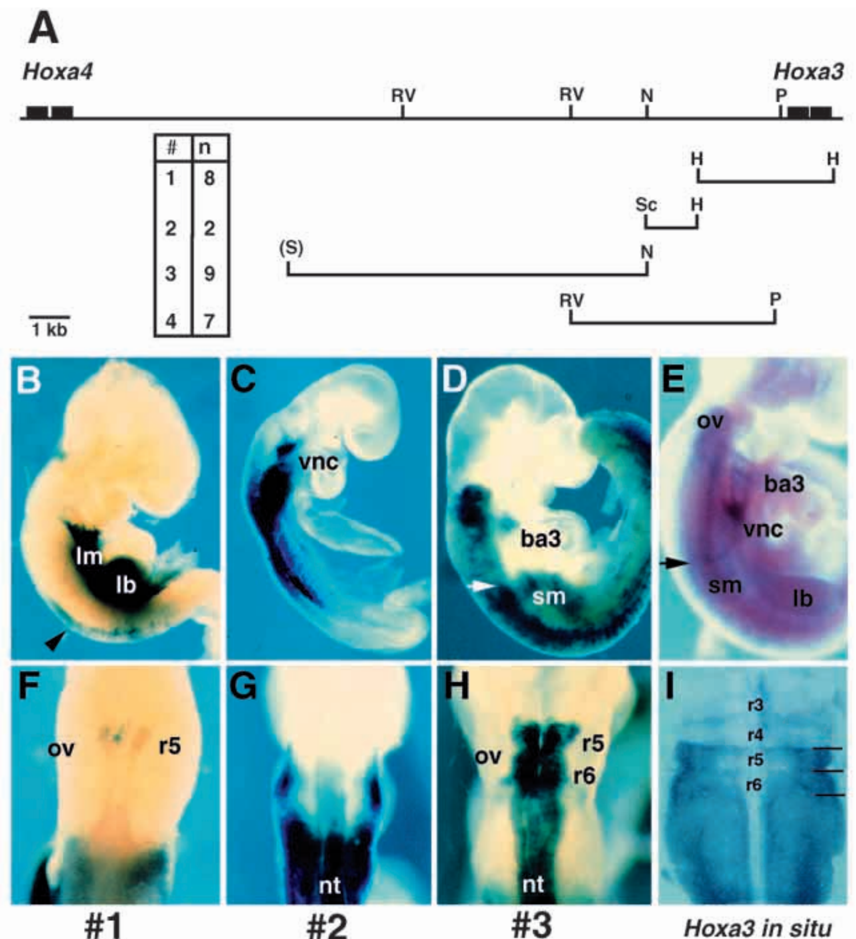

Fig. 1. Reconstruction of the endogenous *Hoxa3* pattern by transgenic analysis. (A) Diagram of the *Hoxa3-Hoxa4* intergenic region from the mouse *HoxA* complex, and the fragments tested by linking to a *lacZ* reporter gene for generation of transgenic embryos. In Figs 1-3, # indicates the construct number, and n indicates the number of embryos obtained showing the same expression pattern with a given construct. H, *Hinc*II; N, *Not*I, P, *Pvu*I; RV, *Eco*RV; (S), *Sal*I, derived from the cosmid vector; Sc, *Sac*II. (B,F) Lateral (B) and dorsal (F) views of embryos with strong expression in lateral plate mesoderm (lm) and limb bud (lb) with construct #1. Note lower expression in r5 and posterior spinal cord (arrowhead in B). (C,G) Lateral (C) and dorsal (G) views of embryos expressing construct #2 in vagal neural crest (vnc) and neural tube (nt). (D,H) Lateral (D) and dorsal (H) views of strong expression in r5, r6 and neural crest migrating into the third branchial arch (ba3) and in somitic mesoderm (sm) at the s4/5 boundary (arrow in D) with construct #3. Note also lower levels in more posterior neural tube regions. (E) Whole-mount in situ hybridization with a *Hoxa3* probe resulted in expression in all of these sites, and the anterior limit of somite expression (arrow in E) was at the same s4/5 boundary as seen with construct #3. (I) A flat mount of the hindbrain showing higher levels of RNA expression in r5 and r6, identical to that seen with construct #3. ov, otic vesicle. All embryos are 9-9.5 dpc.

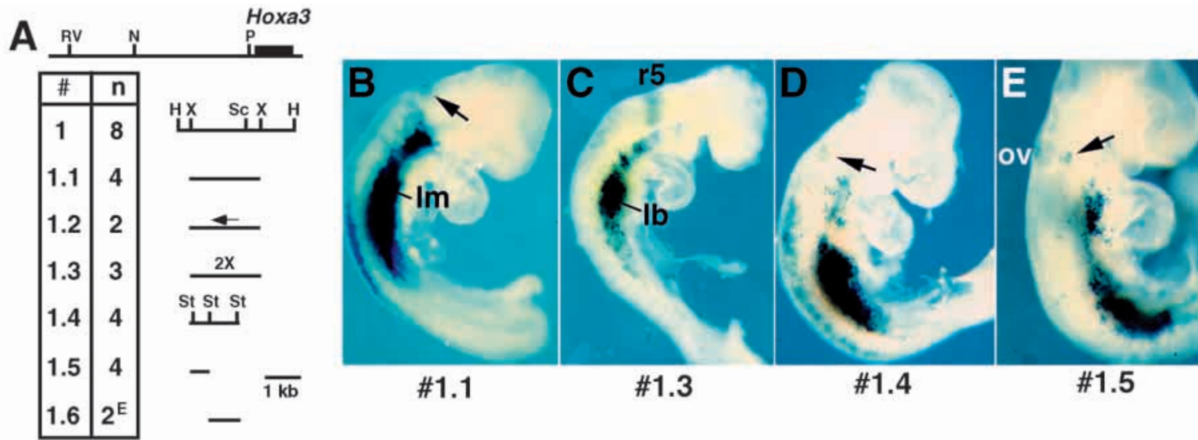

Fig. 2. Deletion analysis of the dorsal r5 enhancer. (A) Diagram of construct #1 and further deletion fragments tested. ^{E} indicates embryos showing ectopic integration-dependant expression; ov, otic vesicle; H, *Hinc*II; Sc, *Sac*II; St, *Sty*I, X, *Xba*I. (B-E) Constructs tested are noted below each panel. Expression in the lateral plate mesoderm (lm), limb bud (lb) and weakly in posterior neural tube is seen with all the constructs of this series. Expression in dorsal r5 is restricted to a small group of cells with constructs #1.1, #1.4 and #1.5 (arrow in B, D, and E), but is expanded throughout r5 when two copies in tandem of a 2.1 *Xba*I fragment are tested (C). All embryos shown are 9-9.5 dpc, and lateral views, except for E, which is dorsolateral.

search for high-affinity Kreml1-binding sites on this fragment. However, under the same conditions used previously to investigate the *Hoxb3* r5 enhancer (Manzanares et al., 1997), and the other *Hoxa3* hindbrain enhancer in this study (see Fig. 5), we detected no specific interactions with Krml1 protein (data not shown). Therefore, other as yet unknown factors appear to be responsible for the weak dorsal r5 activity of this enhancer.

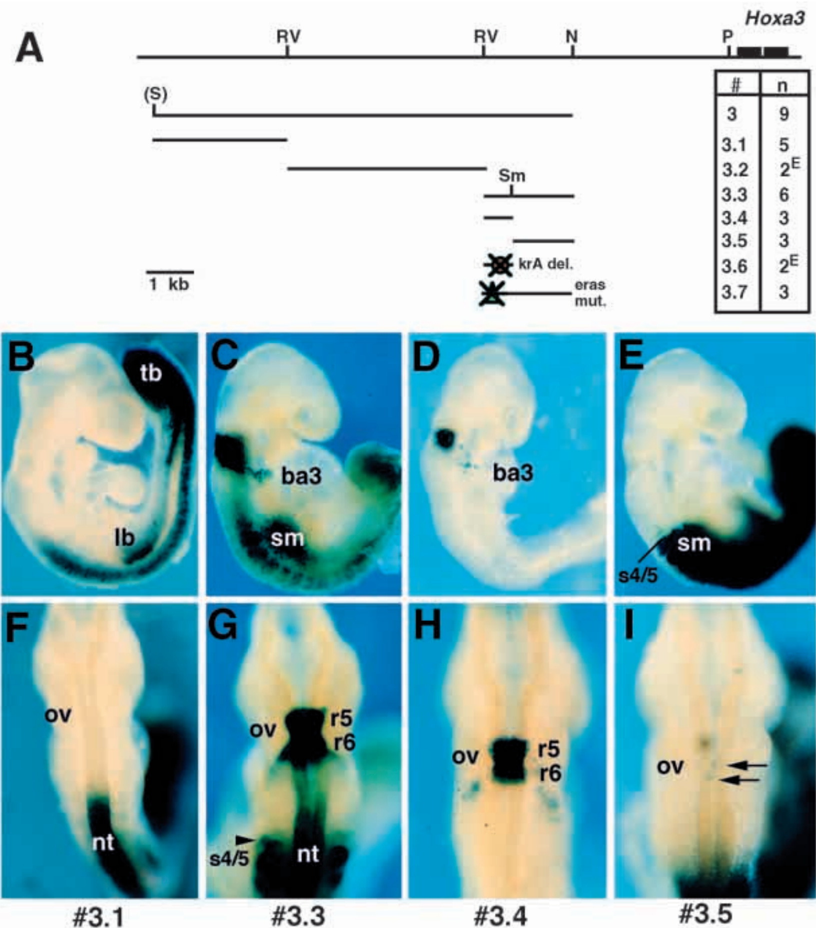

Fig. 3. Deletion analysis of the r5/r6 enhancer. (A) Diagram of construct #3 and further deletion fragments and mutated fragments for the KrA and Ets-related activation site (ERAS) tested. Sm, *Sma*I; RV, *EcoRV*; N, *NcoI* and P, *PstI*. ^{E} indicates embryos showing ectopic integration-dependant expression. (B,F) Lateral (B) and dorsal (F) views of expression in posterior neural tube (nt) and tail bud (tb) with construct #3.1. (C,G) Lateral (C) and dorsal (G) views of segmental expression in the hindbrain (r5, r6, and third arch neural crest, ba3) and somitic mesoderm (sm, at the s4/5 boundary, arrowhead in G) with construct #3.3. (D,H) Construct #3.4 contains the r5/6 hindbrain enhancer activity (D, lateral; H, dorsal). (E,I) Construct #3.5 posses the enhancer which regulates somitic expression up to s4/5 boundary (E, lateral; I, dorsal). In I, the arrows point to a few *lacZ*-positive cells in the ventral part of r5 that are detected with construct #3.5. ov, otic vesicle. All embryos shown are 9.5 dpc.

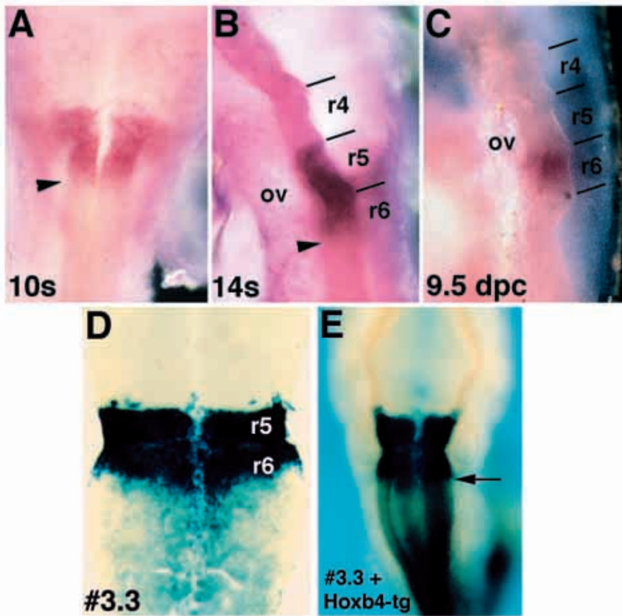

Fig. 4. Comparison of *kreisler* and *Hoxa3* enhancer expression in r5 and r6. (A-C) Whole-mount in situ hybridization with a *kreisler* RNA probe at 10 somites (A), 14 somites (B) and 9.5 dpc (C). Note that in A and C expression is strong throughout r5 with a sharp r4/5 anterior boundary, while staining in r6 is slightly weaker and has a more diffuse posterior limit at r6/7 (arrowhead in A and B). At 9.5 dpc (C), expression is lost in r6 but remains strong in r6. (D) Flat-mounted hindbrain of an embryo transgenic for construct #3.3 showing strong staining in r5 and r6. Note that the anterior r4/r5 boundary is sharp but the posterior boundary is more diffuse. (E) Double transgenic embryos carrying the *Hoxa3* r5/r6 enhancer (#3.3) and a *Hoxb4/lacZ* reporter that mediates expression up to the r6/7 boundary. Note there is no gap in the staining indicating that expression of the r5/r6 enhancer extends through all of r5 and r6. D and E are 9.5 dpc.

Transgenic analysis of the r5/r6 enhancer

Next we performed a deletion analysis on the 9.5 kb fragment, to identify regions involved in mediating segmental expression in the hindbrain (#3-#3.5; Fig. 3A). A region capable of directing expression in the spinal cord, forelimb bud and tailbud (#3.1) was mapped to the most 5′ 2.9 kb of construct #3 (Fig. 3B,F). Expression in the spinal cord did not extend into the hindbrain but there was a sharp anterior limit in the mid-thoracic region. The adjacent 4.8 kb *Eco*RV fragment (#3.2) displayed no activity, while the 3′ 1.8 kb *Eco*RV-*Not*I region (#3.3) mediated expression in r5/r6, somites, third arch crest, neural tube and lateral mesoderm (Fig. 3C,G). Further subdivision of construct #3.3 separated the hindbrain and neural crest component from the other activities, as a 600 bp *Eco*RV-*Sma*I fragment (#3.4) directs expression in r5/r6 and neural crest (Fig. 3D,H).

The most 3′ 1.2 kb region (#3.5) contains an enhancer(s) responsible for the mesodermal and posterior neural domains of expression. While we noted that this enhancer also stimulated weak expression in a small number of ventrally located cells in r5 (arrows in Fig. 3I), the pattern was highly variable and staining was never detected throughout all cells in r5. The strong reporter staining in somites with construct #3.5 displays the same anterior boundary at s4/5 as the endogenous gene (Fig. 3G,E), suggesting it contains the major enhancer(s) directing appropriate somitic expression of *Hoxa3*.

Because we have individually identified and characterised two different regions capable of directing segmental expression in the hindbrain, the dorsal r5 and the r5/r6 enhancers, we wanted to examine if their activities were additive or synergistic. Therefore a construct spanning both of these enhancers (#4; Fig. 1A) was generated and assayed in transgenic embryos. In all cases (n=7), within the hindbrain expression was observed in r5 and r6 (data not shown) in a pattern identical to that seen with constructs containing only the r5/r6 enhancer (#3, #3.3 and #3.4). Since there is no change in reporter expression with respect to timing and spatial restriction, the r5/r6 enhancer appears to be the major control region that directs segmentally restricted *Hoxa3* expression. However, this does not exclude the dorsal r5 enhancer or other regions from contributing to the levels or subsets of r5/r6 expression.

Constructs #3.3 and #3.4 identify a region responsible for mediating reporter expression in r5, r6 and neural crest cells migrating into the third branchial arch at 9.0 dpc. This pattern is very similar to that of *kreisler* in the hindbrain (Cordes and Barsh, 1994). In mouse, *kreisler* is strongly expressed in r5 but the timing and extent of expression in r6 has not been examined in detail. Therefore we performed in situ analysis between 8.25 and 9.5 dpc and found that *kreisler* is initially expressed at the 10- to 14-somite stages in a domain with a sharp r4/5 anterior boundary and a diffuse r6/7 posterior limit (Fig. 4A,B). *kreisler* expression then becomes progressively downregulated in r5 and is only present in r6 by 9.5 dpc (Fig. 4C) and is completely absent in later stages. Transgene expression for a line carrying construct #3.3 also shows reporter staining with a sharp r4/5 boundary and a more diffuse boundary in caudal r6 (Fig. 4D). To test if this staining covered the entire r6 region, we mated the #3.3 line with a *Hoxb4/lacZ* line, which expresses up to the r6/7 boundary (Whiting et al., 1991). Double transgenic embryos showed no gap in expression indicating that the Hoxa3 enhancer mediated expression in all of r5 and r6 (Fig. 4E).

Identification of a Kml1-binding site in the r5/r6 enhancer

In light of the similarity between the expression of the transgene and *kreisler*, we performed in vitro analysis to search for potential interactions with Kml1 protein. Initially, we found that the DNA-binding region of the Kml1 protein fused to maltose-binding protein (MBP-Kr) specifically complexed with the 1.8 kb *Eco*RV-*Not*I fragment (#3.3) in EMSA experiments (Fig. 5A). Binding was blocked by addition of an excess of double-stranded oligonucleotides containing a consensus binding site for Maf proteins (T-MARE), but was not affected by addition of excess random oligonucleotides. Furthermore, this Kml1 protein-DNA complex was disrupted by addition of 10 or 100-fold molar excess of competitor oligonucleotides containing the high-affinity Kml1-binding site (Kr1 site) from the *Hoxb3* r5 enhancer, but was not affected when the lower affinity Kr2 site was used (Fig. 5A).

Sequence analysis of both the 1.8 kb and the 600 bp *Hoxa3* r5/r6 enhancer revealed a single potential Kml1-binding site with similarity to both the T-MARE and the *Hoxb3* Kr sites

(see Fig. 7A). Using a 253 bp fragment spanning this sequence, we confirmed in EMSA experiments that Kml1 bound specifically to this region (Fig. 5B). Furthermore, in DNAseI footprinting experiments, MBP-Kr protein specifically protected a 28 bp region (KrA site) in this fragment that included the putative binding site predicted by DNA sequence analysis (Fig. 5C,D). Wild-type double-stranded oligonucleotides spanning this KrA site disrupt Krml1 binding to both the *Hoxb3* r5 and the *Hoxa3* r5/r6 enhancers, but mutations in the T-MARE consensus (Fig. 5D) fail to compete (data not shown). Together this data indicates that within the *Hoxa3* enhancer there is a single high-affinity Krml1-binding site (KrA) which is comparable in in vitro analysis to the Kr1 site from the *Hoxb3* r5 enhancer.

Multimers of KrA mediate a *kreisler*-like pattern

To further investigate the properties of the KrA site, we generated a series of constructs carrying multimerized double-stranded oligonucleotides spanning the motif linked to a *lacZ* reporter. Four copies of this sequence generated a pattern of reporter staining virtually identical to that of endogenous *kreisler* RNA (compare Fig. 6A-C with D-F). At 8.25 dpc, expression appears in future r5/r6 and early migrating neural crest cells (Fig. 6A,D) and, by 9.5 dpc, in addition to these sites expression is also detected in the dorsal roof plate over the anterior hindbrain and the dorsal spinal cord (Fig. 6B,C,E,F). Sixmers of this motif also direct a *kreisler*-like pattern (Fig. 6G). The finding that the KrA multimers are sufficient to direct expression in a manner that parallels endogenous *kreisler* with respect to timing and spatial domains suggests that this site functions in vivo as a *kreisler* response element.

The dorsal roof plate expression directed by multimers of KrA represents an additional domain of endogenous *kreisler* expression, but we had not previously observed this pattern with multimers of the two *Hoxb3* Krml1 sites (Manzanares et al., 1997). In our earlier study, only 4 copies of the Kr1 site were tested; however, when 8 copies are used we now observe dorsal roof staining in addition to the r5/r6 expression (Fig. 6H). This shows that the *Hoxa3* KrA site also functions in vivo in a comparable manner to the high-affinity Kr1 site from *Hoxb3*, except that KrA is more efficient at lower copy numbers in directing a full *kreisler* pattern. Furthermore, by generating more transgenic embryos with six copies of the Kr2 site, we occasionally observed that it also had the ability to direct dorsal staining over the roof plate, but never any r5/r6 expression (Fig. 6I). These differences between the various Krml1 sites suggests that *kreisler* has different regulatory requirements for mediating r5/r6 versus dorsal roof plate expression.

The KrA site is required for enhancer activity

Based on the in vitro Krml1-binding analysis and multimer experiments, we deleted 7 bp of the core consensus motif in the KrA site (see boxed region in Fig. 7A) within the context of the 600 bp r5/r6 enhancer, to test if it is required in vivo. This mutation (#3.6) abolished the enhancer activity in transgenic embryos but, in a few cases, we obtained ectopic

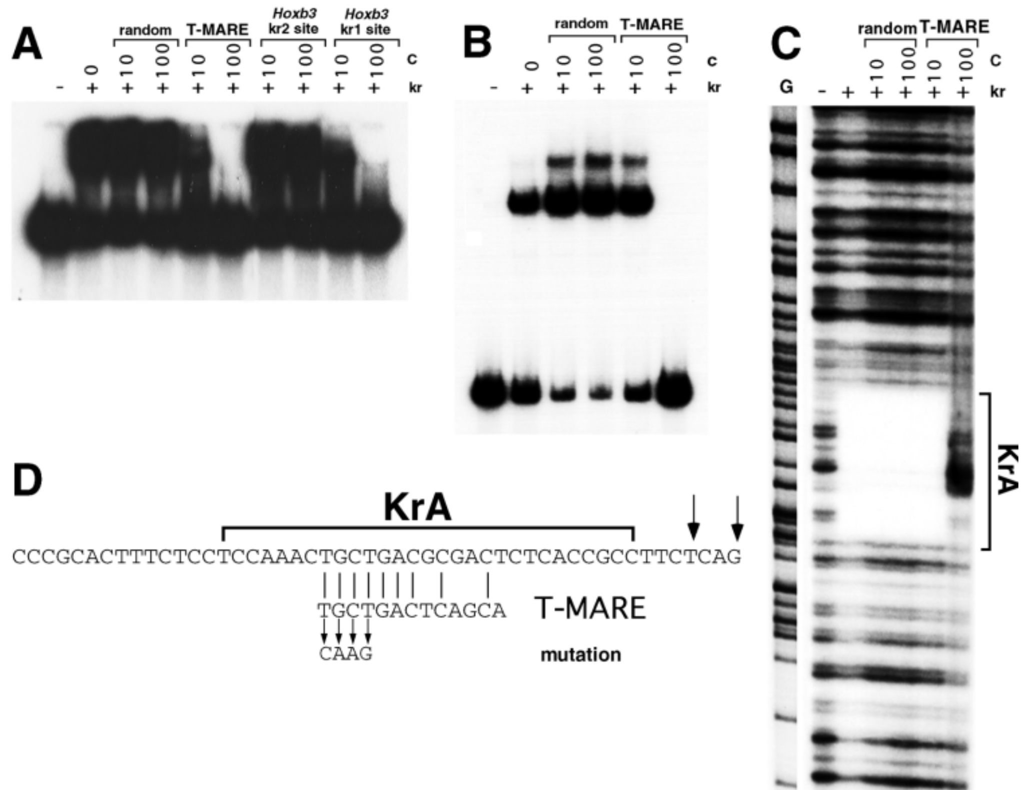

Fig. 5. In vitro analysis of Krml1 binding to the r5/r6 enhancer. (A) Binding of a Krml1-MBP fusion protein to the 1.8 kb *Eco*RV-*Not*I fragment (#3.3). Specific binding is observed that can be competed by the addition of excess oligonucleotides for the T-MARE consensus or the Kr1 site from *Hoxb3*, but not with a random sequence. Oligos for the Kr2 site from *Hoxb3* do not compete as efficiently, indicating that they represent lower affinity binding sites for Krml1 protein. (B) EMSA on a 253 bp fragment from the *Hoxa3* r5/r6 enhancer that contains the putative Krml1-binding site, which is competed by excess T-MARE but not a random oligo. (C) DNAseI protection assay on the same fragment as B, showing a clear footprinted region, which is only competed by T-MARE oligos. Alongside is a G sequencing reaction used as size marker. (D) Sequence of the footprinted region aligned with the T-MARE consensus. Note how only half of the consensus is present in the KrA site. Arrows indicate DNAseI hypersensitive site, and below is shown the mutation introduced in oligonucleotides that blocks the ability of it to compete when used in EMSA. c, fold molar excess of competitor; kr, presence (+) or absence (–) of Krml1-MBP protein.

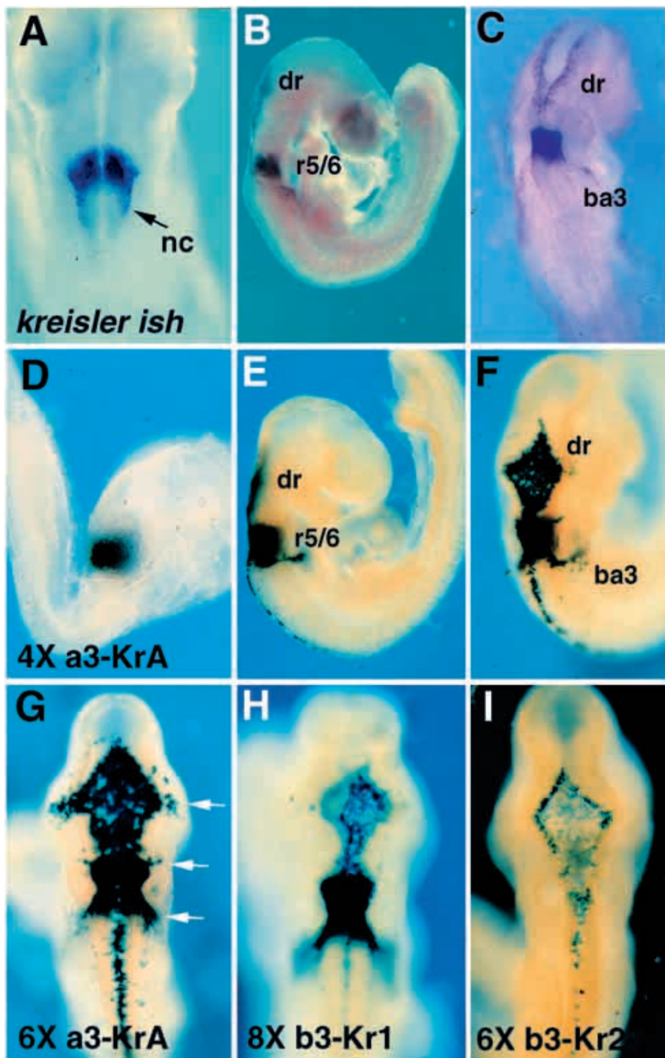

Fig. 6. Analysis of in vivo regulatory activities of individual Krml1-binding sites from *Hoxa3* and *Hoxb3*. (A-C) Expression of *kreisler* as detected by whole-mount in situ. Expression is detected at 7.5 dpc in the neural plate in the prospective r5/r6 region and in neural crest streaming from there (arrow and nc in A). At 9.0 (C) and 9.5 (B), in addition to the strong expression in the hindbrain and neural crest migrating into the third branchial arch (ba3), another domain is also visible in the dorsal roof (dr) over the anterior hindbrain and midbrain. (D-F) Transgenic embryos for a reporter construct with four copies of an oligonucleotide spanning the KrA site from the *Hoxa3* r5/r6 enhancer, showing a pattern that mimics that of endogenous *kreisler*. At 7.5 dpc (D), a unique domain is seen in the hindbrain but at 9.5 dpc (E,F), staining is also visible in the dorsal roof and in dorsal spinal cord. (G) An identical pattern seen with six copies of the *Hoxa3* KrA site. (H) Eight copies of an oligonucleotide for the Kr1 site from *Hoxb3* display similar staining in r5/6 and the dorsal roof. (I) Six copies of the Kr2 site from *Hoxb3* do not direct reporter staining in the hindbrain, but there is expression in the dorsal roof.

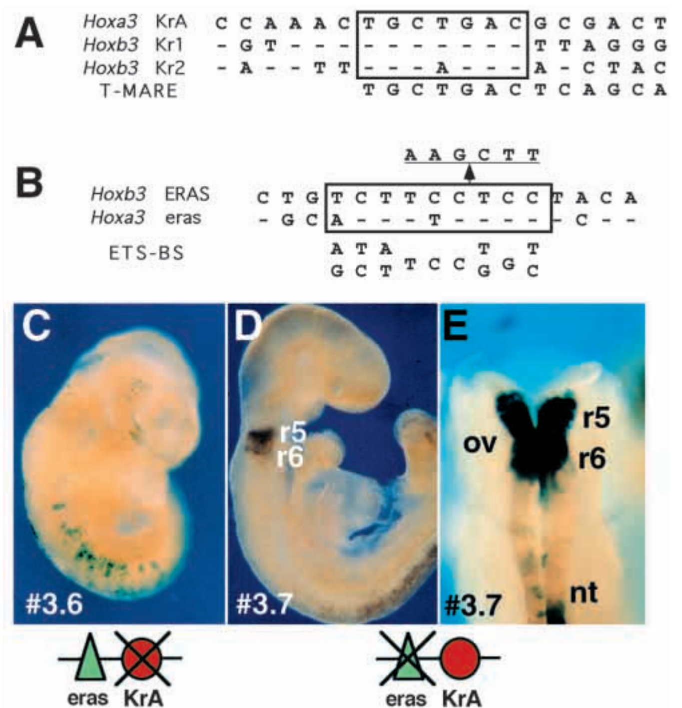

Fig. 7. Mutational analysis of the KrA site and the putative ERAS from the *Hoxa3* r5/r6 enhancer. (A) Sequence comparison of the Krml1-binding sites and flanking sequences from the hindbrain elements of *Hoxa3* (KrA) and *Hoxb3* (Kr1 and Kr2). Boxed is the half site of the T-MARE consensus that has been deleted for the in vivo analysis. In all three cases, only this half site is conserved, and very little similarity is seen for the other half site. There is also high similarity in 5′ flanking sequences between all three sites.
(B) Sequence comparison of the ERAS site from *Hoxb3* with the putative ERAS from *Hoxa3*. Boxed is the predicted Ets-binding site, above is the mutation introduced for the in vivo assays, and below is shown a consensus for Ets domain family binding. The three last residues (CCA) from the *Hoxa3* ERAS correspond to the first three residues from the KrA site shown in A. (C) Deletion of 7 bp corresponding to the T-MARE half site from KrA (construct #3.6) abolishes expression in transgenic embryos. Shown is an example of an embryo with expression in sites not related to the element under study, but dependent on the position of the integration. No expression is detected in the hindbrain at the level of r5 and r6. (D,E) Mutation of the *Hoxa3* ERAS (construct #3.7) has no effect on enhancer function. Expression in r5 and r6 of transgenic embryos carrying a construct with the mutation in the ERAS described in B is identical to that observed with a wild-type construct (#3.3, Fig. 3C,G). ov, otic vesicle. All embryos shown are 9.5 dpc.

integration-dependent expression in other sites, showing that the reporter is still functional (Fig. 7C). This demonstrates that the single high-affinity KrA site is indeed required for the ability of the *Hoxa3* r5/r6 enhancer to mediate segmental expression.

Given that the *Hoxb3* r5 enhancer requires two Krml1-binding sites for activity, we were surprised that the *Hoxa3* r5/r6 enhancer contained only the single KrA site. Therefore we performed a sequence comparison between these enhancers to search for additional conserved motifs that might be functionally relevant. The only significant block of identity found is in the sequence immediately 5′ to the KrA site, and it is similar to the ERAS present in the *Hoxb3* enhancer (Fig. 7B). This is interesting because we previously demonstrated that the ERAS is required for both the activation and restriction of the *Hoxb3* enhancer activity to r5 (Manzanares et al., 1997). To determine if this element has a similar role in the *Hoxa3* enhancer, we introduced 6 bp changes in the core of the motif

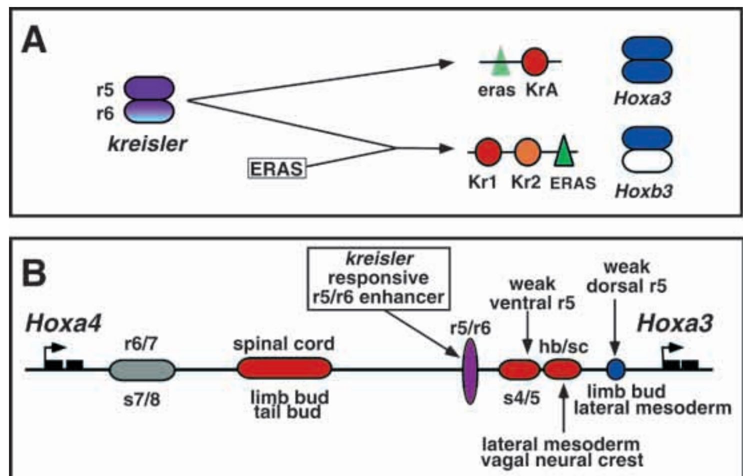

Fig. 8. (A) Control of *Hox* genes mediated by *kreisler*. *kreisler* differentially regulates segmental expression of two *Hox* genes in the hindbrain. *Hoxa3* is upregulated in r5 and r6 (where *kreisler* is also expressed) and this is dependent on a unique Krml1-binding site (KrA). A putative ERAS does not have a role in segmental restriction. In contrast, *Hoxb3* is only upregulated in r5, and this is dependent on two different Krml1 sites (Kr1 and Kr2) and an additional ERAS. Therefore, *kreisler* activity on this enhancer is restricted only to r5, and not active in r6, through factors at present unknown. (B) Genomic organization of regulatory elements from *Hoxa3*. Diagram of the intergenic region between *Hoxa3* and *Hoxa4* from the mouse *HoxA* complex (not to scale) showing the enhancer elements identified in this study. Shown in grey is an element from the *Hoxa4* gene that directs neural expression up to the r6/7 boundary and somitic expression up to s7/8 (Morrison et al., 1997). hb/sc, the hindbrain-spinal cord boundary; s4/5, the somite 4-5 boundary.

(Fig. 7B) identical to those used to inactivate this site in the *Hoxb3* enhancer (Manzanares et al., 1997). However, the construct with this variant (#3.7) directed a pattern of segmental expression in transgenic embryos identical to that of the wild-type version (Fig. 7D,E). Hence, this ERAS-like motif is not required for *Hoxa3* enhancer activity and any additional components involved in potentiating this enhancer appear not to be conserved with *Hoxb3*.

The r5/r6 enhancer responds to and requires *kreisler*

In order to examine the dependence of the *Hoxa3* r5/r6 enhancer upon *kreisler*, we generated two independent lines using construct #3.3. Reporter expression in both of these lines closely parallels the r5/r6 upregulation of endogenous *Hoxa3* in the hindbrain, and is identical to that observed at 9.5 dpc in transient founder embryos (Fig. 3C,G). We crossed these lines of mice with another transgenic line, in which an r3/r5 enhancer from the *EphA4* gene (Theil et al., 1998) is used to ectopically express a full-length *kreisler* cDNA. The mis-expression of *kreisler* in r3 trans-activates both the *Hoxa3* r5/r6 reporter and the endogenous gene in this new location (data not shown and Theil et al., 1999). Furthermore, we observed that enhancer activity is dependent upon endogenous *kreisler*, as r5/r6 expression of the transgene is specifically lost when the reporter lines are mated into the *kreisler* mutant background (data not shown). These findings show that the *Hoxa3* enhancer, containing the KrA-binding site, does function in vivo as a *kreisler*-dependent control region.

DISCUSSION

In this study, we have investigated the regulation of segmental *Hoxa3* expression in the developing mouse hindbrain. Several lines of evidence have shown that the *kreisler* gene plays a major role in this process. We identified an r5/r6 enhancer that contains a unique high-affinity *kreisler*-binding site, which is both necessary and sufficient for directing reporter expression in the hindbrain. Activity of the r5/r6 enhancer is lost in a *kreisler* mutant background and induced by ectopic *kreisler* expression showing that the enhancer is directly dependent upon *kreisler*. Together this data leads us to conclude that *Hoxa3* is a direct target of *kreisler* and raises a number of interesting points with respect to genetic regulation of hindbrain patterning.

kreisler and control of segmental identity

An emerging idea is that *kreisler* functions in multiple steps of segmental patterning in the mouse hindbrain by playing roles in both the specification of segments (Cordes and Barsh, 1994; M. M. and others, unpublished data) and the regulation of segmental identity (Manzanares et al., 1997; Theil et al., 1999). The experiments in this study provide support for the idea that *kreisler* has a role in the control of segmental identity in r5 by showing that it directly regulates rhombomeric expression of at least two group 3 *Hox* genes in the developing hindbrain. The recent finding that ectopic *kreisler* expression in r3 is sufficient to induce both *Hoxa3* and *Hoxb3* and transform its morphological identity into an r5-like character (Theil et al., 1999) is in agreement with this hypothesis. It appears that *kreisler* does not exert its influence on segmental processes solely through *Hoxa3*, *Hoxb3*, or even *Hoxd3* as single and multiple mutant analyses with loss-of-function alleles from these genes have not revealed any defects in the number or identity of hindbrain segments (Chisaka and Capecchi, 1991; Manley and Capecchi, 1995, 1997, 1998). However, *kreisler* might also influence segmental identity by participating in the regulation of other *Hox* genes expressed in r5, such as *Hoxb2* and *Hoxa2*.

Differences in expression and segmental regulation of *Hoxa3* and *Hoxb3*

While we have shown that *Hoxa3* and *Hoxb3* are both direct targets of *kreisler,* it is interesting that in the mouse *Hoxa3* is expressed in r5 and r6 but *Hoxb3* is upregulated only in r5. We have also observed this same difference in rhombomeric expression between these genes in chick embryos (data not shown), indicating that it is conserved in higher vertebrates. Our regulatory analysis has revealed that the difference in segmental expression between these genes can be accounted for by underlying differences in their ability to respond to *kreisler*. Fig. 8A summarises the organisation of the regulatory motifs in these enhancers. *Hoxa3* has a single high-affinity Krml1-binding site (KrA) and its upregulation depends upon and parallels endogenous *kreisler*, which leads to r5/r6

expression. However, *Hoxb3* has a high (Kr1) and low (Kr2) affinity Krml1 site neither of which is sufficient for enhancer activity (Manzanares et al., 1997). In addition to these Krml1 sites, further factors interacting with the ERAS are required to potentiate and restrict the *kreisler*-dependent activity of the enhancer to r5. There is a similar ERAS motif in the *Hoxa3* enhancer, but it is not required for spatially restricted expression. However, we can not rule out other roles for this sequence in regulation of *Hoxa3*, and certainly the conservation of a similar motif between *Hoxa3* and *Hoxb3* points in this direction.

The presence of the associated Krml1 and ERAS motifs in both enhancers most likely reflects a common origin from the ancestral *Hox* cluster followed by duplication and divergence. In this regard, it is interesting that, in lower vertebrates, the expression of *Hoxb3* can vary. In frog embryos *Hoxb3* is expressed in r5 (Godsave et al., 1994; Ruiz i Altaba, 1994), but in zebrafish embryos it is upregulated in r5 and r6 (Prince et al., 1998). This parallels the r5/r6 expression of *valentino* itself in zebrafish embryos (Moens et al., 1998), and more closely resembles the patterns that we observe with mouse and chick *Hoxa3*. This suggests that, early in vertebrate evolution, both *Hoxb3* and *Hoxa3* were expressed in r5 and r6 in direct response to *kreisler/valentino.* Subsequent divergence of the regulatory motifs in the *Hoxb3* enhancer made it dependent upon other factors to potentiate *kreisler* activity, resulting in its restriction to r5. This serves as a good example of how small changes in regulatory motifs during vertebrate evolution can result in subtle changes to *Hox* expression domains (Gellon and McGinnis, 1998).

Krml1-binding sites and enhancer activity

Large Maf proteins such as Krml1 are generally believed to bind as dimers with other bZIP proteins on bipartite palindromic repeats (Blank and Andrews, 1997). This has led to the identification of a consensus site for Maf binding, referred to as the T-MARE. However, our in vivo and in vitro analysis of both the *Hoxa3* and *Hoxb3* enhancers, revealed that the three essential Krml1 sites corresponded to only one half of the T-MARE consensus motif (Fig. 7A). In the case of *Hoxb3,* both half sites are required for enhancer activity and dimers could form through the utilisation of the two half sites even though they are separated by 96 bp (Manzanares et al., 1997). In contrast, we have shown here that the *Hoxa3* r5/r6 enhancer depends upon the single high-affinity KrA half site. Additional support for the relevance of half sites comes from recent findings on the regulation of *Interleukin-4* by c-Maf and *crystallin* genes by L-Maf (Ho et al., 1996; Ogino and Yasuda, 1998). The putative targets of both these proteins contain only one half site clearly matching the consensus. Together this suggests that large *Maf* family members such as *kreisler* do not need a complete palindromic T-MARE consensus for in vivo function. Another feature of these Maf proteins is their ability to interact with a range of other transcription factors, such as Ets and NF-AT family members, to potentiate their activity (Blank and Andrews, 1997; Ho et al., 1996; Sieweke et al., 1996). Therefore, the presence of only a single high-affinity KrA half site in the *Hoxa3* enhancer may reflect the fact that it can recruit or interact with other factors for transcriptional activity. In this regard, it is interesting that an ERAS motif is present adjacent to a Krml1 site in both the *Hoxa3* and *Hoxb3* enhancers (Fig. 7B). This characterisation of T-MARE half sites and associated motifs will be helpful in defining in vivo relevant *kreisler* response elements in searching for other potential targets of *kreisler* in hindbrain patterning, such as *Fgf3, Hoxa2, Hoxb2* or *Krox20.*

In examining the relative activities of the individual Krml1 sites from the *Hoxb3* and *Hoxa3* enhancers, in vitro DNA-binding analysis showed that KrA and Kr1 serve as high-affinity sites, but that Kr2 has a lower affinity (Fig. 5A). In vivo analysis also demonstrated that only KrA and Kr1 are capable of directing reporter expression in r5/r6, but that all three sites can stimulate dorsal roof plate expression (Fig. 6G-I). Hence, Kr2 appears to be a lower affinity site but it is still able to mediate a *kreisler* response in dorsal roof plate cells. These results imply that there are different requirements for activation by *kreisler* in r5/r6 versus the dorsal roof plate.

Multiple components in *Hoxa3* regulation

The *kreisler*-responsive r5/r6 enhancer directs a pattern of expression in the hindbrain that closely parallels endogenous *Hoxa3*. Furthermore, we have also recently observed that the *Hoxa3* r5/r6 reporter line responds in an identical manner to the endogenous *Hoxa3* gene in both gain- and loss-of-function *kreisler* mutants (M. M. and others, unpublished data; Theil et al., 1999). This leads us to conclude that this enhancer is the major component responsible for segmental regulation of *Hoxa3* in the hindbrain. Two other regions, defined by constructs #1.5 and #3.5, displayed some ability to stimulate weak dorsal (Fig. 2E) or patchy ventral (Fig. 3I) expression in the region of r5. In a construct containing all three of these regions with hindbrain activity (#4), we observed no synergy or changes in expression when compared to the r5/r6 enhancer alone. Therefore, while these other regions are not required for the activation or spatial restriction of the r5/r6 enhancer, they could contribute to the endogenous pattern by maintaining expression in later stages or modulating levels of expression in subsets of cells.

This study has focused on the basis of segmental regulation of hindbrain expression, but the transgenic analysis of the *Hoxa3-Hoxa4* intergenic region has identified a number of other enhancers that contribute to both neural and mesodermal expression. The sum of these elements generates a pattern of expression that very closely resembles that of endogenous *Hoxa3* in most tissues. Fig. 8B summarises the position and activities encoded by these enhancers. With respect to neural expression outside of r5/r6, we identified four separate enhancers with activity in the posterior hindbrain and spinal cord. All of these same regions also displayed enhancer activities in some other tissues, such as somites, lateral plate mesoderm, limb buds, tail bud or vagal neural crest. For example, adjacent to the r5/r6 enhancer, we found a region that directed somitic expression up to the same s4/5 (s, somite) boundary as the endogenous gene. Further analysis will be required to examine the basis of these neural and mesodermal patterns and to determine if they are separable from each other.

In conclusion, this regulatory analysis of segmental expression of *Hoxa3* has demonstrated that it is a direct target of *kreisler* in hindbrain patterning. *kreisler* regulates at least two different group 3 *Hox* genes in the hindbrain, which is analogous to the regulation of group 2 *Hox* genes by *Krox20*

in r3 and r5. This points to the key roles of *kreisler* and *Krox20* in initiating segmental identity. Since *kreisler* has an earlier role in the specification of r5, it might also be involved in regulating *Krox20* itself. This makes it important to identify the basis of the segmental expression of *kreisler* and *Krox20* to build a better picture of the regulatory cascade controlling hindbrain segmentation in vertebrates.

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