Krox20 and kreisler co-operate in the transcriptional control of segmental expression of *Hoxb3* in the developing hindbrain

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In the segmented vertebrate hindbrain, the *Hoxa3* and *Hoxb3* genes are expressed at high relative levels in the rhombomeres (r) 5 and 6, and 5, respectively. The single enhancer elements responsible for these activities have been identified previously and shown to constitute direct targets of the transcription factor kreisler, which is expressed in r5 and r6. Here, we have analysed the contribution of the transcription factor Krox20, present in r3 and r5. Genetic analyses demonstrated that Krox20 is required for activity of the *Hoxb3* r5 enhancer, but not of the *Hoxa3* r5/6 enhancer. Mutational analysis of the *Hoxb3* r5 enhancer, together with ectopic expression experiments, revealed that Krox20 binds to the enhancer and synergizes with kreisler to promote *Hoxb3* transcription, restricting enhancer activity to their domain of overlap, r5. These analyses also suggested contributions from an Ets-related factor and from putative factors likely to heterodimerize with kreisler. The integration of multiple independent inputs present in overlapping domains by a single enhancer is likely to constitute a general mechanism for the patterning of subterritories during vertebrate development.

Keywords: hindbrain/Hox/kreisler/Krox20/pattern formation

Introduction

The vertebrate hindbrain is organized into segmental compartments, termed rhombomeres, that provide a basic ground plan for generating regional diversity of structures

during craniofacial development (Lumsden, 1990). Several different gene classes have been shown to be associated with the generation and maintenance of these segmental territories and with the specification of their antero-posterior (A–P) identity. Among these, the transcription factors Krox20, kreisler, Hox and retinoic acid receptors display rhombomere-restricted patterns of expression that have been shown to be functionally important in the segmental processes (Lumsden and Krumlauf, 1996; Schneider-Maunoury *et al.*, 1998; Trainor *et al.*, 2000). Through a variety of approaches, a picture of the interactions existing between these genes and of the regulatory hierarchy of events governing hindbrain segmentation is beginning to emerge (Maconochie *et al.*, 1996; Trainor and Krumlauf, 2000; Trainor *et al.*, 2000).

Krox20 encodes a zinc finger transcription factor, expressed in r3 and r5, that is essential for development and maintenance of these segments (Wilkinson *et al.*, 1989; Schneider-Maunoury *et al.*, 1993, 1997; Swiatek and Gridley, 1993; Giudicelli *et al.*, 2001). *Krox20* has also been shown to play an essential role in the specification of odd- versus even-numbered rhombomere identity by controlling the expression of a number of downstream regulatory genes (Seitanidou *et al.*, 1997; Mechta-Grigoriou *et al.*, 2000; Giudicelli *et al.*, 2001; Voiculescu *et al.*, 2001). In particular, *Krox20* directly regulates the transcription of *Hoxa2* and *Hoxb2* in rhombomeres (r) 3 and 5 through distinct combinations of Krox20-binding sites in their 5'-flanking regions (Sham *et al.*, 1993; Nonchev *et al.*, 1996b). These Krox20-binding sites are also found in association with conserved sites for other factors that differentially contribute to the segmental regulation of these *Hox* genes (Vesque *et al.*, 1996; Maconochie *et al.*, 2001). Hence, this Krox20-dependent regulatory mechanism is highly conserved during vertebrate evolution, as evidenced by the similarity in both patterns of expression and the organization of regulatory elements (Nieto *et al.*, 1991; Nonchev *et al.*, 1996a; Vesque *et al.*, 1996). Finally, Krox20 also directly regulates the expression of the *EphA4* tyrosine kinase receptor gene in r3 and r5, contributing to the control of segregation or mixing between cells from adjacent segments (Theil *et al.*, 1998; Voiculescu *et al.*, 2001).

Another gene that plays an important role in the early formation of segments is *kreisler* (*kr*), which encodes a Maf/basic leucine zipper (b-ZIP) protein (Krm11) that is expressed in r5 and r6 (Cordes and Barsh, 1994). Mutational analyses have shown that it is necessary for the formation of r5 in mouse and for the proper development of r5 and r6 into mature segments in zebrafish (Frohman *et al.*, 1993; McKay *et al.*, 1994; Moens *et al.*, 1996, 1998; Manzanares *et al.*, 1999b). *kreisler* also plays a later role in controlling rhombomere identity through the

Hox genes, since conserved *kreisler*-binding sites in segmental enhancers from *Hoxa3* and *Hoxb3* are essential for initiating rhombomere-restricted expression of these genes (Manzanares *et al.*, 1997, 1999a, 2001).

Hox genes themselves play multiple roles in regulating segmental processes with a major input into A–P specification, but they are also involved in the formation of segmental territories, cell mixing, generation of cranial neural crest and dorso-ventral specification (Chen and Ruley, 1998; Davenne *et al.*, 1999; Gavalas *et al.*, 2001). Detailed analyses of single and compound mutants have revealed complex phenotypes, with evidence for synergy within domains of overlap and defects outside of the domains of expression (Carpenter *et al.*, 1993; Dolle *et al.*, 1993; Mark *et al.*, 1993; Gavalas *et al.*, 1997, 1998, 2001; Helmbacher *et al.*, 1998; Studer *et al.*, 1998). This shows that the segmental control genes exert their influences at multiple steps through a combination of both direct and indirect interactions. *Hox* genes are major targets of the upstream pathways, such as *Krox20* and *kreisler*, that regulate segmentation and morphogenesis (Krumlauf, 1994; Lumsden and Krumlauf, 1996; Maconochie *et al.*, 1996; Trainor and Krumlauf, 2000). However, mechanistically, the way in which the information from such pathways is integrated at the level of *Hox* genes is still poorly understood, and represents an important unresolved issue. In particular, we need to know whether the critical *cis*-acting regulatory regions are modular in nature, with discrete elements that independently receive inputs from a variety of components, or whether the transcriptional output depends upon a complex balance of direct synergistic or antagonistic interactions in association with other factors (Mann and Affolter, 1998; Affolter and Mann, 2001).

In this regard, segmental expression in r3 and r5 of the members of *Hox* paralogy groups 2 and 3 is of particular interest, as it represents an example where there are complex overlaps and differences between the expression and function of *Krox20*, *kreisler* and *Hox* genes. As detailed above, the group 2 genes (*Hoxa2* and *Hoxb2*) are expressed in r3 and r5 through direct transcriptional activation by *Krox20*, although there are differences in the nature of the *Krox20*-dependent control of these two genes (Sham *et al.*, 1993; Nonchev *et al.*, 1996b; Maconochie *et al.*, 2001). Similarly, the group 3 genes are directly up-regulated early in hindbrain segmentation by the action of *kreisler* (Manzanares *et al.*, 1997, 1999b). However, they are differentially maintained in later stages through the presence of an auto/cross-regulatory loop that acts on *Hoxa3* but not *Hoxb3* (Manzanares *et al.*, 1997, 2001). There are also differences in the pattern of initiation of the group 3 genes. While *Hoxa3* is activated in both r5 and r6, reflecting the normal segmental domains of *kreisler*, the *Hoxb3* enhancer is active only in r5, representing a subset of the *kreisler* domain. This indicates that in addition to *kreisler*, the segmental restriction of *Hoxb3* expression to r5 must be mediated by other *cis*-elements and/or factors in the r5/r6 region, which modulate the activity of the *Hoxb3* enhancer.

It is possible that *Krox20* could participate in this additional input to segmental restriction, based on the timing and overlapping patterns of expression of *Krox20* and *kreisler* in r5 and genetic evidence suggesting that

Krox20 function is required for proper *Hoxb3* expression (Seitanidou *et al.*, 1997). Therefore, in the present study, we have investigated the possibility that *Krox20* and *kreisler* might act together on common *Hox* targets in r5. We have performed a detailed analysis of the regulatory elements involved in *Hoxb3* and *Hoxa3* transcriptional control by using *Hox/lacZ* reporter lines, targeted *Krox20* mutants, mutational analysis in transgenic mice and *in ovo* electroporation in chick. We demonstrate that *kreisler* and *Krox20* indeed co-operate to activate the *Hoxb3* enhancer within their common domain of expression, i.e. r5, while the *Hoxa3* enhancer is independent of *Krox20* activity. Furthermore, our data reveal the presence and importance of other regulators that work in conjunction with *kreisler* and *Krox20* to generate the complex information needed to restrict *Hoxb3* expression in r5.

Results

Differences in the *Krox20* dependence of *Hoxb3* and *Hoxa3* segmental enhancers

The *Hoxb3* and *Hoxa3* genes are expressed at high relative levels in r5 and r5/r6, respectively, and at lower levels in more posterior regions of the neural tube (Hunt *et al.*, 1991; Manzanares *et al.*, 2001). We previously have identified *cis*-acting regulatory elements responsible for the segmentally restricted expression of group 3 *Hox* genes in the hindbrain, and shown their dependence on direct binding of the b-ZIP transcription factor *kreisler* that is expressed in r5 and r6 (Manzanares *et al.*, 1999a). While the activity of the *Hoxa3* element in r5 and r6 reflects that of *kreisler*, the response of the *Hoxb3* element is restricted to r5 and must be modulated by additional factors. One candidate potentially involved in mediating this difference is the zinc finger transcription factor *Krox20*.

To investigate genetically the involvement of *Krox20* in the activity of these *Hox* enhancers, a *lacZ* reporter coupled to a minimal promoter under the control of the *Hoxb3* r5 enhancer element or the *Hoxa3* r5/r6 enhancer was introduced into a *Krox20* null background (Swiatek and Gridley, 1993). In a wild-type background, reporter expression under the control of the *Hoxb3* r5 enhancer is clearly visible as a single stripe in the region of the otic sulcus after 8.0 days post-coitum (d.p.c.), when rhombomere territories are being established (Figure 1A). Expression gradually increases until rhombomeres are morphologically visible, being restricted exclusively to r5 (Figure 1B and C). In contrast, in *Krox20*^{−/−} embryos no expression was ever detected in the hindbrain (Figure 1F–H), even at stages when the prospective r5 territory was still present (Figure 1F and G; Schneider-Maunoury *et al.*, 1993; Swiatek and Gridley, 1993). The other domains of expression of the transgene, such as the posterior lateral mesoderm, are not affected in the mutant. Expression of *lacZ* in the *Hoxa3*-r5/6 transgenic line is initiated at a similar time (Figure 1D), but in a broader domain that presumably corresponds to prospective r5 and r6 (Figure 1E). In *Krox20*^{−/−} embryos, expression at 8.5 d.p.c. is identical to that of wild-type littermates (Figure 1I), but by 9.5 d.p.c. it is reduced to a single rhombomere width (Figure 1J). This latter effect is the result of the loss of the r5 territory in *Krox20*^{−/−} embryos at

Fig. 1. Analysis of the genetic requirement of *Krox20* for the segmental activity of the *Hoxb3* and *Hoxa3* enhancers in the mouse hindbrain. Stable transgenic lines carrying the *lacZ* gene under the control of the *Hoxb3* r5 enhancer (A–C and F–H) or the *Hoxa3* r5/r6 enhancer (D, E, I and J) were crossed into a *Krox20* mutant background. Wild-type littermates (A–E) or *Krox20* homozygous mutant (F–J) embryos were examined by X-gal staining at the indicated stages (d.p.c.). (A–C and F–H) Expression in r5 driven by the *Hoxb3* element is eliminated in the homozygous mutant background. (D and I) In contrast, the activity of the *Hoxa3* element is not affected in r5 or r6 at 8.5 d.p.c. (E and J) At the 9.5 d.p.c. stage, the r5 territory has disappeared in the *Krox20* homozygous mutant, but the activity of the *Hoxa3* enhancer is maintained in r6.

this stage of development (Schneider-Maunoury *et al.*, 1993, 1997; Swiatek and Gridley, 1993).

These genetic experiments establish that the activity of the *Hoxb3* r5 enhancer depends on the presence of a functional *Krox20* protein even to activate early expression in r5. In contrast, expression directed by the *Hoxa3* r5/r6 enhancer does not require *Krox20*. These data demonstrate the existence of a fundamental difference in the mechanisms leading to establishment and restriction of the segmental pattern of expression of *Hoxa3* and *Hoxb3* in the mouse hindbrain.

Synergistic activation of the *Hoxb3* r5 enhancer by *Krox20* and *kreisler*

To determine whether the involvement of *Krox20* in the activity of the *Hoxb3* r5 enhancer requires co-operation with *kreisler*, we performed ectopic expression experiments using *in ovo* electroporation of the chick hindbrain (Itasaki *et al.*, 1999). A construct consisting of the *lacZ* reporter driven by a minimal human β -globin promoter and carrying the *Hoxb3* r5 enhancer was co-electroporated together with an empty expression vector or with *Krox20* and/or *kreisler* expression constructs. Electroporation was performed at stages HH10–12, and β -galactosidase activity was assayed 24 h later by X-gal staining. When the

reporter was co-electroporated with the empty expression vector (pAdRSV; Giudicelli *et al.*, 2001), only a few cells weakly positive for X-gal were observed and they were all restricted to r5 (Figure 2A). Co-electroporation of the reporter construct with a *Krox20* expression plasmid (pAdRSVKrox20; Giudicelli *et al.*, 2001) led to a significant activation of the reporter construct in r5 and r6 (Figure 2B). Co-electroporation of the reporter with a *kreisler* expression construct (pAdRSVKreisler) led to strong and reproducible activation of the reporter construct in r3 and to a lower extent in r5, together with weaker and more variable activation in other regions of the electroporated neural tube (Figure 2C). Finally, co-electroporation with both the *Krox20* and *kreisler* expression constructs led to massive activation of the reporter construct throughout the electroporated neural tube (Figure 2D). Activation of the *lacZ* reporter in these experiments was dependent on the presence of the *Hoxb3* enhancer, since similar co-electroporations of a control reporter construct without enhancer did not lead to any activation (data not shown).

In conclusion, these data indicate that when either *Krox20* or *kreisler* are expressed ectopically in the chick hindbrain, they lead to preferential activation of the *Hoxb3* enhancer in the territories where the other partner is

Fig. 2. Krox20 and kreisler co-operate on the *Hoxb3* r5 enhancer. Chick embryos were electroporated into the left side of the neural tube with a *lacZ* reporter construct. β -galactosidase activity was detected subsequently by X-gal staining. In the reporter, *lacZ* is placed under the control of the human β -globin minimal promoter and of the *Hoxb3* r5 enhancer. (A) Co-electroporation of the reporter construct with an empty expression vector (pAdRSV). Weak *lacZ* expression is detected in a few cells restricted to r5. (B) Co-electroporation with a *Krox20* expression vector. Activation of the reporter is observed in r5 and r6. (C) Co-electroporation of the reporter with a *kreisler* expression vector. Strong activation of the reporter is observed reproducibly in r3 and to a lower extent in r5. X-gal-positive cells are also seen occasionally in other areas of the electroporated region. (D) Co-electroporation with both the *Krox20* and *kreisler* expression vectors. Massive induction of the reporter is observed throughout the electroporated region.

endogenously expressed: r5/6 upon expression of *Krox20*, and r3 and r5 upon expression of *kreisler*. Furthermore, co-expression of both genes leads to ubiquitous activation of the enhancer. These data clearly demonstrate that *Krox20* and *kreisler* can co-operate synergistically to activate the *Hoxb3* r5 enhancer. Furthermore, they suggest that, in this assay, ectopic expression of *Krox20* and *kreisler* is sufficient to promote efficient activation of the enhancer in a large part of the neural tube, including the entire hindbrain.

***Krox20* binds *in vitro* to two distinct sites within the *Hoxb3* r5 enhancer**

In light of the above data, we investigated the possibility that *Krox20* was directly controlling the activity of the *Hoxb3* r5 enhancer. For this purpose, a 400 bp *NcoI*–*HindIII* fragment containing the enhancer activity

Fig. 3. The *Hoxb3* r5 enhancer contains two Krox20-binding sites. (A) *In vitro* binding of Krox20 to the 400 bp *NcoI*–*HindIII* fragment carrying the *Hoxb3* r5 enhancer, analysed by electrophoretic mobility shift assay. Lane 1, control protein extract from bacteria transfected with the empty expression vector. Lanes 2–4, protein extracts from *Krox20*-expressing bacteria. Two retarded complexes are observed in lane 2 (B1 and B2), suggesting the existence of two Krox20-binding sites. In lanes 3 and 4, protein–DNA incubation was performed in the presence of a 50-fold molar excess of competitor oligonucleotides containing a high affinity Krox20-binding site or a mutated version of this site, respectively. Complex formation is competed specifically by the Krox20 oligonucleotide (lane 3). P, free probe. (B) DMS interference analysis performed on a 120 bp *Sfe*–*HindIII* subfragment containing the two Krox20-binding sites. Lane 1, G + A scale; lanes 2–5, G scales of the probe before electrophoresis (lane 2), of the free probe (lane 3) and of the probe extracted from complex B1 (lane 4), and from complex B2 (lane 5). This analysis reveals two regions where guanine methylation prevents complex formation. The stronger interference observed in the case of B2 suggests that this corresponds to a ternary complex in which both sites are occupied by Krox20. Two sequences with homology to the Krox20 consensus binding site (KroxA and KroxB) are centred within the regions of interference.

was analysed for its capacity to bind Krox20 in a gel retardation assay. In the presence of bacterially produced Krox20, two retarded complexes were observed (Figure 3A). The formation of these complexes was prevented by addition of an oligonucleotide containing a high affinity Krox20-binding site (5′-GCGTGGGCG-3′), but not by addition of a related oligonucleotide, containing a mutation in this sequence known to abolish Krox20 binding (5′-GCGTCGGCG-3′, Figure 3A, lines 3 and 4; Nardelli *et al.*, 1991). These data suggested the existence of two Krox20-binding sites within the enhancer fragment, and this was confirmed by a dimethylsulfate (DMS) interference analysis of the fragment, which revealed two protected regions (Figure 3B). Examination of the nucleotide sequence of these regions indicated that each contained a sequence with similarity to a consensus Krox20-binding site (Chavrier *et al.*, 1990; Nardelli *et al.*, 1991; Swirloff and Milbrandt, 1995). Hence, these sites were named KroxA (5′-CTGTAGGAG-3′) and KroxB (5′-ATGTAGGTG-3′) (Figure 3B).

The *Hoxb3* r5 enhancer contains two blocks of conserved sequences between mouse and chick, one of 19 bp that includes the *kreisler*-binding site Kr1, and another of 46 bp that contains the *kreisler*-binding site Kr2 and an Ets-related activation site (ERAS) (Figure 4; Manzanares *et al.*, 1997). Deletion of any of the *kreisler*-

Fig. 4. Mutational analysis of the *Hoxb3* r5 enhancer. (A) The top line shows the 2.2 kb *Bam*HI–*Hind*III genomic fragment in the context of which all transgenic analyses of mutated versions were carried out. The advantage of this fragment is that, apart from the r5 element, it includes other enhancers responsible for posterior expression (Manzanares *et al.*, 1997) that may be used as an internal control for transgenesis and *lacZ* activity. Under this is shown the sequence of the 46 bp conserved block from the *Hoxb3* r5 enhancer where the kreisler-binding site Kr2 and the Ets-related activation site (ERAS) previously had been mapped (Manzanares *et al.*, 1997), and where Krox20-binding sites KroxA and KroxB have been located. Sites are boxed, highlighting the overlaps between Kr2 and KroxB on one hand, and between ERAS and KroxA on the other. Below are indicated the nucleotide changes introduced in the different mutated versions. B, *Bam*HI; H, *Hind*III. (B) *In vitro* and *in vivo* behaviour of the different mutated versions of the *Hoxb3* r5 enhancer. Results for Kr1Δ, Kr2Δ and ERAS-m1 were described in Manzanares *et al.* (1997). +, positive binding or transgene expression; –, lack of binding or transgene expression; +/-, reduced transgene expression; ++, increased binding; nt, not tested; N, number of transgenic embryos showing a reproducible pattern.

binding sites or extensive mutation of the ERAS resulted in the loss of expression in r5 (Manzanares *et al.*, 1997). Both of the Krox20-binding sites identified by the *in vitro* assay are located within the 46 bp sequence (Figure 4A) and partially overlap with the ERAS (KroxA) and the Kr2 sites (KroxB). Thus, the mutations tested previously [Kr2Δ and ERAS-m1; see Figure 4A and Manzanares *et al.* (1997)] could have also interfered with *Krox20* input on the *Hoxb3* r5 enhancer. Therefore, we constructed a series of new point mutations within the 46 bp fragment to assess independently the binding and function of kreisler, Krox20 and Ets proteins on the enhancer, and correlate these with their *in vivo* roles in the segmental expression of *Hoxb3* (Figure 4A and B).

The KroxA site is necessary for activity of the *Hoxb3* r5 element

The original ERAS-m1 mutation modified four out of nine base pairs of the KroxA site (Figure 4A), so it was likely

that this mutation would affect binding of both Krox20 and Ets proteins, and this was confirmed experimentally (Figure 4B and data not shown). To separate the Ets and Krox activities, two additional mutations within the non-overlapping portions of the ERAS and KroxA sites (ERAS-m2 and KroxA-m1) were therefore generated in this region (Figure 4A). Direct *in vitro* binding studies of Krox20 were then performed on a 23 bp oligonucleotide spanning the ERAS and KroxA sites (see Materials and methods). They revealed the formation of a unique retarded complex (Figure 5A), presumably corresponding to binding of Krox20 to the KroxA site. Consistent with this interpretation, introduction of the KroxA-m1 mutation abolished binding, while the ERAS-m2 mutation had no effect (Figures 4B and 5A). Competition experiments performed with oligonucleotides confirmed these results. Oligonucleotides carrying a high affinity Krox20-binding site (5'-GCGTGGGCG-3'; Figure 5B, lanes 2–4), the wild-type version of the KroxA site (Figure 5B, lanes 7–9) or the ERAS-m2 version of the 23 bp oligonucleotide (Figure 5B, lanes 13–15) effectively competed binding of Krox20 protein to the wild-type 23 bp oligonucleotide. In contrast, oligonucleotides carrying a mutated version of the high affinity site (5'-GCGTCGGCG-3'; Figure 5B, lanes 5 and 6) or the m1 version of the KroxA site (Figure 5B, lanes 10–12) did not compete efficiently.

Similar binding competition assays were also performed to assess the affinity of the Ets1 protein for wild-type and mutant versions of the 23 bp oligonucleotide. The probe contained an Ets1-binding site (Ets1b, see Materials and methods) and led to formation of low levels of a specific complex with Ets1 (Figure 5C, lane 2). Formation of this complex was competed by oligonucleotides carrying the Ets1b site itself (Figure 5C, lanes 6–8), a different Ets1 site (Ets1a; Figure 5C, lanes 3–5) or the wild-type and KroxA-m1 versions of the 23 bp oligonucleotide (Figure 5C, lanes 12–17). In contrast, a mutated Ets1a site (Etsm; Figure 5C, lanes 9–11) and the ERAS-m2 version of the 23 bp oligonucleotide (Figure 5C, lanes 18–20) could not prevent Ets binding to the probe.

This series of *in vitro* binding experiments showed that the new mutations, KroxA-m1 and ERAS-m2, were able specifically and independently to prevent binding of Krox20 or Ets1, respectively, to the *Hoxb3* r5 enhancer (Figure 4B) and provided a means of discriminating between the roles of these two factors. Therefore, to test the effect of these mutations on the activity of the full 2.2 kb enhancer *in vivo*, transgenic embryos carrying both versions linked to reporter genes were generated and analysed at 9.5 d.p.c. (Figures 4B and 5). The KroxA-m1 mutation completely abolished expression of *lacZ* in r5 (Figure 6B and E). The ERAS-m2 mutation also had an effect on enhancer activity, although less dramatic, as expression in r5 was reduced but not eliminated, compared with the wild-type version (compare Figure 6A and D with C and F). These results imply that the KroxA site of the r5 enhancer element is required for *Hoxb3* transcriptional activation in r5, presumably due to direct Krox20 binding, and that the ERAS site and Ets proteins are necessary to modulate the expression levels mediated by this regulatory element. Hence, both of these sites contribute to the potentiation and restriction of the enhancer activity to r5.

Fig. 5. *In vitro* analysis of the binding of Krox20 and Ets1 to the KroxA/ERAS region of the *Hoxb3* r5 enhancer. (A) Analysis of Krox20 binding by direct gel mobility shift assay. The 23 bp oligonucleotide probes (see Materials and methods) spanning the KroxA and ERAS sites and corresponding to the wild-type (WT), KroxA-m1 and ERAS-m2 mutated sequences were incubated with control (C) or Krox20-containing (K20) bacterial extracts. The KroxA-m1 mutation prevents Krox20 binding, whereas the ERAS-m2 has no effect. (B) Competition gel mobility shift assay performed as in (A), but in the presence of 20- (lanes 2, 7, 10 and 13), 50- (lanes 3, 5, 8, 11 and 14) or 250-fold (lanes 4, 6, 9, 12 and 15) molar excess of competitor oligonucleotides carrying a high affinity Krox20-binding site (lanes 2–4), a mutated version of this site (lanes 5 and 6), the KroxA/ERAS region (lanes 7–9), and this region with the KroxA-m1 mutation (lanes 10–12) and with the ERAS-m2 mutation (lanes 13–15). (C) Competition gel mobility shift assay for Ets1 binding to a 28 bp oligonucleotide (see Materials and methods) carrying an Ets-binding site. The probe was incubated with a control extract (lane 1) or a baculovirus extract containing the Ets1 protein (lanes 2–20) in the presence of 20- (lanes 3, 6, 9, 12, 15 and 18), 50- (lanes 4, 7, 10, 13, 16 and 19) or 250-fold (lanes 5, 8, 11, 14, 17 and 20) molar excess of competitor oligonucleotides carrying two different Ets1 sites (Ets1a and Ets1b, lanes 3–8), a mutated version of the Ets1a site (lanes 9–11), the KroxA/ERAS region (lanes 12–14), and this region with the KroxA-m1 mutation (lanes 15–17) and with the ERAS-m2 mutation (lanes 18–20). P, free probe; B, bound probe.

Mutations affecting the affinity of kreisler for the Kr2 site prevent transcriptional activation in r5

We performed a similar analysis of the region of the *Hoxb3* r5 enhancer containing the overlapping Kr2 and KroxB sites (Figures 4A and B, and 7 and 8). As expected, we first observed that the previously tested Kr2 Δ deletion (Manzanares *et al.*, 1997) interferes with *in vitro* binding of both kreisler and Krox20 (Figure 4B, and data not shown). A new mutation in the Kr2 site was generated, where four base pairs were mutated outside of the KroxB site (Kr2-m1, Figure 4A). In this version, while kreisler *in vitro* binding was still abrogated, Krox20 bound in a normal manner to the adjacent KroxB site (Figure 4B, and

Fig. 6. Effects of mutations in the KroxA/ERAS region on the activity of the *Hoxb3* r5 enhancer. Dorsal (A–C) and lateral (D–F) views of 9.5 d.p.c. transgenic embryos carrying either the wild-type enhancer (A and D) or the KroxA-m1 (B and E) or ERAS-m2 (C and F) mutated versions. The KroxA-m1 mutation abolishes enhancer activity in r5, while the ERAS-m2 mutation significantly reduces the level of expression of the transgene in r5. The black arrowhead in (B) points to the position of r5.

data not shown). In transgenic embryos, the Kr2-m1 mutation abolished the r5 activity of the enhancer (Figure 8B and G), like the Kr2 Δ deletion (Manzanares *et al.*, 1997). These results demonstrate that direct interaction of kreisler with the Kr2 site is necessary for the activity of the *Hoxb3* r5 enhancer, irrespective of Krox20 binding.

In an attempt to assess independently the potential role of Krox20 binding to the KroxB site, we introduced a series of mutations into this site (Figure 4A) and tested their effect both on *in vitro* binding of kreisler and Krox20 and on the activity of the enhancer in mouse transgenic embryos (Figures 7, 8, and 9A and B). An important consideration in this analysis is that kreisler is likely to bind to Kr2 as a dimer and hence would interact with the adjacent 14 bp sequence almost completely covering the KroxB site. In agreement with this idea, we found that three of the four mutations introduced in the KroxB site (KroxB-m1, KroxB-m2 and KroxB-m4) modified kreisler binding. Two of them (KroxB-m1 and KroxB-m4) abolished kreisler binding (Figures 4B and 7). At the same time, KroxB-m1 also prevented Krox20 binding but KroxB-m4 had no effect. Consistent with these *in vitro* data, these two mutations also eliminated the *in vivo* activity of the enhancer in r5, while the other domains of expression were unaffected (Figure 8C, E, H and J). These data therefore reinforced our observation on the necessity of kreisler binding to Kr2 for r5 enhancer activity.

The third mutation, KroxB-m2, showed an unexpected behaviour. It significantly increased the affinity for kreisler, without affecting Krox20 binding (Figure 7A

Fig. 7. *In vitro* analysis of the binding of Krox20 and kreisler to wild-type and mutated versions of the Kr2/KroxB region of the *Hoxb3* r5 enhancer. The binding of each protein was assayed by direct gel mobility shift assay using probes consisting of variants of a 25 bp oligonucleotide spanning the Kr2/KroxB region and carrying the different mutations (see Materials and methods). (A) A comparison of kreisler binding to the wild-type (WT) and mutant KroxB-m1, -m2 and -m3 sequences, as indicated. Note that KroxB-m1 eliminates binding, whereas KroxB-m2 significantly increases it. (B) A similar analysis for Krox20 binding. Mutations KroxB-m1 and -m3 abolish binding. (C) An analysis of kreisler and Krox20 binding to the KroxB-m4 sequence as compared with the wild type. This mutation prevents kreisler binding without affecting the interaction with Krox20. In the last lane on the right, the wild-type probe was exposed simultaneously to the kreisler and Krox20 proteins. No tertiary complex is observed, suggesting that kreisler and Krox20 binding on their respective sites are exclusive. P, free probe; B, bound probe.

Fig. 8. *In vivo* analysis of mutated versions in the Kr2/KroxB region of the *Hoxb3* r5 enhancer. Dorsal (A–E) and lateral (F–J) views of 9.5 d.p.c. transgenic embryos carrying either the wild-type enhancer (A and F) or mutant versions. (B and G) A 4 bp change in the kreisler site Kr2, which does not interfere with Krox20 binding to KroxB (Kr2-m1, see Figure 4B), results in loss of *lacZ* expression in r5. Three different mutations in the KroxB site (KroxB-m1, C and H; KroxB-m2, D and I; KroxB-m4, E and J) also result in loss of r5 expression, although their effect on kreisler *in vitro* binding is diverse (see Figures 4B and 7). The white arrow in (D) points to a positive cell in the hindbrain of a transgenic embryo for the KroxB-m2 mutated construct; however, no consistent expression in r5 is observed with this version of the *Hoxb3* r5 enhancer. The black arrowheads in (A–E) point to the position of r5.

and B). However, when tested in transgenic embryos, this mutation surprisingly led to a loss of r5 expression (Figure 8D and J). To investigate this effect of increased binding and decreased activity, we examined whether there were any synergistic, co-operative or inhibitory interactions in binding of kreisler and Krox20 to the Kr2/KroxB region of the *Hoxb3* r5 element. Assays of binding to this sequence in the presence of both proteins did not result in formation of a tertiary complex (Figure 7C). These data suggest that Krox20 binding to KroxB and

kreisler binding to Kr2 may be exclusive of each other. They do not explain the loss of activity associated with the KroxB-m2 mutation, unless this change also interferes with the binding of other factors required *in vivo* for kreisler or enhancer activity.

A point mutation in the KroxB site expands the activity of the *Hoxb3* enhancer to r6

The fourth mutation introduced in the KroxB site, KroxB-m3 (Figure 4A), showed the *in vitro* behaviour

Fig. 9. Effects of the KroxB-m3 mutation on the activity of the *Hoxb3* r5 enhancer. Dorsal (A–C) and lateral (D–F) views of 9.5 d.p.c. transgenic embryos carrying different enhancer constructs with the KroxB-m3 mutation. In the context of the wild-type *Hoxb3* enhancer, the KroxB-m3 mutation results in an expansion of *lacZ* expression from r5 only to r5 and r6 (A and D, compare with wild type in Figure 8A and F). (B and E) Four copies of a 32 bp oligonucleotide spanning the Kr2/KroxB region (see Materials and methods) and containing this mutation also acts as an r5/r6 enhancer, while an equivalent wild-type oligonucleotide only drives *lacZ* expression in r5 (Manzanares *et al.*, 1997). (C and F) The combination of the Kr1Δ and KroxB-m3 mutations maintains expression in r5, while the Kr1Δ mutation on its own results in loss of r5 expression (Figure 4B; Manzanares *et al.*, 1997). nc, neural crest; dr, dorsal roof; ba3, third branchial arch.

that we initially were looking for in that Krox20 binding was abolished, whereas kreisler binding was not affected (Figures 4B, and 7A and B). The analysis of the activity of the enhancer carrying this mutation in transgenic embryos led to a further surprising result. This enhancer was active not only in r5, but also in r6 (Figure 9A and D), and this behaviour was never observed with the wild-type or other mutated enhancer constructs (Figures 6 and 8; Manzanares *et al.*, 1997). Therefore, by modifying the KroxB site, expression mediated by the *Hoxb3* element is no longer restricted to r5, but extends to r5 and r6 in a manner that reflects the normal segmental domain of *kreisler* expression in the hindbrain.

We further examined the regulatory potential of the KroxB-m3 site in r6 by generating a *lacZ* expression construct containing four copies of an oligonucleotide corresponding to the 5' first 32 nucleotides of the 46 bp block (Figure 4A) carrying this specific mutation (see Materials and methods). This construct directed robust expression in r5, r6 and the roof plate (Figure 9B and E), in a pattern similar to that obtained with multimerized oligonucleotides containing the Kr1 site of *Hoxb3* (Manzanares *et al.*, 1997) or the KrA site of *Hoxa3* (Manzanares *et al.*, 1999a). In contrast, a similar construct

with a wild-type oligonucleotide did not result in expression in the hindbrain, although occasionally there was modest expression in the roof plate (Manzanares *et al.*, 1997, 1999a). This assay suggests that the KroxB-m3 site apparently functions more effectively as a kreisler response element *in vivo*.

We also examined if the KroxB-m3 mutation influenced the dependence of the 2.2 kb enhancer on the upstream Kr1 kreisler-binding site (Figure 4A). In normal circumstances, both the Kr1 and Kr2 sites are required for activity, and a deletion of the Kr1 site (Kr1Δ) abolishes expression in r5 (Manzanares *et al.*, 1997). However, a construct carrying the KroxB-m3+Kr1Δ mutations directed expression in r5 of transgenic embryos (Figure 9C and F). This rescue of r5 activity in the combined mutant is different from the outcome associated with the two mutations tested individually, where in the case of Kr1Δ there is no expression in the hindbrain, and in the case of KroxB-m3 there is expression in r5 and r6. Together, these results on the KroxB-m3 mutation suggest that it is more effective at potentiating the activity of the Kr2 site in r5, so the Kr1 site is not essential for r5 expression. However, co-operation between both kreisler sites, Kr1 and Kr2, is still needed for enhancer activity in r6.

The KroxB-m3 mutation does not alter the dependence of the *Hoxb3* enhancer on Krox20

One reason why the enhancer carrying the double mutant (KroxB-m3+Kr1Δ) might be active in r5 and not r6 is that Krox20 continues to be required through the KroxA site. However, since the *Hoxb3* enhancer carrying the KroxB-m3 mutation is active in r6, where Krox20 is absent, it is possible that Krox20 binding to this site is no longer required for enhancer activity in r5. To distinguish between these possibilities, we combined the KroxB-m3 and the KroxA-m1 mutations within the *Hoxb3* r5 enhancer. Analysis of this mutated enhancer in transgenic embryos (Figure 10) revealed that it was still active in r6, but that *lacZ* expression in r5 was eliminated (Figure 10C). This suggests that while kreisler can activate this enhancer in r6 through the Kr1 and Kr2 sites independently of Krox20, Krox20 is still required for r5 activity of the KroxB-m3 variant enhancer. Therefore, the co-operation between kreisler and Krox20 in r5 and the ability of the Kr1 and Kr2 sites to interact effectively with kreisler in r5 and r6 determine whether this enhancer is active in r5, or in r5 and r6.

Discussion

Through a combination of genetic and molecular approaches, we have performed a detailed analysis of the regulation of *Hox* group 3 genes in r5 and r6. Our data allowed us to reveal differences in the control of *Hoxa3* and *Hoxb3* expression that demonstrate the importance of co-operation between Krox20 and kreisler for direct transcriptional activation of *Hoxb3*. Our findings show how complex, independent inputs can be integrated by a single enhancer element for the generation of novel expression patterns.

Fig. 10. The r5 activity of the KroxB-m3 mutant *Hoxb3* enhancer is dependent on Krox20. Lateral (A), dorsal (B) and flat mount (C) views of a 9.5 d.p.c. transgenic embryo carrying the *Hoxb3* r5 enhancer with a combination of the KroxA-m1 and KroxB-m3 mutations. This results in loss of expression in r5, but not in r6. The black arrowheads in (B) and (C) point to a group of positive cells at the level of the r4–r5 boundary. The white asterisk in (C) indicates staining in the ventral midline of r5. ov, otic vesicle.

Differential requirement for Krox20 in the control of Hox group 3 expression

Involvement of Krox20 in the regulation of *Hoxb3* expression in r5 had been proposed previously on the basis of modifications of its expression pattern in *Krox20* homozygous mutant embryos (Seitanidou *et al.*, 1997). In the present work, we have shown that the enhancer mediating *Hoxb3* expression in r5 requires Krox20 for its activity. Furthermore, we have identified two Krox20-binding sites within this element, KroxA and KroxB, and shown that integrity of KroxA is required for enhancer activity. This indicates that Krox20 is a direct transcriptional activator necessary but not sufficient for expression of *Hoxb3*. In contrast, the activity of the r5/r6 *Hoxa3* enhancer is not dependent on Krox20. Together, these data highlight differences in the segmental regulation of the two *Hox* group 3 genes (Manzanares *et al.*, 1999a, 2001). These enhancers differ not only in their domains of activity, *Hoxa3* in r5/r6 and *Hoxb3* in r5, but also in their molecular control in r5. These two differences may be intimately linked and are likely to reflect the duplication and divergence of *cis*-acting regulatory sequences in group 3 paralogous genes during vertebrate evolution. In the zebrafish embryo, *Hoxa3* and *Hoxb3* are both expressed in r5 and r6 (Prince *et al.*, 1998), suggesting that in other vertebrates both of these genes may recapitulate the *kreisler/valentino* expression pattern. The involvement of Krox20 in activity of the *Hoxb3* enhancer, which may have appeared during amphibian evolution (Godsave *et al.*, 1994; Ruiz i Altaba, 1994), is likely to have resulted from changes in the *cis* elements that took advantage of the ability of Krox20 to potentiate this site. This resulted in its restriction to r5 based on the overlapping domains of Krox20 and *kreisler* expression.

Co-operation between kreisler and Krox20 integrates multiple inputs in segmental patterning

In combination with previous studies (Manzanares *et al.*, 1997), the present work establishes that *Hoxb3* expression in r5 requires binding of both *kreisler* and Krox20 to the r5 enhancer. Two *kreisler*-binding sites, Kr1 and Kr2, have been identified within the enhancer, and the integrity of each of them is required for full enhancer activity. In

particular, we have shown here that in the case of Kr2, which partially overlaps with the KroxB site, a mutation only affecting *kreisler* binding is sufficient to abolish enhancer activity (Figure 4). The other Krox20-binding site, KroxA, overlaps with an Ets-related activation site, and we have shown that a mutation specifically affecting Krox20 binding to KroxA eliminates expression directed by the enhancer (Figure 4). These data indicate that binding of *kreisler* to Kr1 and Kr2, and of Krox20 to KroxA, is required for enhancer activity. Therefore, these two transcription factors co-operate in a synergistic manner to promote *Hoxb3* transcription. Effective co-operation between Krox20 and *kreisler* for the activation of the *Hoxb3* r5 enhancer was confirmed by ectopic expression experiments performed in chick embryo hindbrain (Figure 2). Consistently, ectopic expression of *kreisler* in r3 in transgenic mice led to activation of the endogenous *Hoxb3* gene in this rhombomere (Theil *et al.*, 2002).

The molecular basis of this co-operation has not yet been unravelled. In particular, we do not know whether it involves synergistic binding to the three sites or interactions between *kreisler* and Krox20 with other proteins involved in transcriptional activation. Although members of the Maf family of transcription factors, to which *kreisler* belongs, have been shown to be able to interact with a variety of other transcription factors (Sieweke *et al.*, 1996; Blank and Andrews, 1997; Kataoka *et al.*, 2001), we have been unable to detect synergistic binding of *kreisler* and Krox20 *in vitro* (Figure 7 and data not shown). However, heterodimerization may require co-translation or specific conditions not provided in bacterial extracts. Definitive resolution of this point will therefore require additional investigation.

The regulation of *Hoxb3* expression provides an example of molecular mechanisms involved in the specification of a novel territory and in the determination of its identity during vertebrate development. The r5 territory corresponds precisely to the intersection between the domains of expression of *kreisler* and *Krox20* in the developing neural tube. Both genes have been shown to be involved in the formation or maintenance of this rhombomere since their inactivation results in its failure

to be generated (Schneider-Maunoury *et al.*, 1993, 1997; Swiatek and Gridley, 1993; Manzanares *et al.*, 1999b). Furthermore, accumulating evidence indicates that these two genes also participate in the regulation of segmental identity. In particular, Krox20 is involved in the transcriptional activation of *Hox* group 2 genes (Sham *et al.*, 1993; Nonchev *et al.*, 1996a,b) and kreisler in that of *Hox* group 3 genes (Manzanares *et al.*, 1997, 1999a). Our present data suggest that specification of r5 identity by kreisler and Krox20 does not result simply from the combination of target genes independently activated separately by each factor. It also involves co-operativity between these factors needed to activate common targets. In the case of *Hoxb3*, the integration of the kreisler and Krox20 inputs is performed directly at the level of *cis* elements in the r5 enhancer. It is likely that similar mechanisms involving integration of multiple inputs through enhancer elements are generally important during vertebrate development. This would allow conversion of overlapping expression domains of regulatory genes into novel subterritories of activity with unique identities, as shown in the *Drosophila* early embryo (Mann and Affolter, 1998; Affolter and Mann, 2001).

Cis analysis reveals additional factors controlling *Hoxb3* expression

The detailed mutagenesis analysis that we have performed has revealed the complex organization of the 46 bp DNA fragment containing the adjacent Kr2 and KroxA sites. This region includes two domains required for *Hoxb3* r5 enhancer activity, and each of these domains contains at least two overlapping binding sites for transcription factors (Figure 4). The ERAS-KroxA domain binds Krox20 and members of the Ets family. We have shown that Krox20 binding is absolutely required for enhancer activity and that a mutation destroying the ERAS without affecting Krox20 binding reduces the *in vivo* activity. This suggests that an Ets-related factor is involved in the modulation of *Hoxb3* expression in r5. The mode of action of this factor, in particular whether it interacts directly with Krox20 or kreisler, will be important to investigate.

The Kr2 domain contains a kreisler-binding site (Kr2) and a Krox20-binding site (KroxB). Because of the very large overlap between the two sites, we introduced several mutations into this domain in an attempt to evaluate the role of each factor. In the cases of the mutations located within KroxB (KroxB-m1 to KroxB-m4, Figure 4), there was no correlation between the effects on Krox20 binding *in vitro* and enhancer activity *in vivo*. Therefore, Krox20 binding to this site appears not to be required for proper *in vivo* function. In contrast, all mutations reducing the affinity of Kr2 for kreisler (Kr2Δ, Kr2-m1, KroxB-m1 and KroxB-m4; Figure 4) abolish r5 reporter gene expression, showing that kreisler binding to this site is essential for enhancer activity. The consequences of the two other mutations affecting Kr2 revealed surprising properties. KroxB-m2 led to an increase of kreisler binding *in vitro*, but to a loss of enhancer activity. KroxB-m3 did not affect kreisler binding *in vitro*, yet it resulted in an expansion of enhancer activity to r6. Furthermore, in r5, the KroxB-m3 mutated enhancer is dependent on Krox20 binding to KroxA but does not require kreisler binding to Kr1. In contrast, in r6, kreisler binding to Kr1 is still necessary for

activity, presumably because Krox20 is not present in this segment.

To explain these data, we have to consider the properties of the Maf family of transcription factors. These factors constitute a growing subclass of b-ZIP proteins which can bind DNA as dimers (homodimers or heterodimers) and recognize 13–14 bp palindromic sequences (Blank and Andrews, 1997). A wide range of heterodimers can be formed since the interaction is not restricted to members of the Maf family and Maf proteins have been shown to dimerize with partners belonging to distinct subgroups of b-ZIP proteins, including the AP-1 and CRE/ATF families. Consensus binding sites have been defined for homodimers or heterodimers between Maf proteins and b-ZIP proteins (T-MARE and C-MARE) and specificity of DNA sequence recognition has been shown to vary slightly according to the particular combination. In the case of the Kr2 site, the 5' half of the putative 14 bp site is very close to the consensus T- or C-MARE (six out of seven nucleotides identical), while the 3' half is more distant.

Together with our results, the features of the Maf factors suggest a model to account for the properties and activity of the *Hoxb3* enhancer. The Kr2 site is not an effective kreisler response element. It interacts poorly with kreisler homodimers and more effectively with a combination(s) of heterodimers between kreisler and other b-ZIP factors. These factors co-operate with Krox20 and the ERAS factors to potentiate expression from the Kr2 site. The KroxB-m2 and KroxB-m3 mutations differentially modify the affinity of the Kr2 site for heterodimeric partners and influence the recruitment of binding partners. In this way, these mutations lead to altered co-operativity between the b-ZIP factors and kreisler at Kr1 and Krox20 at KroxA, and to subsequent modifications in the activity of the enhancer. In the case of the KroxB-m3 mutation, the heterodimers binding to Kr2 may even be different in r5 and r6, accounting for the different requirements of the enhancer in these rhombomeres. This model highlights the importance of investigating the nature of kreisler heterodimerization partners during hindbrain development in order to build a more complete picture of the segmental regulatory cascade.

Materials and methods

DNA constructs

In the mouse, all deletion and mutated forms of the *Hoxb3* r5 enhancer were tested in the context of a 2.2 kb *Bam*HI–*Hind*III fragment linked to an expression vector (construct 8 in Whiting *et al.*, 1991) containing the basal *Hoxb4* promoter, the bacterial β-galactosidase gene and an SV40 polyadenylation signal. Except for the alterations, these are equivalent to the wild-type form of construct 6 in Manzanares *et al.* (1997). Specific mutations in the enhancers were generated by site-directed mutagenesis in m13 (Sculptor IVM System, Amersham) or in pBluescript KS⁺ (Transformer Site-Directed Mutagenesis Kit, Clontech). For analysis of enhancer activity of multimerized oligonucleotides, doubled-stranded oligonucleotides with overhanging *Spe*I-compatible ends were cloned into the *Spe*I site of the pBGZ40 reporter vector, which is an expression construct using the human β-globin promoter linked to *lacZ* (Yee and Rigby, 1993). The sequence of the oligonucleotide used to make the KroxB-m3 multimer was as follows: 5'-AAATTTGCAGACACCGAC-ATTCTTGGCTCCTG-3'. The sequence and the copy number of the cloned multimers were verified by sequencing. For microinjection, all inserts were separated from vector DNA by electrophoresis and purified using Gelase (Epicentre Technologies). For chick electroporation

experiments, a 637 bp *Bam*HI–*Stu*I fragment carrying the *Hoxb3* r5 enhancer (construct 5 of Manzanares *et al.*, 1997) was inserted into a reporter plasmid containing *lacZ* driven by a minimal human β -globin promoter (pGZ40; Yee and Rigby, 1993). The *kreisler* expression plasmid was constructed by cloning the mouse *kreisler* cDNA under the control of the RSV promoter and followed by an SV40 polyadenylation signal (F.Giudicelli, unpublished data). The *Krox20* expression plasmid has a similar structure and was reported previously (Giudicelli *et al.*, 2001).

Transgenic mice and breeding

Transgenic embryos were generated by pronuclear injection into fertilized mouse eggs from an intercross of F₁ hybrids (CBA $\times$ C57Bl6) and stained for *lacZ* reporter activity as described (Whiting *et al.*, 1991). Most constructs were assayed in founder (F₀) transgenic embryos. The reproducibility and criteria for positive (+) or negative (–) cases of transgene expression (N), as detailed in Figure 4, was determined as follows: we scored constructs ‘+’ for r5 or r6 *in vivo* expression 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. ‘+/-’ denotes a case where expression in the r5 was either absent or very weak in all embryos, even though these embryos had strong positive expression in the posterior domain. It is important to note that all construct variants were done in the context of a 2.2 kb *Bam*HI–*Hind*III enhancer fragment that directs expression in the posterior mesoderm by virtue of independent regulatory elements contained in the enhancer fragments that are separate from those that regulate segmental expression (construct 6; Manzanares *et al.*, 1997). 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.

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 regions medially. The previously generated *Hoxa3-r5/r6* (construct 3.3; Manzanares *et al.*, 1999a) and *Hoxb3-r5* (construct 6; Manzanares *et al.*, 1997) reporter lines were used for mating into a *Krox20* mutant background lacking a *lacZ* reporter in the targeted allele (Swiatek and Gridley, 1993). The mutant embryos were genotyped as described previously (Swiatek and Gridley, 1993).

In ovo electroporation

HH10–12 stage chick embryos were electroporated as described previously (Giudicelli *et al.*, 2001), except that six pulses of 25 V and 50 ms were performed. The concentrations of the reporter and expression plasmids were at 0.5 μ g/ μ l. Embryos were collected 24 h after electroporation, fixed in 4% paraformaldehyde (PFA) for 10 min and stained for β -galactosidase activity for 4 h at 30°C.

Electrophoretic mobility shift assays and dimethylsulfate interference analysis

For Krox20 binding analysis, electrophoretic mobility shift assay (EMSA) and preparation of bacterial extracts were performed as described previously (Chavrier *et al.*, 1990). The bacterial Krox20-expressing vector has been described previously (Chavrier *et al.*, 1990). For the initial analysis, the probe was a 400 bp *Nco*I–*Hind*III fragment carrying the *Hoxb3* r5 enhancer. Double-stranded oligonucleotides with the following sequences were also used as probes or competitors: 5'-CTGTGTACGCGTGGGCGGTTA-3', carrying a high affinity Krox20-binding site; 5'-CTGTGTACGCGTGGGCGGTTA-3', a variant of the previous one carrying a point mutation preventing Krox20 binding; 5'-AATTTGCAGACACCTACATTCTTGG-3' a 25 bp oligonucleotide spanning the Kr2/KroxB region and its mutated variants described in Figure 4; and 5'-GGCTCTGTCTTCTCCTACAGG-3' a 23 bp oligonucleotide spanning the KroxA/ERAS region and its mutated variants described in Figure 4. For kreisler binding analysis, EMSA was performed with the purified protein as described previously (Manzanares *et al.*, 1997). Kreisler was expressed in *Escherichia coli* as a fusion protein with a C-terminal tag of six histidine residues using the Pet21 (Novagen) vector and subsequently purified on an Ni²⁺ column using the His-bind Purification kit (Novagen). The probes used for kreisler binding were the 25 bp oligonucleotide spanning the Kr2/KroxB region and its derivatives as above. For Ets1 binding analysis, EMSA was performed as described by Bosselut *et al.* (1990). Ets1-containing extracts were prepared from Sf9 cells infected with an Ets1-expressing baculovirus

vector (Bosselut *et al.*, 1990). Double-stranded oligonucleotides with the following sequences were used as probes or competitors: Ets1a, 5'-TCGGGCTCGAGATAAACAGGAAGTGGTC-3' and Ets1b, 5'-TCGGGCTCGAGATAAACAGGAAGTGGTC-3', which contain Ets1-binding sites; and Etsm, 5'-TCGGGCTCGAGATAAACACCAAGTGGTC-3', a mutated version of Ets1a, which does not bind Ets1 efficiently. For DMS interference analysis, a 120 bp *Sfe*–*Hind*III fragment containing the *Hoxb3* r5 enhancer was subcloned in pBluescript, digested with *Bam*HI and labelled at its 5' extremities. After digestion with *Xho*I, the resulting 145 bp fragment was purified by electrophoresis on a polyacrylamide gel, and methylated on G residues by incubation in 0.5% DMS as described (Chavrier *et al.*, 1990). The methylated fragment was then used as a probe in a Krox20 EMSA. Free and complexed probe were purified from the gel, incubated in 1 M piperidine at 90°C for 30 min and analysed by electrophoresis on an 8% polyacrylamide sequencing gel.

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