

Rostral hindbrain patterning involves the direct activation of a *Krox20* transcriptional enhancer by Hox/Pbx and Meis factors

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The morphogenesis of the vertebrate hindbrain involves the generation of metameric units called rhombomeres (*r*), and *Krox20* encodes a transcription factor that is expressed in *r3* and *r5* and plays a major role in this segmentation process. Our knowledge of the basis of *Krox20* regulation in *r3* is rather confusing, especially concerning the involvement of Hox factors. To investigate this issue, we studied one of the *Krox20* hindbrain cis-regulatory sequences, element C, which is active in *r3-r5* and which is the only initiator element in *r3*. We show that element C contains multiple binding sites for Meis and Hox/Pbx factors and that these proteins synergize to activate the enhancer. Mutation of these binding sites allowed us to establish that *Krox20* is under the direct transcriptional control of both Meis (presumably Meis2) and Hox/Pbx factors in *r3*. Furthermore, our data indicate that element C functions according to multiple modes, in Meis-independent or -dependent manners and with different Hox proteins, in *r3* and *r5*. Finally, we show that the *Hoxb1* and *Krox20* expression domains transiently overlap in prospective *r3*, and that Hoxb1 binds to element C in vivo, supporting a cell-autonomous involvement of Hox paralogous group 1 proteins in *Krox20* regulation. Altogether, our data clarify the molecular mechanisms of an essential step in hindbrain patterning. We propose a model for the complex regulation of *Krox20*, involving a novel mode of initiation, positive and negative controls by Hox proteins, and multiple direct and indirect autoregulatory loops.

KEY WORDS: Hindbrain segmentation, Pattern formation, Transcription factor

INTRODUCTION

The morphogenesis of the vertebrate hindbrain involves a transient segmentation process along the anteroposterior (AP) axis, which leads to the generation of seven or eight metameric units, called rhombomeres (*r*) (Lumsden, 1990; Lumsden and Krumlauf, 1996). This subdivision presages the establishment of a stereotyped pattern of neuronal differentiation (Clarke et al., 1998; Lumsden and Keynes, 1989). Rhombomeres constitute units of both cell lineage restriction (Birgbauer and Fraser, 1994; Fraser et al., 1990) and specific gene expression (Lumsden and Krumlauf, 1996; Rijli et al., 1998).

Among the genes involved in hindbrain patterning, *Krox20* plays a particularly important role. It encodes a zinc finger transcription factor (Chavrier et al., 1989; Chavrier et al., 1988) and is expressed in prospective and established *r3* and *r5* territories (Schneider-Maunoury et al., 1993; Wilkinson et al., 1989). Loss- and gain-of-function experiments have shown that *Krox20* is essential for the formation of these odd-numbered segments, and that it acts by coupling segment delimitation, specification of rhombomere identity and cell lineage restriction (Giudicelli et al., 2001; Schneider-Maunoury et al., 1997; Schneider-Maunoury et al., 1993; Swiatek and Gridley, 1993; Voiculescu et al., 2001). *Krox20*

performs its complex function by up- or downregulating the expression of a number of other regulatory genes (Giudicelli et al., 2001; Mechta-Grigoriou et al., 2000; Seitanidou et al., 1997). These include Hox genes, which are involved in regional and segmental specification in the hindbrain (Lumsden and Krumlauf, 1996; Rijli et al., 1998). Specifically *Krox20* is responsible for the direct transcriptional activation of paralogous group (PG) 2 and 3 genes (*Hoxa2*, *Hoxb2* and *Hoxb3*) in *r3* and *r5*, and in *r5*, respectively (Giudicelli et al., 2001; Manzanares et al., 2002; Nonchev et al., 1996a; Nonchev et al., 1996b; Seitanidou et al., 1997; Sham et al., 1993; Vesque et al., 1996), whereas it represses the PG 1 gene *Hoxb1* (Garcia-Dominguez et al., 2006; Giudicelli et al., 2001).

Given the central role of *Krox20* in hindbrain development, understanding the basis of *Krox20* regulation is of prime importance. Significant progress has been made in this direction in *r5*, because the transcription factors MafB and vHnf1 (Hnf1b – Mouse Genome Informatics, Zebrafish Information Network) have been shown to be necessary for its expression (Hernandez et al., 2004). In *r3*, however, our knowledge is more limited and somehow confusing. Pbx and Meis proteins have been implicated in several studies. Hence, eliminating both maternal and zygotic expression of the *pbx2* and *pbx4* genes in zebrafish embryos leads to a complete transformation of the hindbrain, with no *krox20* expression (Waskiewicz et al., 2002). Loss of Meis function in zebrafish results in phenotypes similar to a single *pbx4* mutation, with loss of the anterior domain of *krox20* expression (Choe et al., 2002; Popperl et al., 2000; Waskiewicz et al., 2001). Pbx and Meis usually act as cofactors for Hox proteins (for a review, see Moens and Selleri, 2006). Pbx proteins form complexes with Hox factors and bind bi-partite Hox/Pbx DNA sequence motifs. Meis or Prep proteins recognize separate DNA-binding sites but directly interact with the Hox/Pbx

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complexes. The involvement of Pbx and Meis factors in *Krox20* regulation therefore suggests an involvement of Hox proteins, but other data are apparently contradictory. According to their expression patterns, PG 2 genes could be involved in *Krox20* regulation in r3. However, in the *Hoxa2/Hoxb2* mouse double mutant, *Krox20* expression is not affected, indicating that PG 2 genes are not required for normal *Krox20* regulation (Davenne et al., 1999). Concerning PG 1 genes, they have been proposed to be expressed up to the r3/r4 presumptive rhombomere boundary (reviewed by Lumsden and Krumlauf, 1996), and have not been reported to overlap with the *Krox20* expression domain in r3. Nevertheless, strong evidence for their involvement in *Krox20* regulation in r3 has been obtained in *Xenopus*, where *Hoxd1* is expressed in the hindbrain in addition to *Hoxa1* and *Hoxb1*, and where knockdown of the complete PG 1 leads to loss of *Krox20* expression (McNulty et al., 2005). In mouse and zebrafish, where only *Hoxa1* and *Hoxb1* are expressed, the situation is less clear. The *Hoxa1* mutation results in a patchy but caudally extended *Krox20* r3 domain (Carpenter et al., 1993; Chisaka et al., 1992; Dolle et al., 1993; Lufkin et al., 1991; Mark et al., 1993), and combined *Hoxa1* and *Hoxb1* impairment further reduces *Krox20* expression in r3, but it does not prevent it (Barrow et al., 2000; Gavalas et al., 1998; McClintock et al., 2002; McNulty et al., 2005; Rossel and Capecchi, 1999).

Altogether, the above data offer a contrasting view of Hox protein involvement in the control of *Krox20* expression in r3. To clarify this issue, we have localized the cis-acting regulatory elements responsible for *Krox20* expression in the developing hindbrain. Three evolutionary conserved transcriptional enhancers, designated elements A, B and C, have been characterized (Chomette et al., 2006). Element A is a direct auto-regulatory element, involved in the maintenance of *Krox20* expression, whereas elements B and C are responsible for the initiation of *Krox20* expression, as indicated by their activity in a *Krox20*-null background (Chomette et al., 2006). Element B is active only in r5, whereas element C is active in the r3-r5 domain. Therefore, element C is the only initiator element in r3. In this paper, we analyze chick element C. We establish that Hox proteins are directly involved in *Krox20* regulation and provide a comprehensive view of a very complex regulatory network essential for hindbrain segmentation.

MATERIALS AND METHODS

DNA constructs and mutagenesis

Mutagenesis was performed using the Transformer Site-Directed Mutagenesis Kit (Clontech) with the primers indicated in Table 1. The mutated versions of element C were cloned into pBGZ40 (Yee and Rigby, 1993). pCMVHoxa1, pCMVHoxb1 and pCMVHoxb9 (gifts from R. Krumlauf, Stowers Institute, Kansas City), pCMVHoxa2 (gift from R. Rezsóhazy, Université Catholique de Louvain) and pCMVMeis2, respectively, contain complete cDNAs of human *HOXA1* (IMAGE Consortium Clone ID 5537563), mouse *Hoxb1*, *Hoxb9* and *Hoxa2* (Matis et al., 2001) and *Meis2* (IMAGE Consortium Clone ID 4191098). For cell extract preparation, mouse *Meis2*, *Hoxb1* and *Pbx1* (IMAGE Consortium

Clone ID 5701148) cDNAs were introduced into pAdRSVSp (Giudicelli et al., 2003), together with a HA epitope-coding sequence immediately before the stop codon.

Generation of transgenic mice and in ovo electroporation

Transgenesis were performed as described (Chomette et al., 2006). In ovo electroporation was performed as described (Giudicelli et al., 2001) at stages HH8-HH10. Each construct or combination of constructs was tested in at least two independent experiments, each involving eight or more embryos. The efficiency of electroporation was controlled by co-electroporation of a GFP reporter.

In situ hybridization, immunolabelling and X-gal staining

Whole-mount in situ hybridization was performed as described (Giudicelli et al., 2001). The probes for in situ hybridizations were as follows: a mouse *Krox20* probe (Wilkinson et al., 1989), a mouse *Meis1* probe (gift from Sonia Garel, Ecole Normale Supérieure), a chick *Meis2* probe (gift from Nadia Mercader, EMBL, Heidelberg), mouse *Meis2*, *Meis3*, *Prep1* and *Prep2* probes synthesized from IMAGE Consortium cDNA clones 5687497, 5121146, 5721441 and 6332968, respectively. Double in situ hybridization was performed as described (Giudicelli et al., 2001). Alkaline phosphatase activity was revealed using the NBT/ or INT/BCIP substrates (Roche). Mouse immunolabelling was performed using rabbit anti-Krox20 (Desmazières et al., 2008) and rat anti-GFP (Nacalai Tesque) antibodies. For double labelling, in situ hybridization was performed as above and rat anti-HA antibody (Roche) was added with the anti-DIG antibody. Labelling for β -galactosidase activity was performed as described (Ghislain et al., 2003).

Protein extracts and band shift assays

Expression plasmids were transfected into COS-7 cells using the FuGene 6 Transfection Reagent (Roche). Cell lysates were prepared as described (Dignam et al., 1983). Nuclear membranes were disrupted by the addition of 0.5% Nonidet P-40, the suspension was brought to 0.4 M NaCl and 0.2 mM EDTA. HA-tagged protein was purified from the supernatant using the Anti-HA Affinity Matrix (Roche). Band shift experiments were performed as described (Chomette et al., 2006), with the following modifications: 2 or 4 μ l of Meis2 or Hoxb1/Pbx1 protein preparations were pre-incubated on ice with 1 μ g of poly(dI-dC) in 10 μ l of buffer (10 mM Tris-HCl pH 7.5, 75 mM NaCl, 1 mM EDTA, 1 mM DTT, 540 ng/ml BSA, 12% glycerol for Meis2; 20 mM HEPES pH 7.9, 1 mM EDTA, 1 mM DTT, 2 mM MgCl₂, 6.25% Ficoll for Hoxb1/Pbx1).

Zebrafish manipulations

In situ hybridization was performed as described (Hauptmann and Gerster, 1994), using *hoxb1a* (Prince et al., 1998) and *krox20* (Oxtoby and Jowett, 1993) probes. For cell-autonomy experiments, capped RNAs were transcribed using the mMessage mMachine Kit (Ambion). *hoxb1aMyc* (gift from V. Prince, University of Chicago) and *meis1.1* RNAs were injected into one cell at the 16- to 32-cell stage at concentrations of 60 and 75 ng/ μ l, respectively. Embryos were embedded in gelatine-sucrose after whole-mount in situ hybridization and cryosectioned. Sections (16 μ m) were treated for immunofluorescence with a primary rabbit anti-Myc antibody (Upstate Biotechnology 06-549, 1/300) and a secondary donkey anti-rabbit antibody (Molecular Probes, 1/400). For chromatin immunoprecipitation (ChIP) analysis, 2 nl of *meis1.1* (80 ng/ μ l), Myc-tagged-*hoxb1a* (60 ng/ μ l) and Myc-tagged-*hoxa2* (60 ng/ μ l, gift from V. Prince) RNAs were injected into one cell at the four-cell stage. ChIP was carried out as described (Havis et al., 2006) at the 100% epiboly stage. Ten μ l of the anti-Myc (9B11, Ozyme) antibody were

Table 1. Oligonucleotides used for the mutagenesis

Mutagenesis site	Primer type	Sequence
HP1 site	Selection primer	5'-cactagttctagaggtcgaccaccgcggtg-3'
	Mutagenesis primer	5'-ccaatgtcgaactaatcactgagtaaacctg-3'
HP2 site	Selection primer	5'-gggtaccgggccccctcgaggtcg-3'
	Mutagenesis primer	5'-gcttatcgataccgtccgataagccttgatttaagtgaagcgttttcaggccatccgtcttggttgtcc-3'
Meis sites	Selection primer	5'-cactagttctagaggtcgaccaccgcggtg-3'
	Mutagenesis primer	5'-gtgaacaactccggatccggtaaatccggaaggaagc-3'

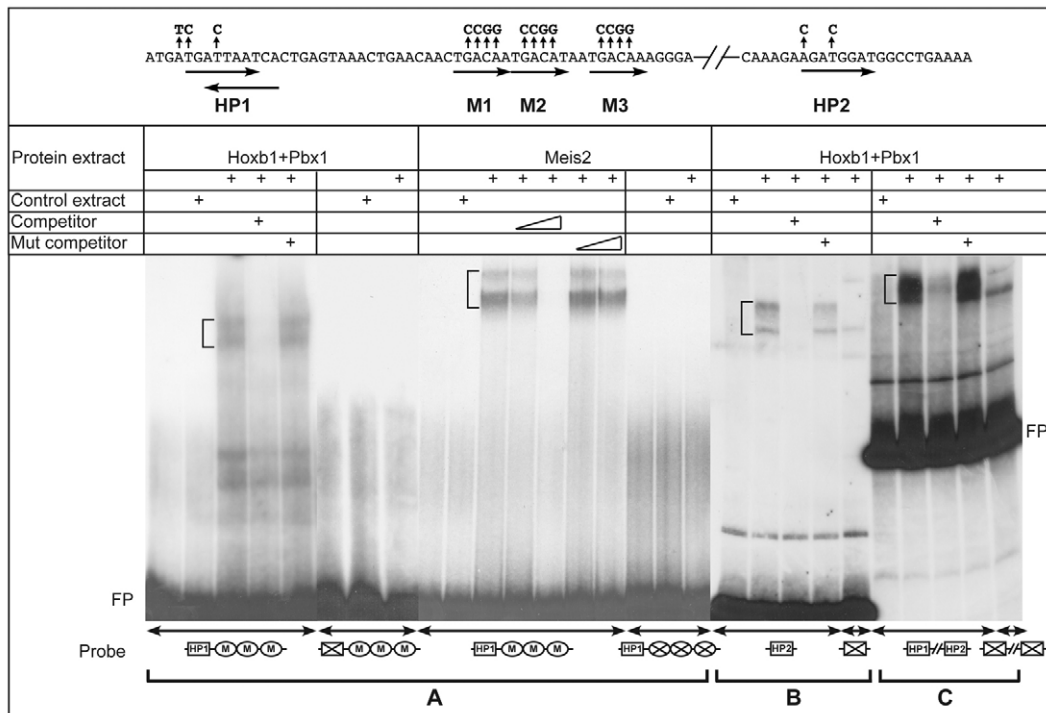


Fig. 1. Identification of Hox/Pbx- and Meis-binding sites within element C. Part of the chick element C sequence is shown, with the different binding sites (horizontal arrows) and the mutations that have been introduced into each of them (vertical arrows). Gel retardation analyses were performed with the probes schematized underneath the gels and the protein extracts indicated above. A cross within a site indicates that it is mutated. The retarded complexes are indicated by brackets and the specificity of the binding was established by competition with oligonucleotides carrying high-affinity Hox/Pbx- or Meis-binding sites (Competitor) or mutated versions unable to bind these factors (Mut competitor). FP, free probe.

used per 10 μ g of sonicated chromatin. Immunoprecipitated DNA was analyzed by PCR using the following primers: element C, 5'-gttaatgacaggaggtcg-3' and 5'-gctctctgggataaagg-3'; and -22.4 kb sequence, 5'-tgcaaccactgcctcac-3' and 5'-gcgctctgttagcctcc-3'.

RESULTS

Enhancer element C contains multiple binding sites for the Hox/Pbx and Meis proteins

As some genetic studies suggest an involvement of Hox proteins and their cofactors in the regulation of *Krox20*, we investigated the possibility of a direct binding of these factors to enhancer C. We therefore scanned the 896-bp chick enhancer sequence for the presence of motifs close to the Hox/Pbx and Meis consensus binding sites (reviewed by Mann and Affolter, 1998). Two overlapping putative Hox/Pbx sites on both strands (HP1), highly conserved among vertebrates, and three clustered putative Meis sites (M1, M2 and M3, the first one being also conserved) were identified within a 70 bp region (Fig. 1; see Fig. S1 in the supplementary material). Another highly conserved putative Hox/Pbx-binding site (HP2) was identified in the 3' part of the enhancer. We investigated the significance of these putative sites by gel retardation with a 64 bp oligonucleotide carrying HP1 and the Meis sites (see Fig. S1 in the supplementary material). This oligonucleotide was incubated with purified proteins prepared from COS-7 cells transfected or co-transfected with HA-tagged mouse *Hoxb1*, *Pbx1* and *Meis2* expression plasmids. Meis2 and the Hoxb1/Pbx1 combination led to the formation of retarded bands, which could be specifically competed out by the addition of oligonucleotides carrying high-affinity Meis- or Hox/Pbx-binding sites, respectively (Fig. 1A). The

relatively efficient binding of Meis2 alone might be explained by the presence of the three sites. To establish that the binding sites were indeed those identified in silico, we introduced mutations separately into HP1 and into the three Meis sites (Fig. 1). Band shift analysis demonstrated that the mutated fragments did not bind Hoxb1/Pbx1 and Meis2, respectively (Fig. 1A). Further experiments performed with the 896 bp fragment demonstrated that mutations of the three Meis sites also abolished Meis2 binding (data not shown), indicating that it does not contain any additional Meis site.

We performed a similar analysis in the case of HP2. An 83 bp oligonucleotide carrying HP2 (see Fig. S1 in the supplementary material) was subjected to gel retardation in presence of Hoxb1 and Pbx1 proteins, leading to formation of specific retarded bands (Fig. 1B). Mutation of the putative Hox/Pbx site eliminated the major band, although some residual binding was maintained, the origin of which was not investigated. To establish that there was no other major Hox/Pbx-binding site within the enhancer, we repeated the band shift analysis with a larger 321 bp fragment that retained element C activity in the chick electroporation assay (data not shown) and that carries both HP1 and HP2 (see Fig. S1 in the supplementary material). Exposure of this fragment to Hoxb1 and Pbx1 led to a strong gel retardation that was dramatically reduced upon mutation of both HP1 and HP2 (Fig. 1C). The residual binding might correspond to a low-affinity binding site(s) that could not be identified by sequence analysis.

In conclusion, these data indicate that element C contains two bona fide Hox/Pbx-binding sites and several Meis sites, and therefore suggest that it could mediate aspects of the *Krox20* regulation by direct binding of these factors.

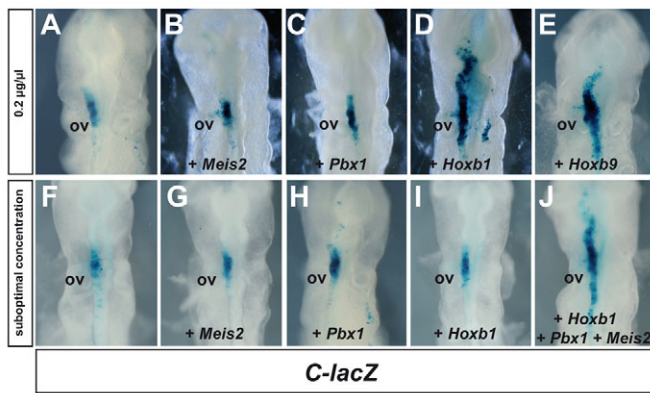


Fig. 2. Synergistic activities of Hox, Pbx and Meis factors on element C. Chick embryos were analyzed by X-gal staining after co-electroporation with a *lacZ* reporter driven by element C and various expression vectors. (A-E) The following expression vectors were used at a concentration of 0.2 µg/µl: (A) empty expression vector, (B) *Meis2*, (C) *Pbx1*, (D) *Hoxb1*, (E) *Hoxb9* expression vectors. (F-J) The following expression vectors were used alone or in combination at the indicated concentration: (F) empty expression vector (0.2 µg/µl), (G) *Meis2* (0.1 µg/µl), (H) *Pbx1* (0.1 µg/µl), (I) *Hoxb1* (0.01 µg/µl), (J) *Meis2* (0.1 µg/µl), *Pbx1* (0.1 µg/µl) and *Hoxb1* (0.01 µg/µl) expression vectors. In G-I, total plasmid concentration was brought to 0.2 µg/µl with empty expression vector. ov, otic vesicle.

Element C mediates Hox, Pbx and Meis synergy

Because element C contains binding sites for Hox/Pbx and Meis proteins, we investigated the possibility that these factors might be able to modulate its activity. For this purpose, a DNA construct in which element C drives a *lacZ* reporter gene (*cC-lacZ*) (Chomette et al., 2006) was electroporated in the chick hindbrain together with various Hox, Meis and Pbx expression vectors. In the first series of experiments, each expression construct was used alone and at a concentration of 0.2 µg/µl. In the absence of exogenous factor, element C drives reporter expression in the r3-r5 domain (Chomette et al., 2006). Co-electroporation with the *Meis2* expression vector did not significantly affect the activity of the enhancer (Fig. 2A,B), and co-electroporation with the *Pbx1* expression vector led to a slight extension of the reporter expression domain (Fig. 2C). By contrast, co-electroporation with *Hoxa1*, *Hoxb1*, *Hoxa2* or *Hoxb9* expression constructs led to major extensions of the expression domain and to increases in the level of reporter gene expression (Fig. 2D,E; data not shown).

These data demonstrated the ability of Hox proteins to activate element C, but surprisingly did not reveal significant roles for the Pbx and Meis co-factors, as Hox proteins were able to act independently of ectopic Pbx or Meis. To investigate a possible synergy between Hox, Pbx and Meis factors, we repeated the co-electroporation experiments, concentrating on *Hoxb1* and using suboptimal concentrations for the *Hoxb1* (0.01 µg/µl) and *Pbx1* and *Meis2* (0.1 µg/µl) expression vectors. In these conditions, co-transfection of the reporter plasmid with any of the *Meis2*, *Pbx1* or *Hoxb1* vectors did not significantly affect *lacZ* expression (Fig. 2F-I). By contrast, combination of the *Hoxb1*, *Pbx1* and *Meis2* expression constructs led to strong activation of the reporter and large extension of its domain of expression (Fig. 2J). These data clearly indicate that the three proteins are able to synergize to activate element C.

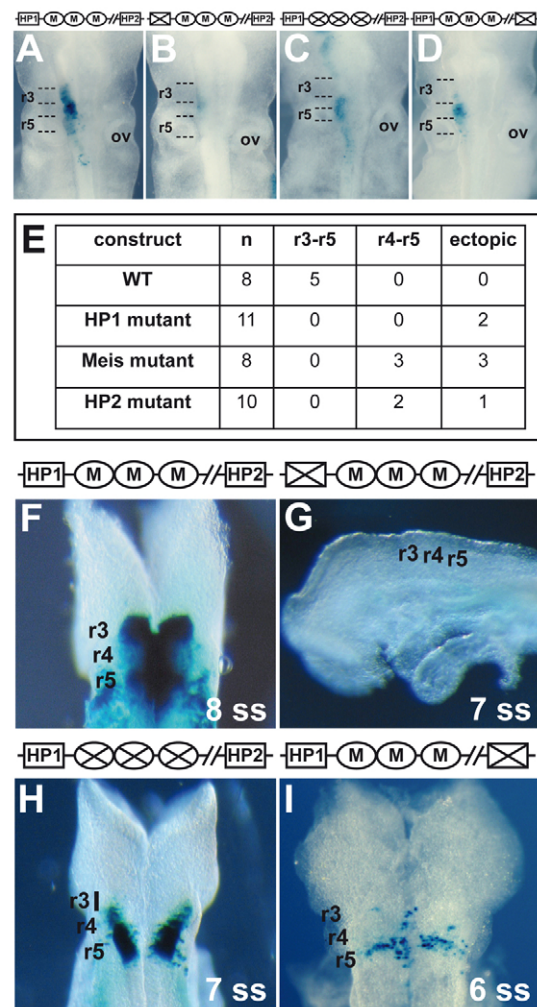


Fig. 3. Hox/Pbx- and Meis-binding sites are essential for element C activity. (A-D) Chick embryos were analyzed by X-gal staining after electroporation with reporter constructs driven by wild-type (A), HP1-site mutant (B), Meis-sites mutant (C) and HP2-site mutant (D) element C (schematized above). (E-I) Constructs driven by wild-type or mutant element C were used to generate mouse transgenic embryos that were analyzed by X-gal staining at around E8.5; the precise somite stage (ss) is indicated. (E) The total number of transgenic embryos obtained with each construct is summarized together with the numbers of embryos showing expression in the r3-r5 region, in r4 and r5 only, or in ectopic locations only. It should be noted that the level of reporter expression in r4/r5 is significantly reduced in the two positive embryos carrying the HP2 mutation. r, rhombomere.

Activation by the Hox/Pbx/Meis complex requires direct binding to element C

To determine whether *Krox20* is under the direct transcriptional control of Hox, Pbx and Meis proteins, we analyzed the consequences of the mutations preventing binding of Hox/Pbx or Meis (Fig. 1) on enhancer activity. This was first investigated by in ovo chick electroporation. Mutation of the Hox/Pbx site HP1 led to complete inactivation of the enhancer (Fig. 3A,B). By contrast, the Meis-binding sites mutant retained its activity in r4 and r5, but lost it in r3 (Fig. 3C). Finally, mutation of the Hox/Pbx site HP2 largely prevented reporter expression in r3, but preserved some activity in r4 and r5 (Fig. 3D).

The effects of the mutations were then investigated by murine transgenesis. Whereas the wild-type enhancer led to specific *lacZ* reporter expression in the r3-r5 region in more than half of the transgenic embryos (Fig. 3E,F) (see also Chomette et al., 2006), the construct carrying the HP1 site mutation was completely inactive in the hindbrain (Fig. 3E,G; $n=11$). Two of these transgenic embryos showed some ectopic *lacZ* expression (Fig. 3E, data not shown), indicating that in these cases the transgene was functional and integrated in a chromatin region compatible with gene expression. These data confirm the results obtained in the chick and strongly suggest that direct binding of the Hox/Pbx complex to site HP1 is absolutely required for element C activity. It should be noted, however, that the relative activity of the enhancer in r3 and r5 compared with r4 is different in the chick and mouse systems (compare Fig. 3A and Fig. 3F). This might reflect the stage of the embryos, as the r4 activity is increased in older mouse embryos (Chomette et al., 2006).

Mutation of the Meis-binding sites in the enhancer also led to a modification of the activity, consistent with the data obtained by electroporation: reporter expression was lost in r3 but maintained in r4 and r5 (Fig. 3H). These data confirm that Meis direct binding to the three sites identified in element C is absolutely required for enhancer activity in r3, but is not required in r5.

Finally, mutation of the HP2 site led to a reduction in the frequency of hindbrain-specific activity (two out of 10 embryos, as compared with five out of eight for the wild-type construct; Fig. 3E). In addition, in the positive embryos the pattern was modified: reporter expression was eliminated from r3 and significantly reduced in r4 and r5 (Fig. 3I, data not shown). Therefore, the HP2 site appears to be essential in r3, and to play a less important role in r4 and r5. Altogether, our work establishes that *Krox20* is a direct transcriptional target of Hox/Pbx and Meis, and reveals different requirements for element C activity in r3, r4 and r5.

Identification of the Meis or Prep protein involved in *Krox20* activation in r3

Because Meis-binding sites are necessary for the activity of *Krox20* element C in r3, we analyzed the expression patterns of the different Meis or Prep family members in the developing hindbrain, to determine which one(s) might be involved in *Krox20* regulation. The family includes five members in the mouse [*Meis1*, *Meis2*, *Meis3*, *Prep1* (Pknx1 – Mouse Genome Informatics) and *Prep2* (Pknx2 – Mouse Genome Informatics)]. A detailed analysis of their expression is presented in Fig. S2 in the supplementary material. Altogether, the data indicate that during the period corresponding to 1 to 5 somite stages (ss), which includes the stage of initiation of *Krox20* expression in the prospective r3 (1 to 3 ss), *Meis2* is the only family member expressed at the level of this rhombomere. Therefore, Meis2 must be the member of the Meis family responsible for the cooperation with the Hox/Pbx complex on element C, and for the initiation of *Krox20* expression in r3. Analysis of chick *Meis2* expression revealed a pattern compatible with a similar role (data not shown). At later stages of development, the continued activity of element C in r3 could also involve other Meis family members, in particular Meis1.

Specificity of *Krox20* activation by Hox proteins

We have shown above that the misexpression of several Hox genes in the chick neural tube leads to general activation of a co-electroporated *lacZ* reporter driven by element C. To investigate whether the endogenous *Krox20* gene would respond in a similar

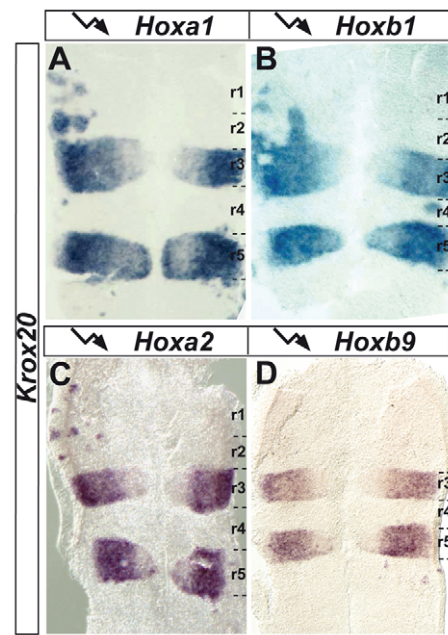


Fig. 4. Specificity of *Krox20* activation by Hox proteins. (A–D) Flat-mounted chick hindbrains in situ hybridized with a *Krox20* probe after electroporation with constructs expressing *Hoxa1* (A), *Hoxb1* (B), *Hoxa2* (C) or *Hoxb9* (D), respectively. The hindbrains are shown rostral up and were electroporated on the left side. r, rhombomere.

manner, we have analyzed its expression by in situ hybridization. The electroporation of *Hoxa1* or *Hoxb1* expression vectors led to ectopic activation of *Krox20* in r1, r2 and the midbrain-hindbrain boundary (MHB), with rostral enlargement of the r3 domain and the presence of homogeneous patches of *Krox20*-positive cells, often connected to the enlarged r3 (Fig. 4A,B). We observed no upregulation of *Krox20* in r3 and r5, nor any activation in other regions of the neural tube (Fig. 4A,B; data not shown). *Hoxa2* misexpression also led to activation of *Krox20* in a restricted domain anterior to r3 (Fig. 4C). By contrast, *Hoxb9* was unable to induce any ectopic *Krox20* expression (Fig. 4D).

Endogenous *Krox20* activation by Hox PG 1 and 2 expression vectors is consistent with the data obtained by co-electroporation, therefore confirming the role of Hox genes in the control of *Krox20* regulation. However, this analysis also revealed differences, suggesting the existence of additional levels of specificity. First, the activation of the endogenous gene is strictly restricted in space, essentially to r1, r2 and the MHB. This restriction is likely to reflect the requirement for Hox proteins to cooperate with other factors or the involvement of repressor molecules. Second, induction of endogenous *Krox20* expression was not observed with *Hoxb9*. The differences observed in the co-electroporation experiments might be explained by the more permissive character of this assay (Chomette et al., 2006; Ghislain et al., 2003; Pouilhe et al., 2007).

Krox20 and *Hoxb1* domain overlap allows the direct activation of *Krox20* by PG 1 proteins

Our data indicating that the initiation of *Krox20* expression in r3 is under direct transcriptional control of Hox proteins raise an interesting issue. The only Hox genes known to be expressed in r3 are the PG 2 genes *Hoxa2* and *Hoxb2*, but, as indicated above, they are not required

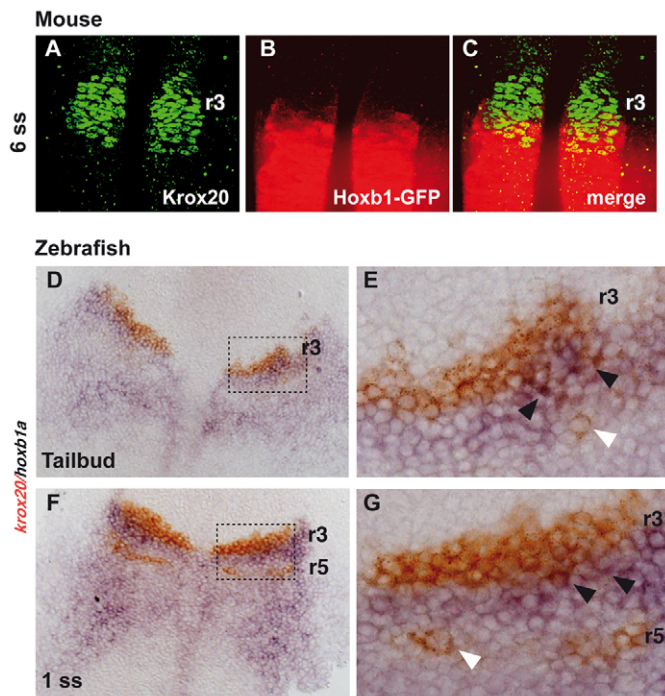


Fig. 5. Overlap between *Krox20* and *Hoxb1* expression domains at the r3-r4 border. (A-C) Optical sections through the hindbrain of a whole-mounted *Hoxb1*^{GFP/+} mouse embryo at the 6-somite stage, immunolabelled for *Krox20* (green) and *Hoxb1*-GFP (red): (A) anti-*Krox20* immunofluorescence, (B) anti-GFP immunofluorescence and (C) merge. (D-G) Dorsal views of flat-mounted zebrafish embryos at the tail bud (D,E) or 1-somite (F,G) stages, hybridized with *krox20* (red) and *hoxb1a* (blue). Black arrowheads point to double-labelled cells at the r3-r4 border. White arrowheads point to cells expressing *krox20* at the level of prospective r5. E and G are higher magnifications of the outlined regions in D and F, respectively.

for its activation. PG 1 genes *Hoxa1* and *Hoxb1* are activated earlier and independently of *Krox20*, but their anterior limits of expression have not been reported to overlap with the r3 *Krox20*-positive domain (Barrow et al., 2000; Lumsden and Krumlauf, 1996). In these conditions, we are confronted with the following alternative possibilities: either *Krox20* is activated in a cell-autonomous manner by PG 1 genes that are at least transiently co-expressed in the same cell, or the activation relies on a non cell-autonomous mechanism. To revisit the issue of an overlap in the territories of expression of PG 1 genes and *Krox20* at the r3-r4 boundary, we made use of a mouse knock-in line in which *GFP* has been inserted into the *Hoxb1* locus and recapitulates its expression pattern (Gaufo et al., 2000). *Hoxb1*^{GFP/+} embryos at around 5-6 ss were subjected to double immunofluorescence analysis using antibodies directed against *Krox20* and GFP. This revealed a clear overlap between the r3 *Krox20* expression domain and the *Hoxb1*-GFP-positive domain (Fig. 5A-C). This suggests that at the r3-r4 boundary *Krox20* is induced in cells that also express *Hoxb1* or that have recently expressed it, as the GFP protein might be more stable than *Hoxb1*. To extend this conclusion to other vertebrates, we performed double in situ hybridization for *krox20* and *hoxb1a*, the zebrafish homolog of mouse *Hoxb1*, at early stages of r3 development in zebrafish. We found that at tailbud and 1-somite stages, a few cells were positive for both *krox20* and *hoxb1a* at the level of the r3-r4 border (Fig. 5D-G, black arrowheads). In

conclusion, the data obtained in the mouse and zebrafish indicate that there is a partial and transient overlap between the *Hoxb1* and *Krox20* expression domains.

We then analyzed the ability of PG 1 protein to activate endogenous *Krox20* in a cell-autonomous manner. For this purpose, an expression vector for an HA-tagged version of *Hoxb1* was electroporated into the chick hindbrain and double labelling was performed by in situ hybridization for *Krox20* and anti-HA immunofluorescence for ectopic *Hoxb1*. In the midbrain and hindbrain region rostral to r3 we observed patches of *Krox20* expression (Fig. 6A-C). Owing to limited resolution of the in situ labelling, we could establish only that at least a fraction of the cells were also positive for *Hoxb1* within these patches. In the latter cells, *Hoxb1* is therefore likely to act in a cell-autonomous manner. The presence of cells possibly showing *Krox20* non-cell autonomous activation would not be surprising, as this gene has been shown to regulate its own expression in this manner (Giudicelli et al., 2001). To avoid this complication, we turned again toward the zebrafish system, which allows the experiment to be performed at an earlier stage in development. In addition, the experiment can be carried out in a *vhnf1*-null background (Sun and Hopkins, 2001), which ensures that *krox20* activation occurs via element C, because element B absolutely requires *vHnf1* binding (Chomette et al., 2006). We co-injected RNAs encoding a Myc-tagged form of *Hoxb1a* and *Meis1.1* (which synergizes with *Hoxb1a*; A.S. and S.S.-M. unpublished) into one cell of 16- to 32-cell stage embryos. The embryos were collected at the 5-somite stage and subjected to combined *krox20* in situ hybridization and Myc immunofluorescence. In injected *vhnf1*^{-/-} embryos, *krox20* was ectopically activated in groups of cells within the neural plate rostral to r3 (Fig. 6D-F). No such ectopic *krox20* expression was observed after injection of *GFP* RNA (52 embryos, data not shown). The large majority of the cells expressing *krox20* ectopically (417/464 cells in eight embryos, three different injection experiments) were positive for *Hoxb1a*-Myc (Fig. 6D-F). The *krox20*-positive, *Hoxb1a*-Myc-negative cells could be, in most cases, explained by quenching of the fluorescence by the strong in situ staining (data not shown). Therefore, in this experiment, *hoxb1a* activates *krox20* in a cell-autonomous manner.

The next question was whether the cell-autonomous activation of *Krox20* by *Hoxb1* involves a direct interaction with element C. To address this issue, we used the zebrafish system, which allows access to larger amounts of material and at earlier embryonic stages, to perform chromatin immunoprecipitation (ChIP) experiments. A conserved orthologous element C is present in the zebrafish genome and has been shown to be active in r3 and r5 (M.A.W., A.S. and S.S.-M., unpublished). Zebrafish embryos were injected into one cell at the four-cell stage with Myc-tagged *hoxb1a* and *meis1.1* RNAs. The embryos were collected at the 100% epiboly stage, when *krox20* is initially activated, and subjected to ChIP with an antibody directed against the Myc tag, followed by PCR amplification. We used two pairs of PCR primers able to amplify the core of element C, or, as a control, an unrelated sequence located within the *krox20* locus. The element C sequence was specifically immunoprecipitated, whereas the control sequence was not (Fig. 6G). These data establish that *Hoxb1a* binds to the element C in vivo, and support a direct involvement of Hox PG 1 proteins in the regulation of *Krox20* via element C.

In conclusion, altogether our data indicate that the transient overlap between the *Hoxb1* and *Krox20* expression domains allows Hox PG 1 proteins to directly activate *Krox20*.

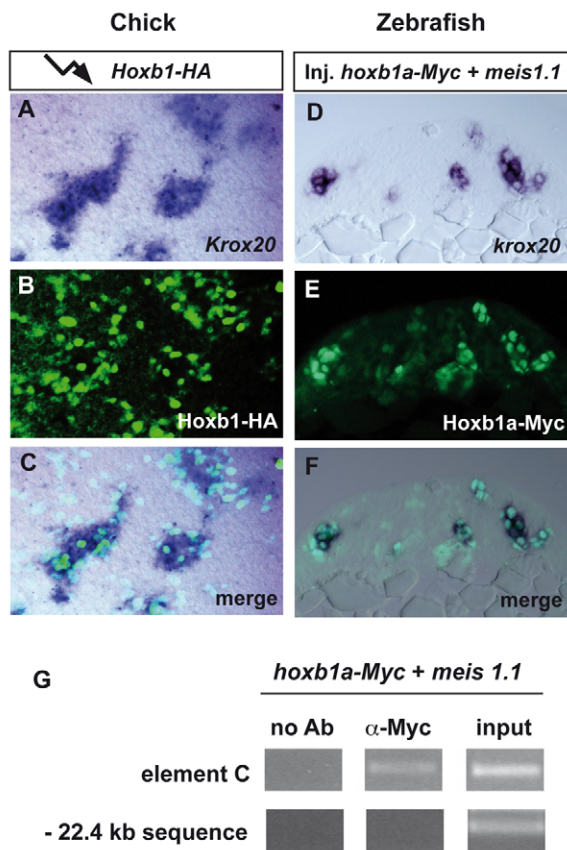


Fig. 6. Cell-autonomous activation of *Krox20* by *Hoxb1*. (A-C) Flat mount, at the level of the mid-hindbrain boundary, of a chick hindbrain electroporated with a construct expressing HA-tagged *Hoxb1*, in situ hybridized for *Krox20* and immunolabelled for *Hoxb1*-HA: (A) *Krox20* staining, (B) anti-HA immunofluorescence and (C) merge.

(D-F) Transverse sections, at the level of the forebrain-midbrain, of 5-somite stage *vhnf1*^{-/-} embryos co-injected with *meis1.1* and *hoxb1aMyc* RNAs, in situ hybridized for *krox20* and immunolabelled for *Hoxb1aMyc*: (D) *krox20* staining, (E) anti-Myc immunofluorescence and (F) merge. (G) ChIP of *Hoxb1a*-bound element C. Chromatin extracted from zebrafish embryos co-injected with *meis1.1* and Myc-tagged *hoxb1a* RNAs was subjected to the ChIP procedure in the absence (first lane) or presence (second lane) of anti-Myc antibody.

Immunoprecipitated DNA was analyzed by PCR using primers designed to amplify the core of element C (297 bp) or an unrelated sequence located -22.4 kb upstream of the transcription start site (422 bp). The third lane shows the PCR products obtained from the input chromatin.

DISCUSSION

In this study, we have performed a detailed analysis of element C that establishes the direct involvement of Hox proteins in *Krox20* regulation, reveals different modes of activity of element C and allows us to propose a model for the establishment and maintenance of *Krox20* expression in r3.

***Krox20* is a direct transcriptional target of Hox/Pbx complexes**

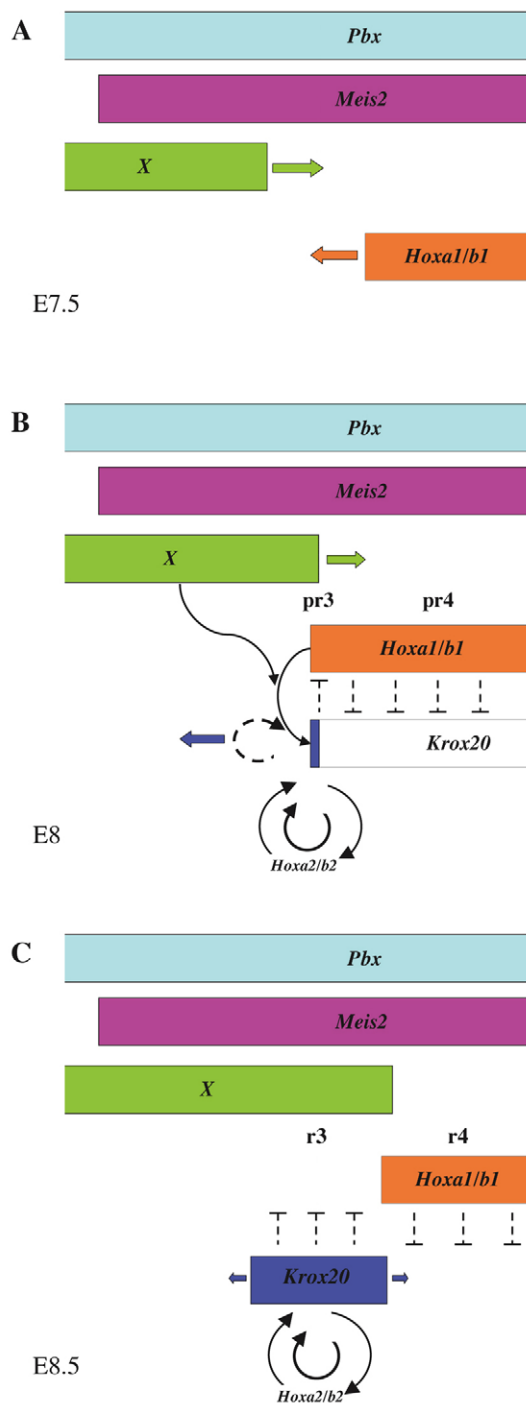
Previous genetic analyses have led to a confusing picture of the involvement of Hox genes in the control of *Krox20* expression in the hindbrain (see Introduction). In this work, we establish their direct role in *Krox20* regulation. We have identified two Hox/Pbx-

binding sites whose integrity is absolutely required for the r3 activity of element C in chick and mouse embryos. Furthermore, we show that *Hoxb1* binds element C in vivo and that Hox, Pbx and Meis proteins cooperate for the enhancer activity. Together, these data strongly suggest that direct binding of Hox/Pbx complexes is required for element C activity in the hindbrain and, consequently, for the initiation of *Krox20* expression in r3. As discussed above, PG 1 gene loss-of-function suggests that these genes could participate in the initiation process. Our observations of an overlap between *Krox20* and *Hoxb1* expression domains in r3 in mouse and zebrafish embryos, and of the capacity of *Hoxb1* to activate *Krox20* expression in a cell-autonomous manner, further supports the implication of PG 1 proteins in *Krox20* initial induction. However, the combined mutations of both PG 1 genes in the mouse do not completely prevent *Krox20* expression in r3, and contrast with the absolute requirement of Hox/Pbx-binding sites for element C activity. This discrepancy raises the possibility that other Hox factor(s) might partially compensate for the absence of PG 1 protein. Early *Krox20*-independent, low-level *Hoxb2* expression up to the r2/r3 boundary (data not shown) raises the possibility that PG 2 might play such a role. To investigate the capacity of Hox PG 2 proteins to bind element C in vivo, we performed a ChIP analysis in zebrafish embryos, similar to the one described above for *Hoxb1a*. We could detect specific binding of *Hoxa2* to element C (see Fig. S3 in the supplementary material), supporting an additional involvement of Hox PG 2 proteins in *Krox20* regulation (see below).

Specificity in transcriptional activation by Hox factors

Besides unravelling the direct involvement of Hox proteins in *Krox20* regulation, our work led to further insights in their mode of action. First, we observed differences among Hox proteins in their activation potential regarding endogenous *Krox20* (Fig. 4), suggesting that different paralogous groups are not functionally equivalent for the activation of *Krox20*. Hence, we have shown that after in ovo electroporation a polymerized HP1 site can drive reporter expression specifically in r4, the posterior hindbrain and the spinal cord (see Fig. S4 in the supplementary material), a pattern very similar to that of *Hoxb1*. This observation suggests that HP1 preferentially responds to Hox PG 1 proteins, consistent with a prominent role of these proteins in the initiation of *Krox20* expression. Finally, we also found that the two Hox/Pbx sites are not equivalent, as mutation of HP1 completely abolishes the activity of the enhancer, whereas mutated HP2 retains limited activity in r4 and r5.

The mutagenesis of the Meis-binding sites (M1-M3) revealed another differential effect: the activity of element C was abrogated in r3, but not in r4 and r5 (Fig. 3). Because analysis of element C by gel retardation did not detect any additional Meis-binding site, this suggests that the Hox/Pbx complex does not require a Meis factor to activate the enhancer in r4 and r5, in contrast to r3. This conclusion is consistent with analyses performed in the zebrafish that have shown that Meis factors are absolutely required for *Krox20* expression in r3 but not in r5 (Choe et al., 2002; Waskiewicz et al., 2001). Variable Meis dependence has been observed among Hox proteins (Choe and Sagerstrom, 2005; Ferretti et al., 2000), and the different requirements of element C in r3 and r5 might reflect the involvement of different Hox proteins and/or the implication of additional accessory factor(s). More generally, our work establishes that element C functions according to different modes in r3 and r5. Finally, our analysis of Meis and Prep gene expression suggests that *Meis2* is a key component of the transcriptional complex in charge of *Krox20* activation in r3.



A model for the control of *Krox20* expression in the developing hindbrain

Krox20 regulation appears to constitute a complex process and we have attempted to amalgamate the observations collected in the present work with previous data to develop a molecular model. Our consistent observations in mouse, chick and zebrafish allow us to combine data obtained in different vertebrate species. We will first envisage the regulation in r3 (Fig. 7). We propose that, in contrast to what was previously thought, at around E8 in the mouse, when *Hoxa1/Hoxb1* neural domains reach their maximal rostral extensions, their limits are located within prospective r3. This point is consistent with recent tracing data indicating that derivatives of

Fig. 7. A model for the establishment of *Krox20* expression in r3. (A-C) The model shows the evolution of the expression domains of *Krox20* and of different established (*Pbx*, *Meis2*, *Hoxa1/b1*) or postulated (X) regulators at different stages of development (A, E7.5; B, E8; C, E8.5). The overlap between the *Hoxa1/Hoxb1* and X domains results in a narrow strip of cells activating *Krox20* and transiently co-expressing PG 1 genes. These cells then shut down PG 1 gene expression, but maintain *Krox20* expression by direct and indirect autoregulatory loops, and r3 extends rostrally by the recruitment of neighbouring cells by non cell-autonomous autoregulation. As the X domain extends posteriorly, the strip of co-expression of *Krox20* and PG 1 genes moves caudally, leading to the progressive extension of r3 at the expense of r4. In the rest of r4, *Krox20* is repressed by mechanisms downstream of *Hoxa1/Hoxb1*. See Discussion for further details. The potential role of Hox PG 2 factors in initial activation is not represented. Expression domain extensions are indicated by coloured arrows. Positive and negative regulations are symbolized by arrows and barred lines, respectively. Direct and indirect regulations are indicated by plain or dotted lines, respectively. r, rhombomere; pr, prospective rhombomere.

Hoxa1-expressing cells are found in r3 (Makki et al., 2007), and is supported by our observation of an overlap between *Krox20* and *Hoxb1* expression domains in r3. In addition, we postulate the existence of another factor (X, unknown), whose expression domain extends caudally (Fig. 7A,B) and will start to overlap with the PG 1 domain around E8. This defines a transversal, narrow stripe of cells where *Krox20* is specifically activated under the synergistic transcriptional activities of factor X, Hox PG 1, *Pbx* and *Meis2* proteins, acting through element C. Interestingly, an essential role of Iroquois transcription factors in the activation of *krox20* in r3 has been recently uncovered (A.S. and S.S.-M., unpublished). Factor X might therefore be an Iroquois transcription factors or it might lie downstream to them in the regulatory cascade. As discussed above, a complementary involvement of Hox PG 2 proteins is also likely, although loss-of-function analyses suggest that the major role is played by PG 1 factors. An important feature of our hypothesis is that it provides an explanation for the characteristic initial expression pattern of *Krox20*, restricted to a very narrow stripe of cells (Schneider-Maunoury et al., 1993). *Krox20* activation will have multiple consequences (Fig. 7B,C). (1) It will lead to the progressive retraction of the rostral limit of Hox PG 1 gene expression to the future r3/r4 boundary. This is consistent with the observations that the *Hoxb1*-positive domain extends within prospective r3 in a *Krox20*-null mutant (Voiculescu et al., 2001) and that ectopic *Krox20* expression results in *Hoxb1* repression (Garcia-Dominguez et al., 2006; Giudicelli et al., 2001). (2) *Krox20* initiates several transcriptional autoregulatory loops that are necessary for the maintenance of its own expression (Schneider-Maunoury et al., 1993). One of them is direct and relies on the binding of *Krox20* to element A (Chomette et al., 2006), whereas the others involve the activation of *Hoxa2* and *Hoxb2* (Nonchev et al., 1996a; Sham et al., 1993), which will replace Hox PG 1 proteins on element C. These autoregulatory mechanisms are likely to be redundant, as the double mutation of *Hoxa2* and *Hoxb2* only marginally affects the r3 domain of *Krox20* expression (Davenne et al., 1999). (3) Expression of *Krox20* also results in its activation in neighbouring *Krox20*-negative cells by non-cell autonomous autoregulation (Giudicelli et al., 2001), a process thought to participate in the extension of r3. The caudal extension

of r3 might also rely on the progression of the front of gene *X* expression. These processes will give rise to a moving stripe of cells co-expressing *Krox20* and *Hoxb1* at the caudal edge of developing r3, as we observe in mouse and zebrafish embryos (Fig. 5). At some point (around E8.5), these processes of extension of r3 at the expense of adjacent rhombomeres will stop, delimiting the final extensions of r2, r3 and r4.

In r5, *Krox20* is under the control of two initiation enhancer elements, B and C (Chomette et al., 2006). The severe loss of *Krox20* expression in r5 upon mutation of *Mafb* or *vHnf1* (Hernandez et al., 2004), and the fact that these factors are likely to act only via element B (M.A.W. and P.C., unpublished), suggests that element B is predominant. In r5, element C functions according to a different mode than in r3: although it still requires binding of a Hox protein, Meis factors are not necessary.

Finally, what happens in r4, where element C is active but *Krox20* is not expressed? To explain this apparent contradiction, we propose that *Krox20*, in addition to the positive regulatory mechanisms discussed above, is subject to a negative regulation, which may lie downstream of the Hox PG 1 genes and prevent *Krox20* expression in r4 (Fig. 7B,C). The existence of such a negative regulation is consistent with the inactivation of *Hoxa1*, which results in an extension of the anterior domain of *Krox20* into prospective r4 (Gavalas et al., 1998; Rossel and Capecchi, 1999), and with the repressive activity of Nlz family members on *Krox20* expression (Runko and Sagerström, 2003; Hoyle et al., 2004).

In conclusion, a particularly interesting feature of this model resides in the initial phase of *Krox20* expression in r3. We propose that a narrow band of cells is defined by the encounter of two domains extending in opposite directions. In these cells, *Krox20* is very transiently activated by Hox PG 1 proteins, which disappear rapidly while *Krox20* expression is maintained and propagated by different molecular mechanisms. We propose to use the term 'ignition' to refer to the role of Hox PG 1 proteins in this novel type of initiation of gene expression, which may occur in other developmental processes.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/135/20/3369/DC1>

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