Development 133, 1253-1262 (2006) doi:10.1242/dev.02289

# *Krox20* hindbrain cis-regulatory landscape: interplay between multiple long-range initiation and autoregulatory elements

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The vertebrate hindbrain is subject to a transient segmentation process leading to the formation of seven or eight metameric territories termed rhombomeres (r). This segmentation provides the basis for the subsequent establishment of hindbrain neuronal organization and participates in the patterning of the neural crest involved in craniofacial development. The zinc-finger gene *Krox20* is expressed in r3 and r5, and encodes a transcription factor that plays a key role in hindbrain segmentation, coordinating segment formation, specification of odd- and even-numbered rhombomeres, and cell segregation between adjacent segments, through the regulation of numerous downstream genes. In order to further elucidate the genetic network underlying hindbrain segmentation, we have undertaken the analysis of the cis-regulatory sequences governing *Krox20* expression. We have found that the control of *Krox20* transcription relies on three very long-range (200 kb) enhancer elements (A, B and C) that are conserved between chick, mouse and human genomes. Elements B and C are activated at the earliest stage of *Krox20* expression in r5 and r3-r5, respectively, and do not require the Krox20 protein. These elements are likely to function as initiators of *Krox20* expression. Element B contains a binding site for the transcription factor vHNF1, the mutation of which abolishes its activity, suggesting that vHNF1 is a direct initiator of *Krox20* expression in r5. Element A contains Krox20-binding sites, which are required, together with the Krox20 protein, for its activity. This element therefore allows the establishment of a direct positive autoregulatory loop, which takes the relay of the initiator elements and maintains *Krox20* expression. Together, our studies provide a basis for a model of the molecular mechanisms controlling *Krox20* expression in the developing hindbrain and neural crest.

**KEY WORDS: Hindbrain segmentation, Pattern formation, Transcriptional enhancers, vHnf1, Mouse, Chick**

## INTRODUCTION

The development of the vertebrate hindbrain involves a transient segmentation process along the anteroposterior (AP) axis, leading to the formation of seven or eight transversal morphological units, called rhombomeres (r) (Vaage, 1969) (reviewed by Lumsden and Keynes, 1989; Lumsden and Krumlauf, 1996; Wingate and Lumsden, 1996). The rhombomeres behave as compartments, constituting units of cell lineage restriction (Fraser et al., 1990; Birgbauer and Fraser, 1994) and domains of specific gene expression (reviewed by Lumsden and Krumlauf, 1996; Rijli et al., 1998). This subdivision presages the metameric pattern of neuronal specification in the hindbrain (Lumsden and Keynes, 1989; Clarke et al., 1998), underlies the pathways of neural crest cell migration into the branchial arches and participates in their patterning (Lumsden et al., 1991; Serbedzija et al., 1992; Birgbauer et al., 1995; Kulesa and Fraser, 2000; Trainor and Krumlauf, 2000; Trainer et al., 2002; Ghislain et al., 2003), thus playing an essential role in craniofacial morphogenesis.

Numerous genes have been implicated at different levels of the segmentation process, including the initial formation of segmental territories (Lufkin et al., 1991; Chisaka et al., 1992; Schneider-Maunoury et al., 1993; Frohman et al., 1993; McKay et al., 1994; Barrow et al., 2000; Waskiewicz et al., 2001; Deflorian et al., 2004; Choe and Sagerstrom, 2004; McNulty et al., 2005), the specification of their AP identities (Rijli et al., 1993; Studer et al., 1996; Seitanidou et al., 1997; Rossel and Capecchi, 1999; Bell et al., 1999) and their stabilisation by restriction of cell intermingling between adjacent rhombomeres (reviewed by Pasini and Wilkinson, 2002), and development of specific cell populations at boundaries (Cheng et al., 2004; Amoyel et al., 2005). Segment formation and specification are highly intricate processes in the hindbrain, with several genes participating in both aspects (Gavalas et al., 1998; Rossel and Capecchi, 1999; Voiculescu et al., 2001).

*Krox20*, which encodes a zinc-finger transcription factor (Chavrier et al., 1988), plays a key role in coupling segmentation and specification of rhombomere identity (Voiculescu et al., 2001). *Krox20* is one of the earliest genes expressed in a segmental pattern in the developing hindbrain, specifically in odd-numbered rhombomeres r3 and r5 (Wilkinson et al., 1989; Schneider-Maunoury et al., 1993). *Krox20* mutation leads to loss of r3 and r5 (Schneider-Maunoury et al., 1993; Swiatek et al., 1993), owing to mis-specification of these territories, which acquire r2 and r4, and r6 identities, respectively (Voiculescu et al., 2001). Krox20 has been shown to exert its function by up- and downregulating the expression of numerous genes, including Hox genes of the paralogous groups 1 to 3, such as *Hoxb1*, *Hoxa2*, *Hoxb2* and *Hoxb3*, which are also involved in the specification of segmental AP identity (Sham et al., 1993; Nonchev et al., 1996a; Nonchev et al., 1996b; Vesque et al., 1996; Seitanidou et al., 1997; Giudicelli et al., 2001; Manzanares et al., 2002) (M. Garcia-Dominguez, P. Gilardi and P.C., unpublished), and the EphA4 receptor gene, which is involved in the segregation of cells between odd- and even-numbered rhombomeres (Theil et al., 1998). Krox20 has also been shown to positively regulate its own expression, both cell-autonomously and non cell-autonomously, and these processes are

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**Accepted 18 January 2006**

thought to play an essential role in the extension and stabilisation of r3 and r5 territories (Schneider-Maunoury et al., 1993; Giudicelli et al., 2001).

Given the central role of *Krox20* in hindbrain development, the efforts invested to elucidate the molecular mechanisms controlling this process have led to the identification of putative upstream Krox20 regulators. Like the other segmentation genes in the hindbrain, *Krox20* appears to be under the control of several signalling pathways involved in long-range patterning, including the Wnt (Nordstrom et al., 2002), retinoic acid (RA) (reviewed by Gavalas and Krumlauf, 2000; Dupe and Lumsden, 2001; Niederreither et al., 2003) and FGF (in r5) (Marin and Charnay, 2000; Maves et al., 2002; Walshe et al., 2002) cascades. It is likely that a large part of the effects of these signalling pathways are indirect and relayed by a series of transcription factors involved in hindbrain segmentation, including paralogous group 1 Hox gene products and their associated factors (Chisaka and Capecchi, 1991; Lufkin et al., 1991; Carpenter et al., 1993; Dolle et al., 1993; Mark et al., 1993; Helmbacher et al., 1998; Rossel et al., 1999; Barrow et al., 2000; Choe et al., 2002; Waskiewicz et al., 2001; Waskiewicz et al., 2002; McNulty et al., 2005), vHNF1 (Sun and Hopkins, 2001; Wiellette and Sive, 2003; Choe and Sagerstrom, 2004; Hernandez et al., 2004) and MafB (Frohman et al., 1993; Cordes and Barsh, 1994; McKay et al., 1994; Moens et al., 1996; Manzanares et al., 1999).

Despite the knowledge of these various genetic connections, no clear picture of *Krox20* regulation has emerged. This is probably due, on the one hand, to the complexity of the network, which involves multiple feedback loops as well as regulators playing variable functions at different times and places; and, on the other hand, to our ignorance of the direct interactions existing between network members. In order to understand the molecular mechanisms controlling *Krox20* expression and to identify its direct regulators, we have initiated an analysis of its cis-acting sequences. This strategy has previously allowed us to unravel the molecular basis of *Krox20* regulation in the neural crest, developing Schwann cells and bone-forming cells (Ghislain et al., 2002; Ghislain et al., 2003; Ghislain and Charnay, 2006) (M.F. and P.C., unpublished). In the present paper, we have screened over 200 kb surrounding the chicken *Krox20* locus and have identified the essential regulatory information controlling *Krox20* expression in the hindbrain. Three cis-acting elements were characterized, designated A, B and C, which are conserved between birds and mammals. These elements, located far upstream of the gene play different, although overlapping roles: B and C are involved in the initiation of *Krox20* expression in r5 and r3/r5, respectively, whereas element A is involved in the maintenance of *Krox20* expression in these rhombomeres. We demonstrate that vHNF1 binds to element B and constitutes a direct transcriptional activator of *Krox20* in r5 and that the maintenance function is achieved by direct autoregulation, with Krox20 binding to multiple sites within element A. Considering the central position occupied by *Krox20* in the complex gene network governing hindbrain segmentation, the present work constitutes an important step towards its elucidation.

## MATERIALS AND METHODS

### DNA constructs and mutagenesis

BAC clones 121 (1) and 27 (2) isolated from a chicken genomic BAC library (Giudicelli et al., 2001) were used as a source of chicken *Krox20* extragenic sequences. BAC121 was digested with *Sal*I to obtain sub-fragments of 42 kb (3) and 65 kb (4) (Fig. 1A). Fragment 3 was inserted in cosmid pTCF using the Gigapack III packaging extract (Stratagene) and used to obtain *Sal*I-*Xba*I fragment 6, *Not*I-*Bam*HI fragment 7, *Bam*HI-*Bam*HI fragment 8, *Bam*HI-*Sal*I fragment 9 (Fig. 2A). The 30 kb fragment 5 was cloned by PCR using primers indicated in Table S1 (see supplementary material) and digested with *Bam*HI to obtain fragments 10 and 11. Fragment 10 was digested with *Afl*II to obtain fragments 12 and 13. Fragment 12 was digested with *Nco*I to obtain fragments 14 and 15 (Fig. 2A). Chicken elements A (*Xcm*I-*Hin*dIII), 12.1 (*Stu*I-*Sca*I), B (*Eco*RI-*Sty*I), 12.3 (*BstYI-BstYI*) and C (*Bgl*II-*Cla*I) were derived from fragments 7, 12 and 14, respectively. Mouse elements A, B and C were cloned by PCR using primers indicated in Table S1 in the supplementary material. Fragments 6-9, 11, 12, 14 and 15, and chicken and mouse conserved elements were cloned into pBGZ40 (Yee and Rigby, 1993) upstream of the minimal *β-globin* promoter/*lacZ* reporter. Mutagenesis of the Krox20-binding sites in element cA was performed using the Transformer Site-Directed Mutagenesis Kit (Clontech) and mutagenesis of the vHnf1-binding site in element cB was performed using the ExSite PCR-Based Site-Directed Mutagenesis Kit (Stratagene).

### Mouse lines, generation of transgenic mice, genotyping and in ovo electroporation

The *Krox20*$^{cre}$ allele (Voiculescu et al., 2000) and the transgenic lines *cA-lacZ*, *cB-lacZ* and *cC-lacZ* generated in this work were maintained in a mixed C57B16/DBA2 background. Fragment purification and transgenesis were performed as described previously (Sham et al., 1993; Ghislain et al., 2002). BAC constructs 1 and 2 were injected as supercoiled plasmids and transgenic embryos were identified by PCR with BAC vector specific primers (see Table S1 in the supplementary material). Transgenic embryos for fragment 4 were identified by PCR using primers specific for the chick sequence (see Table S1). Transgenic embryos for fragments #3, #5, #10 and #13 were obtained by co-injection of equimolar amounts of the respective fragment and of the *Krox20/lacZ* reporter construct (Ghislain et al., 2002). PCR using primers specific for the chick sequence (see Table S1) and *Krox20/lacZ* allele (Schneider-Maunoury et al., 1993) was performed to identify the fragments and reporter, respectively, in transgenic embryos. Transgenic embryos for chick elements A, B and C constructs were identified using the primers indicated in Table S1. Transgenic embryos for the other constructs were identified with primers specific for *lacZ* (Ghislain et al., 2002). In ovo electroporation in the chick neural tube was performed as previously described (Giudicelli et al., 2001) at stages HH8-HH10. Each construct was tested in at least two independent experiments, each involving eight or more embryos. Co-electroporation experiments with pAdRSVKrox20 were carried out as described (Giudicelli et al., 2001).

### In situ hybridization and X-gal staining

Whole-mount in situ hybridization was performed as previously described (Giudicelli et al., 2001; Wilkinson et al., 2002), using a chicken *Krox20* probe (Giudicelli et al., 2001), or a mouse *Hoxb1* probe (Wilkinson et al., 1989). Alkaline phosphatase activity was revealed using the NBT/BCIP substrate (Roche). Simple *lacZ* labelling and double labelling for β-galactosidase activity and *Hoxb1* mRNA of mouse and chick embryos were performed as described previously (Ghislain et al., 2003).

### Protein extracts and band shift assays

The mouse Krox20 protein was expressed in bacteria using the pET3a system (Novagen). Extracts were prepared from *Krox20*-expressing and control bacteria as described previously (Nardelli et al., 1992). The human HNF1β/vHNF1, isoform A protein was prepared from human embryonic kidney HEK 293 cells as described (Cereghini et al., 1992; Barbacci et al., 2004). To prepare the probes, clones of element A in pBS carrying wild-type or mutant Krox20-binding sites were digested with *Hind*III and *Xho*I, and clones of element B in pGEM5 carrying the wild-type or mutant vHnf1-binding sites were digested with *Sph*I and *Spe*I. All fragments were dephosphorylated and labelled using T4 polynucleotide kinase and [γ-$^{32}$P]-ATP. Labelled fragments were purified using Microspin S-200 HR Columns (Amersham) and used in band shift experiments as previously described (Nardelli et al., 1992; Cereghini et al., 1992).

### Nucleotide sequence analyses

Identification of mouse sequences homologous to chicken fragments #7, 8, 12 and 14 and analysis of *Krox20* locus in the chick, mouse and other species were performed using the Sanger Institute (ENSEMBL Project) website.

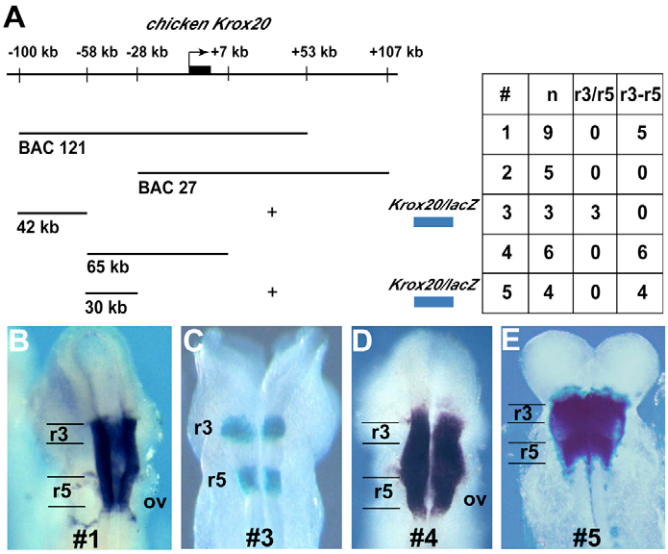
Sequence alignments were performed using the mVista software (Frazer et al., 2004) and identification of putative Krox20-binding sites using the rVista software (Loots et al., 2002).

## RESULTS

### Long-range regulatory elements control *Krox20* expression in the hindbrain

In order to identify cis-acting regulatory elements controlling *Krox20* expression in the developing hindbrain, we analysed the activity of sequences contained within several murine cosmid clones spanning the region extending from –31 kb to +40 kb relative to the transcription start site (Ghislain et al., 2002; Ghislain et al., 2003). Although these studies identified several cis-acting elements involved in the control of various aspects of the *Krox20* expression pattern, including its regulation in the r5-derived neural crest (Ghislain et al., 2003), they did not reveal transcriptional enhancers responsible for *Krox20* expression in r3 and r5, suggesting that such elements were located outside of the tested region. This led us to modify our approach to be able to reach regulatory elements potentially located much farther from the gene: we searched for such regulatory elements within the chicken genome, which is about threefold more compact than the mouse one, and used BAC clones to scan larger regions. This strategy was based on the hypothesis that *Krox20 cis*-acting regulatory sequences would be conserved between the two species, which was supported by the conservation of the expression patterns (Irving et al., 1996; Giudicelli et al., 2001). We isolated two chicken BAC clones (BAC 121 and 27), which together cover the region between –100 kb and +107 kb of the transcription start site (Fig. 1A) (Giudicelli et al., 2001). These clones were introduced by transgenesis into the mouse genome and the expression pattern of the chick *Krox20* gene was analysed by in situ hybridization with a chick specific probe in F0 embryos at embryonic day 8.5 (E8.5). About half of the transgenic embryos obtained with BAC 121 ($n$=9), covering region –100 kb to +53 kb (Fig. 1A, construct #1), strongly expressed chick *Krox20* in the r3-r5 region, with a lower level extending into groups of cells in posterior r2 and anterior r6 (Fig. 1B). By contrast, no transgenic embryo injected with BAC 27 ($n$=5), covering region –28 kb to +107 kb (Fig. 1A, construct #2), showed any expression of the gene in the rhombomeres. This suggests that important element(s) for *Krox20* expression in the hindbrain are located between positions –100 kb and –28 kb.

This possibility was investigated by testing three sub-fragments of BAC 121 (Fig. 1A). Fragment 3 contains the most upstream sequence and is 42 kb long (Fig. 1A). To evaluate its cis-acting activity, it was co-injected with a *Krox20/lacZ* reporter construct consisting of a 11.5 kb mouse genomic fragment containing *Krox20* with an in-frame insertion of *lacZ* in exon 2 (Ghislain et al., 2002). This latter construct is not active in the hindbrain, but responds to transcriptional enhancers, and it leads to synthesis of a chimeric protein with β-galactosidase activity (Ghislain et al., 2002; Ghislain et al., 2003). Transgenic embryos co-injected with fragment #3 and the *Krox20/lacZ* reporter expressed β-galactosidase specifically in r3 and r5 (Fig. 1A,C). Fragment 4 covers the following 65 kb of chick sequence, including the *Krox20* gene (Fig. 1A). The analysis of its cis-acting activity in transgenic embryos was therefore performed by in situ hybridization with the chick probe and revealed a pattern of expression identical to that obtained with the entire BAC 121 in all transgenic embryos (Fig. 1A,D). Fragment 5 contains a 30 kb fragment covering the region of fragment 4 that is not present in BAC 27 (Fig. 1A). In the transgenic embryos co-injected with fragment #5 and the *Krox20/lacZ* reporter, strong β-galactosidase activity was detected in r3 and r5, and lower levels in r4 and in a few cells of the caudal part of r2 and the rostral part of r6 (Fig. 1A,E; data not shown). These data indicate that the original activity observed with BAC 121 is likely to correspond to multiple cis-acting elements present in fragments 3 and 5.




| # | n | r3/r5 | r3-r5 |
|---|---|---|---|
| 1 | 9 | 0 | 5 |
| 2 | 5 | 0 | 0 |
| 3 | 3 | 3 | 0 |
| 4 | 6 | 0 | 6 |
| 5 | 4 | 0 | 4 |

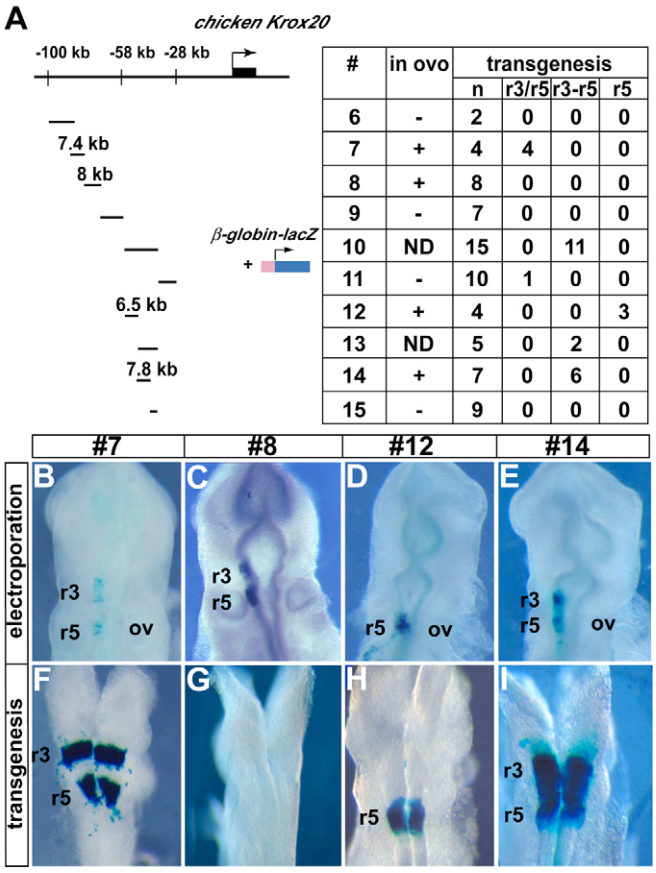
**Fig. 1. Identification of chick genomic regions containing *Krox20* cis-regulatory elements.** (**A**) Schematic representation of the chick *Krox20* locus, BAC clones and subfragments tested by mouse transgenesis. Distances are relative to the start site of transcription of the *Krox20* gene. The BACs (constructs 1 and 2) and the 65 kb fragment (construct 4) were injected alone and transgenic embryos were analysed by in situ hybridization with a chick *Krox20* probe. The 42 kb and 30 kb fragments (constructs 3 and 5), which do not carry the *Krox20* gene, were co-injected with a reporter fusion gene, *Krox20/lacZ*, and transgenic embryos were analysed for β-galactosidase activity by X-gal staining. For each construct, the table indicates the number of E8.5 transgenic embryos obtained (n) and the number of embryos positive in r3 and r5 (r3/r5), or in the r3 to r5 region (r3-r5). (**B-E**) Dorsal views of embryos transgenic for the indicated constructs and analysed as indicated in A. Embryos are rostral side upwards. r, rhombomere; ov, otic vesicle.

### Localisation of three independent hindbrain regulatory elements

To localize the possible cis-acting elements present within fragments 3 and 5, we divided the chicken genomic region between –100 kb and –28 kb upstream of *Krox20* into six fragments (6-11, Fig. 2A). Fragments 6-9 and 11 were cloned upstream of a *lacZ* reporter driven by the human β-globin minimal promoter and analysed by in ovo electroporation in the chicken embryo hindbrain (Giudicelli et al., 2001) and transgenesis in mouse embryos. Two fragments, 7 and 8, were found to drive specific reporter expression in r3 and r5 by in ovo electroporation (Fig. 2A-C), whereas the others were negative (Fig. 2A and data not shown). When the different constructs were tested by mouse transgenesis, construct 7 was active in r3 and r5, consistent with the electroporation data (Fig. 1A,F), whereas construct 8 did not lead to any reporter expression in the hindbrain (Fig. 2A,G), in contrast to the results of electroporation. Fragment 10, because of its large size (20 kb), could be analysed only by transgenesis after co-injection with the *Krox20/lacZ* reporter construct. It led to strong expression in the r3-r5 region in transgenic embryos (Fig. 2A and data not shown). Construct 11, which

presented no activity when tested by electroporation, was active in r3 and r5 in only one transgenic embryo ($n$=10, Fig. 2A). As it was an isolated case, the analysis of this fragment was not pursued.

Fragment 10 was then divided in two pieces (constructs 12 and 13) and subsequently fragment 13 also in two pieces (fragments 14 and 15), which were tested in both systems with the exception of fragment 13, which was tested only by transgenesis because of its large size (15 kb). In transgenic embryos, fragments 13 and 14 presented an activity similar to that of fragment 10 (Fig. 2A,I; data not shown). By electroporation, fragment 14 led to strong reporter expression in r3 and r5, with lower levels in r4 (Fig. 2A,E). Fragment 12 was found to drive specific reporter expression in r5 in both chick and mouse systems (Fig. 2A,D,H). Fragment 15 was not active in transgenic embryos nor in the electroporation assay.




| # | in ovo | transgenesis | | | |
|---|---|---|---|---|---|
| | | n | r3/r5 | r3-r5 | r5 |
| 6 | - | 2 | 0 | 0 | 0 |
| 7 | + | 4 | 4 | 0 | 0 |
| 8 | + | 8 | 0 | 0 | 0 |
| 9 | - | 7 | 0 | 0 | 0 |
| 10 | ND | 15 | 0 | 11 | 0 |
| 11 | - | 10 | 1 | 0 | 0 |
| 12 | + | 4 | 0 | 0 | 3 |
| 13 | ND | 5 | 0 | 2 | 0 |
| 14 | + | 7 | 0 | 6 | 0 |
| 15 | - | 9 | 0 | 0 | 0 |

**Fig. 2. Localization of separate elements carrying hindbrain cis-regulatory activities.** (**A**) Schematic representation of the chick *Krox20* locus and genomic fragments tested by in ovo electroporation and mouse transgenesis. All fragments, except fragments 10 and 13, were cloned upstream of the *lacZ* reporter gene driven by a human β-globin minimal promoter. Fragments 10 and 13 were tested by transgenesis after co-injection with a reporter fusion gene, *Krox20/lacZ*. In all cases, transcriptional activity of the elements was evaluated by X-gal staining of the embryos. The table indicates the construct number, the presence (+) or absence (–) of activity in r3 and/or r5 in electroporated chick embryos (in ovo), the number of E8.5 transgenic embryos analysed (n) and the number of transgenic embryos expressing in r3 and r5 (r3/r5), the r3 to r5 region (r3-r5) or r5 only (r5). (**B-I**) Electroporated chick embryos (B-E) and transgenic mouse embryos (F-I), for the indicated constructs. r, rhombomere; ov, otic vesicle; ND, not determined.

In conclusion, this analysis allowed the isolation of four cis-acting elements which are localized on non-overlapping fragments 7, 8, 12 and 14 and present different functional characteristics. Fragment 8 leads to r3- and r5-specific expression after electroporation into the chick hindbrain, but is totally inactive in transgenic mouse embryos. Fragment 7 drives specific r3 and r5 expression in both systems, similar to fragment 3 (from which it is derived). No other cis-acting element active in transgenesis was found within fragment 3. Fragments 12 and 14 are derived from the original fragment 5 and fragment 14 presents an activity in r3 to r5 similar to fragment 5. By contrast, the activity of fragment 12 is restricted to r5 and was presumably masked in the context of fragment 5 by the enhancer present in fragment 14.

## Functional conservation of three regulatory elements between birds and mammals

The nucleotide sequences of the three chicken fragments active in transgenic experiments (7, 12, 14) were established and compared with the mouse genome, to identify sequences conserved during evolution that might correspond to functional cis-acting elements. The divergence of about 300 million years between the two species suggests that conserved non-coding sequences are likely to have a functional role (Duret and Bucher, 1997). Sequences with significant homology to mouse genomic sequences were identified within each fragment (Fig. 3A). Fragment 7 contained one conserved block of sequence of 410 bp, designated element A (see Fig. S1 in the supplementary material). Fragment 12 contained three conserved blocks: 12.1 (550 bp), element B (480 bp) and 12.3 (200 pb) (see Fig. S1; data not shown). Finally, fragment 14 contained one well-conserved block of 220 bp and a proximal weakly conserved block of 100 bp that, together, correspond to element C (see Fig. S1).

The five chicken conserved elements (cA, c12.1, cB, c12.3 and cC) were then inserted upstream of the β-globin promoter-*lacZ* reporter construct to determine whether they carry the cis-regulatory activities observed with the entire fragments. The constructs were tested both by in ovo electroporation and mouse transgenesis. Elements A and C recapitulated the patterns obtained with fragments 7 and 14, respectively (Fig. 3A-C,E; data not shown). Among the three conserved sequences present in fragment 12, only element B led to specific expression in r5 like the entire fragment (Fig. 3A,B,D and data not shown). The two other elements were negative in the hindbrain (Fig. 3A,B; data not shown). Finally, to establish whether sequence homology was indeed reflecting functional conservation, we cloned the mouse sequences homologous to elements A, B and C (mA, mB and mC) upstream of the β-globin promoter-*lacZ* reporter and tested them by in ovo electroporation. These elements led to β-galactosidase expression patterns very similar to their chick counterparts (Fig. 3F-H and data not shown; compare with Fig. 2B,D,E). In conclusion, using a phylogenetic footprinting approach, we have precisely identified three *Krox20* cis-regulatory elements that show overlapping hindbrain activities and are functionally conserved between birds and mammals.

## Elements B and C are involved in initiation of *Krox20* expression and element A in autoregulation

As the different *Krox20* cis-acting elements lead to overlapping patterns of expression, we wondered whether they might have redundant functions. To address this issue, we performed a time-course analysis of their activities and investigated whether they require the Krox20 protein for their function. Indeed it is known that Krox20 can activate its own expression (Giudicelli et al., 2001) and

the analysis of mutants homozygous for a null *Krox20* allele (*Krox20/lacZ*) shows a loss of *lacZ* expression, rapid in r3 and more gradual in r5 (Schneider-Maunoury et al., 1993). To perform these studies, we generated mouse transgenic lines with the chick A, B and C elements driving the β-globin promoter-*lacZ* reporter. To assess the role of Krox20 on these elements, the transgenes were transferred into a *Krox20*-null background (*Krox20*$^{Cre/Cre}$) (Voiculescu et al., 2000). Element A was shown to be active from at least the six-somite stage (ss) in r3 and r5 in wild-type embryos, and the expression was specifically maintained in these rhombomeres until at least E9.5 (Fig. 4B-D). In *Krox20*-null embryos at 6 ss, a stage when endogenous *Krox20* gene expression can still be detected in mis-specified r3 (r3*) and r5 (r5*) territories (Voiculescu et al., 2001), element A showed no activity (Fig. 4A). This establishes that element A requires the Krox20 protein to drive reporter expression and therefore that it constitutes an autoregulatory element. This conclusion is fully consistent with the pattern generated by element A in wild-type hindbrain, which faithfully reflects the presence of the Krox20 protein (E. Taillebourg, unpublished).

Element B was active specifically in r5 from at least the 7 ss (Fig. 4F). β-Galactosidase could be detected in this rhombomere up to E9.5, although the level of expression was clearly decreasing at this stage, and it had completely disappeared by E10.5 (Fig. 4G,H; data not shown). At the 7 ss, the level of β-galactosidase activity observed in *Krox20*-null embryos in r5* was similar to the wild-type level (Fig. 4E,F), indicating that the Krox20 protein is not required for the activity of element B, which therefore is likely to constitute an initiator element for *Krox20* expression in this rhombomere.

Element C was activated around the 2 ss in r3 and the 5-6 ss in r5 in wild-type embryos (Fig. 4J,K), reflecting the normal pattern of *Krox20* hindbrain expression (Schneider-Maunoury et al., 1993; Wilkinson et al., 1989). This early activation also occurred in *Krox20*-null embryos (Fig. 4I), demonstrating that the activity of element C is independent of the Krox20 protein at these stages.

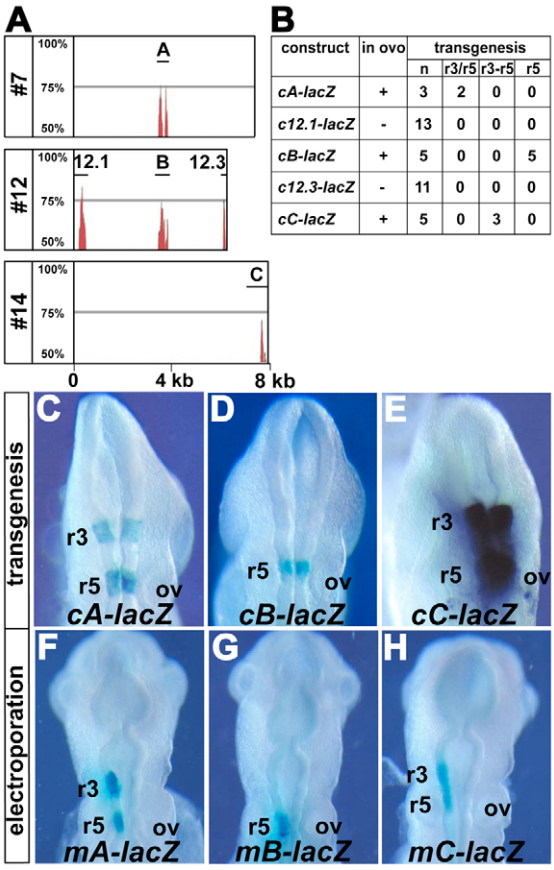



**Fig. 3. Identification and evolutionary conservation of three *Krox20* regulatory elements.** (**A**) Homology plots between chick fragments 7, 12 and 14 and corresponding mouse sequences generated using the VISTA algorithm. The horizontal axis represents the chick sequences with a scale in kilobases and the vertical axis the percentage of homology between mouse and chick sequences in a window of 100 bp with a resolution of 7 bp. Only homology superior to 50% is shown. Genomic chick fragments containing the five conserved elements (A, 12.1, B, 12.3 and C) and indicated by the above bars were cloned upstream of the β-globin promoter-*lacZ* reporter. Each construct was tested by in ovo electroporation and transgenesis in the mouse as indicated in Fig. 2. (**B**) The table indicates the construct names, the presence (+) or absence (–) of activity in r3 and/or r5 in electroporated chick embryos (in ovo), the number of E8.5 transgenic embryos analysed (n) and the number of transgenic embryos expressing in r3 and r5 (r3/r5), the r3 to r5 region (r3-r5) or r5 only (r5). (**C-H**) Transgenic mouse embryos (C-E) and electroporated chick embryos (F-H), for the indicated constructs. r, rhombomere; ov, otic vesicle.

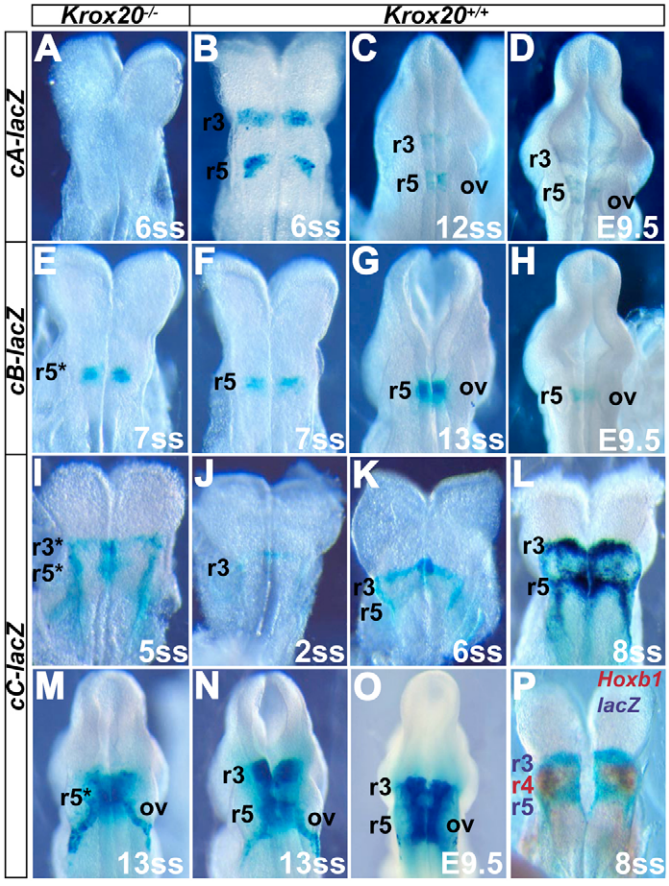



**Fig. 4. Time-course analysis of the activities of elements A, B and C and requirement for Krox20.** Transgenic lines carrying chick element A, B or C driving a β-globin promoter-*lacZ* reporter were analysed in *Krox20* null (**A**,**E**,**I**,**M**) or wild-type backgrounds (**B-D**,**F-H**,**J-L**,**N-P**). The embryos were stained with X-gal and the somite stage (ss) or the embryonic age in day post coitum (E) are indicated. (P) Embryo transgenic for construct *cC-lacZ* analysed by X-gal staining followed by in situ hybridization with a mouse *Hoxb1* probe. r, rhombomere; ov, otic vesicle.

Around the 8 ss, element C led to strong expression in r3 and r5 and in r5-derived neural crest, with an additional patchy expression in r4, where it overlapped with the *Hoxb1*-positive domain (Fig. 4L,P). At the 13 ss, the expression in r4 became homogenous and by E9.5 was throughout the r3 to r5 region (Fig. 4N,O). β-Galactosidase activity persisted in r5 until at least E11.5 (data not shown). In *Krox20*-null embryos at the 13 ss, expression was also observed in r4 and r5* (Fig. 4M; note that at this stage endogenous *Krox20* expression in r3* is lost) (Schneider-Maunoury et al., 1993). Altogether, these data indicate that element C is able to drive reporter expression in r3 and r5 with a time-course very similar to the endogenous *Krox20* gene, both in wild type and *Krox20* null backgrounds. This latter point indicates that this element does not require the Krox20 protein for its activity and therefore can function as an initiator element for *Krox20* expression. However, in contrast to the endogenous gene, it appears to be increasingly active in r4 from the 8 ss.

In conclusion, the characterisation of the different *Krox20* hindbrain cis-acting elements indicates that they have only partially redundant functions: elements C and B are likely to be involved in the initiation of *Krox20* expression in r3 and r5, and in r5, respectively. By contrast, A is an autoregulatory element that is likely to be involved in the maintenance of *Krox20* expression in r3 and r5.

## vHNF1 is a direct transcriptional activator of *Krox20* in r5

The knowledge of *Krox20 cis*-regulatory sequence opens the way to the search for its direct transcriptional regulators. As a first step, we investigated the possibility that vHNF1, which has been implicated in *Krox20* regulation on the basis of loss- and gain-of-function experiments, may constitute such a factor (Sun and Hopkins, 2001; Wiellette and Sive, 2003; Choe and Sagerstrom, 2004; Hernandez et al., 2004). We searched for sequences close to the vHNF1 consensus binding site within elements A, B and C (Tronche et al., 1997). Only one putative site was identified, within element B, in a region completely conserved between mouse and chick (Fig. 5A). In band shifts experiments using element B as a probe, cellular extracts containing vHNF1 produced a slow migrating complex that was absent with control extracts (Fig. 5B). Furthermore the retarded complex could be supershifted with an antibody directed against vHNF1, establishing the presence of vHNF1. Finally, a mutation of a palindromic sequence, which is known to abolish vHNF1 binding, was introduced into the putative vHNF1 binding site of element B (Fig. 5A) (Cereghini et al., 1988). This prevented the formation of a complex with vHNF1. Altogether, these experiments establish that element B contains a unique bona fide vHNF1-binding site.

To determine whether the vHNF1-binding site was playing a role in the enhancer activity of element B, we compared wild-type and mutated versions driving the *lacZ* reporter in the chick electroporation system. Whereas the wild-type enhancer led to specific *lacZ* expression in r5 (Fig. 5C, see also Fig. 3D), the mutated enhancer was completely inactive (Fig. 5D). These data strongly suggest that the initiator activity of element B in r5 requires vHNF1 binding and therefore that *Krox20* constitutes a direct transcriptional target of vHNF1.

## Direct autoregulation driven by element A

As we have shown that element A requires the Krox20 protein for its activity, we wondered whether this element might be activated by ectopic Krox20 and whether Krox20 was acting on this element by

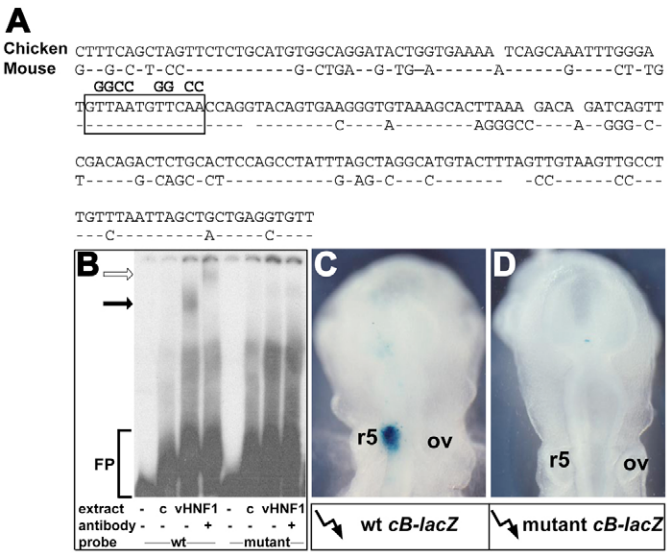



**Fig. 5. Identification of a functional vHNF1 binding site in element B.** (**A**) Alignment of element B chick and mouse nucleotide sequences showing the presence of a putative vHNF1-binding site (boxed) within a highly conserved region. Conserved residues are indicated with a dash in the mouse sequence. The mutations introduced into the vHNF1 site are indicated above the box. (**B**) Bandshift analysis of wild-type and mutant chick elements B (cB). Extracts from control (c) or human *vHNF1*-expressing cells, in the presence or absence of an antibody against vHNF1 were used. The position of the specific complexes is indicated by a black arrow. The supershifted complex is marked by a white arrow. (**C**,**D**) Chick embryos analysed by X-gal staining after electroporation with constructs containing the wild-type (C) or mutant versions (D) of chick element B driving the β-globin promoter-*lacZ* reporter. FP, free probe; ov, otic vesicle.

direct binding. To address these issues, we first co-electroporated the element A-reporter plasmid with a *Krox20* expression construct in the chick neural tube. Whereas in the absence of ectopic Krox20 the reporter expression is limited to r3 and r5 (Fig. 6C), its presence leads to reporter expression along the entire electroporated area, including the whole hindbrain (Fig. 6D). This indicates that element A is able to respond to exogenous Krox20.

To determine if Krox20 can directly regulate element A, we first performed bandshift experiments with element A and bacterially expressed Krox20. This led to the formation of at least two complexes, which could be specifically competed with an oligonucleotide carrying a high affinity Krox20-binding site (Fig. 6B). Having established that Krox20 binds to element A, we analysed its nucleotide sequence in both chicken and mouse to identify conserved putative Krox20-binding sites. Based on the known binding preferences of the protein (Swirnoff and Milbrandt, 1995; Wingender et al., 2000), seven sites were identified (Fig. 6A). To investigate the function of these sites, each was mutated by the introduction of a single substitution at the central position of the site, a mutation that has previously been shown to eliminate Krox20-binding activity both in vitro and in vivo (Nardelli et al., 1992; Sham et al., 1993). As expected, bandshift experiments confirmed that the mutated element A had totally lost Krox20 binding (Fig. 6E). Furthermore, in in ovo electroporation experiments, the reporter construct driven by the mutated element A was completely inactive in r3 and r5 (Fig. 6F) and unresponsive to ectopic Krox20 introduced by co-electroporation (Fig. 6G).

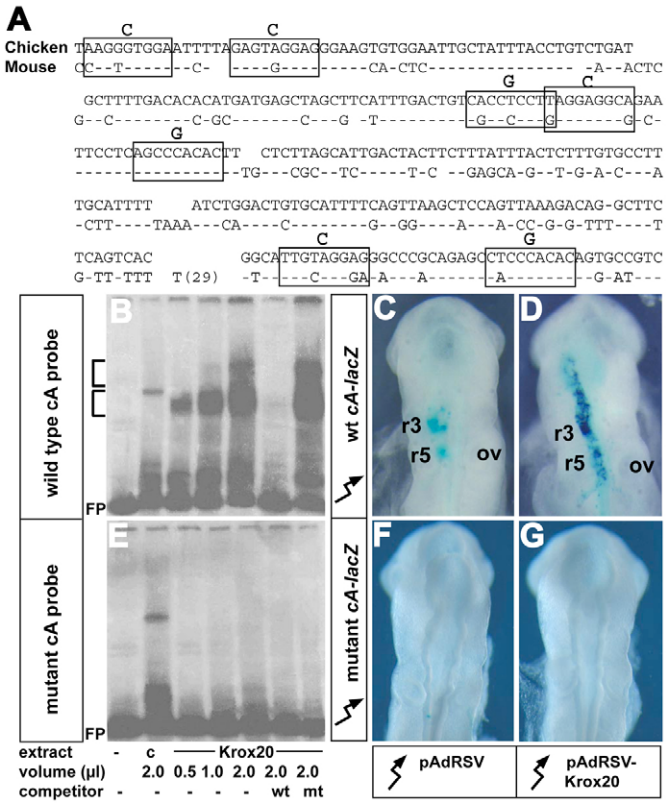



**Fig. 6. Identification of functional Krox20-binding sites in element A.** (**A**) Alignments of chick and mouse nucleotide sequences of element A showing the presence of seven conserved putative Krox20-binding sites (boxed). Conserved residues are indicated with a dash in the mouse sequence. The mutations introduced by site-directed mutagenesis are indicated above each box. (**B**,**E**) Bandshift analysis of the wild-type chick element A (B), and a derivative carrying mutations in the seven Krox20-binding sites (E) using extracts from control (c) or *Krox20*-expressing bacteria. The positions of specific complexes are indicated with brackets. Specific complexes were identified by the addition of oligonucleotides carrying a high-affinity Krox20-binding site (wt) or a mutated version unable to bind the protein (mt). (**C**,**D**,**F**,**G**) Chick embryos analysed by X-gal staining after co-electroporation with constructs containing the wild-type (C,D) or mutant versions (F,G) of chick element A driving the β-globin promoter-*lacZ* reporter together with the empty expression vector (C,F) or the *Krox20* expression vector (D,G). FP, free probe; r, rhombomere; ov, otic vesicle.

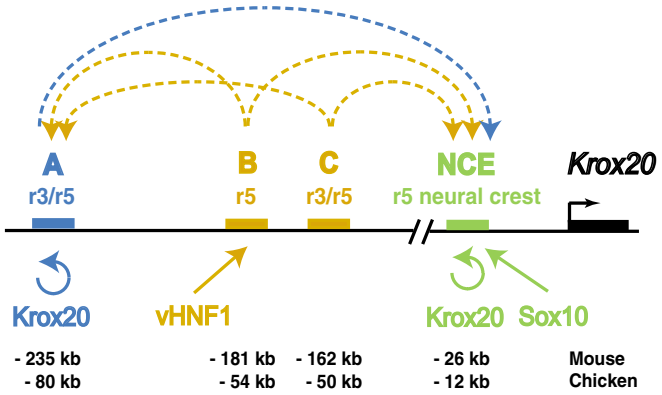



**Fig. 7. Schematic representation of the different cis-acting elements controlling *Krox20* expression in the hindbrain and derived neural crest.** Within the *Krox20* chick and mouse syntenic regions, the different cis-acting elements show an identical general organisation, although the distances relative to the *Krox20* gene vary between species. In this scheme, elements B and C (in orange) are responsible for the initiation of *Krox20* expression in r3 and r5, with a likely redundancy in r5, where vHNF1 participates in the activation of element B. This leads to accumulation of Krox20 that then activates element A (in blue), which is responsible for the maintenance of *Krox20* expression in the neuroepithelium by positive autoregulation. In addition, the combined action of elements A, B and C is responsible for reaching a threshold level of Krox20 protein in the dorsal part of r5, which together with Sox10 initiates another positive autoregulatory loop, the latter involving the NCE element (in green), that maintains *Krox20* expression in migrating r5-derived neural crest cells (Ghislain et al., 2003).

These data demonstrate that element A constitutes a direct transcriptional target of Krox20 and is therefore part of an autoregulatory loop presumably involved in the maintenance of *Krox20* expression.

## DISCUSSION

As a step towards the detailed analysis of an important gene regulatory and patterning network, we have characterized the cis-regulatory sequences and initiated the identification of the trans-acting factors controlling *Krox20* expression. Three different cis-regulatory elements appear responsible for the initiation and maintenance of its expression, this latter aspect involving direct autoregulation by Krox20. Our data provides a basis for a model of the molecular mechanisms controlling *Krox20* expression in the developing hindbrain and neural crest.

### Multiple, long-range and conserved cis-regulatory elements control *Krox20* expression in the hindbrain

By scanning a 200 kb chick genomic region centred around *Krox20*, we identified three short sequence elements that are able to drive hindbrain-specific expression of a reporter gene in both mouse transgenesis and chick hindbrain electroporation assays. These elements are conserved in their sequences and relative positions between chick, mouse, rat and human genomes (Fig. 7; see Fig. S1 in the supplementary material; data not shown), consistent with the high conservation of the *Krox20* expression pattern in the vertebrate hindbrain (Nieto et al., 1991; Oxtoby and Jowett, 1993). In the mouse they are located on chromosome 10, in a gene desert of 290 kb between *Krox20* and *Nrbf2*, the first gene identified upstream of *Krox20*. Their distance from the promoter (in the order of 200 kb in mouse and man) is unusual among mammalian enhancer elements.

Each hindbrain *Krox20* cis-acting element presents specific properties. Element A is active in r3 and r5. It cannot function in the absence of Krox20 and responds to ectopic Krox20 protein. It contains Krox20-binding sites that are absolutely required for its activity. Therefore this element appears as the cis-acting component for a direct, positive autoregulatory loop (see below). By contrast, elements B and C can work independently of *Krox20* and its downstream genes and do not respond to *Krox20* ectopic expression (data not shown). The activity of element B is restricted to r5, whereas that of element C, initially restricted to r3 and r5, extends to r4 as well at later stages of development. These properties suggest that these two elements may be responsible for the initiation of *Krox20* expression in the hindbrain (see below).

In addition to elements A, B and C, our analyses revealed another cis-acting sequence, located on an 8 kb DNA fragment (construct 8), which was active in r3 and r5 in chick electroporation experiments but not in transgenic mouse embryos (Fig. 2A,C,G). We have compared the sequence of this fragment to the mouse genome and found a 200 bp island conserved in sequence and location (data not shown). The 200 bp sequence carries r3/r5 enhancer activity in the chick electroporation assay and this enhancer is strictly dependent on Krox20 binding (data not shown). Our interpretation of these data is that construct 8 carries another direct autoregulatory element, which normally does not function in the hindbrain during segmentation stages, but at another stage and probably in another tissue. Upon electroporation, the constrains normally exerted on this element might be relaxed, so that Krox20, which is necessary for its activity, might become sufficient. We have previously observed that another *Krox20* autoregulatory element, the activity of which is normally restricted to r5-derived neural crest, behaves in a very similar manner upon electroporation (Ghislain et al., 2003). It will therefore be interesting to determine which aspect of *Krox20* expression is normally governed by the novel autoregulatory element present in fragment 8.

## Cell type-specific autoregulatory elements may be required for stringent regulation

Strikingly, our analysis of the *Krox20* cis-regulatory landscape revealed the existence of multiple direct autoregulatory elements with cell type-specific activities. Besides element A and the sequence present in fragment 8, we have so far identified two other such enhancers: the NCE that is required for the maintenance of *Krox20* expression in r5-derived neural crest cells (Fig. 7) (Ghislain et al., 2003) and a bone forming cell-specific element that is responsible for the persistence of *Krox20* expression in this latter cell type (M.F. and P.C., unpublished). This raises the question of the existence of multiple cell type-specific autoregulatory elements versus a unique, global one, as is the case during *Drosophila* embryo segmentation for driving the stripe expression pattern of several pair-rule genes (Dearolf et al., 1989; Han et al., 1998; Small et al., 1996; Andrioli et al., 2002; Riddihough and Ish-Horowicz, 1991). We propose that cell type-specific autoregulatory elements allow for a much more precise regulation of the gene in each situation, as they are not only dependent on Krox20, but also on other transcription factors that bring in additional specificity (as does Sox10 in the case of the NCE) (Ghislain et al., 2003). Specific autoregulatory elements work in association with a particular initiator element(s) and together establish a highly regulated cell type-specific expression (Fig. 7), which may be required in the case of Krox20 because of its key role in several developmental processes (Schneider-Maunoury et al., 1993; Topilko et al., 1994; Levi et al., 1996) (S. Garel, P. Topilko and P.C., unpublished).

## Molecular mechanisms of *Krox20* regulation in the hindbrain and neural crest

The properties of the elements identified in this study allows us to propose a molecular model for *Krox20* regulation in the hindbrain and neural crest (Fig. 7). The characteristics of elements B and C suggest that they are responsible for the initiation of *Krox20* expression in r3 and r5 (initiator elements). In such a case, why is there an apparent redundancy in r5, where both elements are active? It is possible that elements B and C are indeed largely redundant, providing higher security for the system. Alternatively, the two elements might work in a synergistic manner, funnelling information from two separate pathways to reach a level of Krox20 sufficient for starting the autoregulatory loop (see below).

The isolation of elements B and C opens the way to the identification of the transcription factors that are directly acting on them. As a first step in this direction, we have shown that a vHNF1-binding site within element B is absolutely required for its r5-specific enhancer activity. Although vHNF1 involvement in *Krox20* regulation was already known, a direct transcriptional role was not expected: on the basis of the relative *vHnf1* and *Krox20* expression patterns and partial rescue of a *vHnf1* mutation by *val* expression in zebrafish, it was proposed that vHNF1 regulates *Krox20* only indirectly, via val/MafB and other unknown factors (Wiellette and Sive, 2003). Our data, which support a direct involvement of vHNF1 (Fig. 7), force a revision of this hypothesis, at least in mouse and chick.

An intriguing observation with element C is that its activity, although initially restricted to r3 and r5, progressively extends into r4, a territory where *Krox20* is never expressed. The same is true for the larger fragments from which element C is derived, including BAC 121, although in the latter case the chicken *Krox20* gene is present in the construct and this might affect its activity. The simplest hypothesis for explaining such behaviour is that the regulation of *Krox20* involves a repression mechanism in r4 and that in our transgenic analyses this repression does not occur properly, possibly because a necessary cis-acting element is absent from our constructs.

Once elements B and C have established a threshold level of Krox20 in r3 and r5 cells, the autoregulatory element A is likely to take the relay and maintain or amplify this level by a direct, positive feedback loop (Fig. 7). It therefore appears as a maintenance element. There is solid physiological evidence in favour of the importance of such a process: mouse *Krox20* knockout does not affect its initial activation, but leads to rapid downregulation of its expression, without loss of the cells (Schneider-Maunoury et al., 1993; Voiculescu et al., 2001). Furthermore, ectopic expression of *Krox20* in the hindbrain leads to activation of the endogenous gene (Giudicelli et al., 2001). As discussed above, like the other *Krox20* autoregulatory elements, element A is likely to be cell type-specific and therefore rely on additional factor(s) that provide this specificity. The availability of the nucleotide sequence of this element will provide a means to identify this factor. In addition, such a factor might be responsible for switching off the positive feedback loop when *Krox20* expression is downregulated in the hindbrain.

As shown previously, the regulation of *Krox20* in r3 and r5 also involves a non cell-autonomous autoregulatory mechanism, which may be involved in the extension of odd-numbered territories (Giudicelli et al., 2001). As elements B and C are not trans-activated by Krox20, they are not likely to be implicated in this process. By contrast, this could be the case for element A. However, as element A activity is abrogated by mutation of its Krox20-binding sites, this would imply that non cell-autonomous activation of *Krox20* requires the Krox20 protein in the receiving cell. It has been recently shown that some homeodomain transcription factors can be transferred from cell to cell (for a review, see Prochiantz and Joliot, 2003). It will therefore be extremely interesting to investigate whether this could also be the case for Krox20.

Finally, elements A, B and C are also likely to provide the threshold level of Krox20 protein necessary, in conjunction with the crest-specific factor, Sox10, to initiate another positive feedback loop, involving the NCE, at work in the r5-derived neural crest (Fig. 7) (Ghislain et al., 2003). Together, these elements explain the expression of *Krox20* in the r5-derived neural crest and will provide a detailed molecular understanding of its patterning.

We are grateful to Charles Babinet and Pascale Gilardi-Hebenstreit for critical reading of the manuscript. This work was supported by grants from INSERM, MENRT, ARSEP, ARC and AFM.

**Supplementary material**

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/133/7/1253/DC1

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| Table S1. Recapitulative table of primers used in this work | | |
|---|---|---|
| Genotyping | | |
| cA<br>10<br>13 | Sense<br>Antisense | 5′-gtggaattttagagtaggagggaagt-3′<br>5′-gtggtgggaaataaattgtcagagat-3′ |
| cB | Sense<br>Antisense | 5′-ggataatgcatcaagaaactac-3′<br>5′-caatcagcagctaattaaacaaggcaact-3′ |
| cC | Sense<br>Antisense | 5′-agacagtcccgcagttaccatcc-3′<br>5′-gctcgagccacatccaccag-3′ |
| 1<br>2 | Sense<br>Antisense | 5′-cctcgcgggttttcgctatttatga-3′<br>5′-atggcgcctgatgcggtattttc-3′ |
| 3 | Sense<br>Antisense | 5′-ctcactcattaggcaccccaggctttacac-3′<br>5′-agaacgaattgctgccactgacactcaca –3′ |
| 4<br>5 | Sense<br>Antisense | 5′-aggcctataatatgctgagttcacaag-3′<br>5′-tggatcacgttgcctgtcatc-3′ |
| Cloning | | |
| 5 | Sense<br>Antisense | 5′-tgcccagccacttctcattagg-3′<br>5′-aagcagtataacagcagagggag-3′ |
| mA | Sense<br>Antisense | 5′-gggttgtgaatggagccagcggtg-3′<br>5′-gcaagccgaccaaactccgccatg-3′ |
| mB | Sense<br>Antisense | 5′-gtgctggcctgtagatgagccc-3′<br>5′-ctccacagggaaacccttttacac-3′ |
| mC | Sense<br>Antisense | 5′-gacagccacaggaagtgagtcc-3′<br>5′-ctaggagacactttcaaaaggc-3′ |
| Mutagenesis | | |
| mutant cA | Sites 1 and 2<br>Sites 3 and 4<br>Site 5<br>Site 6<br>Site 7<br>Selection | 5′-ccctccgactctaaaattccagccttaaaacacagg-3′<br>5′-ctgccgcctaaggcggtgacagtc-3′<br>5′-gctaagagaagtgtcggctgaggaattctgcc-3′<br>5′-cactgtgtcggaggctctgcgggccctccgacaatg-3′<br>5′-cggcactgtgtcggaggctctgcg-3′<br>5′-cactagttctagaggtcgacccaccgcggtg-3′ |
| mutant cB | Half site sense<br>Half site antisense | 5′-gggcccccaggtacagtgaag-3′<br>5′-aggcccatcccaaatttgctg-3′ |

Sequences of oligonucleotides used as primers for the genotyping, cloning and mutagenesis experiments are indicated. c, chicken; m, mouse.