

Krox-20 patterns the hindbrain through both cell-autonomous and non cell-autonomous mechanisms

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The *Krox-20* gene encodes a zinc finger transcription factor, which has been shown previously, by targeted inactivation in the mouse, to be required for the development of rhombomeres (r) 3 and 5 in the segmented embryonic hindbrain. In the present work, *Krox-20* was expressed ectopically in the developing chick hindbrain by use of electroporation. We demonstrate that *Krox-20* expression is sufficient to confer odd-numbered rhombomere characteristics to r2, r4, and r6 cells, presumably in a cell-autonomous manner. Therefore, *Krox-20*, appears as the major determinant of odd-numbered identity within the hindbrain. In addition, we provide evidence for the existence of a non cell-autonomous autoactivation mechanism allowing recruitment of *Krox-20*-positive cells from even-numbered territories by neighboring *Krox-20*-expressing cells. On the basis of these observations, we propose that *Krox-20* regulates multiple, intertwined steps in segmental patterning: Initial activation of *Krox-20* in a few cells leads to the segregation, homogenization, and possibly expansion of territories to which *Krox-20* in addition confers an odd-numbered identity.

[Key Words: Hindbrain; rhombomere; segmentation; *Krox-20*; Hox; Eph; kreisler]

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The morphogenesis of the vertebrate hindbrain involves a transient segmentation process along the anterior–posterior (AP) axis, which leads to the generation of 7–8 metameres called rhombomeres (r; Vaage 1969; Lumsden 1990). This subdivision presages the differentiation of neurons in segment-specific patterns and underlies the repeated organization of branchiomotor cranial nerves (Lumsden and Keynes 1989). It also participates in the specification of neural crest cells and in the establishment of their pathways of migration, therefore playing a crucial role in craniofacial organization (Bronner-Fraser 1995; Kontges and Lumsden 1996). Segmentation involves a restriction of cell intermingling at rhombomere boundaries due to alternating cell-surface properties that cause r3/r5 cells to be immiscible with r2/r4/r6 cells (Fraser et al. 1990; Guthrie and Lumsden 1991; Guthrie et al. 1993). This restriction of cell lineages is thought to be required for each segment to maintain a specific pattern of gene expression and thus a distinct AP identity.

Numerous genes present spatially restricted patterns of expression along the AP axis in the hindbrain, with limits corresponding to prospective or established boundaries between adjacent rhombomeres (Lumsden

and Krumlauf 1996). Loss-of-function studies have shown that several of these genes play essential roles in the control of hindbrain segmentation (for review, see Schneider-Maunoury et al. 1998). Among them, *Krox-20*, which encodes a zinc finger transcription factor, is activated in two transverse stripes, which prefigure and subsequently coincide with r3 and r5 (Wilkinson et al. 1989). Targeted inactivation of *Krox-20* leads to a progressive elimination of r3 and r5, indicating that this gene is essential to the maintenance of these hindbrain territories (Swiatek and Gridley 1993; Schneider-Maunoury et al. 1993, 1997). In addition, *Krox-20* has been shown to directly activate the transcription of several *Hox* genes in r3 and/or r5 (*Hoxb2*, *Hoxa2*, and *Hoxb3*; Sham et al. 1993; Nonchev et al. 1996; Manzanares et al., unpubl.). Because the combinatorial expression of *Hox* genes is thought to determine AP positional identity (Lumsden and Krumlauf 1996), this suggests that *Krox-20* also plays an important role in the specification of rhombomere identity. Finally, *Krox-20* has been shown to directly activate the transcription of at least one of the Eph tyrosine kinase receptor genes expressed in r3 and r5, *EphA4* (Gilardi-Hebenstreit et al. 1992; Theil et al. 1998). Bidirectional interactions between the Eph receptors and their ephrin transmembrane ligands, which are present in even-numbered rhombomeres, have been implicated in the segregation of cells between odd- and even-numbered rhombomeres (Mellitzer et al. 2000). Therefore,

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Krox-20 is also involved in the control of lineage restrictions in the hindbrain.

To gain further insight into the role of *Krox-20* in the specification of rhombomere identity, we performed gain-of-function experiments in the chick embryo hindbrain using in ovo electroporation. We show that ectopic expression of *Krox-20* can convert even-numbered rhombomere cells into odd-numbered identity (r3 or r5). Unexpectedly, this analysis also revealed that *Krox-20* can propagate its own expression by a non cell-autonomous mechanism. This latter phenomenon is likely to play an important role in the establishment of r3 and r5 territories during hindbrain segmentation.

Results

Krox-20 ectopic expression in the hindbrain neuroepithelium

To ectopically express *Krox-20* in the hindbrain during the period of segmentation, we used the procedure of electroporation in the chick embryo neural tube (Itasaki et al. 1999). This method allows comparison between the electroporated side and the non-electroporated side, which can be used as a control. Because, in the chick, *Krox-20* transcripts were first detected at stage HH8 (Hamburger and Hamilton 1951) in pre-r3 and HH9 in pre-r5 (Nieto et al. 1991; Irving et al. 1996) electroporation was performed at stages HH8–HH10. To set up the conditions, we first electroporated a construct in which the *Escherichia coli* *LacZ* gene is driven by a regulatory element composed of the Rous sarcoma virus long terminal repeat promoter enhanced by a human type 5 adenovirus inverted terminal repeat (pAdRSV β -gal). In these conditions, the presence of β -galactosidase was detected in isolated cells in the neuroepithelium as early as 6 h after electroporation and up to at least 48 h. β -Galactosidase-positive cells were observed only in the electroporated side, in the neuroepithelium, neural crest streams, to a lower extent in non-neural ectoderm, and very rarely in mesodermal tissues (data not shown). They did not show any obvious bias in distribution along the AP axis, often covering the entire hindbrain and part of the midbrain and of the spinal cord, whereas their frequency was usually much higher in the dorsal part of the neural tube (Fig. 1A).

The mouse *Krox-20* gene was placed under the control of the same regulatory elements. Unless otherwise indicated, two constructs, encoding either the wild-type protein or a carboxy-terminal Myc-tagged version, have been used equivalently during the course of this study. No differences were observed in terms of phenotypic consequences between these two constructs. Electroporation of the *Krox-20*-expressing constructs and detection of the protein with an antibody recognizing both the mouse and chick proteins revealed efficient ectopic expression, with patterns largely similar to the β -galactosidase patterns described above (Fig. 1A,B).

Krox-20 activates *EphA4* in a restricted AP domain within the hindbrain

To investigate the consequences of *Krox-20* ectopic expression on hindbrain segmentation and specification, we first analyzed the expression of *EphA4*, known to constitute a direct target of *Krox-20* (Theil et al. 1998). On the electroporated side, *EphA4* mRNA (Fig. 2) and protein (Fig. 1C,F,G) were found outside of the normal expression domain, which is restricted to r3 and r5 (Irving et al. 1996; Hirano et al. 1998). This ectopic pattern presented highly reproducible features: (1) *EphA4* was always expressed at a level similar to that observed in r3 and r5. Consistently, no overexpression was observed in r3 and r5. (2) Cells expressing *EphA4* ectopically were almost never isolated, but rather grouped in large patches. (3) *EphA4* ectopic activation was strictly restricted rostrally to a limit likely corresponding to the r1/r2 boundary according to morphological criteria. (4) The efficiency of *EphA4* activation, in terms of both frequency and size of the patches, generally followed a decreasing rostral to caudal gradient from r2 to r7/r8, with expression in this caudal region observed only in some of the embryos older than HH14+ (Fig. 2D,E,K).

Krox-20 ectopic expression affects the molecular identity of even-numbered rhombomeres

Next, we examined the expression of genes expressed in even-numbered rhombomeres. Around stage HH14, *follistatin* is normally expressed in r2 and r6, and at a lower level in r4 and r5 (Graham and Lumsden 1996). *Krox-20* loss-of-function mutation has been shown previously to lead to ectopic activation of *follistatin* in r3 in the mouse (Seitanidou et al. 1997). Consistently, *Krox-20* electroporation led to a general and drastic downregulation of *follistatin* in even-numbered rhombomeres (Fig. 1D). We also observed a slight reduction of the low level of *follistatin* in r5.

Hoxb1, a major determinant of r4 identity (Studer et al. 1996), is expressed at high levels in r4 and at lower levels in r7/r8 and the spinal cord (Sundin and Eichele 1990) at the stages of our analyses. Following *Krox-20* electroporation, *Hoxb1* expression was dramatically altered, with the appearance of patches of negative cells within r4 and the caudal expression domain (Fig. 1E). Therefore, like in the case of *follistatin*, ectopic expression of *Krox-20* leads to repression of *Hoxb1* transcription.

Because, in even-numbered rhombomeres, *Krox-20* up-regulates an odd-numbered rhombomere marker (*EphA4*) and down-regulates even-numbered rhombomere markers (*follistatin* and *Hoxb1*), it was essential to determine whether these effects involved the same or different cells. Therefore, we recorded the expression of *EphA4* and *Hoxb1* simultaneously by double in situ hybridization and performed a time course analysis. Typical results are shown in Figure 2 and can be summarized as follows: (1) at all stages examined, *Hoxb1* and *EphA4* expression domains remained exclusively in the entire

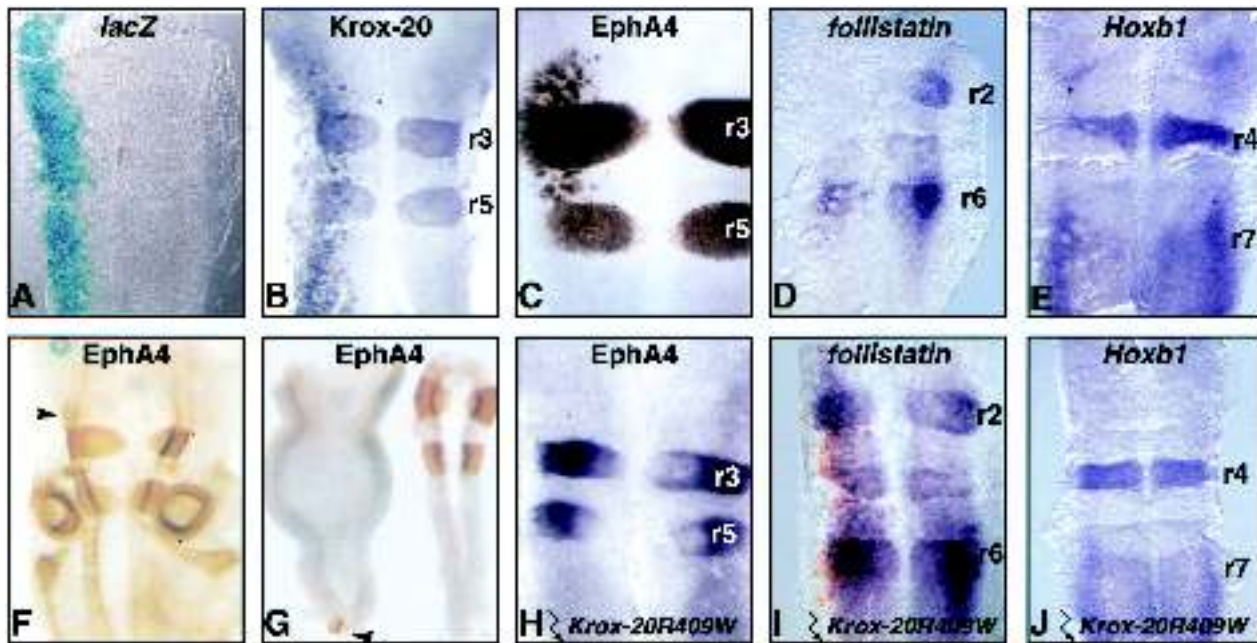


Figure 1. Ectopic *Krox-20* expression leads to *EphA4* induction and *follistatin* and *Hoxb1* repression. Flat-mounted hindbrains (A–E, H–J) or whole mounts (F, G) from chick embryos electroporated with *LacZ* (A), wild-type (B–G), or R409W mutant (H–J) mouse *Krox-20* expressing plasmids between stages HH8 and HH10 (A–E, H–J) or between stages HH10 and HH11 (F, G). The embryos were collected 24 h after electroporation (18 h for D and 16 h for F and G) and the expression of the indicated markers was analyzed by X-Gal staining (A), immunochemistry (B, C, F–H) or in situ hybridization (D, E, I, J). (A) Analysis of β -galactosidase distribution after electroporation with a *LacZ* expression plasmid. (B) Analysis of *Krox-20* expression with an antibody that recognizes both mouse and chicken proteins after electroporation with a *Krox-20* expression plasmid. Note that ectopic *Krox-20* is present in isolated cells with a distribution similar to that of β -galactosidase (A). (C) *EphA4* ectopic expression is detected in large patches of cells in r2 and r4. (D) *follistatin* expression is severely down-regulated upon *Krox-20* ectopic expression in the hindbrain, including r5, where endogenous *Krox-20* is also present. The patchy appearance of the *follistatin*-positive domain on the control side is normal at this stage. (E) *Hoxb1* is repressed following ectopic *Krox-20* expression. Large patches of *Hoxb1*-negative cells are observed within the entire domain of normal expression, including r4 and r7. (F, G) *EphA4* activation in rhombomere 2 (arrowheads) is not due to cell migration from r3 because, in (G), the embryo was cut immediately after electroporation at the level of prospective rhombomere 2 and the two parts were kept separated. (H, I, J) Ectopic expression of the *Krox-20* mutant allele (R409W) does not induce *EphA4* (H), nor does it repress *follistatin* (I) or *Hoxb1* (J). In I, the red staining corresponds to in situ hybridization with a mouse-specific *Krox-20* probe, revealing the transfected cells. Electroporated side is on the left.

hindbrain. (2) In r4, *EphA4* and *Hoxb1* expression domains were strictly complementary in all samples until stage HH14 (Fig. 2A, B). At later stages, the most ventral r4 *Hoxb1*-negative patches down-regulated *EphA4*, a phenomenon reminiscent of the normal *EphA4* down-regulation in ventral r5 (Fig. 2H, M, white arrowheads). In addition, a thin zone negative for both markers appeared at the interface between the *Hoxb1*-positive r4 domains and r3, r5 or the *EphA4*-positive ectopic patches (Fig. 2G, N, black arrowheads). During normal development, such double negative zones were also observed after HH14–HH15 at rhombomere boundaries between r3, r4 and r5 (Fig. 2I–K, control side). (3) In contrast to r4, down-regulation of *Hoxb1* in its caudal expression domain was rarely accompanied by activation of *EphA4*. More precisely, the caudal *EphA4* up-regulation occurred only after stage HH14–HH15 and never completely filled the *Hoxb1*-negative domains, being restricted to a few cells in r7/r8 (Fig. 2E, K).

In conclusion, *Krox-20* ectopic expression leads to the appearance of patches of *EphA4*-positive cells within

even-numbered rhombomeres. These patches are *Hoxb1*-negative in r4 and, therefore, may present molecular characteristics of odd-numbered rhombomeres.

Krox-20 transforms even- into odd-numbered rhombomere identity

To analyze the molecular identity of the *EphA4*-positive patches more precisely, we studied the expression of additional regional markers. *mafB/kr* is normally expressed in r5 and r6 (Eichmann et al. 1997). We performed double in situ hybridizations to detect *mafB/kr* mRNA together with *EphA4* mRNA. The *mafB/kr* pattern was not affected by *Krox-20* ectopic expression (Fig. 3A). Therefore, the *EphA4*-positive patches in r2 and r4 do not express *mafB/kr*, whereas those in r6 still do. Consistently, double in situ hybridization with the *mafB/kr* and *Hoxb1* probes showed that the *Hoxb1*-negative patches in r4 and r7 were also negative for *mafB/kr* (Fig. 3B).

In control embryos, *Hoxa3* is expressed in r5/r6 and at

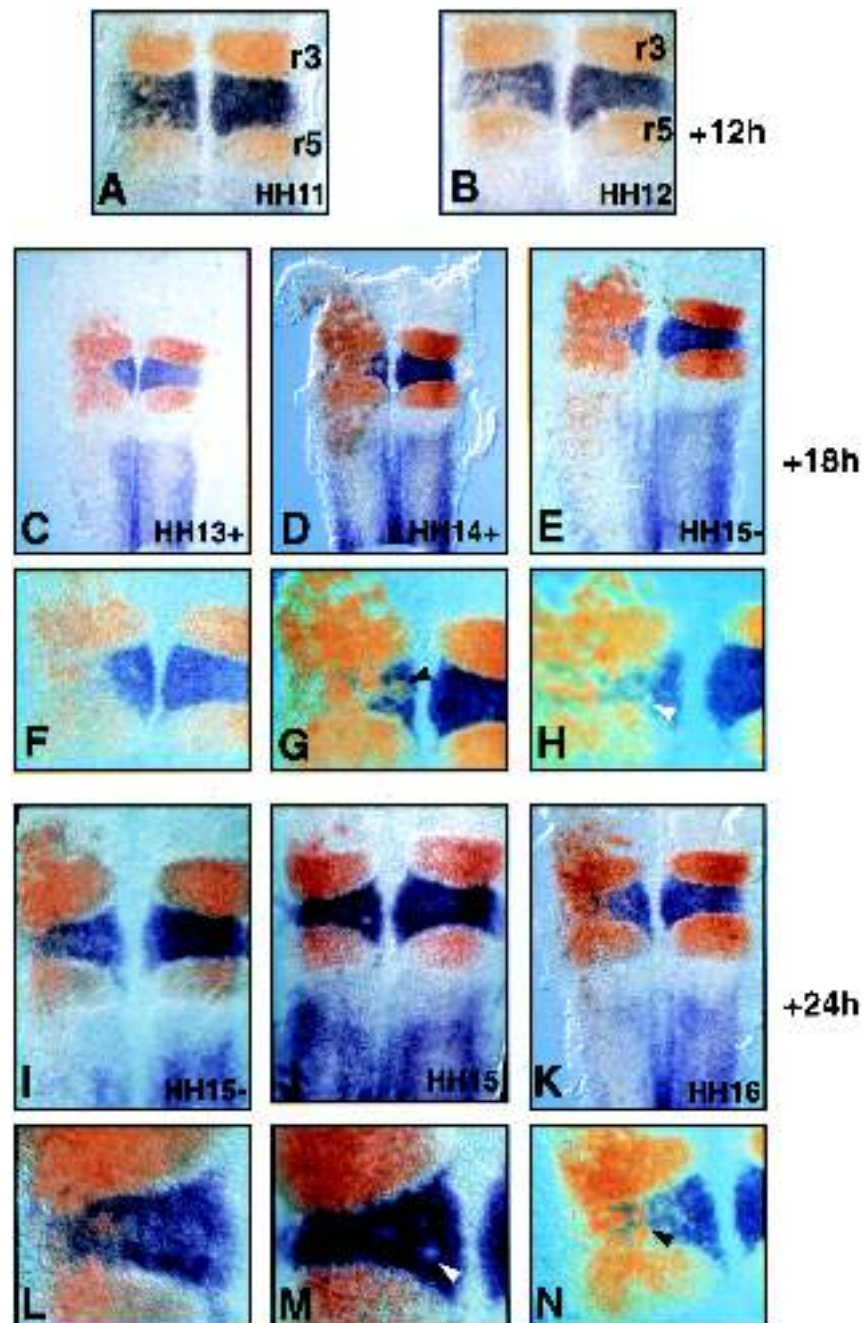


Figure 2. Time-course of the effects of *Krox-20* ectopic expression on *Hoxb1* and *EphA4* expression. Flat-mounted hindbrains from embryos electroporated with *Krox-20*, incubated for the indicated period of time and hybridized in situ with *Hoxb1* (purple) and *EphA4* (red) probes. The developmental stages of the harvested embryos are indicated (bottomright of A–E, and I–K). (F–H, L–N) Higher magnifications of the embryos shown in C–E and I–K, respectively. At early stages, *EphA4* expression fills all *Hoxb1*-negative patches in r4, but not in the caudal domain of *Hoxb1* expression. Later, *EphA4* is down-regulated in basal *Hoxb1*-negative patches in r4 (white arrowheads in H and M). Note the formation of a thin unstained boundary at the interface between adjacent rhombomeres as well as between *EphA4*-positive and *Hoxb1*-positive domains within r4 (black arrowheads in G and N). The apparent overlap in *EphA4* and *Hoxb1* labeling in some areas in M is due to cytoplasmic overlap of different cells. Electroporated side is on the left.

a lower level in r7/r8, whereas *Hoxb3* is expressed in r7/r8 and more caudal and at a much lower level in r5 and r6 (Grapin-Botton et al. 1995). These expression patterns were not significantly modified after electroporation with the *Krox-20* expression construct ($n=15$ and 6 respectively, Fig. 3,C,D). Double in situ hybridization for *Hoxb1* and *Hoxa2*, which is normally expressed from r2 to r7/r8 in the hindbrain, revealed that no modification in *Hoxa2* expression was observed in electroporated embryos and, in particular, that the *Hoxb1*-negative patches were still positive for *Hoxa2* (Fig. 3E).

In summary, our combined data indicate that, in r2 and r4, the *EphA4*-positive patches are positive for

Hoxa2 and negative for *Hoxb1*, *Hoxa3*, *Hoxb3*, *mafB/k*, and possibly also *follistatin*. In r6, the *EphA4*-positive patches are positive for *Hoxa2*, *Hoxa3*, and *mafB/kr* and negative for *Hoxb1*. These expression patterns are consistent with odd-numbered rhombomere molecular identity.

The presence of patches of cells with r3- or r5-like identities in the vicinity of r3 or r5 may have resulted from two different processes: a change of identity of cells within even-numbered rhombomeres, or an inappropriate migration of odd-numbered rhombomere cells into adjacent even-numbered rhombomeres. To resolve this issue, electroporations were performed at stage HH10,

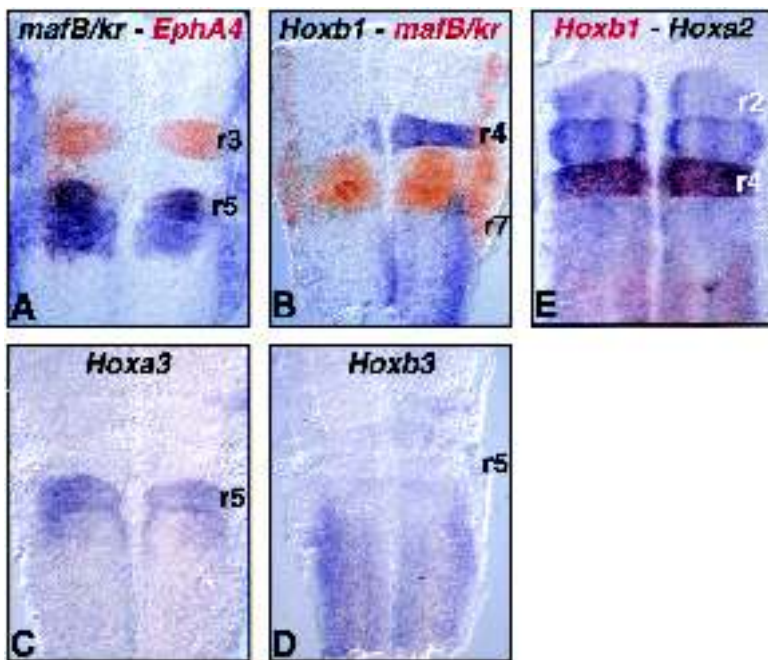


Figure 3. *Krox-20* misexpression does not affect *mafB/kr*, *Hoxa2*, *Hoxa3*, and *Hoxb3* expression. Flat mounts of hindbrains of embryos electroporated with *Krox-20*, incubated for 24 h (18 h in A and B) and hybridized with the indicated probes. (A) *EphA4*-positive patches (red) in r2 and r4 do not express *mafB/kr* (purple) whereas those in r6 maintain this expression. (B) *MafB/kr* expression (red) is not affected by *Krox-20* expression. In particular, the *Hoxb1*-negative domains in r4 and r7 do not activate *mafB/kr*. (C,D) *Krox-20* ectopic expression does not lead to significant modifications in the patterns of *Hoxa3* or *Hoxb3* expression. (E) *Hoxa2* expression (purple) is also maintained in r4 in the patches negative for *Hoxb1* (red). Electroporated side is on the left.

after establishment of lineage restrictions between rhombomeres (Fraser et al. 1990; Birgbauer and Fraser 1994). Immediately afterward, the embryos were sectioned transversally within presumptive r2, and anterior and posterior parts were not allowed to reassociate to prevent any cell migration from r3 into the rostral hindbrain. EphA4 immunodetection was performed after 16 h of incubation, and most embryos that appeared to have been effectively sectioned within r2 showed ectopic expression in the most caudal part of the anterior half of the neural tube, while the entire r3 was clearly present in the posterior half as shown by the EphA4 pattern (Fig. 1G). These data indicate that, in r2, the formation of patches with r3-like identity involves a change of identity induced by *Krox-20* ectopic expression.

Changes of identity require Krox-20 DNA-binding activity

To investigate whether the different consequences of *Krox-20* ectopic expression were actually dependent on the capacity to bind DNA of the protein, we introduced a point mutation resulting in an arginine to tryptophan substitution in the third zinc finger of the DNA-binding domain (R409W). This mutation was identified in a human peripheral myelinopathy (Warner et al. 1998) and leads to a Schwann cell phenotype similar to that observed in *Krox-20* null mice (Topilko et al. 1994). Subsequently, this mutation was shown to completely abolish *Krox-20* DNA binding in vitro (Warner et al. 1999). Chick embryos were electroporated with an R409W mutant *Krox-20* construct and analyzed for the expression of *EphA4* (Fig. 1H), *follistatin* (Fig. 1I), and *Hoxb1* (Fig. 1J). In contrast to the wild-type construct, no alterations in the expression patterns of these genes were observed

with the mutant construct ($n \geq 5$ for each of them), although the levels of ectopic *Krox-20* mRNAs and proteins were similar (cf. Fig. 1I with Fig. 5, below, and data not shown). This demonstrates that the DNA-binding activity of *Krox-20* is required for each of the observed phenotypic consequences of its ectopic expression.

Non cell-autonomous consequences of Krox-20 exogenous expression

A striking and common characteristic of the alterations in gene expression (activation or repression) following ectopic expression of *Krox-20* was their occurrence not in isolated cells but in patches. Because electroporation is expected to hit isolated cells (Fig. 1A), the existence of such patches may be explained by at least three nonexclusive mechanisms: (1) aggregation of transfected cells that may have acquired adhesion properties different from their neighbors; (2) proliferation of transfected cells in the absence of cell intermingling; (3) non cell-autonomous modifications of gene expression around the transfected cells. The presence of cell patches early after electroporation (Fig. 2A,B) argues rather in favor of the last possibility, at least during this early period.

To investigate the issue directly, we performed double labeling experiments to detect both the target genes and the exogenous *Krox-20* mRNA, using an in situ hybridization probe derived from the 3'-UTR of the mouse *Krox-20* mRNA, which did not cross-hybridize with chicken mRNA (Fig. 4A and data not shown). Double in situ hybridization with this probe and the *EphA4* probe on *Krox-20*-electroporated embryos indicated that the mouse *Krox-20* gene is expressed in isolated cells often within or bordering the *EphA4*-positive patches (Fig. 4A). Strikingly, the large majority of the cells expressing

EphA4 ectopically were negative for mouse *Krox-20* mRNA. We then performed double labeling of *Hoxb1* by in situ hybridization and of the exogenous *Krox-20* protein by immunodetection of the Myc tag epitope. In accordance with the previous result, exogenous *Krox-20* was detected in isolated cells within or bordering the *Hoxb1*-negative domains (Fig. 4E). Again, most of the cells repressing *Hoxb1* in r4 or r7 appeared negative for mouse *Krox-20*. Therefore, exogenous *Krox-20* expression appears to enforce modifications in gene expression by a non cell-autonomous mechanism.

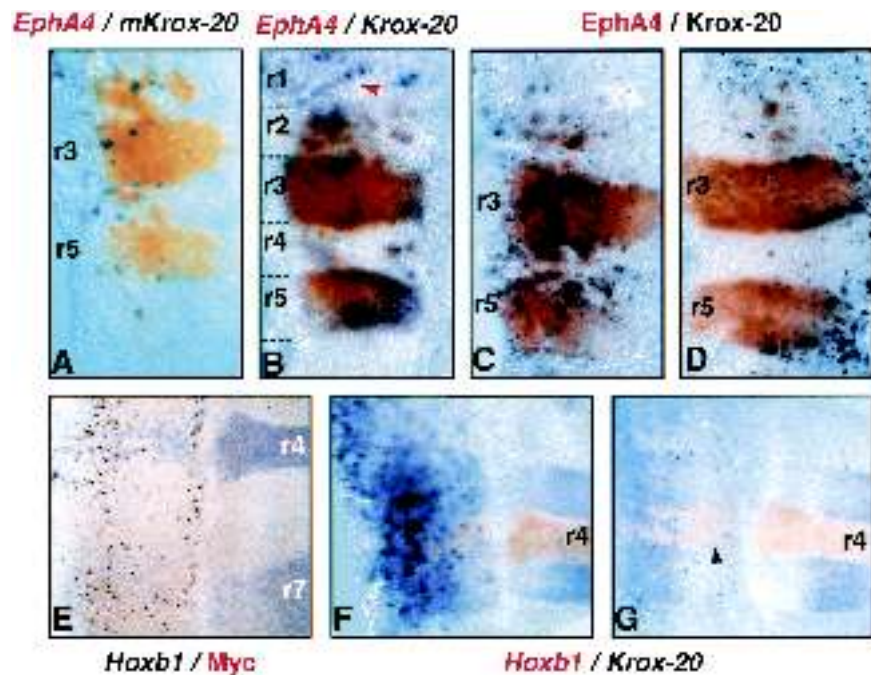
These experiments raised the question of the mediator of the modifications in gene expression in cells that do not express the exogenous *Krox-20* gene. A possibility was that these cells actually expressed the endogenous *Krox-20* gene. To address this question, we first performed another series of double-labeling experiments in which we used an in situ hybridization probe or an antibody recognizing both chicken and mouse *Krox-20* mRNA or protein. Detection of *Krox-20* and *EphA4* transcripts 24 h after *Krox-20* electroporation indicated that the cells in the *EphA4*-positive patches in r2, r4, and r6 expressed *Krox-20*, the uniform brown staining in r3, r5, and the ectopic patches resulting from the superposition of orange/red *EphA4* and purple *Krox-20* stainings (Fig. 4, cf. B with A). Furthermore, this uniform color established that both *EphA4* and *Krox-20* are expressed at similar levels in r3/r5 and in the ectopic patches, apart from isolated cells expressing very high levels of *Krox-*

20. These latter cells presumably correspond to those expressing the exogenous mouse *Krox-20*. As expected from previous experiments, in r1, cells were observed expressing *Krox-20* ectopically but not *EphA4* (Fig. 4B, red arrowhead), confirming that *Krox-20* is not able to activate *EphA4* rostrally to r2. Similar results were obtained when the embryos were incubated for only 14 h (Fig. 4C) or 20 h (Fig. 4D) after *Krox-20* electroporation. In conclusion, these experiments indicate that the cells expressing *EphA4* ectopically also express *Krox-20*, even at early stages of activation, and, for most of them, this expression is likely to result from activation of the endogenous chicken gene.

Consistent with the above data, double in situ hybridization for *Krox-20* and *Hoxb1* demonstrated that *Krox-20* transcripts are present presumably in all cells within *Hoxb1*-negative patches in r4 when the embryos are collected before stage HH14– (Fig. 4F). Similar to what was observed in the case of *EphA4* ectopic expression (Fig. 2H,M), after stage HH14 the *Hoxb1*-negative patches in ventral r4 also appeared mostly negative for *Krox-20* expression (Fig. 4G, black arrowhead), paralleling the down-regulation of endogenous *Krox-20* in basal r3 and r5 (Fig. 4G).

In conclusion, these data suggest that *EphA4* up-regulation in r2, r4, and r6 and *Hoxb1* repression in r4 require the presence of *Krox-20* in a cell-autonomous manner, as the expression of the latter is always observed, at least transiently, in the patches. In contrast, they indicate that

Figure 4. Non cell-autonomous effects on *EphA4* and *Hoxb1* expression. Flat-mounted hindbrains from embryos electroporated with mouse *Krox-20* and revealed by in situ hybridization (A,B,E–G) or immunocytochemistry (C–E) with the indicated probes or antibodies. (A) Double in situ hybridization performed with an *EphA4* probe (red) and a mouse-specific *Krox-20* probe (purple) which labels only electroporated cells. Note that the mouse *Krox-20* probe labels isolated cells within the *EphA4*-positive patches. (B) Double in situ hybridization performed on an embryo harvested 24 h after electroporation with an *EphA4* probe (red) and a *Krox-20* probe (purple), which hybridizes with both the endogenous chicken and the exogenous mouse mRNAs. Note the complete overlap between both labelings (brown color, compare with red/orange staining in A), except in r1, where only *Krox-20* is detected (red arrowhead). (C,D) Double immunocytochemistry performed on embryos harvested 14 h (C) and 20 h (D) following electroporation with antibodies directed against *EphA4* (red) and *Krox-20* (purple). The latter antibody recognizes both the chick and mouse *Krox-20*. Note again that all *EphA4*-positive territories express *Krox-20*. (E) *Hoxb1* in situ hybridization (purple) combined with anti-Myc immunocytochemistry (brown) on an embryo electroporated with the Myc-tagged *Krox-20*. (F,G) Double in situ hybridization performed on embryos harvested at stages HH14– and HH14+ respectively with *Hoxb1* (red) and *Krox-20* (purple) probes. The *Krox-20* probe recognizes both chick and mouse mRNAs. Note the presence of white patches (*Hoxb1*– and *Krox-20*-negative) within basal r4 (black arrowhead in G). Electroporated side is on the left.



ectopic expression of exogenous *Krox-20* results in non cell-autonomous activation of the endogenous *Krox-20* gene.

Spatially and temporally restricted, non cell-autonomous Krox-20 autoactivation

To investigate the autoregulation of *Krox-20*, a chicken-specific probe was required. For this purpose, we screened a chicken genomic BAC library with a *Krox-20* DNA-binding domain cDNA probe (Nieto et al. 1991) and isolated the entire gene. The nucleotide sequence was established (GeneBank accession no. AF291747), and the inferred protein amino acid sequence was shown to present a 65% similarity to the mouse *Krox-20* sequence (data not shown). The chicken *Krox-20* gene was used to derive a 3'-UTR probe presenting no sequence similarity to the corresponding region of the mouse gene,

thus specifically recognizing the chicken mRNA (Fig. 5 and data not shown).

To establish the existence of non cell-autonomous activation of *Krox-20*, we performed double in situ hybridization experiments with mouse- and chicken-specific *Krox-20* probes on *Krox-20*-electroporated embryos. As expected from previous experiments, mouse *Krox-20* labeling was restricted to isolated cells evenly spread along the neural tube, whereas chicken *Krox-20* was expressed at a uniform level in r3/r5 and in large cell patches outside of these rhombomeres (Fig. 5A–D). These patches were often bordered by or contained mouse *Krox-20*-expressing cells. These data strongly suggest that expression of exogenous *Krox-20* in isolated cells leads to activation of the endogenous gene in surrounding, nonelectroporated cells.

To exclude the possibility that these surrounding cells may have also received the plasmid, but subsequently lost it or failed to express the gene at a detectable level,

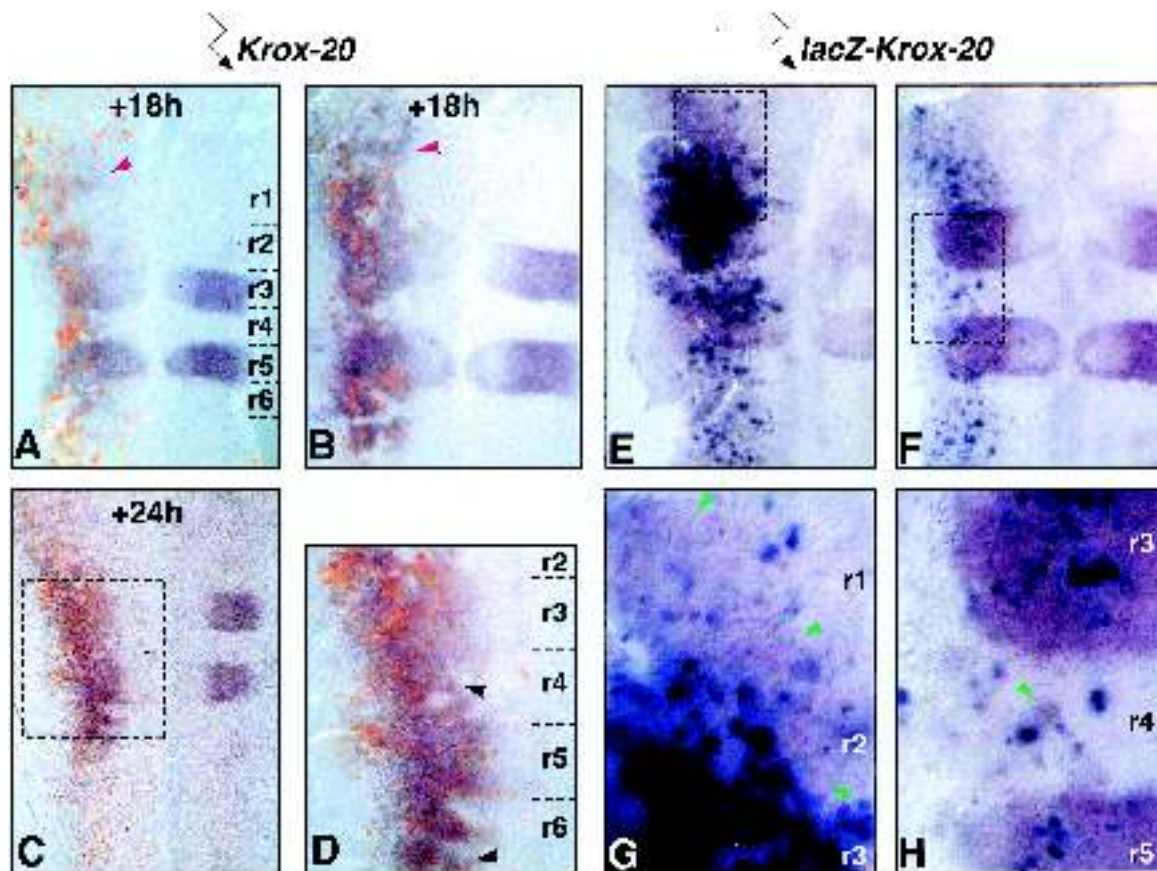


Figure 5. Expression of exogenous *Krox-20* leads to non-autonomous activation of endogenous *Krox-20*. Flat-mounted hindbrains from embryos electroporated with mouse *Krox-20* (A–D) or a construct (pAdRSVβgalKrox20) directing co-transcription of *LacZ* and mouse *Krox-20* (E–H). They were analyzed by in situ hybridization with chicken-specific (purple) and mouse-specific (red, AD) *Krox-20* probes, and Bluo-Gal staining (dark blue, E–H). (A,B) HH13 embryos harvested 18 h after the electroporation. (Red arrowheads) Patches of endogenous *Krox-20* expression in r1. (C) HH15 embryo harvested 24 h after the electroporation. (D) Higher magnification view of the embryo shown in C. While mouse *Krox-20* is expressed in a punctuate manner along the neural tube, chicken *Krox-20* is ectopically activated in cell patches restricted to the r1–r7 region. The extent of chicken *Krox-20* expression appears to broaden at later stages, and positive patches are observed in absence of close mouse *Krox-20*-expressing cells (black arrowheads in D). (E,F) HH13+ embryos harvested 18 h after electroporation. (G,H) Higher magnification views of the embryos shown in E and F. Chicken *Krox-20* is expressed in Bluo-Gal-negative cells (examples are indicated by green arrowheads). Electroporated side is on the left.

we performed an additional experiment. A bicistronic expression vector containing the *LacZ* gene (with a nuclear localization signal) downstream of the RSV promoter, followed by an internal ribosome entry site (IRES) and the *Krox-20* gene, was electroporated into the chick hindbrain. β -Galactosidase activity was revealed by Bluo-Gal staining and endogenous chicken *Krox-20* mRNA by *in situ* hybridization. In these conditions, we observed, within even-numbered rhombomeres, Bluo-Gal-positive cells surrounded by Bluo-Gal-negative, *Krox-20*-positive patches (Fig. 5E–H, green arrowheads). In the hindbrain neuroepithelium, the β -galactosidase protein shows a half-life exceeding 12 h (M. Frain and P. Charnay, unpubl.), and the detection of its activity is very sensitive. In addition, our construct ensures that the first cistron (*LacZ*) is expressed at a higher level than the second one (*Krox-20*; Mizuguchi et al. 2000). Therefore, essentially all cells having expressed exogenous *Krox-20*, even at low levels, should be detected by Bluo-Gal staining. We conclude that ectopic activation of endogenous *Krox-20* expression can occur in cells that never expressed the exogenous gene, establishing the non cell-autonomous character of *Krox-20* ectopic activation.

Interestingly, the distribution of the cell patches expressing endogenous *Krox-20* was different from that observed for the *EphA4*-positive patches (cf Fig. 5A,B with Figs. 1, 3, and 4). While ectopic expression of *EphA4* was restricted to the region caudal to the r1/r2 boundary, induction of endogenous *Krox-20* was observed up to the mesencephalic/metencephalic boundary at least, spreading largely within r1 (Fig. 5A,B, red arrowheads). Endogenous *Krox-20* expression was restricted caudally, in a way similar to ectopic *EphA4*, with a low level expression observed caudally to r6 only in embryos older than HH14–HH15, whereas mouse *Krox-20*-positive cells were observed much more posteriorly (Fig. 5C). These data indicate that the capacity of autoactivation of *Krox-20* through the non cell-autonomous mechanism is approximately restricted to the hindbrain territory.

Comparison of endogenous *Krox-20* patterns between embryos allowed to develop for 18, 24, or 32 h after electroporation suggested an extension of the domains of expression with time, while the number of exogenous *Krox-20*-expressing cells decreased (Fig. 5A–D and data not shown). After 24 or 32 h (Fig. 5C,D, arrowheads, and data not shown), ectopic patches of endogenous expression were often detected in the absence of mouse *Krox-20*-positive cells in their vicinity. These data suggest that some of the electroporated cells die, while endogenous expression is maintained within the patches and possibly extends to previously negative territories. Cell aggregation might also contribute to the increase in size of the *Krox-20*-positive patches.

Krox-20 misexpression affects early neurogenesis

Finally, we asked whether the molecular alterations of rhombomeric identity due to *Krox-20* ectopic expression

were followed by long-term cellular manifestations. In the hindbrain, neurogenesis is regulated in a segment-specific manner. In particular, between HH12 and HH15, r3 and r5 display a marked delay, compared with even-numbered r2, r4, and r6, in the timing of neuronal differentiation and axonal growth. Therefore, we performed an analysis of neurofilament expression, a marker of differentiated neurons, on embryos harvested at around stages HH13–HH15, 24 h after *Krox-20* electroporation. At these stages, the most differentiated neurons are located in the basal plate of even-numbered rhombomeres and extend their axons in the descending medial longitudinal fasciculus (mlf). Other populations, such as post-mitotic dorsal interneurons or basal columnar motor and sensory efferent neurons fated to project toward the dorsolateral cranial nerve exit points, are still largely restricted to even-numbered rhombomeres. In electroporated embryos, neurogenesis was found to be severely delayed and impaired on the experimental side, although not completely prevented. Most noticeable was a significant reduction in the number of neurons in even-numbered, and to a lower extent in odd-numbered rhombomeres (Fig. 6A,B). Co-labeling with an anti-EphA4 antibody demonstrated that the severity of this phenotype correlated with the extent of *EphA4* induction and showed a reduced density of neuronal cell bodies in *EphA4*-expressing territories (Fig. 6C,D). Axons growing from cell bodies located outside of the *EphA4*-positive patches avoided those, to stay within the *EphA4*-negative remnants of even-numbered rhombomeres (Fig. 6D, arrowheads). Finally, the few neurons located in the *EphA4*-positive patches appeared impaired in their ability to develop processes (Fig. 6D, cf. *EphA4*-positive patches with r3/r5 on the control side). These data indicate that the modifications of molecular identity are correlated with defects in neurogenesis timing and axonal progression. They also suggest that the delayed neurogenesis in odd-numbered rhombomeres is a consequence of *Krox-20* expression.

Discussion

In this study, we have used gain-of-function experiments to establish the role of *Krox-20* in the specification of odd-numbered rhombomere identity in the hindbrain. In addition, we have discovered that *Krox-20* is able to promote its own expression non cell-autonomously. We propose a model for hindbrain development in which this mechanism is involved in the homogenization and possibly expansion of odd-numbered rhombomeres.

Krox-20 is sufficient to enforce odd-numbered rhombomere identity in the r2-r6 region in a cell-autonomous manner

Krox-20 misexpression results in marked modifications of patterns of gene expression in the hindbrain. We argue that these patterns primarily reflect changes in cell iden-

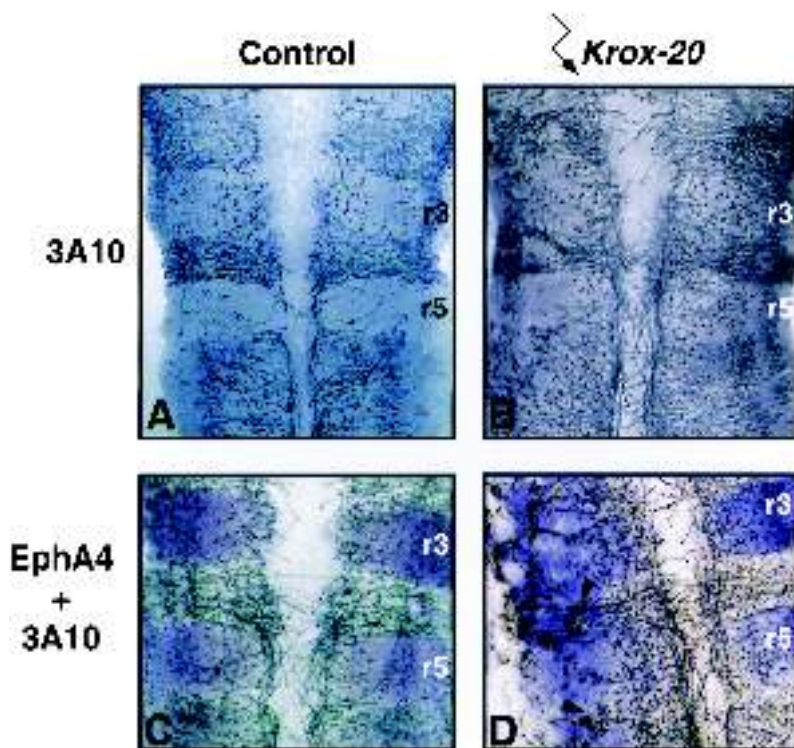


Figure 6. Early neurogenesis is affected by ectopic *Krox-20* expression. Flat-mounted hindbrains from control (A,C) and *Krox-20* electroporated (B,D) embryos. (A,B) Immunohistochemistry with an antibody directed against neurofilaments (3A10), which reveals cell bodies and growing axons of differentiated neurons. The control embryo in A was electroporated with a mutant *Krox-20* allele (R409W). In B, neurogenesis is impaired and delayed on the electroporated side. (C,D) Double labeling with antibodies directed against neurofilaments and EphA4. The embryo in C was not electroporated. In the *Krox-20* electroporated case (D), the density of neurofilament staining is lower in EphA4-positive patches in r4 and r6 and in contrast reinforced in their vicinity (arrowheads). Electroporated side is on the left.

tity rather than inappropriate cell migrations. Indeed we have shown that the presence of *EphA4*-positive patches within r2 cannot be due solely to migration of cells from r3. Our data, however, do not exclude a contribution of cell migration to the formation of the ectopic patches.

Thus, ectopic expression of *Krox-20* in the r2–r6 region is sufficient in even-numbered rhombomeres to convert the gene expression pattern into that of an odd-numbered rhombomere. More precisely, in r2 and r4, the affected cells express *Krox-20*, *EphA4*, and *Hoxa2* and are negative for *Hoxb1*, *Hoxa3*, *Hoxb3*, *mafB/kr*, and *foliastatin*, consistent with an r3-like identity. In r6, the converted cells express *Krox-20*, *EphA4*, *mafB/kr*, *Hoxa2*, and *Hoxa3*, and are negative for *Hoxb1*, consistent with an r5-like identity. Moreover, analysis of neurogenesis in the *EphA4*-positive patches in r2, r4 and r6 indicates that it is delayed, a characteristic of odd-numbered rhombomeres. Together, these data indicate that *Krox-20* can enforce odd-numbered identity in the r2–r6 region. The occurrence of r5 characteristics in r6 patches is likely to result from the presence of the product of the *mafB/kr* gene which, together with *Krox-20*, may specify r5 identity (Manzanares et al. 1999; M. Manzanares, unpubl.). Finally, in r1, ectopic expression of *Krox-20* also leads to the formation of *Krox-20*-positive cell patches, whereas none of the tested *Krox-20* targets is activated.

Krox-20 has been previously implicated in the direct transcriptional activation of several rhombomere-specific genes (*Hoxb2*, *Hoxa2*, *Hoxb3*, and *EphA4*; Sham et al. 1993; Nonchev et al. 1996; Theil et al. 1998; Manzanares et al. unpubl.). The present study is consistent with these data, and, together, they suggest that the

specification of odd-numbered rhombomere identity by *Krox-20* is a cell-autonomous phenomenon. Our analysis also revealed that *Krox-20* can repress *Hoxb1*. This observation was not expected as the inactivation of *Krox-20* does not lead to an extension of r4 (Schneider-Maunoury et al. 1993). In addition, it has been proposed that *Hoxb1* and *Hoxa1* repress *Krox-20* (Barrow et al. 2000). Together, these facts suggest that expressions of *Krox-20* and *Hoxb1* are mutually exclusive, presumably preventing the appearance of cells with an aberrant molecular identity.

Non cell-autonomous *Krox-20* autoregulation

This work has also provided evidence for non cell-autonomous autoactivation of *Krox-20* expression. This conclusion is based on the observation that, following electroporation of the mouse gene, mouse *Krox-20* mRNA is restricted to isolated cells, whereas the endogenous gene appears expressed in large cell patches that can be observed from r1 to r7/r8. Two other mechanisms could have been invoked to explain this observation: cell migration from odd-numbered rhombomeres and loss of exogenous *Krox-20* expression in transfected cells or their progeny. We have already provided arguments against the involvement of cell migration in the early formation of the *EphA4*-positive patches, which are also *Krox-20*-positive. The observation of abundant endogenous *Krox-20* expression in r1 within 14 h after electroporation is also inconsistent with this possibility. Loss of exogenous *Krox-20* expression is also not supported by

our data. Indeed, double labeling experiments after co-transcription of *lacZ* and *Krox-20* in electroporated cells indicate that the cells expressing only the endogenous *Krox-20* gene have not previously expressed the exogenous gene.

As indicated, the formation of the *Krox-20*-positive patches is restricted to the hindbrain, whereas ectopic expression of the mouse gene can be observed beyond these AP limits. This observation can be explained by the existence of positively acting factors restricted to the r1–r7/r8 region or negatively acting factors present outside of the hindbrain and regulating signaling by *Krox-20*-positive cells or responsiveness to the signal by *Krox-20*-negative cells. Caudally, the limits of action of exogenous *Krox-20* appear to correlate in terms of induction of both *EphA4* and endogenous *Krox-20*. In contrast, rostrally, the domain of induction of *EphA4* is more restricted than that of endogenous *Krox-20*, as *EphA4* is never expressed in r1. The same is true for *Hoxa2*, another direct target of *Krox-20* (Fig. 3E). These data suggest that *EphA4* or *Hoxa2* activation by *Krox-20* requires additional factor(s) absent in the part of the CNS rostral to r2 or is subject to repression by factor(s) specifically present in this region.

A model for odd-numbered rhombomere formation

The discovery of the non cell-autonomous autoinduction of *Krox-20* raises the issue of the functional significance of this phenomenon during hindbrain development. We propose that it plays an essential role in the establishment and homogenization of r3 and r5 and present a model for hindbrain patterning in the chick, integrating expression and genetic data derived from the mouse (Fig. 7b). This model accounts for the evolution of the *Krox-20* and *Hoxb1* expression patterns (Fig. 7a) and is depicted in the following steps.

(1) Early after the onset of somitogenesis, *Hoxa1* and *Hoxb1* expression extends within the unsegmented neural plate up to an anterior limit corresponding to the prospective r3/r4 boundary (Fig. 7A; Murphy and Hill 1991; Barrow et al. 2000). At around the 4- to 5-somite stages, *Krox-20* is induced in a narrow stripe of cells immediately rostral to the *Hoxa1* domain (Fig. 7B,b; Schneider-Maunoury et al. 1993; Irving et al. 1996; Barrow et al. 2000). The signals responsible for this activation have not been identified, although FGFs may be involved (Marin and Charnay 2000). *Hoxa1* and *Hoxb1* have been proposed to repress *Krox-20* expression in a cell-autonomous manner (Barrow et al. 2000) and therefore, may antagonize appearance of *Krox-20*-positive cells within their expression domain.

(2) At around the 7-somite stage, a second, more caudal band of sparse cells activates *Krox-20* expression (Fig. 7C,D,b. Schneider-Maunoury et al. 1993). Meanwhile, in prospective r3, *Krox-20*-expressing cells activate the expression of other r3-specific genes and repress *Hoxb1* and *follistatin* expression presumably in a cell-autonomous manner. In addition, *Krox-20*-expressing cells induce neighboring cells to activate *Krox-20* expression in a non

cell-autonomous manner. This recruitment leads to homogenization and possibly extension of r3. This step is in accordance with the analysis of *LacZ* expression in *Krox-20^{LacZ/LacZ}* knock-in mouse mutant embryos, where β -galactosidase-positive cells appear scattered as compared with heterozygous embryos (Schneider-Maunoury et al. 1993). In addition, we have demonstrated previously that in homozygous *Hoxa1* mutant embryos, cell patches with r2 identity are observed within r3, and this phenotype is markedly enhanced when the embryos are also heterozygous for the *Krox-20^{LacZ}* mutation (Helmbacher et al. 1998). We now propose that this genetic interaction involves the promotion, by a signal derived from prospective r4, of the induction of *Krox-20* expression in neighboring cells by *Krox-20*-positive cells. Therefore, signaling from *Krox-20*- and *Hoxa1/Hoxb1*-expressing cells synergizes to enforce recruitment of additional r3 cells. This mechanism may ensure, on the one hand, that the territory immediately rostral to r4 expresses *Krox-20* and, on the other hand, that the expansion of r3 is restricted anteriorly because of limited diffusion of the r4-derived signal.

(3) At around the 9-somite stage, r3 has largely increased in size and is becoming a homogeneous, well-defined, *Krox-20*-positive territory, presumably due to the combination of recruitment of adjacent cells with segregation from even-numbered cells governed by Eph receptors–ephrins interactions, themselves controlled by *Krox-20* (Fig. 7a, E and 7b; Mellitzer et al. 1999; Xu et al. 1999). In prospective r5, levels of *Hoxa1* and *Hoxb1* have decreased, which may allow efficient recruitment of additional *Krox-20*-positive cells by *Krox-20*-expressing cells and progressive homogenization of the rhombomere.

(4) By the 12-somite stage, *Krox-20* has activated the expression of *Nab1* and *Nab2* (Mechta-Grigoriou et al. 2000). These genes encode specific antagonists of *Krox-20* activity and their expression is reinforced at the vicinity of rhombomere interfaces (Mechta-Grigoriou et al. 2000). We propose that this reinforcement may also participate in preventing further expansion of r3 and r5.

(5) After the 20-somite stage, the antagonistic action of the Nab proteins on *Krox-20* may also be involved in enforcing cells located at the level of the r3/r4 and r4/r5 interfaces to adopt specific fates, leading to the formation of *Krox-20*- and *Hoxb1*-negative boundaries.

In conclusion, our work suggests that *Krox-20* is involved in multiples steps in hindbrain patterning, both in proper segmentation and in acquisition of AP identity. The ground-state fate of the prospective r2–r6 region may be to acquire even-numbered rhombomere identity. Activation of *Krox-20* in a few cells leads to the formation, homogenization, extension, and later stabilization of territories, which segregate, from even-numbered domains. In parallel, these prospective odd-numbered rhombomeres also acquire their proper identity under the control of *Krox-20*. Therefore, segmentation and identity specification are largely intertwined in the developing vertebrate hindbrain.

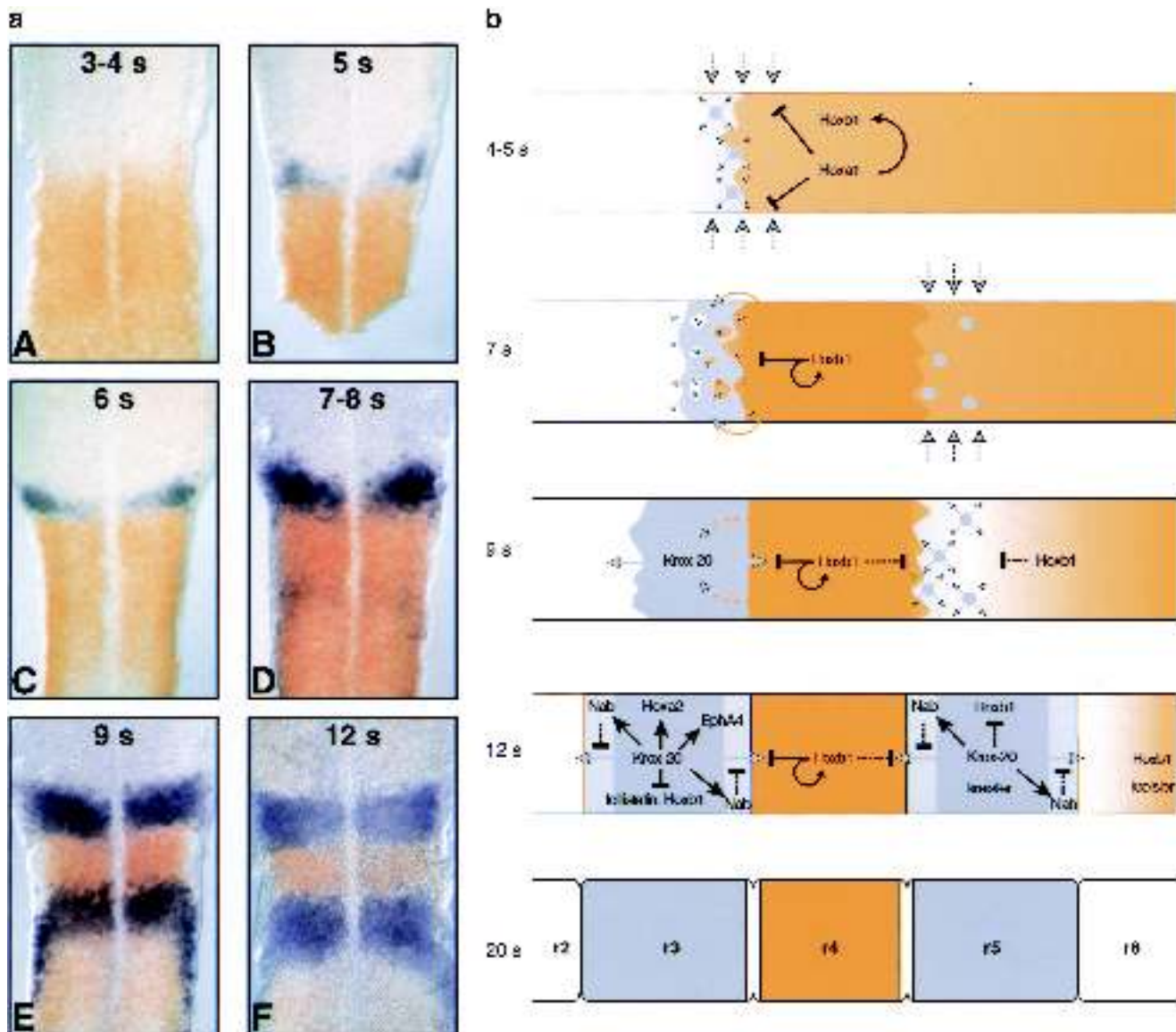


Figure 7. A model for chick hindbrain patterning. (a) Time-course of *Krox-20* and *Hoxb1* expression in the developing chick hindbrain. (A–F) Flat mounts of embryos at the indicated somite stages of development, subjected to double in situ hybridization with chicken *Krox-20* (purple) and *Hoxb1* (red) probes. (b) Schematic representation of the development of *Krox-20* and *Hoxb1* expression and of putative genetic interactions. For a detailed description, see Discussion. *Hoxa1/Hoxb1*- and *Krox-20*-expressing territories are represented in orange and purple-grey, respectively. (Light purple-grey) High level expression of *Nab1* and *Nab2* within the *Krox-20*-positive territories. (Black arrows and bars) Established (solid stem) or putative (dashed stem) cell-autonomous induction and repression/inhibition, respectively. (Purple-grey arrowheads) Different putative signals involved in *Krox-20* activation and originating from unidentified tissues (dashed stem), *Krox-20*-expressing cells (purple-grey stem) and prospective r4 (orange stem). Somite stages are indicated on the left.

Materials and methods

Plasmid constructs

The *Krox-20* expression plasmid, pAdRSVKrox20, was constructed by cloning of a 5-kb *AvrII*–*SpeI* genomic fragment (Chavrier et al. 1989) containing the gene plus 1.2 kb of 3' flanking sequences, into the pAdRSV β gal plasmid (Le Gal La Salle et al. 1993), after removal of the β -galactosidase coding sequence. pAdRSVKrox20Myc differs from pAdRSVKrox20 by the insertion of a Myc epitope coding sequence (5'-GAACAGAACT

TATCTCAGAGGAAGACCTT-3') just before the *Krox-20* STOP codon. The two constructs are expressed at similar levels and equivalently activate transcription as assayed in transiently transfected cells according to Vesque and Charnay (1992; data not shown). pAdRSVKrox20R409W was constructed by directed mutagenesis with the oligonucleotide 5'-GGCCGCAAGTTT GCCTGGAGTGACGAAAGG-3' using the Transformer Site-Directed Mutagenesis Kit (Clontech). pAdRSV β galKrox20 was constructed by cloning of the *Krox-20* gene downstream of the encephalomyocarditis virus IRES (Ghattas et al. 1991) followed

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by insertion of the resulting fragment downstream of the *LacZ* gene in pAdRSV β gal. For all constructs, cloning junctions were checked by sequencing, and the molecular weight of the encoded proteins was verified by immunoblots performed on extracts of transiently transfected 293 cells.

In ovo electroporation

Commercial fertilized hens eggs were incubated typically for 30 h, up to stages HH8–HH10, before injection. DNA was resuspended at a concentration of 1 μ g/ μ L in 10 mM Tris (pH 8) solution, and 0.025% Fast-Green (Sigma) was added. The DNA solution was injected into the embryo neural tube by use of a stretched glass capillary, anteriorward from the level of approximately the third somite. A drop of L15 medium (Life Technologies) was poured onto the egg membrane and electroporation was performed with a BTX820 electroporator (Quantum) and CUY611 platinum-coated electrodes (Tr Tech) as described (Itasaki et al. 1999), using the following parameters: four pulses of 25 V and 50 ms at a frequency of 1 Hz. Embryos were harvested in phosphate buffered saline (PBS) and fixed in paraformaldehyde (PFA, 4% in PBS) for 2–3 h or 8–24 h for whole-mount immunohistochemistry or in situ hybridization respectively, then dehydrated in methanol. For detection of β -galactosidase activity, embryos were fixed in PFA for 45 min and stained with X-Gal as described (Schneider-Maunoury et al. 1993), or with Blue-Gal (Sigma) following manufacturer conditions. When subsequent in situ hybridization was required, staining was performed in 0.2% PFA, and the embryos were post-fixed overnight in 4% PFA. Unless otherwise indicated, the embryos were electroporated between stages HH8 and HH10 and collected 24 h later. In the type of experiment presented in Figure 1G, embryos were electroporated at stage HH10 and immediately cut transversally at the presumed level of r2 (according to morphological landmarks), using a tungsten needle. The resulting anterior part was then slightly rotated to avoid subsequent healing of the two parts of the neural tube.

Whole-mount immunohistochemistry

Immunohistochemical detection of proteins was performed on dissected neural tubes with the following primary antibodies and dilutions: rabbit polyclonal antibodies directed against Krox-20 (BAbCO, 1:500) or EphA4 (Becker et al. 1995; 1:20,000), and mouse monoclonal antibodies directed against EphA4 (Hirano et al. 1998; 1:20), chick neurofilaments, (DSHB 3A10, 1:50) or the Myc epitope (9E10, 1:5). The following secondary antibodies were used at a 1:200 dilution: peroxidase-coupled goat antibody directed against mouse IgG (Sigma), alkaline phosphatase-coupled goat antibody directed against mouse IgG (Vector), biotinylated donkey antibody directed against rabbit IgG (Amersham), or biotinylated hamster antibody directed against mouse IgG (Vector). The biotinylated antibodies were detected using streptavidin-horse radish peroxidase (Amersham, 1:500). Peroxidase activity was revealed with diaminobenzidine (Sigma; brown staining). Occasionally, the staining was enhanced with nickel ammonium (dark blue staining) according to Adams (1981). Alkaline phosphatase activity was detected using 4-nitroblue tetrazolium chloride/5-bromo 4-chloro 3-indolyl phosphate (NBT/BCIP, Roche; purple staining). In some cases, 2-(4-5-(4-nitrophenyl) 3-phenyltetrazolium chloride (INT/BCIP, Roche; orange/red staining) was used instead.

Whole-mount in situ hybridization

In situ hybridization was performed essentially as described (Wilkinson and Nieto 1993), using digoxigenin-labeled ribo-

probes. For double hybridization, one of the probe was labeled with fluorescein-UTP. Digoxigenin and fluorescein were then detected sequentially using alkaline phosphatase-coupled antibodies (Roche, 1:2000). NBT/BCIP (purple) staining was always carried out first, and the antibody was stripped in 0.1 M glycine-HCl (pH 2.2). The embryo was then incubated with the second antibody and stained with INT/BCIP (orange/red). The riboprobes were as follows: *cHoxb1* (Guthrie et al. 1992), *mKrox-20* (Wilkinson et al. 1989), *Krox-20* zinc finger coding region (Nieto et al. 1991), *cEphA4* (Sajjadi and Pasquale 1993), *cfollistatin* (Graham and Lumsden 1996), *cHoxa3*, *cHoxb3* (Grapin-Botton et al. 1995), *cHoxa2* (Prince and Lumsden 1994), *mKrox-20-3'* is a 500-bp *NheI*–*NsiI* fragment derived from the 3'-UTR of mouse *Krox-20* (Chavrier et al. 1988) and presenting no similarity to the chicken gene. *cKrox-20-3'* is a 700-bp *StuI*–*PstI* fragment derived from the 3'-UTR of chicken *Krox-20* and presenting no similarity to the mouse gene (this work).

Isolation of the chicken Krox-20 gene

A chicken genomic BAC library (Crooijmans et al. 2000) provided by the UK HGMP Resource Centre as high-density gridded filters was screened with a 150-bp cDNA probe, presumably of chick origin, encoding part of the Krox-20 DNA-binding domain (Nieto et al. 1991). Three strongly hybridizing clones were identified, and a common 10-kb *HindIII* fragment containing the entire *Krox-20* gene was subcloned into BlueScript (Stratagene) and used for restriction mapping and nucleotide sequencing. This analysis revealed that the original cDNA probe used for BAC screening was only 89% identical to the corresponding sequence of the chicken gene, while it showed 98% identity to the *Xenopus* gene. Therefore, this sequence is more likely to be of *Xenopus* origin.

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References

- Adams, J.C. 1981. Heavy metal intensification of DAB-based HRP reaction product. *J. Histochem. Cytochem.* **29**: 775.
- Barrow, J.R., Stadler, H.S., and Capecchi, M.R. 2000. Roles of Hoxa1 and Hoxa2 in patterning the early hindbrain of the mouse. *Development* **127**: 933–944.
- Becker, N., Gilardi-Hebenstreit, P., Seitanidou, T., Wilkinson, D., and Charnay, P. 1995. Characterisation of the Sek-1 receptor tyrosine kinase. *FEBS Lett.* **368**: 353–357.
- Birgbauer, E. and Fraser, S.E. 1994. Violation of cell lineage restriction compartments in the chick hindbrain. *Development* **120**: 1347–1356.
- Bronner-Fraser, M. 1995. Patterning of the vertebrate neural

Krox-20 autoregulation and hindbrain patterning

- crest. *Perspect. Dev. Neurobiol.* **3**: 53–62.
- Chavrier, P., Zerial, M., Lemaire, P., Almendral, J., Bravo, R., and Charnay, P. 1988. A gene encoding a protein with zinc fingers is activated during G0/G1 transition in cultured cells. *EMBO J.* **7**: 29–35.
- Chavrier, P., Janssen-Timmen, U., Mattei, M.G., Zerial, M., Bravo, R., and Charnay, P. 1989. Structure, chromosome location, and expression of the mouse zinc finger gene Krox-20: Multiple gene products and coregulation with the proto-oncogene c-fos. *Mol. Cell. Biol.* **9**: 787–797.
- Crooijmans, R.P., Vrebalov, J., Dijkhof, R.J., van der Poel, J.J., and Groenen, M.A. 2000. Two-dimensional screening of the Wageningen chicken BAC library. *Mamm. Genome* **11**: 360–363.
- Eichmann, A., Grapin-Botton, A., Kelly, L., Graf, T., Le Douarin, N. M., and Sieweke, M. 1997. The expression pattern of the mafB/kr gene in birds and mice reveals that the kreisler phenotype does not represent a null mutant. *Mech. Dev.* **65**: 111–122.
- Fraser, S., Keynes, R., and Lumsden, A. 1990. Segmentation in the chick embryo hindbrain is defined by cell lineage restrictions. *Nature* **344**: 431–435.
- Ghattas, I.R., Sanes, J.R., and Majors, J.E. 1991. The encephalomyocarditis virus internal ribosome entry site allows efficient coexpression of two genes from a recombinant provirus in cultured cells and in embryos. *Mol. Cell. Biol.* **11**: 5848–5859.
- Gilardi-Hebenstreit, P., Nieto, M.A., Frain, M., Mattei, M.G., Chestier, A., Wilkinson, D.G., and Charnay, P. 1992. An Eph-related receptor protein tyrosine kinase gene segmentally expressed in the developing mouse hindbrain. *Oncogene* **7**: 2499–2506.
- Graham, A. and Lumsden, A. 1996. Interactions between rhombomeres modulate Krox-20 and follistatin expression in the chick embryo hindbrain. *Development* **122**: 473–480.
- Grapin-Botton, A., Bonnin, M.A., McNaughton, L.A., Krumlauf, R., and Le Douarin, N.M. 1995. Plasticity of transposed rhombomeres: Hox gene induction is correlated with phenotypic modifications. *Development* **121**: 2707–2721.
- Guthrie, S. and Lumsden, A. 1991. Formation and regeneration of rhombomere boundaries in the developing chick hindbrain. *Development* **112**: 221–229.
- Guthrie, S., Muchamore, I., Kuroiwa, A., Marshall, H., Krumlauf, R., and Lumsden, A. 1992. Neuroectodermal autonomy of Hox-2.9 expression revealed by rhombomere transpositions. *Nature* **356**: 157–159.
- Guthrie, S., Prince, V., and Lumsden, A. 1993. Selective dispersal of avian rhombomere cells in orthotopic and heterotopic grafts. *Development* **118**: 527–538.
- Hamburger, V. and Hamilton, H.L. 1951. A series of normal stages in the development of the chick embryo. *J. Morphology* **88**: 49–92.
- Helmbacher, F., Pujades, C., Desmarquet, C., Frain, M., Rijli, F.M., Chambon, P., and Charnay, P. 1998. Hoxa1 and krox-20 synergize to control the development of rhombomere 3. *Development* **125**: 4739–4748.
- Hirano, S., Tanaka, H., Ohta, K., Norita, M., Hoshino, K., Meguro, R., and Kase, M. 1998. Normal ontogenic observations on the expression of Eph receptor tyrosine kinase, Cdk8, in chick embryos. *Anat. Embryol.* **197**: 187–197.
- Irving, C., Nieto, M.A., DasGupta, R., Charnay, P., and Wilkinson, D.G. 1996. Progressive spatial restriction of Sek-1 and Krox-20 gene expression during hindbrain segmentation. *Dev. Biol.* **173**: 26–38.
- Itasaki, N., Bel-Vialar, S., and Krumlauf, R. 1999. ‘Shocking’ developments in chick embryology: Electroporation and in ovo gene expression. *Nat. Cell Biol.* **1**: E203–E207.
- Kontges, G. and Lumsden, A. 1996. Rhombencephalic neural crest segmentation is preserved throughout craniofacial ontogeny. *Development* **122**: 3229–3242.
- Le Gal La Salle, G., Robert, J.J., Berrard, S., Ridoux, V., Stratford-Perricaudet, L.D., Perricaudet, M., and Mallet, J. 1993. An adenovirus vector for gene transfer into neurons and glia in the brain. *Science* **259**: 988–990.
- Lumsden, A. 1990. The cellular basis of segmentation in the developing hindbrain. *Trends Neurosci.* **13**: 329–335.
- Lumsden, A. and Keynes, R. 1989. Segmental patterns of neuronal development in the chick hindbrain. *Nature* **337**: 424–428.
- Lumsden, A. and Krumlauf, R. 1996. Patterning the vertebrate neuraxis. *Science* **274**: 1109–1115.
- Manzanares, M., Trainor, P.A., Nonchev, S., Ariza-McNaughton, L., Brodie, J., Gould, A., Marshall, H., Morrison, A., Kwan, C.T., Sham, M.H., Wilkinson, D.G., and Krumlauf, R. 1999. The role of kreisler in segmentation during hindbrain development. *Dev. Biol.* **211**: 220–237.
- Marin, F. and Charnay, P. 2000. Hindbrain patterning: FGFs regulate krox20 and mafB/kr expression in the otic/preotic region. *Development* **127**: 4925–4935.
- Mechta-Grigoriou, F., Garel, S., and Charnay, P. 2000. Nab proteins mediate a negative feedback loop controlling Krox-20 activity in the developing hindbrain. *Development* **127**: 119–128.
- Mellitzer, G., Xu, Q., and Wilkinson, D.G. 1999. Eph receptors and ephrins restrict cell intermingling and communication. *Nature* **400**: 77–81.
- . 2000. Control of cell behaviour by signalling through Eph receptors and ephrins. *Curr. Opin. Neurobiol.* **10**: 400–408.
- Mizuguchi, H., Xu, Z., Ishii-Watabe, A., Uchida, E., and Hayakawa, T. 2000. IRES-dependent second gene expression is significantly lower than cap-dependent first gene expression in a bicistronic vector. *Mol. Ther.* **1**: 376–382.
- Murphy, P. and Hill, R.E. 1991. Expression of the mouse labial-like homeobox-containing genes, Hox 2.9 and Hox 1.6, during segmentation of the hindbrain. *Development* **111**: 61–74.
- Nieto, M.A., Bradley, L.C., and Wilkinson, D.G. 1991. Conserved segmental expression of Krox-20 in the vertebrate hindbrain and its relationship to lineage restriction. *Development* (Suppl.) **59**–62.
- Nonchev, S., Vesque, C., Maconochie, M., Seitaniidou, T., Ariza-McNaughton, L., Frain, M., Marshall, H., Sham, M.H., Krumlauf, R., and Charnay, P. 1996. Segmental expression of Hoxa-2 in the hindbrain is directly regulated by Krox-20. *Development* **122**: 543–554.
- Prince, V. and Lumsden, A. 1994. Hoxa-2 expression in normal and transposed rhombomeres: Independent regulation in the neural tube and neural crest. *Development* **120**: 911–923.
- Sajjadi, F.G. and Pasquale, E.B. 1993. Five novel avian Eph-related tyrosine kinases are differentially expressed. *Oncogene* **8**: 1807–1813.
- Schneider-Maunoury, S., Topilko, P., Seitandou, T., Levi, G., Cohen-Tannoudji, M., Pournin, S., Babinet, C., and Charnay, P. 1993. Disruption of Krox-20 results in alteration of rhombomeres 3 and 5 in the developing hindbrain. *Cell* **75**: 1199–1214.
- Schneider-Maunoury, S., Gilardi-Hebenstreit, P., and Charnay, P. 1998. How to build a vertebrate hindbrain. Lessons from genetics. *C.R. Acad. Sci. Iii* **321**: 819–834.
- Seitaniidou, T., Schneider-Maunoury, S., Desmarquet, C., Wilkinson, D.G., and Charnay, P. 1997. Krox-20 is a key regulator of rhombomere-specific gene expression in the de-

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- veloping hindbrain. *Mech. Dev.* **65**: 31–42.
- Sham, M.H., Vesque, C., Nonchev, S., Marshall, H., Frain, M., Gupta, R.D., Whiting, J., Wilkinson, D., Charnay, P., and Krumlauf, R. 1993. The zinc finger gene *Krox20* regulates *HoxB2* (*Hox2.8*) during hindbrain segmentation. *Cell* **72**: 183–196.
- Studer, M., Lumsden, A., Ariza-McNaughton, L., Bradley, A., and Krumlauf, R. 1996. Altered segmental identity and abnormal migration of motor neurons in mice lacking *Hoxb-1*. *Nature* **384**: 630–634.
- Sundin, O.H. and Eichele, G. 1990. A homeodomain protein reveals the metameric nature of the developing chick hindbrain. *Genes Dev.* **4**: 1267–1276.
- Theil, T., Frain, M., Gilardi-Hebenstreit, P., Flenniken, A., Charnay, P., and Wilkinson, D.G. 1998. Segmental expression of the *EphA4* (*Sek-1*) receptor tyrosine kinase in the hindbrain is under direct transcriptional control of *Krox-20*. *Development* **125**: 443–452.
- Topilko, P., Schneider-Maunoury, S., Levi, G., Baron-Van Evercooren, A., Chennoufi, A.B., Seitanidou, T., Babinet, C., and Charnay, P. 1994. *Krox-20* controls myelination in the peripheral nervous system. *Nature* **371**: 796–799.
- Vaage, S. 1969. The segmentation of the primitive neural tube in chick embryos (*Gallus domesticus*). A morphological, histochemical and autoradiographical investigation. *Ergeb Anat. Entwicklungsgesch* **41**: 3–87.
- Vesque, C. and Charnay, P. 1992. Mapping functional regions of the segment-specific transcription factor *Krox-20*. *Nucleic Acids Res.* **20**: 2485–2492.
- Warner, L.E., Mancias, P., Butler, I.J., McDonald, C.M., Keppen, L., Koob, K.G., and Lupski, J.R. 1998. Mutations in the early growth response 2 (*EGR2*) gene are associated with hereditary myelinopathies. *Nat. Genet.* **18**: 382–384.
- Warner, L.E., Svaren, J., Milbrandt, J., and Lupski, J. R. 1999. Functional consequences of mutations in the early growth response 2 gene (*EGR2*) correlate with severity of human myelinopathies. *Hum. Mol. Genet.* **8**: 1245–1251.
- Wilkinson, D.G. and Nieto, M.A. 1993. Detection of messenger RNA by in situ hybridization to tissue sections and whole mounts. *Methods Enzymol.* **225**: 361–373.
- Wilkinson, D.G., Bhatt, S., Chavrier, P., Bravo, R., and Charnay, P. 1989. Segment-specific expression of a zinc-finger gene in the developing nervous system of the mouse. *Nature* **337**: 461–464.
- Xu, Q., Mellitzer, G., Robinson, V., and Wilkinson, D.G. 1999. In vivo cell sorting in complementary segmental domains mediated by Eph receptors and ephrins. *Nature* **399**: 267–271.



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