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Hindbrain patterning: *Krox20* couples segmentation and specification of regional identity

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SUMMARY

We have previously demonstrated that inactivation of the *Krox20* gene led to the disappearance of its segmental expression territories in the hindbrain, the rhombomeres (r) 3 and 5. We now performed a detailed analysis of the fate of prospective r3 and r5 cells in *Krox20* mutant embryos. Genetic fate mapping indicates that at least some of these cells persist in the absence of a functional Krox20 protein and uncovers the requirement for autoregulatory mechanisms in the expansion and maintenance of *Krox20*-expressing territories. Analysis of even-numbered rhombomere molecular markers demonstrates that in *Krox20*-null embryos, r3 cells acquire r2 or r4 identity, and r5 cells acquire r6 identity. Finally, study of embryonic chimaeras between *Krox20* homozygous mutant and wild-type cells shows that the mingling properties of r3/r5 mutant cells are changed towards those of even-numbered rhombomere cells. Together, these data demonstrate that *Krox20* is essential to the generation of alternating odd- and even-numbered territories in the hindbrain and that it acts by coupling the processes of segment formation, cell segregation and specification of regional identity.

Key words: Segmentation, Hindbrain, Rhombomere, *Krox20*, Cre recombinase, Fate mapping, Mouse

INTRODUCTION

The establishment of functional diversity in the vertebrate central nervous system (CNS) largely relies on its early patterning. This involves the subdivision of the neural tube into distinct territories along its anteroposterior (AP) and dorsoventral (DV) axes during early embryogenesis. These territories are defined by the expression of specific regulatory genes and their limits often correspond to morphological landmarks and/or compartment boundaries, suggesting that they constitute developmental units (Lumsden and Krumlauf, 1996; Shimamura et al., 1997). A striking illustration of such a patterning process is provided by the hindbrain, which is transiently segmented along its AP axis into seven or eight bulges called rhombomeres. Rhombomeres constitute units of specific gene expression and several Hox genes have been shown to participate in the specification of their identity (Zhang et al., 1994; Alexandre et al., 1996; Goddard et al., 1996; Studer et al., 1996; Gavalas et al., 1997; Gavalas et al., 1998; Bell et al., 1999; Jungbluth et al., 1999). Sharp limits of gene expression at rhombomere boundaries are thought to result, at least in part, from the acquisition of different mingling properties by cells from adjacent rhombomeres, leading to the sorting of even- and odd-numbered rhombomere cells (Guthrie and Lumsden, 1991; Guthrie et al., 1993; Wizenmann and Lumsden, 1997). Members of the Eph family of receptor tyrosine kinase and their ephrin ligands have been shown to be involved in this segregation process (Xu et al., 1999). Evidence that rhombomeres also constitute functional patterning units has come from the analysis of several hindbrain neuronal populations, including those for branchiomotor nerves, which originate in pairs of rhombomeres (Lumsden and Keynes, 1989; Clarke and Lumsden, 1993). Finally, rhombomeres also participate in the patterning of neural crest cells and in the control of their migration towards the branchial arches and cranial ganglia, thereby playing an additional crucial role in the establishment of craniofacial organisation (Kontges and Lumsden, 1996; Trainor and Krumlauf, 2000).

A fundamental question concerning functional and developmental units within the CNS is how they are established. Several genes have been demonstrated, mainly using loss-of-function mutations in the mouse, to be required for the establishment of such territories (Joyner, 1996; Schneider-Maunoury et al., 1998; Acampora et al., 1999). These mutations generally lead to the loss of whole brain territories. In the hindbrain, this is the case for mutations in *Mafb/kr*, *Krox20/Egr2*, *Hoxa1* and *Gbx2*, which result in disappearance of specific rhombomeres (Schneider-Maunoury et al., 1998). Although such phenotypes illustrate the importance of these genes in the patterning process, very little has been revealed about their precise function at the cellular level. An important issue in this respect is the fate of the cells that constitute the affected territories. Different hypotheses have been proposed to explain loss of brain territories: cell death, impairment of cell proliferation or early changes in cell specification leading to the incorporation of the cells into adjacent territories (McMahon et al., 1992; Carpenter et al., 1993; Dolle et al., 1993; Wurst et al., 1994). In no case, however, has the issue been clearly resolved.

We have investigated this question in the case of *Krox20* loss of function. *Krox20* encodes a zinc-finger transcription factor and is expressed in the hindbrain in two non-adjacent stripes that prefigure and then coincide with r3 and r5 (Wilkinson et al., 1989a), and in the neural crest originating from r5 and r6 (Wilkinson et al., 1989a; Nieto et al., 1995). *Krox20* has been shown to be a major regulator of gene expression in these rhombomeres, controlling the expression of numerous downstream regulatory genes (Seitanidou et al., 1997; Schneider-Maunoury et al., 1998; Giudicelli et al., 2001). Among these, the Hox genes *Hoxa2*, *Hoxb2* and *Hoxb3*, and the receptor tyrosine kinase gene *Epha4* have been demonstrated to constitute direct targets (Sham et al., 1993; Nonchev et al., 1996; Theil et al., 1998) (M. Manzanares, J. Nardelli, P. Gilardi-Hebenstreit, H. Marshall, M.-T. Martinez-Pastor, R. Krumlauf and P. C., unpublished).

To study the function of *Krox20* in hindbrain development, we had previously produced a mutant allele, *Krox20^{lacZ}*, by inserting the *E. coli lacZ* open reading frame into the *Krox20* gene. Analysis of these mice showed that *Krox20* is required for the maintenance of r3 and r5 (Schneider-Maunoury et al., 1993; Schneider-Maunoury et al., 1997). Thus, in *Krox20$^{lacZ/lacZ}$* embryos at 10.5 days post coitum (dpc), the total length of the hindbrain is reduced and only four rhombomeres instead of six are morphologically conspicuous. Moreover, the motoneuronal component normally derived from r3 and r5 is absent. The loss of r3 and r5 in *Krox20$^{lacZ/lacZ}$* embryos is progressive: when assayed by whole-mount detection of β-galactosidase activity (X-gal staining), the two stripes appear at the right time and position along the AP axis, but disappear much more rapidly than in *Krox20$^{lacZ/+}$* embryos (Schneider-Maunoury et al., 1993).

We have performed a detailed analysis of the behaviour of r3 and r5 cells in *Krox20*-null embryos, and in embryonic chimaeras between wild-type and *Krox20* mutant cells. Besides the *Krox20^{lacZ}* allele described above, we made use of a *Krox20^{Cre}* allele (Voiculescu et al., 2000), which carries a Cre recombinase gene insertion into the locus. This *Krox20^{Cre}* mouse line, when crossed with transgenic mice carrying appropriate reporter genes, allowed tracing of the derivatives of *Krox20*-null cells normally programmed to express this gene. Our results indicate that at least some of these cells survive. They prematurely lose *Krox20* expression, adopt an even-numbered rhombomere identity, segregate from odd-numbered cells and mix with even-numbered cells. These data demonstrate that Krox20 plays multiple roles in hindbrain patterning, being involved in the early establishment and later maintenance of odd-numbered territories, in the specification of their identity and in the preservation of their integrity.

MATERIALS AND METHODS

Mouse lines and genotyping

All the mouse lines used in this study were maintained in a mixed C57Bl6/DBA2 background, except for ES cell line derivations (see below). For r2-specific human placental alkaline phosphatase (AP) staining, *r2-HPAP* (thereafter named *r2AP*) transgenic mice (Studer et al., 1996; Helmbacher et al., 1998) were used. For fate mapping analyses, four Cre-excision reporter lines (collectively termed *lox* mice) were used: the *CAG-CAT-Z* (Araki et al., 1995), *ACZL* (Akagi et al., 1997), *R26R* (Soriano, 1999) and *Z/AP* (Lobe et al., 1999) lines. They varied in the reporter gene used (*E. coli lacZ* or AP gene), the delay in the activation of the reporter gene and the intensity of staining.

To analyse Cre-induced reporter expression in the absence of a functional Krox20 protein, *Krox20$^{Cre/Cre}$ lox* embryos were initially produced by crossing *Krox20$^{Cre/+}$ lox* mice with *Krox20$^{Cre/+}$* mice. Surprisingly, most of the embryos resulting from these crosses were totally blue after X-gal staining. In addition, some of these had no *Krox20^{Cre}* allele, suggesting that recombination had occurred in the germline, before meiosis. This is probably due to a low level expression of *Krox20* in the germline, leading to expression of Cre and recombination of the *lox* reporter in the germline of *Krox20$^{Cre/+}$ lox* mice. To overcome this problem, *lox* mice were crossed with *Krox20$^{lacZ/+}$* mice, *Krox20$^{lacZ/+}$ lox* mice were then crossed to *Krox20$^{Cre/+}$* mice, and the expression of reporter was analysed in *Krox20$^{Cre/+}$ lox* and *Krox20$^{lacZ/Cre}$ lox* embryos. The mouse lines and embryos were genotyped by PCR as described in the original papers.

In situ hybridisation, X-gal, AP and antibody staining

Whole-mount in situ hybridisation (ISH) was performed as described previously (Wilkinson, 1992). The NBT/BCIP and INT/BCIP substrates (Roche) were used to obtain purple or orange precipitates, respectively. Antisense RNA probes were prepared from *Krox20* (Wilkinson et al., 1989a), *Hoxb1* (Wilkinson et al., 1989b), cadherin 6 (*Cdh6*) (Padilla et al., 1998) and *Mafb/kr* (Cordes and Barsh, 1994) cDNAs, and the *NeoR* gene. Whole-mount X-gal staining was performed as described (Schneider-Maunoury et al., 1993). For 14.5 to 16.5 dpc embryos, free-floating 90 μm parasagittal brain sections were obtained with a freezing microtome and were processed for X-gal staining. For combined X-gal staining and ISH, 0.2% paraformaldehyde was added to the X-gal reaction mixture (Houzelstein and Tajbakhsh, 1998). AP detection in the *r2AP* and *Z/AP* transgenic embryos was performed as described (Helmbacher et al., 1998). Krox20 immunohistochemistry was performed as described (Schneider-Maunoury et al., 1993), using a polyclonal antibody directed against the N-terminal part of Krox20 (Babco).

Cell death and cell proliferation analyses

Apoptotic cells in the hindbrain were detected by terminal-UTP-nick-end-labelling (TUNEL) staining (Gavrieli et al., 1992) adapted to whole-mount embryos (Conlon et al., 1995). To detect dying cells in live embryos, Nile Blue Sulphate (1/1000 w/v in water) was injected into the ventricles of 9.5 dpc embryos dissected in PBS. Injected embryos were incubated for 20 minutes at 37°C and then washed rapidly in phosphate-buffered saline (PBS). The hindbrain was opened dorsally and pictures were taken immediately. For bromodeoxyuridine pulse, pregnant females were injected intraperitoneally with 50 mg/g 5-bromo-2′-deoxyuridine (BrdU, Sigma) in 0.9% NaCl. The mice were sacrificed 1 hour later, and the collected embryos were fixed and processed for X-gal staining and then for paraffin sections. BrdU immunohistochemistry was performed as described (Garel et al., 1997). Sections were counterstained with nuclear Fast Red to allow the detection and counting of the total number of cells in r5. For M-phase staining, immunohistochemistry on whole embryos and paraffin sections was performed as previously described (Schneider-Maunoury et al., 1997), with an antibody directed against phosphorylated histone

H3 (Upstate Biotechnology). For counting M-phase cells, the number of H3P-positive cells in each area (X-gal-positive r5 territory, or whole hindbrain) was reported relative to the surface of the area in stage-matched embryos.

Generation of chimaeric embryos

To obtain *Krox20*$^{lacZ/lacZ}$ ES cells, blastocysts resulting from crosses of *Krox20*$^{lacZ/+}$ mice maintained in the inbred 129 background were set in culture as described (Kress et al., 1998). To obtain chimaeric embryos between *Krox20*$^{lacZ/+}$ (*Krox20*$^{lacZ/lacZ}$) and wild-type cells, *Krox20*$^{lacZ/+}$ (*Krox20*$^{lacZ/lacZ}$) ES cells were injected into wild-type C57Bl/6J blastocysts, and the resulting embryos were reimplanted into foster mothers. To obtain chimaeras between wild-type and *Epha4/lacZ* transgenic cells (Theil et al., 1998), or between wild type and *Krox20*$^{lacZ/Cre}$ *R26R* cells, morulae from appropriate crosses were aggregated in vitro, as described (Hogan et al., 1994). Resulting embryos were reimplanted into foster mothers.

RESULTS

Fate mapping of *Krox20*-expressing cells in the hindbrain and the neural crest

To follow r3 and r5 cells after the downregulation of *Krox20* gene expression, we produced a *Krox20*^{Cre} mutant allele, in which the Cre recombinase gene is inserted in place of the *Krox20*-coding sequence (Voiculescu et al., 2000). In order to trace the progeny of *Krox20*-expressing cells, *Krox20*$^{Cre/+}$ mice were crossed with different Cre-excision reporter (*lox*) mouse lines (Araki et al., 1995; Akagi et al., 1997; Lobe et al., 1999; Soriano, 1999). These *lox* mice carry a reporter gene (encoding *E. coli* β-galactosidase or human alkaline phosphatase (AP)) under the control of a ubiquitous promoter, and this reporter is permanently activated on excision of a cassette flanked by loxP sites. We have previously shown that in *Krox20*$^{Cre/+}$ *lox* mice, the pattern of activation of the *lox* reporter faithfully recapitulates that of *Krox20* in several tissues, and that expression is maintained in the progeny of *Krox20*-expressing cells (Voiculescu et al., 2000). In the present study, we have performed a detailed analysis of reporter activity in the hindbrain of *Krox20*$^{Cre/+}$ *lox* embryos.

Fig. 1 presents a comparison of reporter patterns obtained with two *lox* lines, *R26R* (Soriano, 1999) and *Z/AP* (Lobe et al., 1999), based on *lacZ* and *AP* expression, respectively, with the *lacZ* expression pattern in *Krox20*$^{lacZ/+}$ embryos. In *Krox20*$^{Cre/+}$ *lox* embryos, expression of the reporter was first detected around the six-somite (s) stage (data not shown). At the 10 s stage, in *Krox20*$^{lacZ/+}$ embryos, *lacZ* expression is strong in r3 and r5, and in a few isolated cells in even-numbered rhombomeres (Fig. 1A), as previously described for *Krox20* expression (Irving et al., 1996). In *Krox20*$^{Cre/+}$ *lox* embryos at the same stage, reporter expression is fainter and less homogeneous in r3 and r5 (Fig. 1B,I). This is likely to reflect a delay in the activation of the reporter in *Krox20*$^{Cre/+}$ *lox* embryos, owing to the time required for accumulation of the Cre recombinase. At the 14-15 s stage, the pattern of expression of the *lacZ* reporter gene in *Krox20*$^{Cre/+}$ *R26R* embryos (Fig. 1D) is very similar to that of *Krox20*$^{lacZ/+}$ embryos (Fig. 1C), whereas expression of the AP reporter is still less homogeneous in *Krox20*$^{Cre/+}$ *Z/AP* embryos (Fig. 1J). At this stage, a few isolated *lacZ*-positive cells are occasionally detected in even-numbered rhombomeres in *Krox20*$^{Cre/+}$ *R26R* embryos (data not shown), but not in *Krox20*$^{lacZ/+}$ embryos, except in the most dorsal, neural crest-generating region (Fig. 1C). This suggests that these cells have downregulated *Krox20* expression, as proposed by Irving et al. (Irving et al., 1996). From 9.5 dpc onwards, when downregulation of *lacZ* expression in *Krox20*$^{lacZ/+}$ embryos is first initiated in r3 (Fig. 1E) and then in r5 (Fig. 1G), expression of the *lox* reporter gene is maintained in r3 and r5 derivatives in *Krox20*$^{Cre/+}$ *lox*

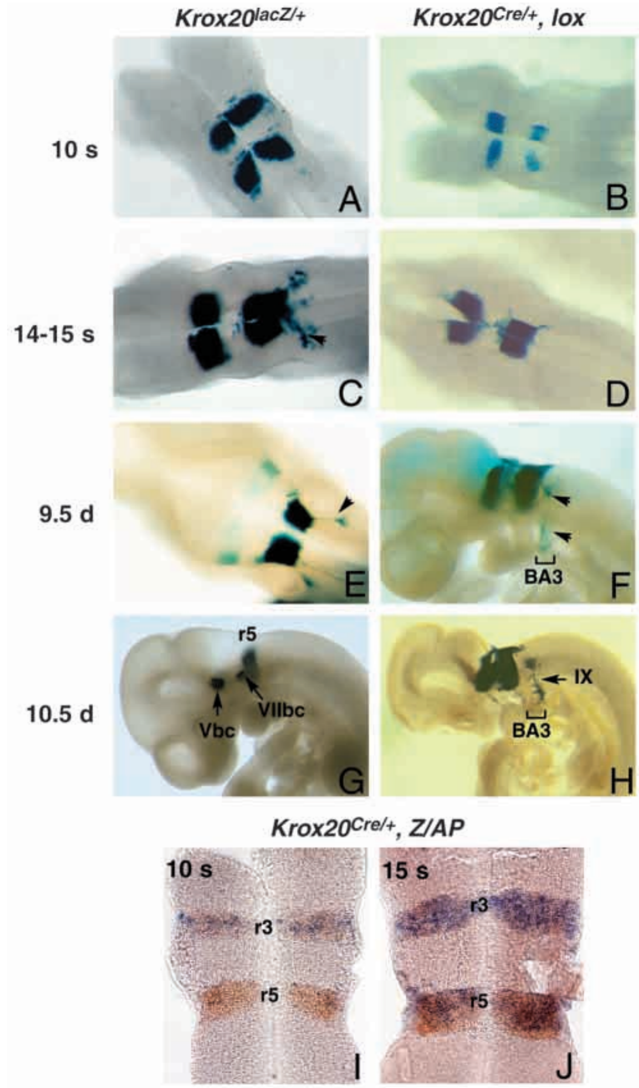

Fig. 1. Comparison of reporter gene expression in *Krox20* heterozygous embryos. Reporter gene expression was assayed by X-gal staining in *Krox20*$^{lacZ/+}$ (A,C,E,G) and *Krox20*$^{Cre/+}$ *R26R* (B,D,F) embryos, and by AP staining in *Krox20*$^{Cre/+}$ *Z/AP* embryos (H-J). Embryos shown in I,J were processed for *Krox20* ISH (brown) after AP staining. (A-H) Hindbrains of whole-mounted embryos with rostral towards the left. (I,J) Flat-mounted hindbrains with rostral towards the top. From the 14-15 s stage onward, neural crest cells exiting r5 are positive for the reporter gene only in the *Krox20*$^{lacZ/+}$ embryo (arrowhead in C). Derivatives of the r5 neural crest are labelled in the *Krox20*$^{Cre/+}$ *R26R* embryo at 9.5 dpc in the third branchial arch (BA3) (arrowheads in F), and at 10.5 dpc in BA3 and in the superior ganglion of the glossopharyngeal nerve (IX) (arrow in H). At 10.5 dpc, in the *Krox20*$^{lacZ/+}$ embryo (G), *lacZ* expression is detected in the boundary cap cells of the trigeminal (Vbc) and facial (VIIbc) nerves (arrow in G).

embryos (Fig. 1F,H). This expression of the *lox* reporter gene is maintained at later stages of embryogenesis and after birth (Fig. 2G,H; data not shown). *lox* reporter gene expression is also maintained in the derivatives of r5 and r6 neural crest which express *Krox20* (Fig. 1F,H; data not shown).

In conclusion, these data show that reporter gene expression in *Krox20*^{Cre} *lox* mice can be used to follow faithfully the progeny of *Krox20*-expressing cells in the hindbrain.

Directionality of *Krox20* activation in r3 and r5

In mouse embryos, *Krox20* gene expression is first activated in a narrow stripe of scattered cells in prospective r3 at the 0 s stage, and dorsally in a small triangular territory in prospective r5 at the 5 s stage (Irving et al., 1996). These domains then homogenise, expand and develop sharp limits, in order to form transverse territories corresponding to r3 and r5 at the 12 s stage. We took advantage of the delayed activation of the reporter gene in *Krox20*$^{Cre/+}$ *lox* embryos to analyse the directionality of *Krox20* activation in r3 and r5. For this purpose, we combined AP staining and *Krox20* in situ hybridisation (ISH) on *Krox20*$^{Cre/+}$ *Z/AP* embryos between the 10 s and 17 s stages. In these embryos, *Krox20* ISH labels cells as soon as they activate the gene, while AP staining marks only cells that activated *Krox20* several hours before. In r3, at the 10 s stage, AP-positive cells were found as a line of scattered cells at the centre of the *Krox20*-expressing territory (Fig. 1I). By the 15 s stage, they had filled the whole r3 territory but still showed a salt-and-pepper distribution (Fig. 1J). In r5, AP-positive cells were also scattered, and formed a triangle covering the anterior part of the rhombomere (Fig. 1I,J). At later stages, AP staining in r3 and r5 becomes homogeneous and covers the *Krox20* expression domains (data not shown). These data show that *Krox20* expression in r3 and r5 is established by expansion at the expense of adjacent territories, and that r3 expands both anteriorly and posteriorly, whereas r5 expansion occurs only posteriorly. In addition, both r3 and r5 show progressive homogenisation of *Krox20* expression.

Fate mapping of r3 and r5 cells in *Krox20* null embryos

To follow the fate of r3/r5 cells in the absence of a functional Krox20 protein, we could not use *Krox20*$^{Cre/Cre}$ *lox* embryos because activation of the *lox* reporter occurs in the germline of *Krox20*$^{Cre/+}$ *lox* mice (see Materials and Methods). As the *Krox20*^{Cre} allele is associated with the same phenotypes as the *Krox20*^{lacZ} allele and constitutes a null allele (data not shown), we analysed reporter activity in compound heterozygous *Krox20*$^{lacZ/Cre}$ embryos. At the 13-14 s stage, when β-galactosidase activity has almost completely disappeared at the level of r3 in *Krox20*$^{lacZ/lacZ}$ embryos (Fig. 2A), a variable, low number of scattered cells strongly positive for X-gal are present at this level in *Krox20*$^{lacZ/Cre}$ *R26R* embryos (Fig. 2B). These data demonstrate that in *Krox20*-null embryos, cells derived from prospective r3 are still present at this stage, but have downregulated *Krox20* gene expression.

At 9.5 dpc, X-gal staining in the hindbrain of *Krox20*$^{lacZ/lacZ}$ embryos is restricted to a few dorsal cells at the level of r5 (Fig. 2C). By contrast, in *Krox20*$^{lacZ/Cre}$ *R26R* embryos, a stripe of strong and homogeneous labelling was observed in r5, whereas at the level of r3 a variable, low number of X-gal-positive cells were detected (Fig. 2D). In 10.5 to 16.5 dpc *Krox20*$^{lacZ/Cre}$ *lox* embryos, lox reporter (X-gal or AP)-positive cells were also detected in the hindbrain (Fig. 2E,F; data not shown). Some of these cells had differentiated into neurones as indicated by the AP staining of axonal processes (Fig. 2E). At all stages examined, the number of lox reporter-positive cells in

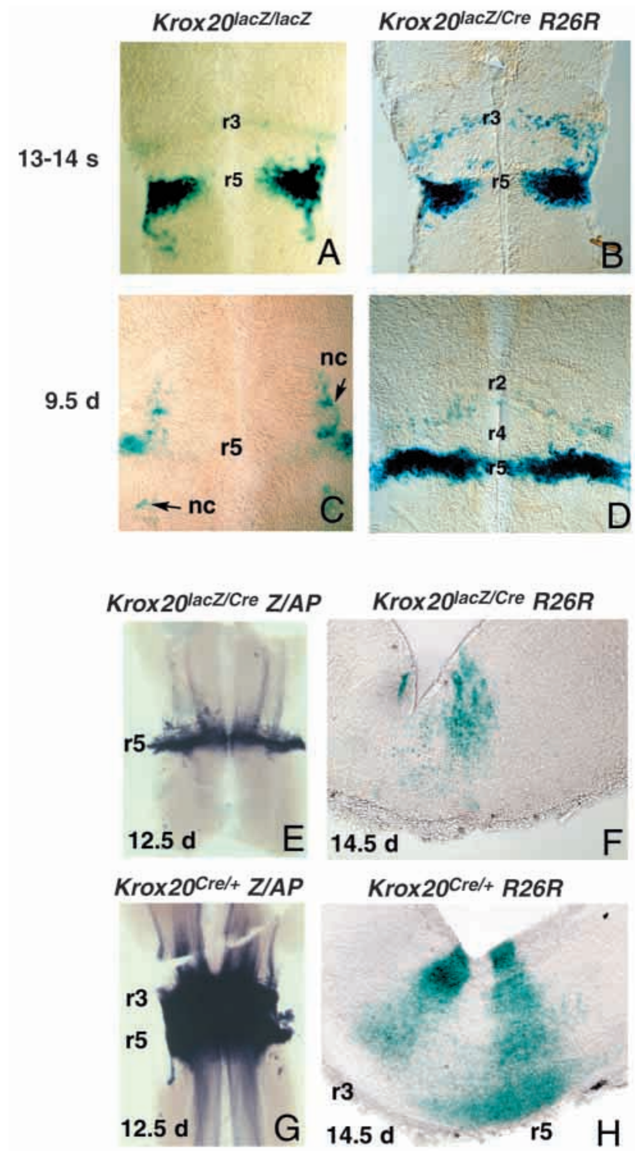

Fig. 2. Fate mapping in *Krox20*-null embryos. Reporter gene expression was assayed by X-gal staining in *Krox20*$^{lacZ/lacZ}$ (A,C), *Krox20*$^{lacZ/Cre}$ *R26R* (B,D,F) and *Krox20*$^{Cre/+}$ *R26R* (H) embryos and by AP staining in *Krox20*$^{lacZ/Cre}$ *Z/AP* (E) and *Krox20*$^{Cre/+}$ *Z/AP* (G) embryos of the indicated stages. (A-E,G) Flat-mounted hindbrains with rostral towards the top; (F,H) Parasagittal sections of the hindbrain region with rostral towards the left. In B, expression from the *Krox20*^{lacZ} allele and from the R26R reporter are superimposed in r5. In C, labelled neural crest (nc) cells that migrate rostrally and caudally to r5 have not been totally removed. In D, note that r3 blue cells are localised both rostral and caudal to the r2/r4 boundary (visible on the right side). In G, note that an unstained territory corresponding to r4 is present but is not observed on the picture because the rhombomeres are not perfectly perpendicular to the AP axis at this stage.

Krox20$^{lacZ/Cre}$ *lox* embryos (Fig. 2B,D-F) was reduced when compared with *Krox20*$^{Cre/+}$ *lox* embryos (Fig. 1D,E; Fig. 2G,H), especially at the level of r3.

In conclusion, our data indicate that, in the absence of a functional Krox20 protein, at least a subset of r3 and r5 cells persist until 16.5 dpc at least and have prematurely downregulated *Krox20* expression. However, the number of cells detected with the *lox* reporter gene was significantly reduced in *Krox-20*$^{lacZ/Cre}$ *lox* embryos when compared with *Krox20*$^{Cre/+}$ *lox* embryos. This led us to investigate the possible involvement of cell death or impaired cell proliferation in this process.

Cell death and proliferation in the hindbrain of *Krox20* null embryos

To analyse cell proliferation in the hindbrain, the embryos were first immunostained with an antibody directed against a phosphorylated form of histone H3 (H3P), which detects cells in M-phase. H3P and combined X-gal/H3P staining was performed on 8.5-9.5 dpc embryos. No significant difference was observed in the number of M-phase cells between the hindbrains of 8-14 s control (227±36, n=4) and *Krox20*$^{lacZ/lacZ}$ (235±72, n=2) embryos and within r5 (X-gal-positive) in heterozygous (27±5, n=3) and homozygous (32±12, n=2) *Krox20* embryos (r3 cannot be analysed owing to the absence of X-gal labelling at these stages in *Krox20*-null embryos) (Fig. 3A-C). Similarly, at 9.5 dpc, no significant difference was observed in the number of M-phase cells between the hindbrains of control (670±77, n=4) and *Krox20*$^{lacZ/lacZ}$ (630±64, n=3) embryos, and within r5 between heterozygous (102±17, n=3) and homozygous (117±12, n=3) embryos. To extend this analysis,

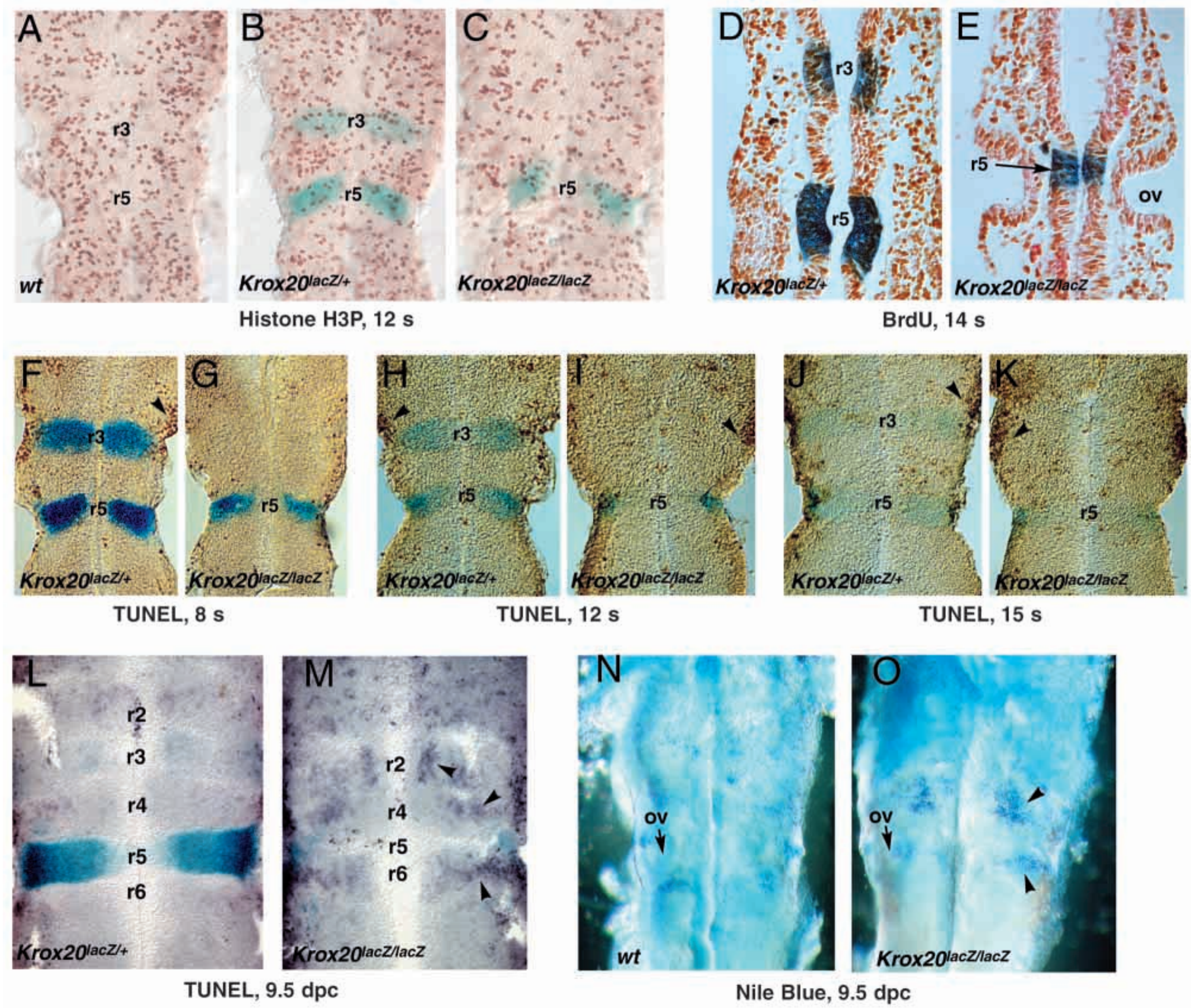

Fig. 3. Cell death and proliferation in hindbrains of wild type and *Krox20* mutant embryos. (A-C) Flat mounted hindbrains of 12 s wild type (A), *Krox20*$^{lacZ/+}$ (B) and *Krox20*$^{lacZ/lacZ}$ (C) embryos stained with X-gal (blue) and processed for H3P immunohistochemistry (brown). (D,E) Coronal sections of 14 s *Krox20*$^{lacZ/+}$ (D) and *Krox20*$^{lacZ/lacZ}$ (E) embryos whose mothers were injected with BrdU 1 hour before sacrifice. The sections were stained with X-gal (blue) and processed for BrdU immunohistochemistry (brown). (F-M) Flat mounted hindbrains of 8 s (F,G), 12 s (H,I), 15 s (J,K) and 9.5 dpc (L,M) *Krox20*$^{lacZ/+}$ (F,H,J,L) and *Krox20*$^{lacZ/lacZ}$ (G,I,K,M) embryos stained for β-galactosidase activity (blue), and for cell death by the TUNEL method (brown or purple). (N,O) Dissected hindbrains of 9.5 dpc wild-type (N) and *Krox20*$^{lacZ/lacZ}$ (O) embryos stained for cell death with Nile Blue Sulphate. Black arrowheads in F,H,I,J,K,M,O point to regions of intense cell death. ov, otic vesicle. Rostral is towards the top.

combined X-gal/BrdU staining was performed on 8 s (not shown) and 14 s (Fig. 3D,E) *Krox20*$^{lacZ/+}$ and *Krox20*$^{lacZ/lacZ}$ embryos, to allow detection of cells in S-phase. No significant difference was observed in the proportion of BrdU-positive cells in r5, as defined as the X-gal-positive territory, between homozygous (62.5%±0.7%, *n*=2) and heterozygous (62.5%±2.1%, *n*=2) embryos. Together, these data indicate that there is no major defect in cell proliferation in r5 in *Krox20*-null embryos, as a significant increase in the length of the cell cycle is expected to result in a reduction in the proportion of cells in S- or M-phase. This suggests that an impairment of cell proliferation cannot be responsible for the apparent reduction of the r5 territory observed in the genetic fate mapping in *Krox20*-null embryos. However, a slight reduction in the proliferation rate would not have been detected by our analysis.

To determine whether cell death contributes to the reduction of the r3 and r5 territories, we performed TUNEL staining of apoptotic cells combined with X-gal labelling in embryos from the 5 s stage up to 9.5 dpc. Between the 5 s and 15 s stages, we did not observe any significant increase in the number of TUNEL-positive cells in *Krox20* homozygous embryos (*n*=17) (Fig. 3G,I,K) when compared with wild type (*n*=13) (not shown) or heterozygous (*n*=28) (Fig. 3F,H,J) siblings. By contrast, at 9.5 dpc, several areas of increased cell death were detected in even-numbered rhombomeres in *Krox20*-null embryos (*n*=4) (arrowheads in Fig. 3M), when compared with control embryos (*n*=9) (Fig. 3L). The position of these areas varied from embryo to embryo, and did not correlate with r3 and r5 remnants. Nile Blue staining, which labels dying cells in live embryos, confirmed these data (Fig. 3N,O). Our results therefore suggest that cell death is not responsible for the disappearance of r3 or r5 territories in *Krox20*-null embryos. They do, however, point to a possible involvement of apoptosis in the reduction of the size of the hindbrain observed in these embryos at 10.5 dpc (Schneider-Maunoury et al., 1993; Schneider-Maunoury et al., 1997).

r3 and r5 cells acquire even-numbered rhombomere characters in *Krox20*-null embryos

As cells normally programmed to contribute to r3 and r5 are still present in the hindbrain of *Krox20*-null embryos, it is possible that they are incorporated into adjacent even-numbered rhombomeres. Indeed, previous studies have shown that Krox20 regulates the expression of several Hox genes that are essential for the acquisition of rhombomere and/or neural crest cell regional identity (Sham et al., 1993; Nonchev et al., 1996; Theil et al., 1998). Therefore, we sought to determine whether the inactivation of *Krox20* leads to perturbations in the rhombomeric identity of r3 and r5 cells. For simplicity, we will refer to r3 and r5 cells when considering those that, according to their position, should contribute to r3 and r5, irrespective of their actual rhombomeric identity. In *Krox20*-null embryos, these cells are identified by the fact that they express or have expressed the *Krox20* gene.

r3 cells adopt r2 or r4 identities

To analyse the identity of r3 cells, we monitored the expression of markers corresponding to adjacent rhombomeres. *Hoxb1* expression was used as an r4 marker and an r2 marker was obtained by introduction of the *Krox20*$^{lacZ/+}$ allele into a transgenic line, r2AP, carrying an AP reporter gene under the control of *Hoxa2* regulatory elements driving r2-specific expression (Studer et al., 1996; Helmbacher et al., 1998). In a first series of experiments, we performed combined staining for AP and *Hoxb1* on whole-mount embryos. At the 8 s stage, in wild-type embryos, the two stained regions corresponding to r2 and r4 are separated by a negative territory corresponding to r3 (Fig. 4A). In *Krox20*$^{lacZ/lacZ}$ embryos, these two stained regions are adjacent and their size is increased (Fig. 4C). In these embryos, r3 cells are still present as indicated by X-gal staining (Fig. 4D), suggesting that these cells have been incorporated into the r2 and/or r4 territories. In addition, we observed that in *Krox20* heterozygous mutants, the distance between r2 and r4 is reduced when compared with

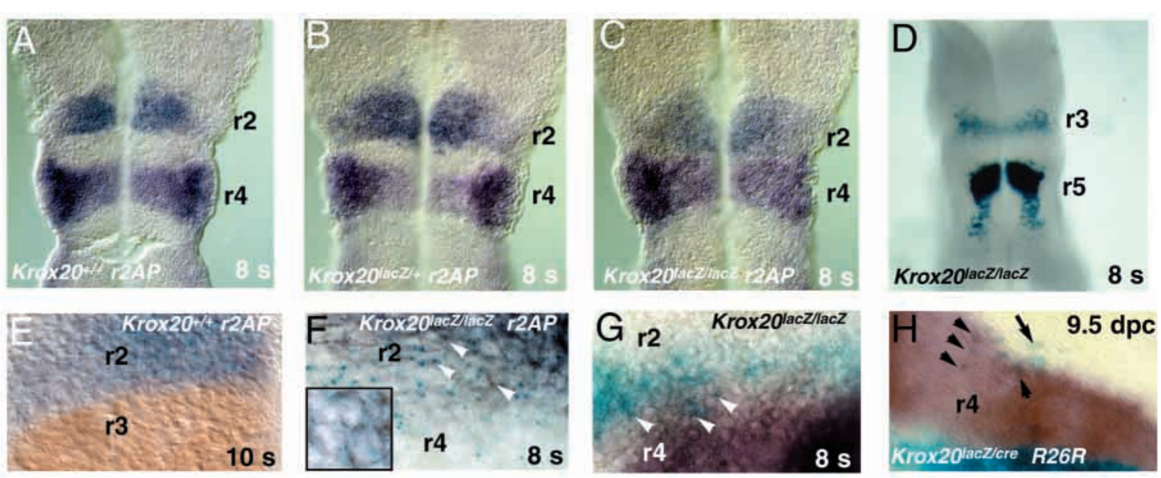

Fig. 4. Modification of rhombomere identity of r3 cells in *Krox20* mutant embryos. (A-C) Flat-mounted hindbrains of 8 s *Krox20*$^{+/+}$ (A), *Krox20*$^{lacZ/+}$ (B), or *Krox20*$^{lacZ/lacZ}$ (C) embryos carrying the AP transgene expressed in r2 (*r2AP*), stained for AP activity and processed for *Hoxb1* ISH (both purple). (D) Hindbrain of an 8 s *Krox20*$^{lacZ/lacZ}$ whole embryo labelled with X-gal for 12 hours to show the presence of r3 cells at this stage. (E) r2-r3 region of the flat-mounted hindbrain of a *Krox20*$^{+/+}$ *r2AP* embryo at the 10 s stage, processed for Krox20 protein immunochemistry (orange) and AP activity (purple). (F) The r2/r4 region of the flat-mounted hindbrain of an 8 s *Krox20*$^{lacZ/lacZ}$ *r2AP* embryo, stained for X-gal (blue) and AP activity (purple). The inset shows a high magnification of three cells, one of which is a double stained cell. (G) The r2/r4 region of the flat-mounted hindbrain of an 8 s *Krox20*$^{lacZ/lacZ}$ embryo stained with X-gal (blue) and processed for *Hoxb1* ISH (brown). (H) r3/r5 region of the flat-mounted hindbrain of a 9.5 dpc *Krox20*$^{Cre/lacZ}$ *R26R* embryo stained with X-gal (blue) and processed for *Hoxb1* ISH (purple). The arrowheads in F-H point to cells labelled by both markers. Rostral is towards the top.

the wild-type situation (Fig. 4B). The ratio of the surface area of the r2 to r4 region in 8-12 s embryos to the surface of the same region in wild-type embryos did not vary according to the genotypes: wild type (100%±11%, *n*=6), *Krox20*$^{lacZ/+}$ (104%±10%, *n*=15) and *Krox20*$^{lacZ/lacZ}$ (97%±4%, *n*=3). This indicates that in homozygous mutant embryos, most r3 cells are present at these stages but have been incorporated into adjacent even-numbered rhombomeres.

To determine whether incorporation of r3 cells occurred preferentially in r2 or in r4, we performed double labelling for either β-galactosidase and AP activities (Fig. 4F) or for β-galactosidase activity and *Hoxb1* expression (Fig. 4G). In *Krox20*$^{lacZ/lacZ}$ embryos, some of the X-gal-positive cells were found in the *r2AP* positive territory (Fig. 4F), whereas the others were in the *Hoxb1*-positive territory (Fig. 4G). In addition, many r3 (X-gal-positive) cells located within r2 expressed *r2AP* (arrowheads and inset in Fig. 4F). By contrast, AP labelling combined with Krox20 protein immunohistochemistry in wild-type embryos carrying the *r2AP* transgene showed that very few co-expressing cells were present (Fig. 4E). Similarly, X-gal positive cells within r4 co-expressed *Hoxb1* (arrowheads in Fig. 4G). To determine whether these r3 cells were maintained within r2 and r4 at later stages, we performed double labelling by X-gal and *Hoxb1* ISH in *Krox-20*$^{lacZ/Cre}$ *R26R* embryos at 9.5 dpc (Fig. 4H). At this stage, r2 and r4 territories are also juxtaposed in *Krox20*-null embryos (data not shown), and the scattered r3 cells detected with the lox reporter are within r2 or r4 (arrows and arrowheads respectively in Fig. 4H). In r4, these cells co-express *Hoxb1* and the lox reporter.

In conclusion, these data indicate that, in *Krox20*-null embryos, cells normally programmed to belong to r3 acquire r2 or r4 identity and are incorporated into these rhombomeres. In addition, experiments performed on *Krox20* heterozygous mutants suggest that the specification of r3 versus r2/r4 identity by the Krox20 protein is dose dependent (Fig. 4B and data not shown).

r5 cells adopt r6 identity

To analyse the identity of r5 cells in *Krox20*-null embryos, we first performed double ISH with probes that labelled adjacent rhombomeres. We used probes for *Hoxb1* and *Cdh6*, *Cdh6* being expressed in r6 at 8.5 dpc (Inoue et al., 1998). As expected, in wild-type embryos at the 8 s stage, a negative region corresponding to r5 was observed in between the stained r4 and r6 domains (Fig. 5A). In *Krox20*$^{lacZ/lacZ}$ embryos, the r4 and r6 territories appeared larger and much closer, suggesting that a large part of r5 cells had been incorporated into even-numbered rhombomeres (Fig. 5B).

To further analyse the rhombomeric identity of r5 cells in *Krox20* mutants, we followed the expression of the *Mafb/kr* gene. In wild-type embryos, *Mafb/kr* is expressed in r5 and r6 from the 1-2 s stage (Cordes and Barsh, 1994). After the 16 s stage, its expression fades in r5 and in dorsal r6, while it is maintained in a ventral population in r6 (Fig. 5E; data not shown). In *Krox20*$^{lacZ/lacZ}$ embryos at the 13 s stage, X-gal-positive cells are still present in r5 (Fig. 5C). Double staining for β-galactosidase activity and *Mafb/kr* expression indicated that almost all X-gal-positive cells were also positive for *Mafb/kr* (Fig. 5D), suggesting that these cells had not acquired r4 identity. Fate-mapping experiments in *Krox-20*$^{lacZ/Cre}$ *Z/AP* embryos also indicated that, at later stages, very few prospective r5 cells had contributed to r4 (Fig. 4H). Moreover, at the 20 s stage, the size of the territory of higher level *Mafb/kr* expression (r6) was larger in *Krox20*-null than in wild-type embryos (Fig. 5E,F), supporting the idea that a large part of the r5 cells had acquired r6 identity.

In conclusion, our data indicate that in *Krox20*-null embryos cells normally programmed to belong to r5 acquire r6 identity and are incorporated in this latter rhombomere.

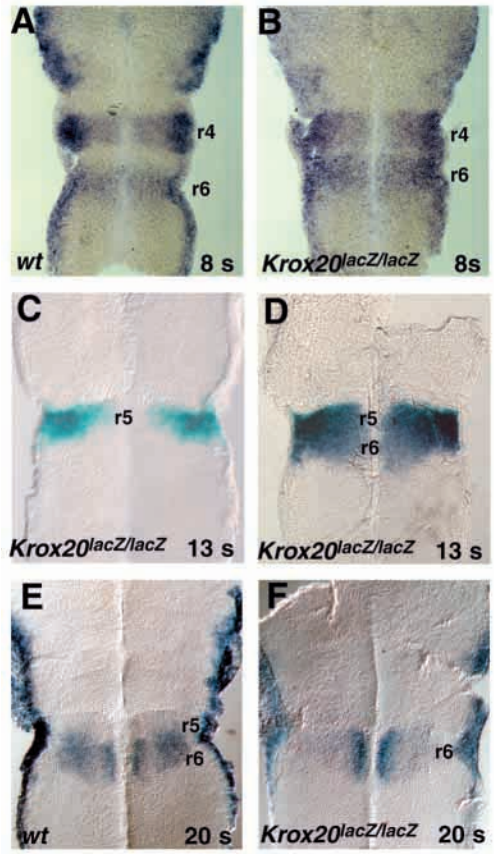

Fig. 5. Modification of rhombomere identity of r5 cells in *Krox-20* homozygous embryos. (A,B) Flat-mounted hindbrains of wild-type (A) and *Krox20*$^{lacZ/lacZ}$ (B) embryos at the 8 s stage processed for ISH with probes for *Hoxb1* and *Cdh6* probes (both purple). (C,D) Flat-mounted hindbrains of 13 s *Krox20*$^{lacZ/lacZ}$ embryos stained with X-gal (C) or stained with X-gal and processed for *Mafb/kr* ISH (D). (E,F) Flat-mounted hindbrains of wild-type (E) and *Krox20*$^{lacZ/lacZ}$ (F) 20 s embryos processed for *Mafb/kr* ISH. Rostral is towards the top.

Krox20 controls cell mingling between odd- and even-numbered rhombomeres

The data presented above could suggest that, in absence of Krox20, prospective r3 or r5 cells can mix with even-numbered cells. To further investigate the involvement of Krox20 in the specification of cell mingling properties in the hindbrain, we generated embryonic chimaeras between *Krox20*$^{lacZ/lacZ}$ and wild-type cells.

We first analysed such chimaeric embryos by X-gal staining combined to ISH for the *NeoR* gene. The *NeoR* probe detects all *Krox20*$^{lacZ/lacZ}$ cells in the embryo, whereas X-gal staining marks only r3- and r5-derived *Krox20*$^{lacZ/lacZ}$ cells. In chimaeric embryos older than 12 s, *Krox20*$^{lacZ/lacZ}$ (purple and/or blue) cells formed patches in r5 and did not mix with wild-type cells (Fig. 6A, *n*=6), suggesting a difference in cell mingling properties. Moreover, r5-derived blue cells were found in adjacent even-numbered rhombomeres, particularly in r6 (white arrowheads in Fig. 6A), suggesting that they had acquired cell mingling properties closer to those of even-numbered rhombomere cells. As controls, we generated chimaeric embryos between wild-type cells and cells from a transgenic line that expresses *lacZ* in r3 and r5 under the control of *cis*-acting regulatory elements of the *Epha4* gene (Theil et al., 1998). In these chimaeric embryos, wild-type and transgenic cells mixed freely in r3 and r5 (Fig. 6C, *n*=2). This indicates that the restriction in cell mixing observed in the previous experiment was due to the *Krox20* mutant allele and not to a bias in cell mixing in chimaeric embryos. We also generated chimaeric embryos between wild-type cells and *Krox20*$^{lacZ/+}$ cells. Interestingly, in this case an intermediate phenotype was observed (Fig. 6B, *n*=5): heterozygous (blue) cells tended to form clusters and did not mix freely with wild-type (white) cells in r3 and r5, but this segregation was less dramatic and occurred later than in wild-type/*Krox20*$^{lacZ/lacZ}$ chimaeras.

Cell segregation in the hindbrain does not occur before the 10-12 s stage, when X-gal-positive cells are largely undetectable in r3 in *Krox20*$^{lacZ/lacZ}$ embryos. To investigate cell mingling properties in r3 in the chimaeric embryos further, we performed combined X-gal staining and ISH for the *Epha4* gene. *Epha4* is a direct target of Krox20 in r3 and r5 (Theil et al., 1998), and in wild-type embryos it is strongly expressed in r3 at the 15 s stage (Fig. 6D). Therefore, we assumed that in chimaeric embryos, the *EphA4*-negative cells in r3 were of *Krox20*$^{lacZ/lacZ}$ genotype. In chimaeric wt/*Krox20*$^{lacZ/lacZ}$ embryos, *Krox20*$^{lacZ/lacZ}$ (*Epha4*-negative) cells formed patches within r3 and did not mix with wild type (*Epha4*-positive) cells (Fig. 6E, *n*=3). Moreover, the limits of the *EphA4* expression domains were often not straight (Fig. 6F), suggesting that patches of *Krox20*$^{lacZ/lacZ}$ cells originating from r3 were repelled toward the boundaries.

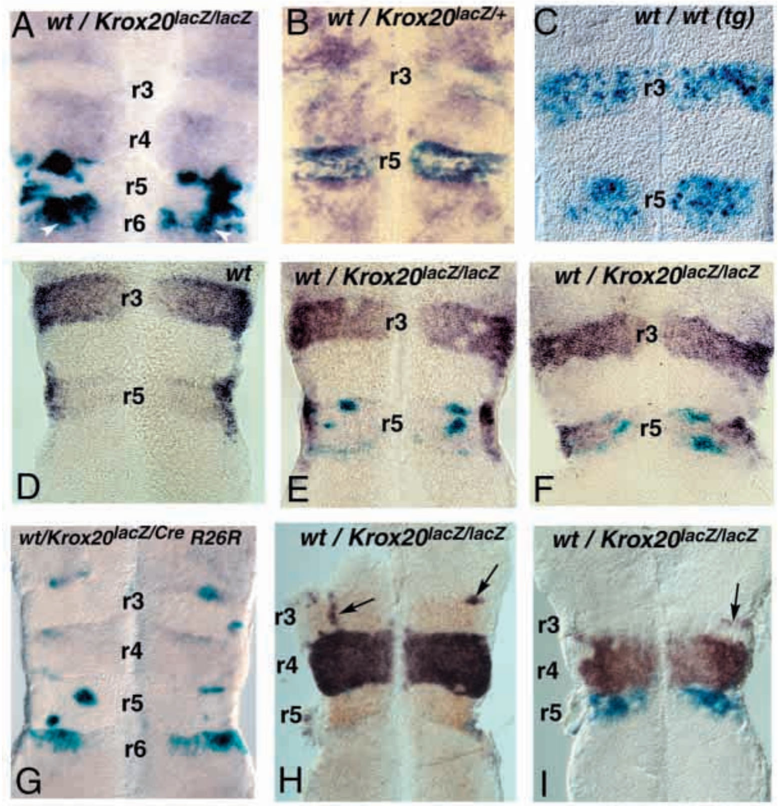

Fig. 6. Analysis of cell identity and cell mingling in embryonic chimaeras. All the pictures show flat-mounted hindbrains. (A) Combined X-gal staining and *NeoR* ISH performed on a 15 s wild-type/*Krox20*$^{lacZ/lacZ}$ chimaeric embryo. The white arrowheads point to groups of blue cells in even-numbered rhombomeres. (B) Combined X-gal staining and *NeoR* ISH performed on a 18 s wild-type/*Krox20*$^{lacZ/+}$ chimaeric embryo. (C) X-gal staining of a 14 s chimaeric embryo between wild-type and *Epha4/lacZ* transgenic lines. (D) *Epha4* ISH performed on a 15 s wild-type embryo. (E,F) Combined X-gal staining and *Epha4* ISH performed on 15 s (E) and 12 s (F) wild-type/*Krox20*$^{lacZ/lacZ}$ chimaeric embryos. (G) Combined X-gal staining and *NeoR* ISH performed on a 9.5 dpc wild-type/*Krox20*$^{lacZ/Cre}$ *R26R* chimaeric embryo. (H) Combined X-gal staining and double *Krox20* (orange) and *Hoxb1* (purple-brown) ISH performed on a 14 s wild-type/*Krox20*$^{lacZ/lacZ}$ chimaeric embryo. (I) Combined X-gal staining and *Hoxb1* ISH performed on a 12 s wild-type/*Krox20*$^{lacZ/lacZ}$ chimaeric embryo.

To determine the behaviour of mutant cells originating from r3 and r5 in older embryos, we generated chimaeras between wild-type and *Krox20*$^{lacZ/Cre}$ *R26R* cells. Analysis of these chimaeras at 9.5 dpc revealed two types of behaviours for X-gal positive cells: within r3 and r5, these cells were grouped in patches, most probably in order to minimise their contacts with wild-type neighbours. By contrast, at the periphery of the rhombomeres, X-gal-positive cells were dispersed and mixed with X-gal-negative cells, invading even-numbered rhombomeres, especially r6 (Fig. 6G). Interestingly, groups of cells at the r4/r5 border did not penetrate r4 efficiently but rather segregated to the r4/r5 boundary (Fig. 6G), suggesting that r5 mutant cells had acquired cell mingling properties closer to those of r6 than to those of r4.

To investigate the rhombomeric identity of the patches of *Krox20*-null cells within r3 or r5 in the wild-type/*Krox20*$^{lacZ/lacZ}$ chimaeras, we analysed the expression of the r4 marker *Hoxb1*. Patches of *Hoxb1*-expressing cells were observed in r3 but not in r5 (Fig. 6H,I, *n*=6). We assume that these cells are r3-derived *Krox20* null cells that have acquired r4 identity. This is consistent with our observation of the acquisition of r4 identity by r3 but not r5 cells in *Krox20*-null embryos and shows that r3 identity cannot be rescued in mutant cells by surrounding wild-type cells.

In conclusion, our data demonstrate that *Krox20*-null and wild-type cells in r3 and r5 have different cell mingling properties and sort from each other. They actually show that r3 and r5 mutant cells have acquired cell mingling properties similar to those of even-numbered rhombomere cells. In addition, this work indicates that Krox20 controls the specification of odd-numbered cell identity in a cell autonomous manner.

DISCUSSION

Fate of r3 and r5 cells in the absence of a functional Krox20 protein

In previous studies, we have shown that in *Krox20*$^{lacZ/lacZ}$ embryos, the X-gal staining in r3 and r5 appears normally, but disappears much more rapidly than in *Krox20*$^{lacZ/+}$ embryos. However, it was not clear whether this reflected an early disappearance of the cells, or a defect in the maintenance of *Krox20/lacZ* fusion gene expression. In this paper, we used even-numbered rhombomere markers to demonstrate that at 8.5 dpc r3 and r5 cells were still present, but were incorporated into adjacent rhombomeres. Moreover, genetic fate mapping allowed us to follow r3 and r5 cells at later stages of development. We show that in *Krox20*$^{lacZ/Cre}$ *lox* embryos, part of the r3 and r5 cells can be detected by *lox* reporter activity staining until at least 16.5 dpc, and these cells are able to form neurones. This demonstrates that the premature disappearance of β-galactosidase activity in *Krox20*$^{lacZ/lacZ}$ embryos is partly due to a defect in the maintenance of *Krox20/lacZ* fusion gene expression, and points to a role of *Krox20* in the maintenance of its own expression in r3 and r5.

The number of r3 and r5 cells detected with the *lox* reporter is reduced in *Krox20*$^{lacZ/Cre}$ *lox* embryos when compared with *Krox20*$^{Cre/+}$ *lox* embryos. This difference is unlikely to be due to defects in survival and/or proliferation of r3 and r5 cells, as shown by our analysis of dying cells and of cells in S- or M-phase. The low number of r3 and r5 cells positive for the *lox* reporter is more likely to originate from a lack of recombination-induced activation of the *lox* reporter gene in many cells, caused by two phenomena. First, the level of Cre protein synthesised in *Krox20* null cells may be too low to support recombination, because the *Krox20* autoregulatory loop is not functional. Second, it is likely that part of the cells never activate *Krox20* gene expression, because the Krox20 protein is required in a non cell-autonomous manner for the activation of the expression of its own gene in these cells. We have recently demonstrated the existence of such a mode of regulation for *Krox20* expression in the chick hindbrain (Giudicelli et al., 2001).

Multiple functions of Krox20 in the formation and specification of r3 and r5

Maintenance of its own expression and expansion of the r3/r5 territories

X-gal staining disappears prematurely in the hindbrain of *Krox20*$^{lacZ/lacZ}$ embryos, whereas many r3 and r5 cells are still present. This demonstrates that the maintenance of *Krox20* gene expression in r3 and r5 involves an autoregulatory loop. This positive feedback loop requires Krox20 in a cell autonomous manner, as premature disappearance of X-gal staining is also observed in embryonic chimaeras between wild-type and *Krox20*$^{lacZ/lacZ}$ cells. In addition, as indicated above, our data are consistent with our previous work performed in the chick (Giudicelli et al., 2001), suggesting that in the mouse the Krox20 protein is also required in a non cell-autonomous manner for the activation of its own gene in adjacent cells.

These cell autonomous and non cell-autonomous autoregulatory mechanisms are likely to be involved in the consolidation, homogenisation and expansion of the r3 and r5 territories (Fig. 7). The incorporation of additional cells in the territories by cell recruitment is consistent with the mode of establishment of *Krox20* expression in r3 and r5 by anterior and posterior expansion, that we have deduced from combined *Z/AP-Krox20* ISH staining of *Krox20*$^{Cre/+}$ *Z/AP* embryos (Fig. 1I,J). This recruitment process may be particularly important for the expansion of r3, which is more dramatically affected than r5 in *Krox20*-null mutants. Finally, the involvement of the non cell-autonomous autoregulatory loop in the maintenance of *Krox20* expression may also explain the loss of *Krox20* expression in isolated cells that have inappropriately activated the gene in even-numbered rhombomeres.

Krox20 represses even-numbered rhombomere identity and promotes r3/r5 identity

Our work suggests that, in the absence of a functional Krox20 protein, prospective r3 and r5 cells adopt an even-numbered rhombomere molecular identity. r3 cells acquire r2 or r4-like identity, as shown by the expansion of the *r2-AP*- and *Hoxb1*-positive territories. r5 cells acquire r6 identity: they express *Cdh6* and *Mafb/kr* and do not express *Hoxb1*. This is in perfect accordance with gain-of-function experiments performed in the chick, which show that r2 and r4 cells ectopically expressing *Krox20* acquire r3 identity, whereas r6 cells expressing *Krox20* acquire r5 identity (Giudicelli et al., 2001).

Therefore, an essential outcome of these data is that one of the initial defects in *Krox20*-null embryos is a mis-specification of *Krox20*-expressing cells. They suggest that, in addition to activating r3/r5 specific genes, a prime role of *Krox20* is to repress even-numbered rhombomere specific genes (Fig. 7). Whether the cells become r3 or r5 cells upon *Krox20* expression must be determined by a repertoire of genes unaffected by *Krox20* expression (e.g. *Mafb/kr*).

Ectopic expression experiments have shown that *Krox20* is able to repress *Hoxb1* expression in r4 (Giudicelli et al., 2001). The present data demonstrate that *Krox20* is necessary to repress *Hoxb1* expression in r3, but not in r5. This suggests that another gene, expressed in r5 independently of *Krox20*, has a redundant function in repressing *Hoxb1* expression. A good candidate for this function is *Mafb/kr* because, as shown in this study, it is still expressed in r5 remnants in *Krox20* homozygous mutants and because in 8.5 dpc *MafB/kr* (*kreisler*) mutants, there is a transient expansion of *Hoxb1* expression caudal to r4 (Frohman et al., 1993; McKay et al., 1994; Manzanares et al., 1999).

In conclusion, these data show that Krox20 is essential for the acquisition of r3/r5 identity. In its absence, hindbrain cells adopt a default state that corresponds to even-numbered rhombomere identity.

Krox20 controls cell mingling properties of r3 and r5 cells

We have shown that in embryonic chimaeras, *Krox20*$^{lacZ/lacZ}$ and wild-type cells cannot intermingle in r3 and r5. Moreover, mutant cells penetrate into even-numbered rhombomeres, showing that they have acquired cell mingling properties close to those of even-numbered rhombomere cells. This change can be considered as another aspect of the acquisition of even-numbered rhombomere identity by r3/r5 homozygous mutant cells, and suggests that *Krox20* is essential for the sorting out of even and odd-numbered cells (Fig. 7). We propose that this

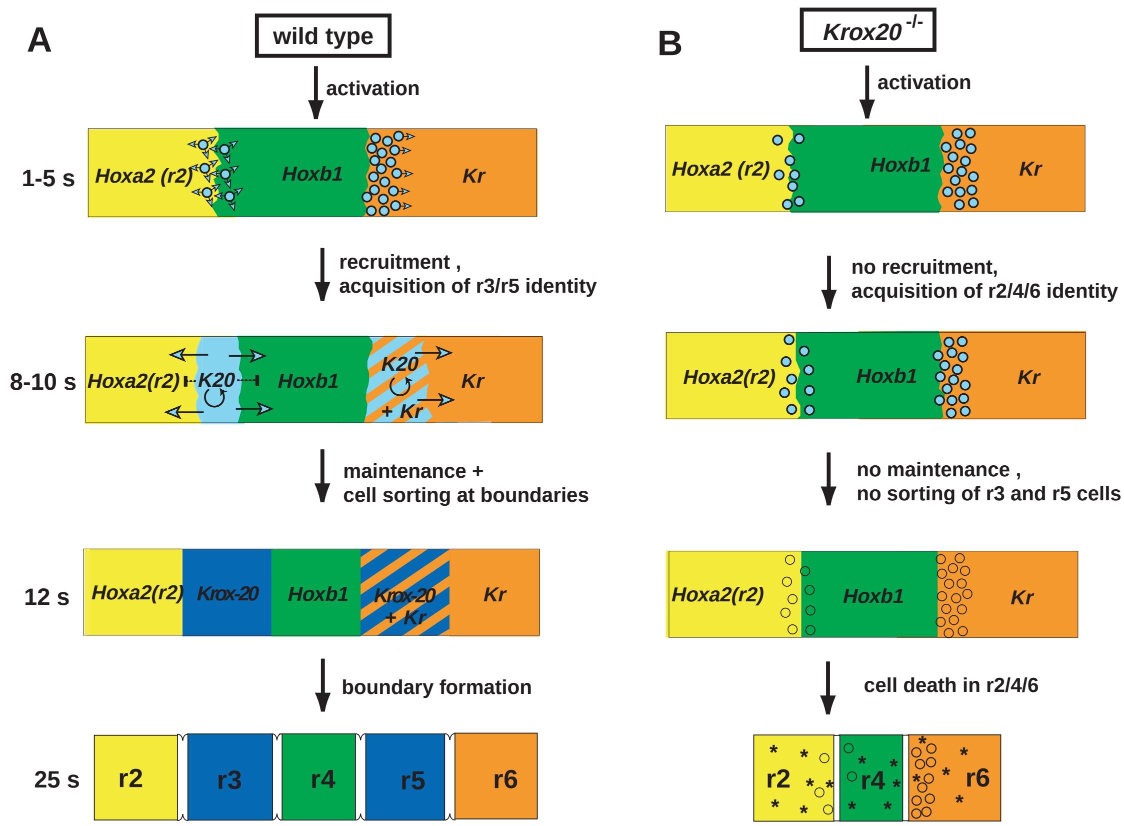

Fig. 7. Schematic model for hindbrain segmentation in wild-type and *Krox20*$^{-/-}$ embryos. (A) Wild-type situation. At the 1-5 s stages, *Krox20* is activated in a few scattered cells in prospective r3, and in a more coherent group of cells in prospective r5 (light blue circles). *Hoxb1* (green) and *Mafb/kr* (*Kr*) (orange), and the *Hoxa2* (*r2*) (yellow) enhancer are also activated. Additional cells are subsequently recruited to express *Krox20*, probably by a non cell-autonomous autoactivation process (light blue arrows). At the 8-10 s stage, prospective r3 and r5 now express *Krox20* homogeneously, and continue to expand by cell recruitment at the expense of r2/r4 (for r3) and of r6 (for r5). In addition, an autoregulatory loop (curved arrows) leads to enhancement of *Krox20* expression, presumably in a cell-autonomous manner. *Krox20* expression in these cells leads to acquisition of r3/r5 molecular identity (*Epha4*, *Hoxa2*-r3/r5 enhancer activation) and repression of even-numbered rhombomere molecular identity (e.g. *Hoxb1*, *Hoxa2*-r2 enhancer repression). At the 12 s stage, r3 and r5 express *Krox20* at high levels (dark blue) and have acquired r3/r5 identity, including cell mingling properties. This leads to the sorting of even- and odd-numbered rhombomere cells, and sharpening of gene expression limits. At the 20-25 s stage, boundaries are morphologically conspicuous. (B) In *Krox20*-null mutants, at the 1-5 s stages, early activation of *Krox20* occurs normally (light blue circles). However, the *Krox20*-expressing territories do not expand, because of defective cell recruitment based on non cell-autonomous autoregulation. At the 8-10 s stage, *Krox20*-expressing cells are still scattered. They have acquired even-numbered rhombomere identity and are incorporated into adjacent even-numbered territories, namely r2 and r4 for cells that should belong to r3, and r6 for cells that should give rise to r5. At the 12 s stage, *Krox20* gene expression is not maintained in these cells, owing to impaired cell autonomous autoregulation. Finally, at the 20-25 s stage, cell death in the even-numbered rhombomeres leads to a significant size reduction of the hindbrain.

property of *Krox20* is mediated by the regulation of the Eph/ephrin system, as it was shown previously that Krox20 is required for the activation of the *Epha4* gene in r3 and r5 (Seitanidou et al., 1997).

A Krox20 dosage effect in the early specification of r3 and r5

Our data indicate that in *Krox20* heterozygous embryos, some of the r3 cells acquire r2/r4 identity (Fig. 4B; data not shown). This is consistent with previous observations indicating that in some *Krox20*$^{lacZ/+}$ embryos at 10.5 dpc, nerve exit points, which are normally present only in even-numbered rhombomeres, are also found in r3 (Helmbacher et al., 1998). In addition, we show here that in embryonic chimaeras, cell mingling properties of *Krox20* heterozygous cells are different from those of wild-type cells in r3 and r5. Finally, in some *Krox20*$^{lacZ/+}$ embryos, the *Krox20*-expressing stripes at 8.5 dpc are smaller than in wild-type embryos (data not shown), suggesting that the expansion of the territories is also affected. Together, these data suggest that a similar threshold level of Krox20 is required for its three functions: expansion of r3 and r5 territories and specification of rhombomeric identity and of cell mingling properties. However, the defects observed in *Krox20*$^{lacZ/+}$ animals must be transient, as they are viable and do not show any obvious phenotype at later stages of development (Schneider-Maunoury et al., 1993).

A model for hindbrain patterning in wild-type and *Krox20* homozygous embryos

Fig. 7 summarises our conclusions and presents a model for the development of the hindbrain in both wild-type and *Krox20*-null embryos. Most of the points of the model have been discussed above and are detailed in the legend. However, an additional issue is worth considering: despite the incorporation of prospective r3 and r5 cells into even-numbered rhombomeres, a shortening of the hindbrain of about two rhombomeres is observed after 9.5 dpc in *Krox20*$^{-/-}$ embryos. The significant increase in cell death detected in this work at the 20-25 s stage in even-numbered rhombomeres is likely to explain this general shortening of the hindbrain as well as a specific size reduction of r4 (Schneider-Maunoury et al., 1993). Cell death may originate from a control mechanism used by even-numbered rhombomeres to regulate their size. An alternative possibility is that cell survival in even-numbered rhombomeres depends on trophic factors produced by odd-numbered rhombomeres.

In conclusion, our work shows that *Krox20* is involved in the expansion, consolidation and homogenisation of *Krox20*-expressing r3 and r5 territories by cell-autonomous and non cell-autonomous auto-activation, in the acquisition of r3/r5 identity and repression of even-numbered identity, and in the segregation between odd- and even-numbered rhombomere cells. The integration of these various steps of segment formation and of specification of regional identity by the *Krox20* gene promotes the formation of alternating odd- and even-numbered territories along the hindbrain.

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