Perinatal lethality and defects in hindbrain development in mice homozygous for a targeted mutation of the zinc finger gene *Krox20*

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***Krox20* is a zinc finger gene expressed in rhombomeres 3 and 5 during hindbrain development in vertebrates. Mice homozygous for a targeted mutation that deletes the majority of the *Krox20* gene, including the zinc finger DNA-binding domain, died shortly after birth. The primary phenotype of the homozygous mutant animals was the loss of rhombomeres 3 and 5. This resulted in fusions of the trigeminal ganglion with the facial and vestibular ganglia, and of the superior ganglia of the glossopharyngeal and vagus nerves. These fusions resulted in a disorganization of the nerve roots of these ganglia as they entered the brain stem. These data demonstrate that *Krox20* plays an essential role during development of the hindbrain and associated cranial sensory ganglia in mice.**

[Key Words: Hindbrain; rhombomeres; segmentation; *Hox* genes]

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Studies over the last several years have unequivocally shown that segmentation is a primary feature of the development of the hindbrain in vertebrates (for review, see Keynes and Lumsden 1990; Lumsden 1990; Wilkinson and Krumlauf 1990; Wilkinson 1993). The segmental pattern underlying vertebrate hindbrain development is indicated in several different ways. Morphologically, the vertebrate hindbrain transiently possesses a series of periodic swellings and constrictions, termed rhombomeres (Vaage 1969), and a number of features relating to neuronal development and hindbrain organization appear to be correlated with specific rhombomeres. Thus, studies in the chick have shown that differentiated neurons first appear in the even-numbered rhombomeres (r) r2, r4, and r6, before appearing in the odd-numbered rhombomeres (Lumsden and Keynes 1989). Structurally, each of the branchial motor nerves arises from a pair of rhombomeres: Cell bodies for the fifth nerve (V) are located in r2/r3, those for the seventh nerve (VII) in r4/r5, and those for the ninth nerve (IX) in r6/r7 (Lumsden and Keynes 1989).

The segmental pattern underlying vertebrate hindbrain development is also apparent at the level of gene expression. A number of genes have segmentally restricted patterns of expression in the developing vertebrate hindbrain. These genes include *Hox* homeo box genes (Murphy et al. 1989; Wilkinson et al. 1989b; Fro-

hman et al. 1990; Sundin and Eichele 1990; Hunt et al. 1991), retinoic acid receptors and the cellular retinoic acid-binding proteins (Smith and Eichele 1991; Maden et al. 1992; Ruberte et al. 1992), the receptor-type tyrosine kinase *Sek* (Nieto et al. 1992), the fibroblast growth factor family member *Fgf3* (*int2*; Wilkinson et al. 1988), and the zinc finger gene *Krox20* (Wilkinson et al. 1989a). The limits of expression of these genes coincide with rhombomere boundaries, providing molecular evidence that rhombomeres are true segmental compartments. In addition, the expression patterns of these genes suggest that at least some of them may play roles in establishing pattern and/or segment identity in the hindbrain.

As mentioned above, the zinc finger gene *Krox20* is one of the genes expressed in a rhombomere-restricted fashion in the hindbrain. *Krox20* was originally cloned as an immediate-early response gene isolated from serum-stimulated fibroblasts (Chavrier et al. 1988). The protein encoded by the *Krox20* gene contains three Cys₂His₂-type zinc fingers, and several lines of evidence indicate that it acts as a transcription factor. *Krox20* protein binds to DNA in a sequence-specific manner (Chavrier et al. 1988; 1990) and is able to *trans*-activate a promoter containing multiple *Krox20*-binding sites (Chavrier et al. 1990; Vesque and Charnay 1992). In the mouse, *Krox20* is expressed early during hindbrain development in r3 and r5 (Wilkinson et al. 1989a). Expression in these rhombomeres is down-regulated and is subsequently followed by expression in two columns of cells in the hindbrain, which presumably correspond to specific hind-

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brain nuclei. *Krox20* is also transiently expressed in a population of migrating neural crest cells adjacent to r6/7 and is later expressed in the neural crest-derived boundary cap cells of the cranial ganglia (Wilkinson et al. 1989a).

The *Krox20* gene is evolutionarily conserved in vertebrates, and *Xenopus*, chick, human, and zebrafish *Krox20* homologs have been cloned (Joseph et al. 1988; Nieto et al. 1991; Bradley et al. 1992; Oxtoby and Jowett 1993). In addition to amino acid sequence conservation, most aspects of the patterns of embryonic gene expression are also conserved in the vertebrate *Krox20* homologs, suggesting a conserved role for these genes in hindbrain development (Wilkinson et al. 1989a; Nieto et al. 1991; Bradley et al. 1992; Oxtoby and Jowett 1993). In all vertebrates examined to date, expression of *Krox20* genes is first detected, before the morphological appearance of rhombomeres, in two spatial domains in the hindbrain that are later seen to coincide with r3 and r5 (Wilkinson et al. 1989a; Nieto et al. 1991; Bradley et al. 1992; Oxtoby and Jowett 1993). Work in the chick has established that the appearance of rhombomere boundaries coincides with cell lineage restriction in the rhombomeres (Fraser et al. 1990). Thus, *Krox20* expression is initiated before the appearance of lineage-restricted segmental compartments in the hindbrain. Recent work has demonstrated that *Krox20* regulates the expression of the homeo box gene *Hoxb-2* (*Hox-2.8*) (Sham et al. 1993). This suggests that *Krox20* may exert its role during hindbrain development, at least in part, by regulating the expression of the *Hox* homeo box genes, which also display rhombomere-restricted patterns of expression in the hindbrain (Murphy et al. 1989; Wilkinson et al. 1989b; Frohman et al. 1990; Sundin and Eichele 1990; Hunt et al. 1991).

To begin to examine the role that the *Krox20* gene plays during hindbrain development, we deleted most of

the *Krox20* gene by homologous recombination in embryonic stem (ES) cells and used these cells to generate mutant mice. Mice homozygous for this mutation die shortly after birth and exhibit major defects in the formation of the cranial sensory ganglia. In situ hybridization analysis indicated that these defects resulted from the loss of r3 and r5 in the homozygous mutant animals. This work establishes that the *Krox20* gene plays an essential role during development of the hindbrain and associated cranial sensory ganglia in mice.

Results

Targeted disruption of Krox20 and germ-line transmission of the mutated allele

A *Krox20* genomic clone isolated from a strain 129 genomic library was used for construction of the targeting vector. The vector contained a *neo* expression cassette for positive selection and a herpes virus thymidine kinase expression cassette for negative selection against random integration of the targeting vector (Mansour et al. 1988). The targeting vector contained 2.5 kb of homologous sequence 5' and 3.4 kb of homologous sequence 3' of the *neo* cassette (Fig. 1). This construct deletes the carboxy-terminal 315 amino acids of the *Krox20* protein (Chavrier et al. 1988), which includes the three zinc fingers. We expect that this deletion should act as a null mutation, as the DNA-binding domain (the zinc finger region) (Vesque and Charnay 1992) is deleted. In addition, because the *Krox20* protein binds DNA as a monomer (Nardelli et al. 1991 1992; Pavletich and Pabo 1991), this mutation should not cause a dominant-negative effect. Because our targeting vector deletes the zinc finger region of the *Krox20* gene, we refer to this mutant allele as *Krox20*^{Δzf}.

The linearized targeting vector was electroporated into

Figure 1. Targeted disruption of the *Krox20* gene. (A) Targeting scheme. The top line shows the genomic organization of the *Krox20* gene (Chavrier et al. 1989). Exons are indicated by stippled boxes. The three zinc fingers in the second exon are indicated by a solid box. The middle line represents the structure of the targeting vector. An 800-bp deletion was created in exon 2 that removes the carboxy-terminal 315 amino acids of the *Krox20* protein, including the zinc finger DNA-binding domain. Shown at the bottom is the predicted structure of the *Krox20* locus following homologous recombination of the targeting vector. Hybridization probes used for Southern blot analysis are indicated, as well as the expected sizes of hybridizing restriction fragments. Restriction enzymes: (B) *Bam*HI; (E) *Eco*RI; (H) *Hind*III; (S) *Sfi*I.

CJ7 ES cells (see Materials and methods for derivation of the cell line), and the electroporated cells were subjected to positive/negative selection (Mansour et al. 1988) using G418 and FIAU. Two independently targeted clones were identified by Southern blot screening, which represented a targeting frequency of $\sim 1/125$ double-selected clones. Mutant clones were expanded and confirmed by Southern analysis with various restriction enzymes (see Fig. 2). Mutant clones were injected into C57Bl/6 recipient blastocysts, and male founder animals exhibiting extensive coat-color chimerism were crossed to C57Bl/6 females. Because the ES cells were derived from 129/Sv mice, progeny of the chimeric founders were F_1 hybrids of the two strains. Germ-line transmission of the *Krox20*^{*Azf*} allele was obtained for both targeted clones. *Krox20*^{*Azf*} alleles passed through the germ line were analyzed by Southern blotting to confirm that they contained the expected replacement-type homologous recombination event (Fig. 2; also see Materials and methods). F_1 mice heterozygous for the *Krox20*^{*Azf*} mutation appeared phenotypically normal.

Mice homozygous for the Krox20^{Azf} mutation die shortly after birth

To examine whether mice homozygous for the *Krox20*^{*Azf*} mutation were viable, heterozygous F_1 animals were intercrossed and the genotypes of F_2 progeny were determined. Breeding and phenotypic analysis were carried out for both independently targeted *Krox20*^{*Azf*} alleles. No differences in the mutant phenotype described below were noted between mice derived from the two clones. Most mice ($\sim 70\%$) homozygous for the *Krox20*^{*Azf*} mutation died during the first 24 hr after birth (Table 1). These animals appeared morphologically normal and were able to breathe but did not feed. A smaller proportion ($\sim 30\%$) of the homozygous animals lived for 5–10 days. These surviving *Krox20*^{*Azf*} homozygotes, however, were severely runted compared with heterozygous or wild-type

littermates. No *Krox20*^{*Azf*} homozygous animal has survived longer than 20 days, despite attempts to maximize their chances for survival (Table 1). This analysis was performed on a genetically segregating F_2 generation. Although lethality was completely penetrant in *Krox20*^{*Azf*} homozygotes, there was some variability in the time that the lethality occurred. At present, we do not know whether the variable penetrance in the time of lethality that we have observed would be reduced if the *Krox20*^{*Azf*} mutation was maintained on the inbred 129/Sv background. We have not determined a specific cause of death for the *Krox20*^{*Azf*} homozygous animals, although it is likely that impaired ability to feed plays some role in the observed lethality (see Discussion).

Mice homozygous for the Krox20^{Azf} mutation exhibit defects in cranial nerves and ganglia

Given the expression pattern of the *Krox20* gene and the perinatal lethality observed in *Krox20*^{*Azf*} homozygous animals, we sought to determine whether there were detectable neuronal defects in the *Krox20*^{*Azf*} homozygotes. To assess the development of cranial nerves and ganglia, embryos from intercrosses of *Krox20*^{*Azf*}/+ heterozygous mice were isolated at 9.5 and 10.5 days postcoitum (dpc) and were assayed by whole-mount immunohistochemistry with a monoclonal antibody to the 155-kD neurofilament protein (Dodd et al. 1988). In wild-type embryos at 9.5 dpc, separate primordia for the trigeminal (V) and the combined facial/acoustic (VII/VIII) ganglia could be discerned clearly (Fig. 3A). At this stage of gestation the facial/acoustic ganglion complex appeared more condensed than the trigeminal ganglion, and it was clearly separated from the trigeminal by a zone free of neurofilament-positive cells (Fig. 3A). In *Krox20*^{*Azf*} homozygous mutant embryos at 9.5 dpc, the most striking defect was an expansion of the domain of the trigeminal ganglion so that it overlapped that of the facial/acoustic ganglion complex (Fig. 3B). In addition, the facial/acoustic ganglion complex of *Krox20*^{*Azf*} homozygotes appeared less condensed than that of wild-type embryos. These defects were bilateral in the *Krox20*^{*Azf*} homozygous mutant embryos.

In wild-type embryos at 10.5 dpc, in addition to the primordia of the cranial ganglia, axons associated with several of the cranial nerves were clearly visible (Fig. 3C). The three divisions of cranial nerve V (the ophthalmic, maxillary, and mandibular divisions) could be seen emanating from the trigeminal ganglion. The facial (VII) nerve could be seen innervating the second branchial arch, and a zone free of neurofilament-positive cells still clearly separated the trigeminal ganglion from the facial/acoustic ganglion complex (arrowhead in Fig. 3C). In addition, axons of the glossopharyngeal (IX) and vagus (X) nerves and their associated ganglia were then visible (Fig. 3C). In *Krox20*^{*Azf*} homozygous mutant embryos at 10.5 dpc, the zone free of neurofilament-positive cells that separates the trigeminal and facial/acoustic ganglia was no longer present, and these ganglia appeared to overlap one another (Fig. 3D,E). More distally, the seventh cra-

Figure 2. Southern blot analysis. DNA isolated from progeny of the intercross of *Krox20*^{*Azf*}/+ heterozygous mice was digested with the indicated restriction enzymes, blotted, and hybridized with the indicated probes (Fig. 1). Sizes of hybridizing fragments are indicated. Genotypes of progeny are indicated at the top of the lane.

Table 1. Genotypes of progeny of F_1 intercrosses of $Krox20^{\Delta zf/+}$ heterozygous mice

Clone	Number of litters	Total mice	Genotypes				
			+/+	+/-	-/- survive <24 hours	-/- survive 5–10 days	-/- survive >2 weeks
2-4A	8	55	18	22	10	4	1 ^a
3-5F	3	17	3	11	3	0	0

^aThis animal died at day 20.

nial nerve (VII) and the three branches of the fifth nerve (V) appeared unaffected in the $Krox20^{\Delta zf}$ homozygous mutant embryos. An additional defect observed in $Krox20^{\Delta zf}$ homozygotes at 10.5 dpc was a fusion of the glossopharyngeal (IX) nerve with the vagus (X) nerve complex (Fig. 3D,E). All of these defects were observed on both sides of the $Krox20^{\Delta zf}$ homozygous mutant embryos.

Histological sections of $Krox20^{\Delta zf}$ homozygous mutant embryos and control littermates were also examined. Embryos isolated at 12.5 dpc were sectioned in transverse and frontal planes, and embryos isolated at 15.5 dpc were sectioned in transverse, frontal, and sagittal planes. This analysis revealed that all $Krox20^{\Delta zf}$ homozygous mutant embryos exhibited the same basic defect: The trigeminal (V) ganglion of the mutants overlapped and intermingled with the facial (VII) and vestibulocochlear (VIII) ganglia. At 12.5 dpc, the trigeminal ganglion was seen to overlap the rostral portion of the vestibulocochlear ganglion (Fig. 4A,B). More caudally at 12.5 dpc, the vestibulocochlear ganglion is beginning to separate into distinct vestibular and cochlear components. This partitioning of the vestibulocochlear ganglion was unaffected in mutant embryos (Fig. 4C,D). The trigeminal ganglion also overlapped and appeared to intermingle (or fuse) with the facial ganglion (Fig. 4C–F). Formation of the seventh nerve (VII), however, appeared unaffected in the $Krox20^{\Delta zf}$ homozygous mutant embryos (Fig. 4C–F). Entry of the trigeminal nerve roots into the brain stem appeared very disorganized in mutant embryos (Fig. 4E,F).

Essentially the same phenotype was observed in mutant embryos isolated at 15.5 dpc (Fig. 5). The trigeminal ganglion overlapped with both the facial ganglion and the vestibular ganglion, which is distinct from the cochlear ganglia, and the nerve roots of these ganglia appeared disorganized where they entered the brain stem (Fig. 5C,D). Despite the fusion of the glossopharyngeal (IX) nerve with the vagus (X) nerve complex observed in mutant embryos by anti-neurofilament immunohistochemistry (Fig. 3D,E), we did not detect by histological analysis of sectioned material any obvious abnormalities of the ninth and tenth cranial nerves (IX and X, respectively) or their associated superior and inferior ganglia (data not shown).

Krox20^{Δzf} homozygous mutant embryos lack r3 and r5

To determine the cause for the fusions of cranial sensory

ganglia observed in $Krox20^{\Delta zf}$ homozygous mutant mice, we performed in situ hybridization analyses with probes for several genes that are expressed in a segmentally restricted fashion in the developing hindbrain. Sham et al. (1993) recently reported convincing evidence that the *Krox20* gene regulates expression of the *Hoxb-2* (*Hox-2.8*) gene. At 8.5 dpc, *Hoxb-2* is expressed at a uniform level in neuroepithelium, with an anterior boundary of expression that corresponds to the future r2/r3 boundary. By 9.5 dpc, however, *Hoxb-2* expression is up-regulated in r3, r4, and r5. Krumlauf and colleagues have identified two enhancer elements in the *Hoxb-2* gene responsible for this up-regulation (for review, see Krumlauf 1993). One element is responsible for the up-regulation in r4 (Marshall et al. 1992); the second element is responsible for the up-regulation in r3 and r5, and confers r3/r5-restricted expression to a heterologous promoter in transgenic mice. They further identified *Krox20*-binding sites in this enhancer and demonstrated that the r3/r5-restricted expression conferred by this enhancer was lost if the *Krox20*-binding sites were mutated (Sham et al. 1993).

We analyzed *Hoxb-2* expression by nonradioactive whole-mount in situ hybridization in $Krox20^{\Delta zf}$ homozygous embryos and wild-type littermates isolated at 9.0 dpc (14–16 somites) (Fig. 6). *Hoxb-2* expression in wild-type embryos appeared identical to the pattern described previously (Sham et al. 1993). Wild-type embryos had an anterior limit of *Hoxb-2* expression at the r2/r3 boundary (Fig. 6A). Expression in r3, r4, and r5 was higher than in more posterior regions of the neural tube, and a column of neural crest cells expressing *Hoxb-2* was observed migrating from r4 into the second branchial arch (Fig. 6A). In $Krox20^{\Delta zf}$ homozygous embryos, no *Hoxb-2* expression was detectable in the region of the neural tube corresponding to r3 (Fig. 6B,C). *Hoxb-2* expression in r4 and in the column of neural crest cells migrating from r4 was unaffected. *Hoxb-2* was still expressed in the region of the neural tube corresponding to r5 in the $Krox20^{\Delta zf}$ homozygous embryos (Fig. 6B,C), although the level of expression was reduced compared with the intense band of *Hoxb-2* expression seen in r5 in wild-type embryos (Fig. 6A; also cf. Fig. 1, C and D, in Sham et al. 1993).

Two possibilities could explain the *Hoxb-2* expression pattern observed in the $Krox20^{\Delta zf}$ homozygous mutant embryos. The first possibility is that r3 and r5 are present in the homozygous mutants, but *Hoxb-2* expression is down-regulated because of the lack of its transcriptional

Figure 3. The *Krox20*^{Δzf} mutation affects the formation of cranial ganglia. Whole-mount immunohistochemistry with an anti-neurofilament monoclonal antibody of *Krox20*^{Δzf} homozygous mutant embryos (*B,D,E*) and wild-type littermates (*A,C*). (*A,B*) 9.5 dpc embryos. A zone free of neurofilament-positive cells separating the condensing trigeminal ganglion from the facial/acoustic ganglion complex, which is present in the wild-type embryo (solid arrowhead in *A*), is absent in the *Krox20*^{Δzf} homozygous mutant embryo. In addition, the facial/acoustic ganglion complex in the mutant embryo is less condensed and is stained more lightly by the monoclonal antibody than the facial/acoustic complex of the wild-type littermate. (*C–E*) 10.5-dpc embryos. The zone free of neurofilament-positive cells (solid arrowhead in *C*) still clearly separates the trigeminal ganglion from the facial/acoustic ganglion complex in the wild-type embryo (*C*). This zone is absent from the *Krox20*^{Δzf} homozygous mutant embryos and the two ganglia overlap (*D,E*). Distal portions of the facial nerve (arrows in *C–E*) and the three branches of the trigeminal nerve appear identical in the mutant and wild-type embryos. An additional defect observed in the *Krox20*^{Δzf} homozygotes was a fusion of the glossopharyngeal (IX) nerve with the vagus (X) nerve complex (open arrowhead, *D,E*). Cranial nerves and ganglia are designated with Roman numerals as follows: (V) Trigeminal; (VII/VIII) facial/acoustic; (IX) glossopharyngeal; (X) vagus. (ov) otic vesicle.

regulator. One problem with this interpretation is that *Hoxb-2* expression is entirely absent at the expected position of r3, whereas it is present, albeit at a lower level, at the position of r5. A second possibility is that r3 and r5

are missing in the *Krox20*^{Δzf} homozygous mutant embryos. In this case, the *Hoxb-2* expression observed at the position of r5 would actually be the expression normally observed in r6.

Figure 4. Histological analysis of *Krox20 $\Delta z f$* homozygous embryos and control littermates at 12.5 dpc. Transverse (A–D) and frontal (E,F) sections through *Krox20 $\Delta z f$* homozygous mutant embryos (B,D,F) and wild-type (A,C) or heterozygous (E) littermates. (A,B) Note that the neuron-free zone (solid arrowhead in A) present between the trigeminal ganglion and the vestibulocochlear ganglion complex in the wild-type embryo is absent in the *Krox20 $\Delta z f$* homozygous mutant embryo. (C,D) More caudal sections of the same embryos shown in A and B. Note the fusion of the trigeminal and facial ganglia in the mutant embryo. Partitioning of the vestibulocochlear ganglion into distinct vestibular (VG) and cochlear (CG) components is unaffected in the mutant embryo, as is formation of the facial nerve (n7). (E,F) Note again the fusion of the trigeminal and facial ganglia in the mutant embryo. The entry of the nerve roots of the trigeminal ganglion into the brain stem (nr5 in E) is very disorganized in the *Krox20 $\Delta z f$* homozygous mutant embryos, and the trigeminal nerve roots appear to intermingle with nerve roots from the facial ganglion as they enter the brain stem. (F) Bar, 100 μ m (applies to A–E). (CG) Cochlear component of the vestibulocochlear ganglion; (G5) trigeminal ganglion; (G7) facial ganglion; (n7) facial nerve; (nr5) nerve roots of the trigeminal nerve entering the brain stem; (t) tubotympanic recess; (VCG) vestibulocochlear ganglion; (VG) vestibular component of the vestibulocochlear ganglion.

To help distinguish between these possibilities, we performed *in situ* hybridization on embryo sections with radioactively labeled probes for several genes expressed

in a segmentally restricted fashion in the developing hindbrain (Fig. 7). We chose to use radioactive probes on sectioned material for this analysis to enable us to test

Figure 5. Histological analysis of *Krox20*^{Δzf} homozygous embryos and control littermates at 15.5 dpc. Sagittal (A–D) and transverse (E,F) sections through *Krox20*^{Δzf} homozygous mutant embryos (B,D,F) and wild-type (A,C,E) littermates. (A,B) Note that the enlarged domain (solid arrowhead, in B) of the trigeminal ganglion present in the *Krox20*^{Δzf} homozygous mutant embryo overlaps the domain of the facial ganglion. The two ganglia are quite distinct in the wild-type embryo. (C,D) More medial sections of the same embryos shown in A and B. Note the well-organized entry of the proximal portion of the fifth cranial nerve (V) into the brain stem (open arrow, C) in the wild-type embryo and the distinct facial and vestibular ganglia. In the *Krox20*^{Δzf} homozygous mutant embryo the trigeminal, facial, and vestibular ganglia all overlap, and the entry of these nerve roots into the brain stem appears disorganized (solid arrow in D). Note also that the cochlear (spiral) ganglia are unaffected in the mutant embryo. (E,F) These panels represent similar planes of section to those in Fig. 4, C and D. Note again the fusion of the trigeminal and facial ganglia in the mutant embryo. (F) Bar, 200 μm (applies to A–F). (CG) Cochlear (spiral) ganglion; (G5) trigeminal ganglion; (G7) facial ganglion; (n5) trigeminal nerve; (n7) facial nerve; (t) tubotympanic recess; (VG) vestibular ganglion.

several different probes on each embryo. The probes used were cellular retinoic acid-binding protein I (*CRABP I*), *Hoxb-1*, the receptor-type tyrosine kinase *Sek*, *Fgf3* (*int-2*), and a sense strand probe for *Hoxb-3*. The latter probe was used as a result of the serendipitous finding that the

sense strand of *Hoxb-3* was expressed in r4 and r6 and was absent from r5 (see Discussion). We therefore used hybridization of the sense strand of *Hoxb-3* as a marker for r4, r5, and r6. *Hoxb-1* served as a marker for r4 (Murphy et al. 1989; Wilkinson et al. 1989b), whereas *CRABP*

Figure 6. *Krox20*^{Δzf} homozygous embryos exhibit altered expression of *Hoxb-2*. (A–C) Whole-mount in situ hybridization with a *Hoxb-2* probe of *Krox20*^{Δzf} homozygous mutant embryos (B,C) and a wild-type littermate (A). Note in all embryos *Hoxb-2* expression in r4 and in a column of neural crest cells migrating from r4 into the second branchial arch. The wild-type embryo exhibits *Hoxb-2* expression in r3 (open arrowhead, A) which is absent in the *Krox20*^{Δzf} homozygous embryos (solid arrowheads, B and C). In addition, the intensity of *Hoxb-2* expression in r5 in the *Krox20*^{Δzf} homozygous embryos is reduced compared with the wild-type littermate.

I was particularly informative as a marker for r3 because in the ventral portions of the neural tube *CRABP I* expression diminishes in r3, while it is expressed at moderate levels in r2 and at high levels in r4–r6 (Maden et al. 1992; Ruberte et al. 1992). *Sek* expression was informative as a marker for several rhombomeres, as it is expressed at high levels in r3 and r5, at moderate levels in r2 and r6, and absent from r4 (Nieto et al. 1992). *Fgf3* (*int-2*) was informative as a marker for r5 and r6 (Wilkinson et al. 1988).

Examination of this in situ hybridization data indicated that r3 and r5 are missing from *Krox20*^{Δzf} homozygous mutant embryos by the 14-somite stage. The loss of r3 is demonstrated clearly by *CRABP I* hybridization (Fig. 7A,B). In the mutant embryo, the moderate expression domain of r2 directly abuts the high expression domain of r4, with no intervening domain of reduced expression (r3). In addition, *CRABP I*-expressing neural crest cells can be seen migrating from the adjacent expression domains of r2 and r4 in the mutant embryo (Fig. 7B), with no crest-free zone separating them. Loss of r5 is demonstrated clearly by hybridization of the *Hoxb-3* sense strand probe (Fig. 7I,J). The high *Hoxb-3s* expression domains of r4 and r6 abut directly in the mutant, with no intervening nonexpressing domain (r5 in the wild-type embryo). Loss of both r3 and r5 is also supported by *Sek* hybridization (Fig. 7E,F). The moderate expression domains of r2 and r6 abut the nonexpression domain of r4. The high-level *Sek* expression domains of r3 and r5 are lost in the mutant. Hybridization of *Fgf3* (*int-2*) (Fig. 7G,H) also supports the loss of r5, as in the mutant the expressing domain appears shorter and the

rhombomeric constriction separating r5 and r6 in the wild-type embryo is absent in the mutant. In summary, the data presented in Figures 6 and 7 indicate that, by 9.0 dpc (14–16 somites), r3 and r5 are absent from *Krox20*^{Δzf} homozygous mutant embryos.

Discussion

In this report we have demonstrated that *Krox20* plays an essential role during development of the hindbrain and associated cranial sensory ganglia in mice. Mice homozygous for a targeted mutation that deletes the majority of the protein coding capacity of the *Krox20* gene, including the zinc finger DNA-binding domain, die shortly after birth. These mice exhibit major defects in the formation of the cranial sensory ganglia. The primary phenotype observed in *Krox20*^{Δzf} homozygous mutant embryos was an overlap and partial fusion of the trigeminal ganglion with the facial/acoustic ganglion complex. In addition, the proximal nerve roots emanating from these ganglia were disorganized and appeared to intermingle as they entered the brain stem. *Krox20*^{Δzf} homozygous mutants also displayed a fusion of the glossopharyngeal nerve with the vagus nerve complex. We demonstrated further that the probable cause for this fusion of the cranial nerves and ganglia was the loss of r3 and r5 in *Krox20*^{Δzf} homozygous mutant embryos.

This interpretation is supported by findings from a set of rhombomere transplantation experiments performed recently in the chick, as well as by findings from the treatment of vertebrate embryos with retinoic acid. Kuratani and Eichele (1993) found that when they removed

Figure 7. r3 and r5 are missing in *Krox20*^{Δzf} homozygous embryos at 9.0 dpc. In situ hybridization analysis of *Krox20*^{Δzf} homozygous mutant embryos (B,D,F,H,I) and a wild-type littermate (A,C,E,G,I) are shown. Coronal sections through the hindbrain of embryos isolated at 9.0 dpc (14–16 somites) were hybridized with antisense riboprobes for *CRABP I* (A,B), *Hoxb-1* (C,D), the receptor-type tyrosine kinase *Sek* (E,F), *Fgf3* (*int-2*; G,H), and a sense-strand probe for *Hoxb-3* (*Hoxb-3s*; I,J). In all panels, anterior is to the right and the position of the otic vesicle is marked with an asterisk (*). Two homozygous mutant embryos (one in B and D; the other in F, H, and J) were used. In the wild-type embryo, rhombomere boundaries are marked with arrowheads and are numbered. For the homozygous mutant embryos, rhombomere boundaries are marked with arrowheads and our interpretation of the rhombomere identity, based on marker gene expression patterns, is indicated as the prime of the rhombomere number (e.g., 4' indicates r4 of the homozygous mutant embryo).

Figure 7. (See facing page for legend.)

r3 from chick embryos and grafted r4 in its place, fusion of the trigeminal and facial/acoustic ganglia occurred. This result is strikingly similar to the phenotype observed in *Krox20^{Azf}* homozygous mutants. Alternatively, when r3 was grafted in place of r7 in the chick experiments, a nerve-free gap resulted within the glossopharyngeal/vagus nerve root (Kuratani and Eichele 1993). r3, therefore, will apparently inhibit the formation of neurofilament-positive cells in the periphery, and this property is autonomous to the neuroepithelium of r3, as it is not altered upon transplantation. Further support for our interpretation of the phenotype of *Krox20^{Azf}* homozygous mutants comes from the analysis of embryos treated with retinoic acid. Retinoic acid treatment of vertebrate embryos can lead to fusions of cranial nerves and ganglia and also causes alterations in the expression patterns of *Krox20* and some of the *Hox* homeo box genes (Morriss-Kay et al. 1991; Papalopulu et al. 1991; Conlon and Rossant 1992; Marshall et al. 1992; Sundin and Eichele 1992; Kessel 1993; for reviews, see Maden and Holder 1992; Morriss-Kay 1992). Recent work analyzing the effects of retinoic acid administration on embryonic development in mice suggested that r2/r3 adopted an r4/r5 identity in treated embryos. This transformation was indicated by the duplication of r4/r5 expression markers in r2/r3, as well as by the morphological transformation of the cranial nerve emanating from r2/r3 from a trigeminal to a facial identity (Marshall et al. 1992; for review, see Krumlauf 1993). Thus, retinoic acid administration may also lead to a loss of r3 and can lead to fusions of the cranial ganglia similar to that observed in *Krox20^{Azf}* homozygous mutant embryos.

Interestingly, a mutation in another gene expressed in the hindbrain can also lead to fusions of cranial sensory ganglia. One of the groups that made mice mutant in *Hoxa-1* (*Hox-1.6*) reported fusions in mutant embryos of both the trigeminal and facial/acoustic ganglia and the glossopharyngeal and vagus complexes, a phenotype very similar to that observed in *Krox20^{Azf}* homozygous mutant embryos (Chisaka et al. 1991). The other group that made a targeted mutation of *Hoxa-1* did not report such fusions (Lufkin et al. 1991).

The formation of r3, r4, and r5 may be pivotal for proper development of the hindbrain. In the chick, where it has been studied most extensively, formation of rhombomere boundaries takes place in a defined sequence (Vaage 1969). r3 is the first rhombomere for which both anterior and posterior boundaries form (Vaage 1969; see Fig. 2 in Lumsden 1990), and the next boundary to form completes the central set of r3, r4, and r5. A large number of observations indicate that r3 and r5 have different properties than other rhombomeres. For example, the appearance of differentiated neurons is delayed in r3 and r5 compared with the even-numbered r2, r4, and r6 (Lumsden and Keynes 1989). r3 and r5 may also have cell-surface properties that differ from those of even-numbered rhombomeres. In grafting experiments in the chick, Guthrie and Lumsden demonstrated that the juxtaposition of r3 with r5 did not generate a new boundary, even though the juxtaposition of either of these rhombomeres

with an even-numbered rhombomere did generate a boundary (Guthrie and Lumsden 1991; Lumsden and Guthrie 1991). r3 and r5 have been found to coincide with localized areas of cell death, as assayed by Nile blue sulfate staining (Lumsden et al. 1991; Jeffs et al. 1992). In addition, it has been reported that in the chick, r3 and r5 do not produce migratory neural crest cells (Lumsden et al. 1991), although recent work in several vertebrate species contradicts these results (Bradley et al. 1992; Serbedzija et al. 1992; Oxtoby and Jowett 1993; Sechrist et al. 1993; Sham et al. 1993). Our results demonstrate that expression of the *Krox20* gene is required for development of r3 and r5.

The discovery that the nominal noncoding strand (i.e., detected with hybridization of the sense strand in situ hybridization probe) of the *Hoxb-3* gene is expressed in r4 and r6 is a curious finding. Extensive analysis of the *Hoxb-3* transcription unit had shown that multiple alternatively spliced transcripts from the coding strand were present (Sham et al. 1992). Examination of the noncoding strand of the mouse *Hoxb-3* cDNA reveals the presence of a 169-amino-acid open reading frame (data not shown), most of which is conserved in the human *Hoxb-3* gene (Acampora et al. 1989). This open reading frame, however, does not have any significant homology to any entries in the nucleic acid and protein sequence data bases. Experiments are in progress to learn more about this transcript from the noncoding strand. Presently, however, this transcript serves as a very useful marker for the presence or absence of r4, r5, and r6.

We have not determined a specific cause of death for the *Krox20^{Azf}* homozygous animals. However, the cranial nerves and ganglia provide both motor and sensory innervation for the head and neck, as well as autonomic control over visceral functions such as breathing, heart rate, and swallowing (for review, see Role and Kelly 1991). Examination of *Krox20^{Azf}* homozygous mutant embryos revealed major defects in several cranial ganglia and the connection of the nerve roots of these ganglia to the brain stem. The trigeminal and facial ganglia were affected most severely. Among its many functions, the trigeminal nerve carries the sensory innervation of most of the oral mucosa and the anterior portion of the tongue, whereas trigeminal motor neurons innervate muscles responsible for mastication (Williams and Warwick 1980; Dodd and Kelly 1991). The facial nerve is also a mixed nerve and provides sensory innervation of part of the tongue, transmits parasympathetic innervation of the salivary glands, and provides motor innervation for muscles of the face (Williams and Warwick 1980; Role and Kelly 1991). Defects in both of these cranial nerves and ganglia would be expected to severely compromise the ability of the animal to feed. The majority of *Krox20^{Azf}* homozygous mutant animals die during the first 24 hr, without having fed. Mutant animals that survive for a week or two are runted severely at all stages compared with heterozygous and wild-type littermates. Thus, it seems likely that impaired ability to feed strongly contributes to the perinatal lethality observed in *Krox20^{Azf}* homozygous mutant animals.

Materials and methods

Derivation of ES cell lines

For low-passage-number ES cell lines to be used in these experiments, new ES cell lines were derived essentially as described (Robertson 1987). Cell lines were derived both from normal and implantationally delayed blastocysts from agouti 129/Sv mice cultured on feeder layers of mitomycin C-treated primary embryonic fibroblast (PEF) cells in media containing 1000 U/ml of recombinant leukemia inhibitory factor (LIF) (ESGRO; GIBCO). Male ES cell lines were identified by Southern blot analysis using the Y chromosome-specific probe pY2 (Lamar and Palmer 1984) and were tested for germ-line transmission by blastocyst injection. Several cell lines gave good germ-line transmission, and one of these, the CJ7 line, was used for these experiments.

Targeting vector construction

A strain 129 mouse genomic phage library (Stratagene) was screened with a probe encompassing nucleotides 2417–2789 in the 3'-untranslated region of the *Krox20* cDNA (Chavrier et al. 1988). This probe was made by using PCR to amplify this fragment from genomic DNA (Chavrier et al. 1989). Hybridizing phage were purified and phage inserts were subcloned into pGem7Zf(+) (Promega). The identity of hybridizing clones was confirmed by nucleotide sequencing. To construct the targeting vector, we utilized the *neo* expression cassette from PGKneobpa (Soriano et al. 1991). A 2.5-kb *EcoRI*–*Bam*HI fragment from the 5' end of the *Krox20* gene was blunt-end-ligated into a *Cla*I site upstream of the pGKneobpa cassette, and a 3.4-kb *Sfi*I–*Bam*HI fragment just 3' to the *Krox20* gene was blunt-end-ligated into a *Not*I site downstream of the pGKneobpa cassette. This resulted in an 800-bp deletion in exon 2 that removes the carboxy-terminal 315 amino acids of the *Krox20* protein, including the zinc finger DNA-binding domain. To permit negative selection against random integration of the targeting vector, a pMCI–HSVTK cassette (Mansour et al. 1988) was introduced 3' to the *Krox20* *Sfi*I–*Bam*HI fragment.

Electroporation and selection of ES cells

CJ7 cells were routinely cultured on mitomycin C-treated PEF feeder layers in Dulbecco's modified Eagle medium [(DMEM) high glucose formulation; GIBCO] supplemented with 15% fetal bovine serum (FBS) (HyClone), 1000 U/ml of recombinant LIF (ESGRO; GIBCO), and additional supplements (nonessential amino acids, β -mercaptoethanol, nucleosides, and antibiotics) as described (Robertson 1987). CJ7 ES cells at passage 3–4 were trypsinized and resuspended in phosphate-buffered saline (PBS) at 1.2×10^7 cells/ml. The resuspended cells (0.75 ml) were electroporated with 25 μ g of linearized targeting vector DNA using a Bio-Rad Gene Pulser (500 μ F, 240 V). After electroporation, cells were plated into four 6-cm tissue culture dishes containing mitomycin C-treated PEF feeder layers prepared from a transgenic mouse line that constitutively expresses the neomycin resistance gene (Stewart et al. 1992). After 24 hr, media in the dishes were removed and replaced with selection media containing, in addition to the routine supplements, 350 μ g/ml of G418 (GIBCO) (as dry powder) and 0.5 μ M FIAU [1-(2-deoxy, 2-fluoro- β -D-arabinofuranosyl)-5 iodouracil; Bristol-Myers Squibb]. Media on the plates were changed daily. After 4 days, FIAU selection was stopped and media were replaced with selection media containing only 350 μ g/ml of G418. Colonies were picked individually on days 6 and 7, disaggregated in trypsin–EDTA, and plated into wells of 48-well tissue culture plates containing PEF feeder cells.

Screening of G418-resistant colonies

For screening, ES cell clones in the 48-well dishes were trypsinized, and $\frac{1}{3}$ of the well was replica plated into a new 48-well dish without feeder cells and grown for DNA isolation. To the remaining $\frac{2}{3}$ of the ES cell clone in the 48-well dish, an equal volume of $2\times$ freezing solution (20% DMSO, 20% FBS, 60% DMEM prepared without sodium bicarbonate and containing 10 mM HEPES buffer) was added and the plate was frozen at -80°C . DNA for Southern analysis was prepared from colonies in the replica plates by the method of Laird et al. (1991).

Genotyping ES cell lines and mice by Southern analysis and PCR

DNA was isolated from the mutant ES cell lines and tail biopsy samples using standard procedures (Sambrook et al. 1989). Embryos were genotyped using yolk sac DNA. Yolk sacs were incubated under mineral oil in 40–100 μ l of lysis buffer [50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2 mM MgCl_2 , 0.45% NP-40, 0.45% Tween 20, 60 μ g/ml of proteinase K] at 55°C overnight. The proteinase K was inactivated by heating the lysates at 94°C for 30 min, and the lysates were genotyped using PCR or Southern analysis. PCR primers for the wild-type *Krox20* allele were 5'-GCAGAAGGAACGGAAGAGCAG-3', located 3' to the zinc finger domain in a region deleted in the targeting construct, and 5'-ATCAAGGTCCTTTGCCAGATC-3', located downstream of the *Sfi*I site. PCR primers for the *Krox20*^{*Δ*2f} mutant allele were 5'-TCGCAGCGCATCGCCTTCTATCG-3', located in the *neo* gene, and 5'-ATCAAGGTCCTTTGCCAGATC-3', located downstream of the *Sfi*I site in the wild-type *Krox20* locus. The PCR mixture consisted of 20 μ l of lysis buffer, 2 μ l of $10\times$ PCR buffer, [166 mM $(\text{NH}_4)_2\text{SO}_4$, 670 mM Tris-HCl (pH 8.8), 1 mg/ml of BSA], 200 μ M each dNTP, 200 ng of each primer, and 2 U/ μ l of *Taq* polymerase (Cetus) in a final volume of 40 μ l. Temperature cycling conditions were 95°C , 30 sec, 65°C for 30 sec, and 72°C for 90 sec for 40 cycles. PCR products were resolved on 1% agarose gels.

Krox20^{*Δ*2f} alleles passed through the germ line were analyzed by Southern blotting to confirm that they contained the expected replacement-type homologous recombination event. For Southern analysis, 10 μ g of DNA was digested with the restriction enzymes *Eco*RI or *Hind*III. The DNA was fractionated on a 0.8% agarose gel, transferred to ZetaProbe GT membranes (Bio-Rad), and hybridized with the indicated probes (Fig. 1). The 5' probe was a *Hind*III–*Eco*RV genomic subclone. The exon 1 probe was a 155-bp fragment amplified by PCR from genomic DNA (Chavrier et al. 1989), and the *neo* probe was the *Xho*I fragment of pGKneobpa (Soriano et al. 1991). Both the 5' and 3' sides of the recombinant *Krox20*^{*Δ*2f} alleles were checked using enzymes that flanked the integration event. The 5' side was checked with the 5' and exon I probes (Fig. 2). The 3' side was checked by hybridization of *Eco*RI-digested DNA with the *neo* probe. This gave the expected 5.5-kb hybridizing band present only on the *Krox20*^{*Δ*2f} chromosome (data not shown). Hybridization and washing conditions were as described by the membrane supplier.

Blastocyst injection and animal breeding

Wells from the replica plate that screened positive for homologous recombination at the *Krox20* locus were identified, and the corresponding well on the master plate was thawed and the mutant ES cell clone was expanded on PEF feeder cells for injection and DNA isolation. Mutant ES cells were trypsinized, centrifuged, and resuspended in injection medium (DMEM pre-

pared without sodium bicarbonate and containing 10 mM HEPES buffer and 10% FBS). Ten to fifteen ES cells were injected into the blastocoel cavity of 3.5-dpc blastocysts from C57Bl/6J mice. Injected blastocysts were surgically transferred into the uteri of pseudopregnant B6CBAF1/J (Jackson Laboratories) recipients at 2.5 dpc. Male chimeras with extensive ES cell contribution to the coat were bred with C57Bl/6J females to test for germ-line transmission of the dominant agouti coat color marker. F₁ animals heterozygous for the *Krox20*^{Δ_{2f}} allele were intercrossed.

Histological analysis

Embryos were dissected and DNA was prepared from the yolk sacs for genotyping by PCR or by Southern blot analysis. Embryos for histological analysis were fixed in Bouin's solution. Embryos for in situ hybridization were fixed overnight at 4°C in 4% paraformaldehyde in PBS. Fixed embryos were dehydrated through graded alcohols, embedded in paraffin, sectioned at 6–10 μm (depending on age), and stained with hematoxylin and eosin.

Whole-mount immunohistochemistry

Whole-mount immunohistochemistry with the monoclonal antibody 2H3, which recognizes a 155-kD neurofilament protein (Dodd et al. 1988) (obtained from the Developmental Studies Hybridoma Bank, maintained by the Department of Biology, University of Iowa, Iowa City, IA, under contract from the National Institute of Child Health and Human Development (NICHD)), was performed according to a protocol kindly supplied by Dr. Andrew Lumsden (Lumsden and Keynes 1989). Embryos at 9.5 and 10.5 dpc were dissected from the decidua, and DNA was prepared from the yolk sacs for genotyping. Embryo heads were opened along the dorsal midline to allow penetration of reagents, and embryos were fixed in 4% paraformaldehyde in PBS for 4 hr at 4°C. Embryos were washed three times for 60 min in PBS. Endogenous peroxidases were blocked by incubating the embryos overnight at 4°C in PBS containing 0.1% hydrogen peroxide, 1.0% Triton X-100, 5% calf serum. Embryos were then washed three times for 60 min in PBS containing 1.0% Triton X-100, 5% calf serum. For incubation of embryos with 2H3 monoclonal antibody, fresh hybridoma supernatant was mixed with an equal volume of DMEM (without sodium bicarbonate) containing 10 mM HEPES, 10% FCS, 1.0% Triton X-100, and 0.02% sodium azide. Embryos were incubated for 2 days at 4°C and washed three times for 60 min at 4°C in PBS containing 1% normal goat serum, 1.0% Triton X-100. For incubation with the secondary antibody, embryos were incubated in a 1 : 100 dilution in PBS containing 1% normal goat serum, 1.0% Triton X-100 of peroxidase-conjugated goat anti-mouse IgG (Sigma) at 4°C overnight. Embryos were then washed three times for 60 min at 4°C in PBS containing 1% normal goat serum, 1.0% Triton X-100. To detect the peroxidase reaction, embryos were incubated in diaminobenzidine (500 μg/ml in Tris-buffered saline) for 3 hr at 4°C in the dark. To activate the diaminobenzidine, hydrogen peroxide was added to 0.03% (vol/vol), and embryos were observed at room temperature under a dissecting microscope until cranial ganglia and nerves were visible (~5 min). Embryos were then washed repeatedly in large volumes of PBS, and photographs were taken immediately on an Olympus stereoscope.

In situ hybridization

Whole-mount in situ hybridization was performed according to the method of Wilkinson (1992), with the following modifica-

tions. For 9.0-dpc embryos the time of the proteinase K treatment was reduced to 7 min. The time for preblocking the embryos with normal sheep serum was increased to 2.5 hr. In addition, to stop the histochemical reaction we found it necessary to store the embryos overnight in low pH (pH 5.5) PBS–0.1% Tween 20. The *Hoxb-2* probe (Sham et al. 1993) was the gift of Robb Krumlauf (Institute for Medical Research, London, UK). The new *Hox* gene nomenclature is from Scott (1992).

In situ hybridization of embryo sections was performed as described previously (Franco del Amo et al. 1992; Smith and Gridley 1992). Plasmids were linearized with the appropriate enzymes, and ³⁵S-labeled transcripts were generated by runoff transcription. The probe for the *CRABP I* gene consisted of a 750-bp cDNA clone containing the entire coding sequence (Stoner and Gudas 1989; gift of Joe Grippo, Hoffman-La Roche, Inc., Nutley, NJ). The sense-strand probe for the *Hoxb-3* gene was transcribed from the *Bam*HI–*Hind*III fragment just downstream of the homeo box (Sham et al. 1992; gift of Robb Krumlauf). The probe for the *Fgf3 (int-2)* gene was as described (Wilkinson et al. 1988; gift of David Wilkinson, Institute for Medical Research, London, UK). In situ hybridization probes for *Hoxb-1* (Frohman et al. 1990) and *Sek* (Nieto et al. 1992) were made by reverse transcriptase–polymerase chain reaction (RT-PCR). The *Hoxb-1* probe was a 435-nucleotide fragment from the 3' untranslated region of the gene. The *Sek* probe consisted of nucleotides 1848–2250 of the *Sek* cDNA (Nieto et al. 1992). Double-exposure photographs (dark field with red filter; bright field with blue filter) were taken on a Zeiss Axioplan microscope.

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