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|2, 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, Frohman 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-
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<sup>neo<sup>.

The linearized targeting vector was electroporated into
C57 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 ~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 F1 hybrids of the two strains. Germ-line transmission of the Krox20 (Krox20) allele was obtained for both targeted clones. Krox20 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]. F1 mice heterozygous for the Krox20 mutation appeared phenotypically normal.

**Mice homozygous for the Krox20 mutation die shortly after birth**

To examine whether mice homozygous for the Krox20 mutation were viable, heterozygous F1 animals were intercrossed and the genotypes of F2 progeny were determined. Breeding and phenotypic analysis were carried out for both independently targeted Krox20 alleles. No differences in the mutant phenotype described below were noted between mice derived from the two clones. Most mice (~70%) homozygous for the Krox20 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 (~30%) of the homozygous animals lived for 5–10 days. These surviving Krox20 homozygotes, however, were severely runted compared with heterozygous or wild-type littermates. No Krox20 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 F2 generation. Although lethality was completely penetrant in Krox20 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 mutation was maintained on the inbred 129/Sv background. We have not determined a specific cause of death for the Krox20 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 mutation exhibit defects in cranial nerves and ganglia**

Given the expression pattern of the Krox20 gene and the perinatal lethality observed in Krox20 homozygous animals, we sought to determine whether there were detectable neuronal defects in the Krox20 homozygotes. To assess the development of cranial nerves and ganglia, embryos from intercrosses of Krox20 +/+ 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] ganglion 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 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 homozygotes appeared less condensed than that of wild-type embryos. These defects were bilateral in the Krox20 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 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-
nial nerve (VII) and the three branches of the fifth nerve (V) appeared unaffected in the Krox20azf null embryos. An additional defect observed in Krox20azf 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 Krox20azf homozygous mutant embryos.

Histological sections of Krox20azf 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 Krox20azf 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 Krox20azf 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].

Krox20azf homozygous mutant embryos lack r3 and r5 To determine the cause for the fusions of cranial sensory ganglia observed in Krox20azf 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 (Hoxb-2) 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 Krox20azf 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 Krox20azf 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 Krox20azf 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 Krox20azf 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
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+/- 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* mutant embryos and control littermates at 12.5 dpc. Transverse (A–D) and frontal (E,F) sections through Krox20* homozygous mutant embryos (B,D,F) and wild-type (A,C) or heterozygous (E) littermates. (A,B) Note that the neuron-flee 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* 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* 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 µm [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<sup>−/−</sup> homozygous embryos and control littermates at 15.5 dpc. Sagittal (A–D) and transverse (E,F) sections through Krox20<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> 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 1 (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
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 Krox20AB/ 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 Krox20AB 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 Krox20AB 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. Krox20AB 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 Krox20AB 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...
Hindbrain defects in Krox20 mutant mice

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<sup>hpr</sup> 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 neurolamin-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<sup>hpr</sup> 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<sup>hpr</sup> 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<sup>hpr</sup> 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<sup>hpr</sup> 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<sup>hpr</sup> 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<sup>hpr</sup> 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<sup>hpr</sup> 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 PGKneoeba (Soriano et al. 1991). A 2.5-kb EcoRI-BamHI fragment from the 5’ end of the Krox20 gene was blunt-end-ligated into a ClaI site upstream of the pGKneoeba cassette, and a 3.4-kb SfiI-BamHI fragment just 3’ to the Krox20 gene was blunt-end-ligated into a NotI site downstream of the pGKneoeba 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 SfiI-BamHI 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 μg of linearized targeting vector DNA using a Bio-Rad Gene Pulser (500 μF, 240 V). After electroporation, cells were plated into four 6-cm tissue culture plates containing PEF feeder cells.

For screening, ES cell clones in the 48-well dishes were plated into the method of Laird et al. (1991).

Screening of G418-resistant colonies

For screening, ES cell clones in the 48-well dishes were trypsinized, and ⅓ of the well was replica plated into a new 48-well dish without feeder cells and grown for DNA isolation. To the remaining ⅓ of the ES cell clone in the 48-well dish, an equal volume of 2X 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 μl of lysis buffer [50 mm KCl, 10 mm Tris-HCl [pH 8.3], 2 mm MgCl2, 0.45% NP-40, 0.4% Tween 20, 60 μg/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’-GCAGAAGGAAAGGAACGGAC-3’, located 3’ to the zinc finger domain in a region deleted in the targeting construct, and 5’-ATCAAGGTCTTTCGCCAGATC-3’, located downstream of the Shfl site. PCR primers for the Krox20mutant allele were 5’-TCGACACCGGATCCCTTCTATCG-3’, located in the neo gene, and 5’-ATCAAGGTCTTTCGCCAGATC-3’, located downstream of the Shfl site in the wild-type Krox20 locus. The PCR mixture consisted of 20 μl of lysis buffer, 2 μl of 10× PCR buffer, [166 mm (NH4)2SO4, 670 mm Tris-HCl [pH 8.8], 1 mg/ml of BSA], 200 μM each dNTP, 200 ng of each primer, and 2 U/μl of Taq polymerase [Cetus] in a final volume of 40 μl. 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.

Krox20mutant 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 μg of DNA was digested with the restriction enzymes EcoRI or HindIII. 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 HindIII–EcoRV 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 XhoI fragment of pGKneoeba [Soriano et al. 1991]. Both the 5’ and 3’ sides of the recombinant Krox20mutant 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 EcoRI-digested DNA with the neo probe. This gave the expected 5.5-kb hybridizing band present only on the Krox20mutant 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 blastocoeal cavity of 3.5-dpc blastocysts from C57Bl/6j mice. Injected blastocysts were surgically transferred into the uterus of pseudopregnant B6CBAF1/J (Jackson Laboratory) recipients at 2.5 dpc. Male chimeras with extensive ES cell contribution to the gnoto was bred with C57Bl/6j females to test for germ-line transmission of the dominant agouti coat color marker. F1 animals heterozygous for the Krox20 (mot) 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. Histological analysis of embryos was performed according to methods described previously (Franco do Amo et al. 1992, Smith and Gridley 1992).

**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. Embryonic heads were opened along the dorsal midline to allow penetration of reagents, and embryos were fixed 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 protocol. We thank Drs. Phil Soriano for the PGK-neobpa plasmid, Colin Stewart for breeding stock for the 129/Sv and the transgenic G418-resistant mouse strains, and Andrew Lumsden for the whole-mount immunohistochemistry protocol. We also thank David Wilkinson for the Fg3 (int-2) in situ hybridization probe, Robb Krumlauf for the Hoxb-3 probes, and Maureen Gendron-Maguire for help with the histology.

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