

Specification of the anterior hindbrain and establishment of a normal mid/hindbrain organizer is dependent on *Gbx2* gene function

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SUMMARY

Analysis of mouse embryos homozygous for a loss-of-function allele of *Gbx2* demonstrates that this homeobox gene is required for normal development of the mid/hindbrain region. *Gbx2* function appears to be necessary at the neural plate stage for the correct specification and normal proliferation or survival of anterior hindbrain precursors. It is also required to maintain normal patterns of expression at the mid/hindbrain boundary of *Fgf8* and *Wnt1*, genes that encode signaling molecules thought to be key components of the mid/hindbrain (isthmic) organizer. In the absence of *Gbx2* function, isthmic nuclei, the cerebellum, motor nerve V, and other derivatives of rhombomeres 1-3 fail to form. Additionally, the posterior midbrain in the mutant embryos appears to be extended caudally and displays abnormali-

ties in anterior/posterior patterning. The failure of anterior hindbrain development is presumably due to the loss of *Gbx2* function in the precursors of the anterior hindbrain. However, since *Gbx2* expression is not detected in the midbrain it seems likely that the defects in midbrain anterior/posterior patterning result from an abnormal isthmic signaling center. These data provide genetic evidence for a link between patterning of the anterior hindbrain and the establishment of the mid/hindbrain organizer, and identify *Gbx2* as a gene required for these processes to occur normally.

Key words: anterior hindbrain, cerebellum, *Gbx2*, midbrain, mid/hindbrain organizer, mes/met junction, rhombomeres, mouse

INTRODUCTION

Embryogenesis can be viewed as a sequential process in which expression of genes that confer positional information results in subdivision of the embryo into differently specified territories. In many regions of the body this leads in turn to the formation of groups of cells (organizers) producing secreted signaling molecules that function to specify new positional information. The developing vertebrate neuraxis provides an excellent experimental paradigm for studying these processes (reviewed by Lumsden and Krumlauf, 1996; Rubenstein and Shimamura, 1997). At the earliest step in its development, cells in the pluripotent embryonic ectoderm adopt a neural fate and acquire some degree of anterior-posterior (A-P) positional information, which is reflected by gene expression in restricted domains along the length of the primitive neuroectoderm. In the mouse, such region-specific gene expression is first

detected at the late-streak stage (approx. embryonic day [E] 7.5). As development proceeds, additional genes are expressed in the neuroepithelium and their expression domains become more restricted and refined, leading to precise specification of positional information along the main axes of the neural tube. Many genes involved in this process are vertebrate homologs of *Drosophila* homeobox genes.

Between E8.5 and E9.5 the anterior end of the neuraxis is partitioned into three vesicles, each of which develops into a major subdivision of the brain: the prosencephalon forms the forebrain; the mesencephalon (mes) develops into the midbrain; and the rhombencephalon, which is transiently subdivided into a series of swellings known as rhombomeres (r), forms the hindbrain. Transplantation studies have indicated that during this developmental period an organizer is established at the boundary between the mesencephalon and the metencephalon (met; the anterior rhombencephalon, including

r1 and r2) (Martinez et al., 1991; reviewed by Puelles et al., 1996). This mid/hindbrain organizer becomes localized within a morphological feature of the neural tube known as the isthmus constriction. It is thought to be responsible for producing signals that act over a relatively long range to pattern tissue both rostral and caudal to it.

Some of the genes required for the development of the region encompassing the mesencephalon and metencephalon have been identified (reviewed by Bally-Cuif et al., 1995; Joyner, 1996). *En1* and *En2*, vertebrate homologs of the *Drosophila engrailed* gene, are broadly expressed in the mes/met, and are required from at least E9.5 for development of the midbrain and cerebellum (Wurst et al., 1994; Hanks et al., 1995). In addition, studies in chick embryos have provided evidence that *En* genes play a later role in patterning the midbrain, possibly by regulating the genes that encode the ligands for EPH-related receptor tyrosine kinases, RAGS/AL1 and ELF1 (reviewed by Rétaux and Harris, 1996). Members of the PAX gene family (*Pax2*, *Pax5*, and *Pax8*), vertebrate relatives of the *Drosophila* paired box genes, are also expressed in the mes/met, and appear to be direct upstream regulators of *En2* (Song et al., 1996). Mutations in *Pax* genes cause deletions in the mid/hindbrain region (Urbanek et al., 1994; Brand et al., 1996; Favor et al., 1996). Recent studies have indicated that specification of the mesencephalon and establishment of a normal mid/hindbrain organizer also require a minimum dosage of *Otx* genes (Suda et al., 1996; A. Simeone, pers. comm.), vertebrate homologs of the *Drosophila orthodenticle* gene, which are expressed in the forebrain and midbrain (Simeone et al., 1992, 1993).

Two secreted proteins, WNT1 and FGF8, are known to play key roles in mid/hindbrain organizer function, and their expression domains in the mid/hindbrain boundary region therefore serve as markers for this signaling center. WNT1 is encoded by the vertebrate homolog of the *Drosophila wingless* gene, which is a regulator of *engrailed* in *Drosophila* (reviewed by Siegfried and Perrimon, 1994). After E9.5, *Wnt1* is expressed in a sharp transverse ring just rostral to the mes/met junction. In mice homozygous for *Wnt1* loss-of-function alleles, *En1*-expressing cells are rapidly lost (McMahon et al., 1992), and mutant embryos lack a midbrain and cerebellum (McMahon and Bradley, 1990; Thomas and Capecchi, 1990) as do mice deficient for both *En1* and *En2* (W. Wurst and A. L. J., unpublished observations). Expression of *En1* under the control of regulatory elements from the *Wnt1* gene is sufficient to substantially rescue early midbrain and cerebellum development in *Wnt1*-deficient embryos (Danielian and McMahon, 1996), suggesting that a major function of WNT1 signaling is to maintain *En* expression.

FGF8, a member of the Fibroblast Growth Factor family, is normally produced in a transverse ring of cells immediately caudal to the *Wnt1*-expressing cells at the mes/met junction (Heikinheimo et al., 1994; Ohuchi et al., 1994; Crossley and Martin, 1995; Mahmood et al., 1995). Evidence that FGF8 plays a role in regulating midbrain and cerebellum development comes from experiments showing that beads containing FGF8 protein can induce the formation of an ectopic midbrain and cerebellum when inserted into the posterior prosencephalon or anterior mesencephalon of an early chick embryo (Crossley et al., 1996; P. Crossley, J. L. R. R., G. R. M. and S. M., unpublished data) and from studies of transgenic mice in which *Fgf8* is ectopically expressed in the developing midbrain (Lee et al., 1997).

Gbx2, a vertebrate homeobox gene (Matsui et al., 1993; Chapman and Rathjen, 1995) related to the *Drosophila unplugged* gene (Chiang et al., 1995), is expressed at very early stages of neuraxis development (Bouillet et al., 1995; von Bubnoff et al., 1995; M. Frohman and G. R. M., unpublished observations). In the mouse embryo, *Gbx2* RNA is detected at approx. E7.5 in all three germ layers in a domain that extends rostrally from the posterior end of the embryo into the prospective hindbrain. By E9.5, *Gbx2* RNA is detected in a sharp transverse ring that is immediately caudal to the midbrain; *Fgf8* is also expressed in this domain. At this stage, *Gbx2* is also expressed in two longitudinal columns in the hindbrain and spinal cord. Later in development it is expressed in two domains in the forebrain (Bulfone et al., 1993). Outside the central nervous system (CNS), *Gbx2* RNA is detected at various sites including the developing otocysts, branchial arches, tail bud and limb bud. By producing and analyzing embryos homozygous for a *Gbx2* loss-of-function allele, we have identified *Gbx2* as an essential component of the network of genes required at the earliest stages of brain morphogenesis for development of the anterior hindbrain and the establishment of a normal mid/hindbrain organizer.

MATERIALS AND METHODS

Production of mice carrying a mutant allele of *Gbx2*

Because *Gbx2* is expressed during gastrulation and also later in embryogenesis, it was possible that homozygosity for a null allele might result in lethality at an early stage of development, thereby precluding analysis of *Gbx2* function at later stages. We therefore modified the *Gbx2* gene in such a way that its inactivation would be conditional upon DNA recombination mediated by the site-specific DNA recombinase, Cre (reviewed by Kilby et al., 1993). We isolated approx. 9 kb of *Gbx2* DNA from a strain 129 genomic library (Stratagene, La Jolla, CA) and inserted a *neomycin (neo)* resistance gene cassette (from pKJ1; Tybulewicz et al., 1991) and a *loxP* site, the recognition site for Cre, into a *Bam*HI site in the *Gbx2* intron, and a second *loxP* site into an *Apa*I site just downstream of the stop codon (Fig. 1A,B). The resulting construct, in which part of the *Gbx2* coding sequence was 'floxed' (i.e. flanked by *loxP* sites), was cloned into pTKa (a plasmid containing the HSV thymidine kinase (*tk*) gene (kindly provided by K. Jones, UCSF), to generate the full targeting construct, pGbx2^{lox} (Fig. 1A).

A homologous recombinant cell line was produced by electroporating *NotI* linearized pGbx2^{lox} into R1 embryonic stem cells (Nagy et al., 1993) followed by selection with G418 and Gancyclovir (Wurst and Joyner, 1993), but the latter had no significant effect on the number of stable integrants obtained. A PCR assay was used to identify homologous recombinants, and correct targeting was verified by Southern blot analysis using a 250 bp *Gbx2* genomic DNA fragment upstream of, and a *SalI-EcoRI* fragment downstream of the sequences included in the targeting vector (see Fig. 1B). Homologous recombinants were detected at a frequency of 1 per approx. 1000 clones screened.

A mouse line carrying the *Gbx2*^{lox} allele (Fig. 1C) was produced by injecting homologous recombinant cells into C57BL/6 blastocysts and mating the resulting chimeric males to B6DBA2 females. *Gbx2*^{lox} heterozygotes are phenotypically indistinguishable from their wild-type littermates, but *Gbx2*^{lox} homozygotes die shortly after birth. A detailed analysis of these mice has not been performed, but they exhibit abnormalities in anterior hindbrain development. Aberrantly spliced *Gbx2* transcripts have been detected in mice carrying the *Gbx2*^{lox} allele, suggesting that the presence of the *neo* cassette interferes with normal expression of *Gbx2* (data not shown).

To obtain mice in which the floxed region of the *Gbx2* gene is deleted,

we crossed *Gbx2^{fllox/+}* mice to B6DBA2 or FvB/N transgenic mice that express a *cre* DNA recombinase gene in the egg or early embryo under the control of regulatory elements from the mouse *Zp3* (Lewandoski et al., 1997) or the human β -*actin* (M. L. and G. R. M., unpublished data) gene, respectively. This produced an allele, *Gbx2^{Δhb}*, that lacks the homeobox (Fig. 1D). *Gbx2^{Δhb/+}* mice are phenotypically indistinguishable from their wild-type littermates (data not shown).

Genotype analysis

Genotyping was performed by PCR and/or Southern blot analysis using genomic DNA samples prepared from tails or embryonic tissue. The PCR assay used to detect *Gbx2^{fllox}* or *Gbx2^{Δhb}* was performed using a 5' primer that anneals to sequences in the *Gbx2* gene upstream of the genomic segment used in the targeting vector (5'TGACCTATATAACAATGCC3') and a 3' primer that anneals to sequences within the *neo* gene cassette (5'GTTCTAAGTACTGTGGTTCC3') (see Fig. 1). These same primers were employed in the PCR assay used to identify homologous recombinant ES cells. The *Gbx2^{fllox}* allele was also identified by PCR analysis, using oligonucleotide primers that anneal to *Gbx2* sequences on either side of the downstream *loxP* site (5'TCACGTTAGCAGGTTCCG3') and (5'GGTGCCTCTGAGTTCTGC3'). Southern blot analysis was performed on DNA digested with *EcoRI*, using a 250 bp fragment containing sequences upstream of the *Gbx2* coding region as a probe (see Fig. 1B).

Histological, histochemical and RNA in situ hybridization analyses

Mid-day of the day on which the vaginal plug was detected was considered as E0.5 in the timing of embryo collection. Embryos were dissected and staged according to morphological criteria (Kaufman, 1994). For histological analysis, embryos were fixed in Bouin's fixative, and 8 μ m wax sections were stained with hematoxylin and eosin. Motor nuclei and the locus coeruleus were detected using a histochemical assay for acetylcholinesterase performed on frozen sections of brains, as previously described (Karnovsky and Roots, 1964). For skeletal preparations, the embryos were processed as described by McLeod (1980).

For whole-mount RNA in situ hybridizations, embryos were isolated, fixed in 4% paraformaldehyde at 4°C, and then processed following the protocol of Henrique et al. (1995). To prepare flat mounted samples, the neural tube was dissected from the head, the mesencephalic/rhombencephalic region was isolated, and a cut was made along the floor plate to open the ventricular lumen and expose the dorsal midline. The samples were then flattened and mounted in a 25% solution of glycerol in PBS.

For RNA in situ hybridizations of wax-embedded sectioned samples, the protocol of A. Neubüser and R. Balling

(personal communication) was used. Antisense riboprobes were labeled with UTP-digoxigenin and detected with alkaline phosphatase-coupled anti-digoxigenin antibodies using BM purple (Boehringer Mannheim, Indianapolis, IN) as the substrate. Alternatively, frozen sections were hybridized with ³⁵S-labeled antisense riboprobes using a protocol similar to the one described by Hui and Joyner (1993). Probes for in situ hybridization were prepared using previously published mouse sequences: *En2* (Davis and Joyner, 1988); *Fgf8* (Crossley and Martin, 1995); *Gbx2* (Bouillet et al., 1995); *Hoxb1* (Frohman et al., 1990); *Krox20* (Wilkinson et al., 1989a); *Otx2* (Simeone et al., 1993); *Rags/Al-1* (Flenniken et al., 1996); *Wnt1* (McMahon et al., 1992).

RESULTS

Anterior hindbrain development is abnormal in embryos homozygous for a deletion allele of *Gbx2*

To study the function of the *Gbx2* gene, we produced mice

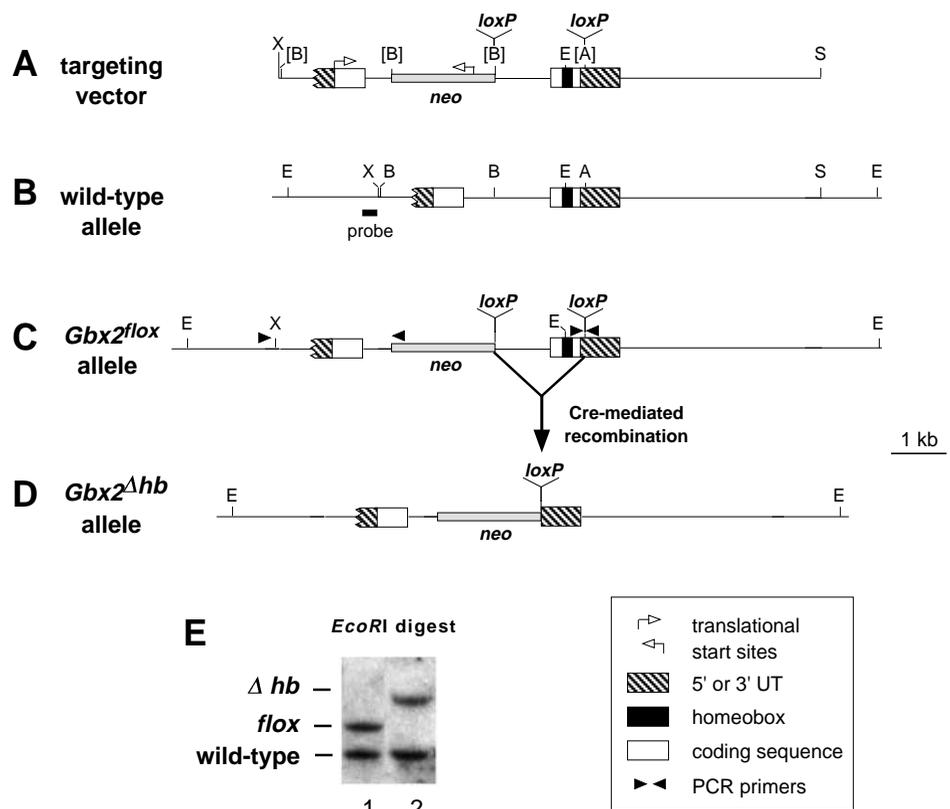


Fig. 1. Production of two mutant alleles of *Gbx2*. (A–D) Schematic representation of (A) the targeting vector; (B) the *Gbx2* wild-type allele; (C) the *Gbx2^{fllox}* allele produced by gene replacement at the *Gbx2* locus; and (D) the *Gbx2^{Δhb}* allele, obtained by mating mice carrying the *Gbx2^{fllox}* allele with mice that produce Cre in the egg or early embryo. A horizontal line is used to represent *Gbx2* genomic DNA. The two exons of the *Gbx2* gene are represented by boxes, shaded as shown to indicate the 5' and 3' untranslated (UT) regions, the protein coding sequences and the homeobox. The *neo* expression cassette is represented by a light gray bar. The probe used for genotype analysis by Southern blot is illustrated as a black bar. The *tk* expression cassette that was included at the 3' end of the targeting vector is not illustrated. The positions of all *EcoRI* (E) sites are shown, whereas other restriction enzyme sites are indicated only if they were used in the construction of the targeting vector: A, *ApaI*; B, *BamHI*; S, *Sall*; X, *XbaI*. Brackets indicate restriction sites that were destroyed in the cloning process. (E) Genotype analysis of mice heterozygous for the *Gbx2^{fllox}* or *Gbx2^{Δhb}* alleles. The Southern blot was performed on *EcoRI*-digested tail DNA isolated from (lane 1) a mouse heterozygous for the *Gbx2^{fllox}* allele or (lane 2) a mouse heterozygous for the *Gbx2^{Δhb}* allele.

carrying a mutant allele, *Gbx2^{Δhb}*, which lacks the homeobox and is thus expected to be a null allele (see Fig. 1, Materials and Methods). When mutant heterozygotes were intercrossed, no *Gbx2^{Δhb}* homozygotes were found among several hundred offspring genotyped 3–4 weeks after birth. Examination of the litters within a day of birth (postnatal day [P] 0–1), revealed that some progeny had already died and others were failing to nurse. Genotype analysis confirmed that most of the dead and abnormal newborns were *Gbx2^{Δhb}* homozygotes. When fetuses were genotyped at E18.5, just prior to parturition, all four categories of offspring were present at normal Mendelian frequency: 26.5% (26/98) *Gbx2^{Δhb}/Gbx2^{Δhb}*; 44.9% (44/98) *Gbx2^{Δhb}/+*, and 28.5% (28/98) *+/+*. Thus embryos homozygous for *Gbx2^{Δhb}* survive to birth, but die soon afterwards.

A gross morphological analysis of the embryos at E18.5 revealed that brain development was affected in the mutant embryos. The most obvious abnormality was absence of a normal cerebellum (Fig. 2A,B). Histological analysis at E17.5–P0 ($n=5$) showed that a small amount of amorphous tissue, which varied in size and appearance among individuals, was present in the region of the mutant brain in which the cerebellum would normally be found (dorsal anterior hindbrain). Although the cerebellar markers Calbindin (Wassef et al., 1985) and Calretinin (Abbott and Jacobowitz, 1995) were detected in very lateral sections, none of the tissue in the mutant anterior hindbrain had the morphological features of a normal cerebellum (Fig. 2C,D, and data not shown). The choroid plexus was present caudal to this amorphous tissue, but

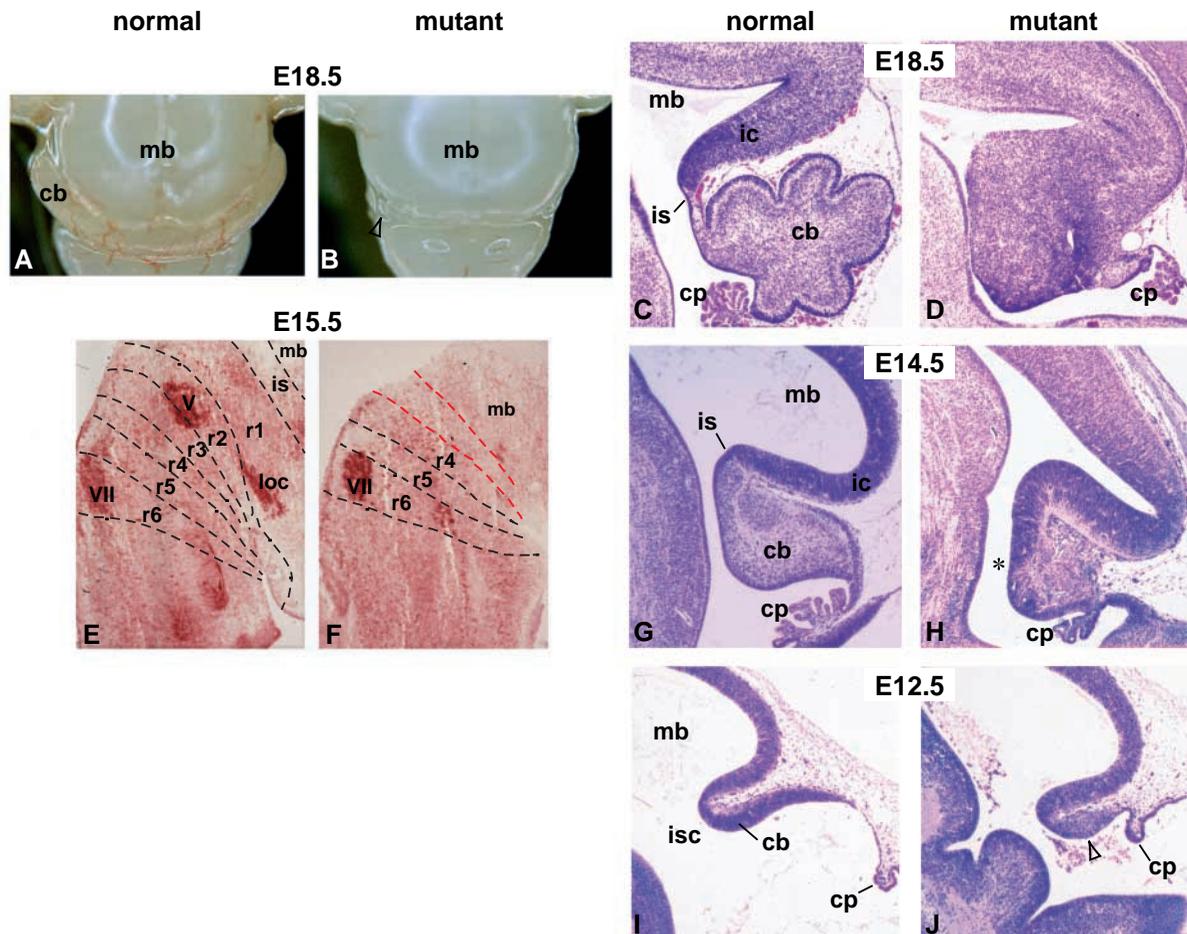


Fig. 2. Morphological analysis of the mid/hindbrain region in normal and *Gbx2^{Δhb}* homozygous mutant embryos at late and mid-gestation stages of development. Brains of wild-type embryos and their homozygous mutant littermates are shown as indicated. (A,B) Dorsal view of the mid/hindbrain region of intact E18.5 brains. Note the absence of a normal cerebellum (cb) caudal to the midbrain (mb) in the mutant embryo. The small amount of amorphous tissue found in place of the cerebellum is particularly visible laterally (open arrowhead). In C–J, anterior is at the top, ventral is to the left. (C,D) Sagittal sections of E18.5 brains. Note, in the mutant embryo, the abnormal morphology of the tissue located in the region where the cerebellum and the inferior colliculus (ic) would normally be found, and the abnormally small choroid plexus (cp). (E,F) Motor neurons and the locus coeruleus (loc) in sagittal sections of E15.5 brains. The loc and the V motor nucleus are not detected in the mutant embryo, whereas the VII motor nucleus is present. Note that at this stage the VII motor nucleus is located in r6, whereas at earlier stages it is located in r4/r5 (see Fig. 7). Dashed lines represent the approximate locations of the boundaries that separate the derivatives of the rhombomeres (r), the isthmus region (is), and the midbrain in the normal embryo. Comparable approximate boundaries are indicated in the mutant embryo, with those that flank the abnormal region shown in red. (G,H) Sagittal sections of E14.5 brains. The abrupt thinning of the ventricular layer that normally marks the isthmus is detected at a more caudal position in the mutant embryo (indicated by * in H). (I,J) Sagittal sections of E12.5 brains. The open arrowhead (in J) points to neuroepithelium on the posterior side of the isthmus constriction (isc) that normally gives rise to the cerebellar anlage.

appeared smaller and simpler in structure than normal (Fig. 2C,D). Ventrally, the wall of the rostral pontine region was unusually thin and its cytoarchitecture was very abnormal (data not shown).

Histological analysis of E14.5-E16.5 embryos, showed that the region in which the developing cerebellum would normally be located was morphologically abnormal and reduced in size in all mutant brains examined ($n=8$; Fig. 2E-H, and data not shown). The junction between the midbrain and developing cerebellum (isthmus), which is marked by an abrupt thinning of the neuroepithelium, was found in a more caudal position in the mutant brain (marked by * in Fig. 2H) than in the normal brain. In addition, the developing choroid plexus appeared to be fused to the amorphous tissue in the mutant embryos. Histochemical staining for acetylcholinesterase revealed that the IV motor nucleus, locus coeruleus, and V motor nucleus, which are derived from the isthmus (Is), were absent in mutant homozygotes. In contrast, the III motor nucleus in the midbrain, and the VII motor nucleus, which forms in r4/r5 but subsequently migrates to the r6 region in mice (Gilland and Baker, 1993; S. M., unpublished observations) are present and appear normal (Fig. 2E,F, and data not shown). At even earlier stages (E12.5-E13.5), the region caudal to the midbrain that includes the cerebellar anlage was again found to be morphologically abnormal: it was both reduced in A-P length, and displayed a disorganized cellular architecture ($n=5$) (Fig. 2I,J, and data not shown). Together, these data indicate that normal derivatives of the isthmus (isthmus nuclei; IV motor nucleus) and r1-3 (cerebellum, locus coeruleus, V motor nucleus) fail to develop in the mutant homozygotes, and suggest that defects in this region, which will hereafter be referred to as Is-r3, are detectable by at least E12.5. No obvious abnormalities were detected in the brain or spinal cord derivatives of regions posterior to r3 in mutant embryos collected from E12.5 through P0 (data not shown).

The Is-r3 defects were observed in all mutant homozygotes examined. Forebrain development was also severely perturbed in some mutant homozygotes. This incompletely penetrant forebrain phenotype will be the subject of a future study. In addition to these abnormalities in the brain, a few abnormalities were detected in other parts of the head. The supraoccipital bone appeared to be smaller than normal or was absent in some mutant embryos, and the development of the dorsal membranous labyrinth, which gives rise to the vestibular organ in the ear, was found to be very abnormal (data not shown). The ear phenotype is being analyzed in detail. No other gross morphological abnormalities were

observed in mutant homozygous embryos or newborns (data not shown).

Precursors of the anterior hindbrain are affected from early stages of mutant brain development

To further study the anterior hindbrain abnormalities we analyzed embryos at E9.5-E10.5 (Figs 3, 4). At these stages, it was evident in both intact and sectioned embryos that the region containing the anterior hindbrain was significantly reduced in size along the A-P axis in the mutant embryos. (Note for example the abnormal proximity to the midbrain of the otocysts, which also appear smaller than normal and are laterally displaced in the mutant embryos; see Fig. 3G-J). Other

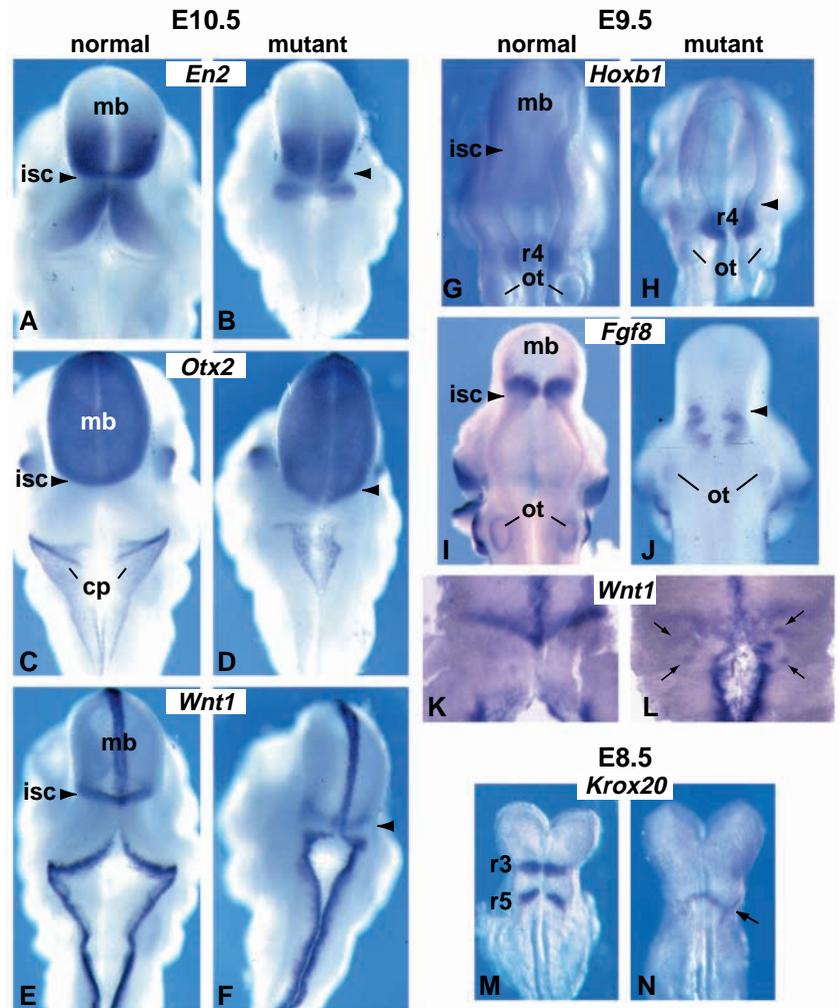


Fig. 3. Whole-mount RNA in situ hybridization analysis of gene expression in normal and mutant embryos at E10.5-E8.5. Normal (wild-type or heterozygous) embryos and their homozygous mutant littermates were hybridized with probes for the genes indicated. In A-J, showing dorsal views of the embryos, an arrowhead indicates the approximate position of the isthmus constriction (isc) in normal embryos, and the approximate position of the comparable constriction in mutant embryos. (A,B) *En2*; (C,D) *Otx2*. The *Otx2*-positive progenitors of the choroid plexus (cp) are indicated in C. (E,F) *Wnt1*; (G,H) *Hoxb1*; (I,J) *Fgf8*. The otocysts (ot) are indicated. (K,L) Flat mount preparations (ventral midline cut) of brains of E9.5 embryos hybridized with a probe for *Wnt1*. Arrows in L point to ectopic patches of *Wnt1*-expressing cells. (M,N) *Krox20*. In the normal embryo, *Krox20* expression marks prospective r3 and r5. The arrow in N points to *Krox20*-expressing cells in prospective r5.

morphological abnormalities were also apparent. For example, a constriction analogous to the isthmic constriction that normally causes the neuroepithelium at the mid/hindbrain junction to bulge into the ventricle of the brain is observed in the mutant embryo, but the neuroepithelium on its posterior side is morphologically abnormal when compared with the tissue in the same region of a normal embryo (Fig. 4A,B). The ventral wall of the anterior hindbrain is also morphologically abnormal (bar in Fig. 4B), indicating that both the alar and basal plates of the anterior hindbrain are affected.

Gene expression in the mid/hindbrain region was studied at E9.5 and E10.5 by RNA in situ hybridization analyses of embryos in whole mount (Fig. 3A-L) and of serial sagittal sections (Fig. 4A-H). At these stages, *En2* is normally expressed in a continuous domain that extends from a region in the anterior midbrain to the r1/r2 boundary (Davis and Joyner, 1988). In mutant homozygotes the *En2* expression domain caudal to the isthmic constriction appears to be substantially reduced (Fig. 3A,B). *Otx2* expression is normally detected throughout the midbrain as well as in the prospective choroid plexus (roof of the IVth ventricle), but not in the rest of the anterior hindbrain (Simeone et al., 1993; Millet et al., 1996). In the mutant homozygotes the non-*Otx2*-expressing region in the anterior hindbrain is severely reduced in size (Figs 3C,D, 4E,F and data on E9.5 embryos, not shown). *Wnt1* RNA is normally detected in a transverse band at the posterior end of the midbrain, and in a stripe along the dorsal midline of the midbrain and of the hindbrain posterior to the cerebellar anlage; *Wnt1* is not expressed in r1-r2 (McMahon et al., 1992). However, in the mutant homozygotes the non-*Wnt1*-expressing r1-r2 region is severely reduced in size (Fig. 3E,F,K,L). *Hoxb1* RNA is normally detected in r4 at some distance caudal to the isthmic constriction (Wilkinson et al., 1989b; Frohman et al., 1990), but in mutant embryos *Hoxb1* RNA was detected in cells within and near the constriction (Figs 3G,H, 4C,D). The observation that an r4 marker is expressed in cells very close to the posterior end of the midbrain is consistent with the conclusion that the region affected by loss of *Gbx2* function includes r3. Together, these data demonstrate that by E9.5 the region between the posterior end of the midbrain and r4 (i.e. the anterior hindbrain) is severely reduced in length in the mutant homozygotes (Figs 3A-H, 4A-F, and data not shown).

To determine whether the anterior hindbrain is affected at an even earlier stage, we assayed embryos at E8.5 using a probe for *Krox20* RNA, which is an early marker for specification of r3. In the normal embryo, *Krox20* RNA is detected in prospective r3 cells at the 1-3 somite stage (approx. E8.25) and a short time later *Krox20* expression is also detected in prospective r5 cells (Wilkinson et al., 1989a). At E8.5, we detected *Krox20* RNA in two transverse stripes, representing prospective r3 and r5, in the neuroepithelium of all wild-type or *Gbx2*^{Δhb/+} embryos examined (Fig. 3M). In their mutant homozygote littermates, only a single stripe of *Krox20*-expressing cells was detected in cells at the level of the otocysts, which presumably are the precursors of r5 ($n=3$; Fig. 3N). The level of *Krox20* expression appeared to be lower in the r5 stripe in the mutant homozygotes than in their normal littermates, perhaps reflecting a small developmental delay in the mutant embryos. However, since expression in r3 normally precedes that in r5, delayed development of the mutant homozygotes cannot account for the lack of *Krox20* expression in r3. These data

show that by E8.5, cells in the mutant anterior hindbrain region do not express a normal marker of r3, and therefore are already mis-specified or absent.

Gene expression at the mid/hindbrain junction is perturbed in mutant homozygotes

The analysis of gene expression described above not only helped to visualize the morphological abnormalities of the anterior hindbrain at E9.5-E10.5, but also demonstrated that the mid/hindbrain boundary region has abnormal molecular characteristics. Specifically, in mutant homozygotes *Fgf8* RNA was detected in a diffuse, expanded domain that extended caudally from the end of the midbrain, rather than the sharp transverse stripe at the anterior boundary of the hindbrain that is seen in normal embryos. The intensity of the *Fgf8* signal in individual cells in this region appeared to be reduced compared with normal, although the level of *Fgf8* expression was apparently normal in the forebrain and other regions of the embryo (Figs 3I,J, 4G,H; and data not shown). In addition, ectopic patches of *Fgf8* RNA were detected laterally, both rostral and caudal to this domain in different individual embryos (Fig. 3J and data not shown).

There were also abnormalities in *Wnt1* expression, which were most easily visualized in flat mount preparations (Fig. 3K,L). The transverse stripe of *Wnt1*-expressing cells, which in normal embryos is located at the posterior midbrain boundary, was more diffuse in the mutant homozygotes, and lacked the sharp boundaries typical of the normal *Wnt1* mes/met expression domain. The intensity of the *Wnt1* signal in this stripe was significantly reduced in mutant embryos, whereas the level of *Wnt1* expression appeared normal in the stripe along the dorsal midline (see Fig. 3E,F). Furthermore, patches of *Wnt1*-expressing cells were observed throughout the region immediately caudal to the *Wnt1* stripe (Fig. 3K,L), an expression pattern that is never observed in the normal embryo. In addition to the obvious abnormalities in *Fgf8* and *Wnt1* expression, differences in *Otx2* expression between normal and mutant embryos were observed at E9.5-10.5 (Figs 3C,D, 4E,F; and data not shown). The posterior boundary of the *Otx2* expression domain appeared less sharp in mutant embryos, apparently because a few *Otx2*-expressing cells could be found caudal to the normal limit of the *Otx2* expression domain. Moreover, patches of *Otx2*-expressing cells were often found in the same region as the ectopic patches of *Wnt1*-expressing cells. Thus a number of genes including *Fgf8*, *Wnt1*, *Otx2* and *Hoxb1* are expressed in the region caudal to the mid/hindbrain boundary in the mutant homozygotes, giving this region a very abnormal molecular character.

The results of a similar analysis at E12.5 (Fig. 4I-P) indicated that the abnormalities in gene expression detected in younger embryos persisted and became more severe. The *Wnt1* and *Otx2* expression domains in mutant embryos were found to extend caudally through the constriction at the mid/hindbrain junction and into the region in which the cerebellar anlage is normally found (Fig. 4K-N). The *Fgf8* expression domain was also expanded caudally (Fig. 4O,P). Thus the *Wnt1* and *Otx2* expression domains overlap with the *Fgf8* expression domain, a situation that does not normally occur. In addition, caudal to the domain in which all three genes are expressed, patchy ectopic expression of *Wnt1* and *Otx2* in the same cells was frequently detected laterally (Fig.

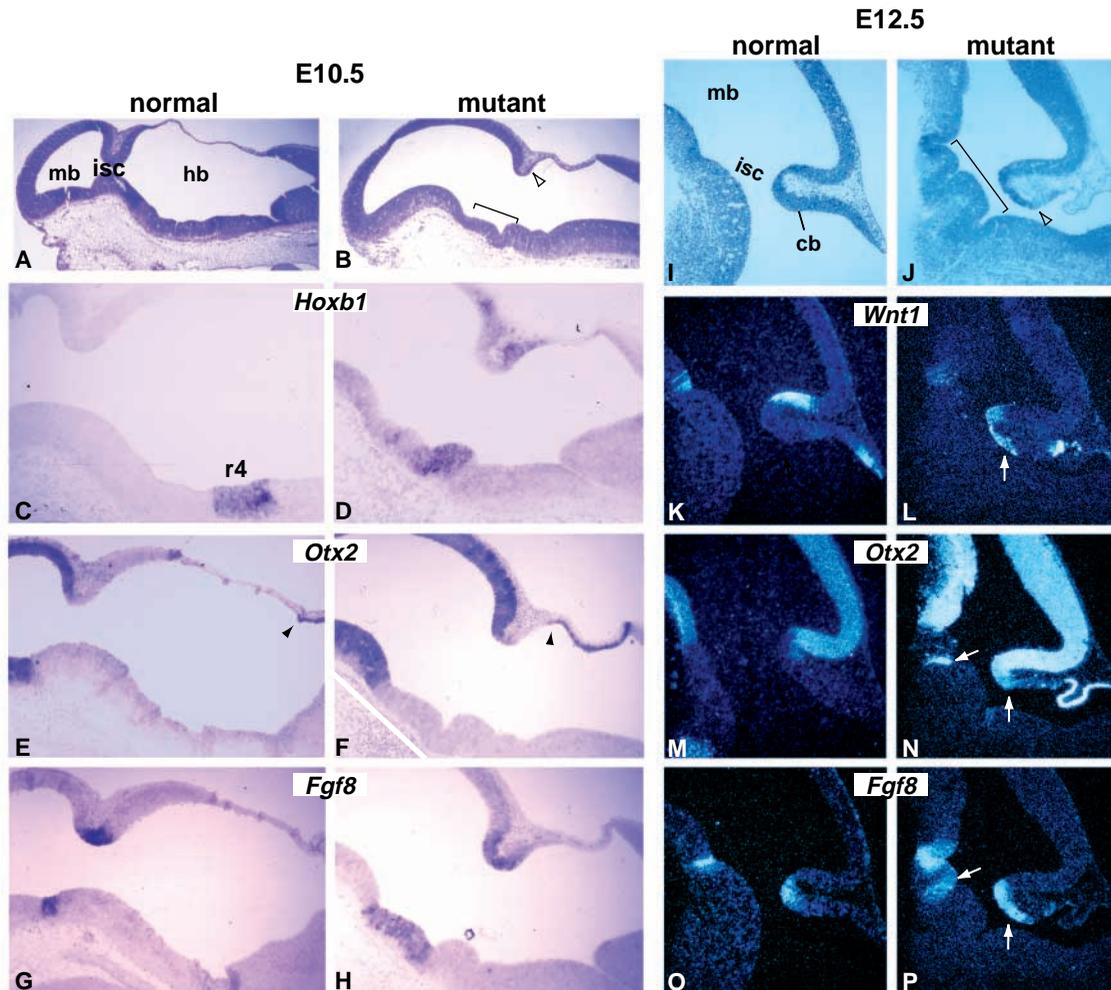


Fig. 4. Analysis of gene expression in sagittal sections of normal and mutant embryos at E10.5 and E12.5. Sections of normal (wild-type or heterozygous) embryos and their homozygous mutant littermates were hybridized with probes for the genes indicated. In B and J, the region of the ventral hindbrain (hb) wall that is morphologically abnormal is indicated by a bar, and the region on the dorsal posterior side of the constriction that has abnormal cytoarchitecture is indicated by an open arrowhead. (A,B) parasagittal sections of E10.5 embryos stained with hematoxylin and eosin. Note the decreased length of the roof of the hindbrain in the mutant homozygote. The remaining panels show near-adjacent sections hybridized with digoxigenin-labeled probes for (C,D) *Hoxb1*. Note that although signal is detected on the rostral side of the constriction in the section shown in D, this was not observed in sections of another embryo. (E,F) *Otx2*. Solid arrowheads indicate the anterior end of the *Otx2* expression domain in the roof of the hindbrain that marks the prospective choroid plexus. (G,H) *Fgf8*. (I,J) midsagittal sections of E12.5 embryos stained with toluidine blue, bright-field illumination. The remaining panels show near-adjacent sections, viewed in dark-field illumination, hybridized with ^{35}S -labeled probes for (K,L) *Wnt1*; (M,N) *Otx2*; (O,P) *Fgf8*. In L, N, P, the arrows pointing up indicate the caudal extension of the gene expression domain in the dorsal wall of the constriction in the mutant embryos, and the arrows pointing to the left indicate ectopic patches of *Fgf8*- or *Otx2*-expressing cells in the ventral wall.

4K-N, and data not shown). Similar *Otx2* and *Wnt1* co-expression has also been observed in embryos homozygous for *Swaying*, a frame-shift mutation in *Wnt1* that is thought to impair the formation of the mid/hindbrain junction, and lead to the mixing of cells on either side of the boundary (Bally-Cuif et al., 1995). However, whereas *Swaying* mutant embryos contain patches of *Otx2*-negative cells in the midbrain, this was not observed in the *Gbx2* mutant embryos.

Abnormalities in the midbrain of mutant homozygotes

Histological analysis of mutant homozygotes revealed that in addition to the abnormalities detected in the anterior hindbrain, the posterior midbrain was also abnormal. At E14.5 the normal

dorsal midbrain is a laminar cortical structure that is subdivided into the superior colliculus (anterior approx. 2/3) and the inferior colliculus (posterior approx. 1/3). At E14.5 the inferior colliculus is normally thinner than the superior colliculus (Figs 2G, 5A). In *Gbx2* ^{Δ hb} homozygotes the region in which the inferior colliculus would normally be located, particularly the rostral part of this region, contains tissue that is dramatically thickened, and thus resembles the superior colliculus. In addition, the abrupt transition from thick to thin neuroepithelium that normally marks the mid/hindbrain junction is found caudal to its normal location (Figs 2H, 5B), suggesting that the posterior midbrain may extend caudally as a consequence of loss of *Gbx2* function.

To determine the molecular nature of the abnormally

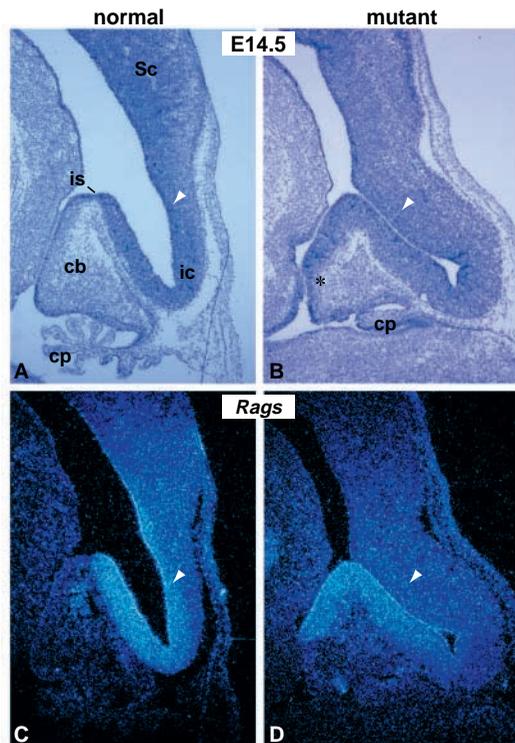


Fig. 5. Analysis of *Rags/All* expression in normal and mutant embryos at E14.5. In situ hybridization analysis of *Rags/All* expression in serial sagittal sections of normal and mutant embryos at E14.5. Anterior is at the top in all panels. An arrowhead indicates the region in which the anterior part of the inferior colliculus (ic) is normally located. (A,B) Bright-field illumination. In the mutant embryo, the posterior limit of the inferior colliculus (*) appears to be shifted caudal to its normal location. (C,D) Dark-field illumination.

thickened and extended inferior colliculus, we examined *Rags/All* and *En2* expression in the mutant embryos. At E14.5, *Rags/All* and *En2* are normally expressed at high levels throughout the inferior colliculus and in a gradient that decreases from posterior to anterior in the superior colliculus (Donoghue et al., 1996; Davis and Joyner, 1988). In mutant homozygotes at E14.5, *Rags/All* and *En2* expression levels were high in the caudally extended posterior midbrain (Fig. 5C,D and data not shown). Thus, despite its abnormal thickness, this tissue has molecular characteristics of the inferior colliculus: high *Rags/All* and *En2* expression. In contrast, the thickened tissue located where the anterior inferior colliculus would normally be found contained relatively low levels of *Rags/All* and *En2* RNA compared to a similar region in the normal embryo (Fig. 5D and data not shown). In addition, in medial sagittal sections of this region of the midbrain, we detected numerous Calbindin- and Calretinin-producing cells, which are normally abundant only in the superior colliculus (data not shown). Thus, this tissue has both morphological and molecular characteristics similar to those of superior colliculus, suggesting that either there is a caudal extension of the superior colliculus or that part of the posterior midbrain has taken on a more anterior character in the mutant homozygotes.

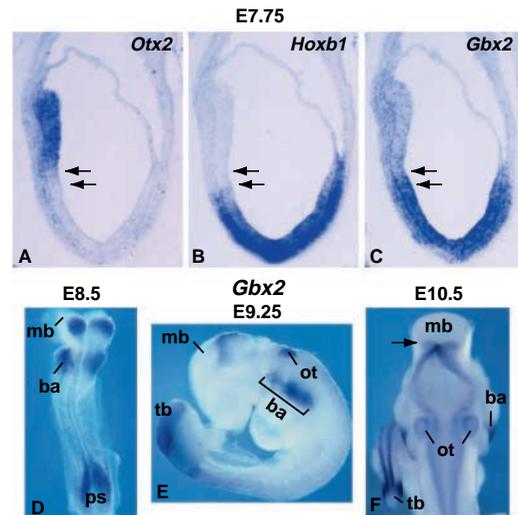


Fig. 6. Analysis of the normal pattern of *Gbx2* expression at E7.5-E10.5. (A-C) Analysis of gene expression in near-adjacent serial sections of a normal embryo at E7.75 hybridized with probes for (A) *Otx2*. The posterior border of the *Otx2* expression domain is designated by the top arrow in A-C. (B) *Hoxb1*. The anterior border of the *Hoxb1* expression domain is designated by the bottom arrow in A-C. (C) *Gbx2*. Faint non-specific background staining is observed in the anterior region of the embryo. (D-F) Detection of *Gbx2* RNA by whole-mount RNA in situ hybridization analysis at the stages indicated. The arrow in F points to the stripe of *Gbx2* expression at the mid/hindbrain boundary. Note the absence of *Gbx2* expression in the midbrain. Abbreviations: ba, branchial arches; ot, otocyst; ps, primitive streak; tb, tail bud.

The mid/hindbrain abnormalities in mutant homozygotes may result from a lack of *Gbx2* function during gastrulation

The data described above demonstrate that *Gbx2* function is required for normal development of both the anterior hindbrain and posterior midbrain. The absence in E8.5 mutant homozygotes of *Krox20*-expressing cells in the region in which r3 would normally be found (Fig. 3N), indicates that anterior hindbrain development is already abnormal in *Gbx2^{Δhb}* homozygotes by that stage, and suggests that *Gbx2* function may be required at an earlier stage. It has been reported that *Gbx2* is expressed as early as E7.5 (Bouillet et al., 1995), but it is not known whether it is expressed in anterior hindbrain (r1-r3) precursors at that stage. To precisely define the *Gbx2* expression domain in the anterior neuroectoderm, we performed RNA in situ hybridization analysis on serial sagittal sections of E7.75 mouse embryos using a probe for *Gbx2*, and probes for *Otx2* and *Hoxb1*, which serve as regional markers in the presomite embryo. In the neural tube, the posterior boundary of the *Otx2* expression domain marks the posterior end of the midbrain and the anterior boundary of the *Hoxb1* expression domain marks the anterior end of r4; the same relationship presumably exists at the early neural plate stage. If so, then the gap between the *Otx2* and *Hoxb1* expression domains would contain the precursors of r1-r3. We sought to determine whether *Gbx2* is expressed in those cells (Fig. 6A-C).

As previously reported, we found that at E7.75, *Otx2* expression in the neuroectoderm is restricted to an anterior

domain (Fig. 6A; see also Simeone et al., 1993; Ang et al., 1994; Acampora et al., 1995). The *Hoxb1* expression domain extends rostrally from the posterior end of the embryo to a position relatively close to the posterior end of the *Otx2* expression domain (Fig. 6B; see also Frohman et al., 1990). We detected *Gbx2* RNA in all three germ layers in a domain similar to that of *Hoxb1*, but extending slightly further rostrally and apparently abutting the posterior boundary of the *Otx2* expression domain (Fig. 6C; see also Bouillet et al., 1995), as previously suggested by studies in *Xenopus* (von Bubnoff et al., 1995). Therefore, there is a stripe of cells in the neuroectoderm at E7.75 in which *Gbx2* but not *Otx2* or *Hoxb1* is expressed. As development proceeds, the level of *Gbx2* RNA dramatically decreases in the region caudal to r2, and by E8.5, *Gbx2* RNA is abundant only in a broad stripe in the most anterior region of the hindbrain, in the primitive streak at the posterior end of the embryo, and in the branchial arches (Fig. 6D, and data not shown; see also Bouillet et al., 1995). Together these data indicate that *Gbx2* is expressed at high levels in the precursors of Is-r3 at E7.75, and by E8.5 the level of *Gbx2* expression is very low in r3 and the neuroectoderm caudal to it. On the basis of these observations, we hypothesize that it is the lack of *Gbx2* function at E7.75 that is responsible for the defects observed at later stages in the anterior hindbrain of *Gbx2^{Δhb}* homozygotes.

Another conclusion from the observation that the *Gbx2* and *Otx2* expression domains do not overlap at E7.75 is that *Gbx2* is not expressed in the precursors of the midbrain. Analysis of *Gbx2* expression in embryos at progressively later stages (Fig. 6D-F and data not shown; see also Bouillet et al., 1995) confirmed that *Gbx2* is never expressed in the midbrain. For example, at E9.25 the *Gbx2* expression pattern is similar to that at E8.5, except that at the later stage expression was also detected in the otocysts (Fig. 6E). By E10.5, *Gbx2* RNA was detected in a sharp transverse ring immediately caudal to the midbrain (arrow in Fig. 6F), in longitudinal stripes that run the length of the hindbrain and spinal cord, and in the developing limb bud, otocysts, branchial arches and tail bud (Fig. 6F, and data not shown). The conclusion that *Gbx2* expression is never detected in the midbrain or its precursors, indicates that the abnormalities in midbrain A/P patterning detected in *Gbx2^{Δhb}* homozygotes must be an indirect consequence of the loss of *Gbx2* function.

DISCUSSION

We have focused our analysis on the defects in mid/hindbrain development in *Gbx2^{Δhb}* homozygotes, which die shortly after birth. At late gestation stages, the mutant embryos lack CNS structures normally derived from the isthmic constriction and rhombomeres 1-3, including the cerebellum, motor nuclei IV and V and locus coeruleus. Our data show that anterior hindbrain defects can be traced back to an early stage of neuraxis development (at least E8.5) and are likely due to the absence of *Gbx2* function as early as E7.75. By E9.5-E10.5 the region between the midbrain and r4 (i.e. Is-r3) is dramatically reduced in size in the mutant embryos, and the cells in this region display aberrant patterns of gene expression (e.g. of *Wnt1*, *Fgf8*, *Hoxb1* and *Otx2*). In recognition of its abnormal and ill-defined character, we designate this region 'zone X'.

The morphological and molecular abnormalities of zone X at E10.5 are summarized in Fig. 7. At later stages of development, abnormalities in midbrain development become apparent.

Gbx2 function is required at early stages for normal development of the anterior hindbrain

At present we can only speculate on the precise role played by GBX2 protein in the development of Is-r3 in the normal embryo. Since it is a homeodomain protein, GBX2 is presumably a transcription factor that is likely to regulate the expression of genes involved in establishing early A/P pattern in the neural plate. Such positional information may be required for Is-r3 precursor cells to become competent to respond to the signals that normally stimulate their proliferation or promote their survival. Loss of *Gbx2* function would therefore result in a failure of the Is-r3 precursor cell population to be properly specified, and consequently it would either fail to proliferate or die.

The hypothesis that *Gbx2* is required to provide patterning information required for cell proliferation or survival suggests several possible explanations of the origin of zone X cells in mutant homozygotes. One possibility is that they are mis-specified descendants of the Is-r3 precursor cell population, which have failed to expand properly in the absence of *Gbx2* function. Another possibility is that Is-r3 precursors or a subset of those cells may die as a consequence of loss of *Gbx2* function, and the cells in zone X might be derivatives of cell populations that normally flank the missing region. In that case, the observed abnormalities in gene expression in zone X might

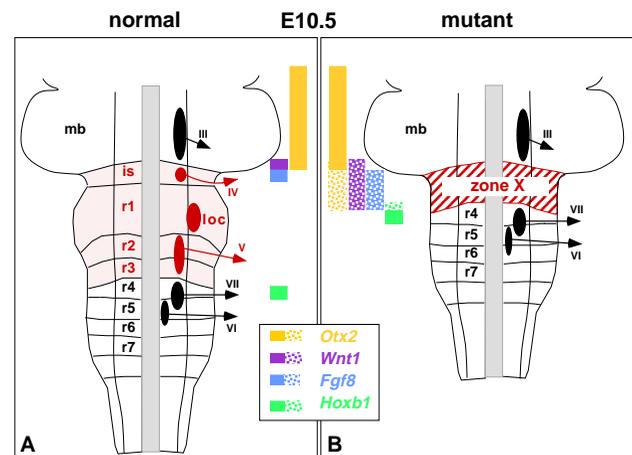


Fig. 7. Summary of abnormalities in morphology and gene expression in mutant embryos at E10.5. Schematic diagram of a flat-mount preparation (dorsal midline cut) of the mid/hindbrain region of normal and mutant embryos at E10.5. Grey bar represents the floor plate. Colored bars represent the domains of expression of the genes indicated; stippling indicates regions in which expression is patchy. Circles and ovals indicate the normal locations of progenitors of the motor nuclei and the locus coeruleus (loc) at this stage. Structures that were found at later stages to be missing in the mutant embryo are indicated in red in the diagram of the normal embryo. The region between the midbrain (mb) and r4 in the mutant embryo, which corresponds to Is-r3 in the normal embryo, is designated zone X to indicate its abnormal morphological characteristics and patterns of gene expression.

result from abnormal inductive interactions that occur when prospective midbrain and r4 cells are juxtaposed as a consequence of the loss of the intervening cell population.

Fate mapping studies in the chick (Köntges and Lumsden, 1996) and studies in the mouse (Osumi-Yamashita et al., 1996) have indicated that neural crest cells derived from the region that encompasses Is-r3 migrate into the first branchial arch and give rise to specific skeletal elements. Remarkably, those skeletal elements (e.g. malleus, incus, squamosal, tympanic and alisphenoid) appear to be normal in *Gbx2* mutant homozygotes (data not shown), even though loss of *Gbx2* function affects development of the Is-r3 region at a very early stage, as evidenced by the lack of *Krox20*-expressing cells in the region in which r3 would normally be found. It is possible that early development of the region affected by loss of *Gbx2* function is sufficiently normal to allow formation of neural crest cells. Alternatively, neural crest cells from adjacent, unaffected regions in mutant embryos may substitute for the cells normally derived from Is-r3. Recent studies in the chick embryo have demonstrated that such replacement can occur: following removal of the region spanning from the mid-mesencephalon to r8 and replacement with quail caudal mesencephalon only, quail neural crest cells were found to have populated the first branchial arch and given rise to neural crest derivatives that normally originate from r1 to r3 (Couly et al., 1996).

Loss of *Gbx2* function results in abnormalities of the mid/hindbrain organizer

In addition to defects in anterior hindbrain development, abnormalities were detected in the posterior midbrain of mutant homozygotes despite the fact that *Gbx2* is not expressed there. In the normal embryo, midbrain patterning is apparently regulated by signaling molecules such as WNT1 and FGF8, key components of the mid/hindbrain organizer that are first expressed in the mid/hindbrain region at approx. E8.0 and approx. E8.5, respectively (McMahon et al., 1992; Crossley and Martin, 1995). Although loss of *Gbx2* function does not result in a lack of *Fgf8* or *Wnt1* expression, a normal mid/hindbrain signaling center, in which *Wnt1*- and *Fgf8*-expressing cells are found in tight, closely apposed, but non-overlapping transverse stripes, is not formed in the mutant embryos. Instead, from E9.5 *Fgf8* and *Wnt1* RNAs were both detected in a diffuse expanded domain that extended caudally from the end of the midbrain into the anterior hindbrain (i.e. zone X). Since *Gbx2* expression is not detected in the midbrain or its precursors, the failure to establish a normal mid/hindbrain organizer is likely to be the cause of the A/P patterning defects in the posterior midbrain (inferior colliculus), which include abnormal thickening and gene expression patterns typical of more anterior regions of the midbrain (superior colliculus).

It remains to be determined how loss of *Gbx2* function results in failure to form a normal mid/hindbrain signaling center. It has been proposed that organizers form at the boundaries between differently specified territories (Meinhardt, 1983). Thus one possibility is that because the region immediately caudal to the midbrain (i.e. zone X) is mis-specified due to a loss of *Gbx2* function, the normal inductive interactions necessary to establish the organizer do not occur in the mutant embryos. Alternatively, *Gbx2* may play a much more direct role in controlling organizer gene expression. For example,

since the expression domains of *Gbx2* and *Fgf8* in the prospective anterior hindbrain are very similar from E8.5 on, it is possible that GBX2 protein produced in cells at the anterior end of the metencephalon is directly involved in regulating *Fgf8* expression in those cells. FGF8 may in turn function to maintain *Wnt1* expression in the posterior midbrain. One way to distinguish between these two possibilities would be to perform tissue-specific gene inactivation experiments, in which *Gbx2* is inactivated specifically in cells in the anterior hindbrain after E8.5.

Another abnormality in *Gbx2^{Δhb}* homozygotes is the apparent extension of the mutant midbrain caudal to its normal limit. This caudal extension might be due to the early abnormalities in mid/hindbrain organizer function via an effect on *Otx2* expression. Recent studies in chick embryos suggest that application of FGF8 protein represses *Otx2* expression in the mesencephalon (P. Crossley, G. R. M., J. L. R. R. and S. M., unpublished observations), raising the possibility that *Otx2* expression may normally be regulated, perhaps indirectly, by FGF8. Thus the apparent lack of proper regulation of *Otx2* expression in mutant homozygotes might be accounted for by a decrease in the local concentration of FGF8 at the mid/hindbrain junction. In turn, the caudal extension of the *Otx2* expression domain may be more than just a marker of midbrain abnormality in mutant homozygotes; since the level of *Otx* gene expression appears to be an important determinant of midbrain identity (Suda et al., 1996; A. Simeone, personal communication). Alternatively, it has been suggested that the mid/hindbrain boundary might be positioned by a more direct interaction between *Otx2* and *Gbx2* (von Bubnoff et al., 1995). We have shown here that as early as E7.75 the *Otx2* and *Gbx2* expression domains in the neuroectoderm abut. One possibility is that GBX2 normally represses *Otx2* expression in the anterior hindbrain, and thus the caudal extension of the posterior midbrain that is observed in *Gbx2^{Δhb}* homozygotes might result from an early lack of repression of midbrain development due to a loss of *Gbx2* function.

Concluding remarks

Our data provide genetic evidence for a link between specification of cells fated to give rise to Is-r3 and the establishment of the mid/hindbrain organizer, and identify *Gbx2* as a gene that is required for these processes to occur normally. The challenge now is to precisely define the role(s) that the *Gbx2* gene plays in mid/hindbrain development. One provocative finding is that the *unplugged* gene, which appears to be the *Drosophila* homolog of *Gbx2*, is required for formation of the tracheal branches that penetrate the fly CNS (Chiang et al., 1995), a process that depends on FGF gene function (Sutherland et al., 1996). Although, as yet, no link has been established between *Gbx2* and *Fgf* gene function in *Drosophila*, it is possible that future studies will provide evidence for an evolutionarily conserved relationship between them that will help to elucidate their functions in embryogenesis.

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