**Otx2 and Gbx2 are required for refinement and not induction of mid-hindbrain gene expression**

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**SUMMARY**

Otx2 and Gbx2 are among the earliest genes expressed in the neuroectoderm, dividing it into anterior and posterior domains with a common border that marks the mid-hindbrain junction. Otx2 is required for development of the forebrain and midbrain, and Gbx2 for the anterior hindbrain. Furthermore, opposing interactions between Otx2 and Gbx2 play an important role in positioning the mid-hindbrain boundary, where an organizer forms that regulates midbrain and cerebellum development. We show that the expression domains of Otx2 and Gbx2 are initially established independently of each other at the early headfold stage, and then their expression rapidly becomes interdependent by the late headfold stage. As we demonstrate that the repression of Otx2 by retinoic acid is dependent on an induction of Gbx2 in the anterior brain, molecules other than retinoic acid must regulate the initial expression of Otx2 in vivo. In contrast to previous suggestions that an interaction between Otx2- and Gbx2-expressing cells may be essential for induction of mid-hindbrain organizer factors such as Fgf8, we find that Fgf8 and other essential mid-hindbrain genes are induced in a correct temporal manner in mouse embryos deficient for both Otx2 and Gbx2. However, expression of these genes is abnormally co-localized in a broad anterior region of the neuroectoderm. Finally, we find that by removing Otx2 function, development of rhombomere 3 is rescued in Gbx2–/– embryos, showing that Gbx2 plays a permissive, not instructive, role in rhombomere 3 development. Our results provide new insights into induction and maintenance of the mid-hindbrain genetic cascade by showing that a mid-hindbrain competence region is initially established independent of the division of the neuroectoderm into an anterior Otx2-positive domain and posterior Gbx2-positive domain. Furthermore, Otx2 and Gbx2 are required to suppress hindbrain and midbrain development, respectively, and thus allow establishment of the normal spatial domains of Fgf8 and other genes.

Key words: Compartment, Fgf8, Mid-hindbrain organizer, Retinoic acid, Wnt1, Mouse

**INTRODUCTION**

The molecular mechanisms that control development of the midbrain and cerebellum are an excellent paradigm of how stepwise inductive events can lead to patterning of the neuroectoderm along the anteroposterior (AP) axis. The midbrain develops from the mesencephalon (mes), an early morphologically distinct subdivision of the neural tube, and the cerebellum derives from the most anterior region of the hindbrain, the metencephalon (met). Studies of formation of these two distinct brain structures have shown that during embryogenesis, development of the mesencephalon and metencephalon is coordinately regulated. After the initial regionalization of the primitive neuroectoderm, patterning of the mes-met region is thought to be further refined by a local organizing center formed at the mes-met junction. Heterotopic transplantation studies using chick-quail chimeras have demonstrated that the mes-met boundary region can induce an ectopic midbrain with appropriate AP pattern in the posterior forebrain or anterior midbrain, and ectopic cerebellar tissue in the posterior hindbrain (Alvarado-Mallart, 1993; Le Douarin, 1999). Recent studies have demonstrated that Fgf8, which is expressed in the mes-met junction, is an important component of the organizer activity (Joyner et al., 2000). Fgf8-soaked beads placed in the caudal forebrain or anterior midbrain of chick embryos induce ectopic midbrain and cerebellar development (Martinez et al., 1999; Crossley et al., 1996; Shamim et al., 1999). Furthermore, partial loss-of-function mutations in Fgf8 disrupt midbrain and cerebellum development in the mouse and fish (Meyers et al., 1998; Reifers et al., 1998; Brand et al., 1996).

Embryological manipulations in chick embryos have demonstrated that Fgf8 can be induced by a juxtaposition of posterior forebrain or midbrain, and rhombomere 1 (r1) tissues (Irving and Mason, 1999; Hidalgo-Sanchez et al., 1999b). These observations strongly support a model proposed by
Meinhardt that the formation of an organizing center involves initial specification of two populations of cells in adjacent territories and subsequent induction of cells at the common border to express signaling molecules (Meinhardt, 1983). According to Meinhardt’s model, the mid-hindbrain organizer would be established via differential specification of the midbrain and hindbrain.

Previous studies have shown that development of the midbrain and hindbrain requires two homeobox genes, Otx2 and Gbx2. Otx2 and Gbx2 are expressed by the headfold stage in the anterior and posterior neuroectoderm, respectively, and their common border of expression later demarcates the presumptive mid-hindbrain junction (Ang et al., 1994; Bouillet et al., 1995; Wassarman et al., 1997). Mouse embryos lacking Otx2 have gastrulation defects and fail to form the neural structures anterior to r3 (Acampora et al., 1995; Ang et al., 1996; Matsuo et al., 1995), primarily owing to a requirement for Otx2 in the anterior visceral endoderm (Rhinn et al., 1998; Acampora et al., 1998; Kimura et al., 2000; Perea-Gomez et al., 2001). Embryos that lack Otx2 function specifically in the epiblast and its derivatives, however, lack only a forebrain and midbrain (Acampora et al., 1995; Rhinn et al., 1998).

In a complementary manner, Gbx2 mutant embryos fail to develop anterior hindbrain structures including r1-3, and Otx2 expression is expanded caudally at the four- to six-somite stages, showing that the anterior hindbrain is transformed into a midbrain fate (Millet et al., 1999; Wassarman et al., 1997).

Gain-of-function studies have demonstrated that mutual antagonism between Otx2 and Gbx2 determines the position of mid-hindbrain border. Misexpression of Gbx2 leads to repression of Otx2 in the posterior midbrain (Millet et al., 1999; Katahira et al., 2000), and similarly misexpression of Otx2 results in repression of Gbx2 in the metencephalon (Broccoli et al., 1999; Katahira et al., 2000). In both cases, the expression domain of Fgf8 is shifted and situated at the new Otx2-Gbx2 border. In agreement with Meinhardt’s model, Otx2 and Gbx2 could therefore confer differential specification to the mesencephalic and metencephalic cells and subsequent interactions between these two populations of cells could lead to induction of Fgf8 at their common border. Interestingly, in the absence of either Otx2 or Gbx2 alone, Fgf8 is still expressed (Wassarman et al., 1997; Acampora et al., 1998; Rhinn et al., 1998). It could be, however, that juxtaposition of Otx2- or Gbx2-expressing cells with Otx2 or Gbx2 non-expressing cells is sufficient to induce Fgf8.

As the domains of Otx2 and Gbx2 expression position the mid-hindbrain organizer, it is important to determine how their expression domains are established. Otx2 is initially expressed throughout the epiblast of mouse embryos before gastrulation and its expression becomes progressively restricted to the anterior third of the embryo by the headfold stage (Ang et al., 1994). Expression of Gbx2 is first detected at the mid-streak stage in the primitive streak (J. H. L. and A. L. J., unpublished), and as gastrulation proceeds expression extends laterally and anteriorly such that its anterior limit directly abuts the posterior domain of Otx2 (Wassarman et al., 1997; Hidalgo-Sanchez et al., 1999a; Garda et al., 2001). These dynamic and complimentary expression patterns of Otx2 and Gbx2, and the presence of an apparent antagonism between them suggest that an interaction between these two genes could directly regulate their expression domains in vivo. Intriguingly, in mouse embryos that lack Otx2 in the epiblast, Gbx2 expression appears normal at E7.75, based on section in situ analysis, but rostrally expanded at E8.5 (Acampora et al., 1998), while in Gbx2 homozygous mutants, caudal expansion of Otx2 was detected at the four- to six-somite stage but earlier stages were not analyzed (Millet et al., 1999). There are suggestions that Fgf8, which starts to be expressed at the three-somite stage, might actually mediate the apparent opposing interaction between Otx2 and Gbx2 (Acampora et al., 1997; Liu and Joyner, 2001; Wassarman et al., 1997). Therefore, further studies of the timing of gene alterations in Otx2 or Gbx2 mutant embryos at early stages could provide new insights into the molecular mechanism that underlies the interaction between Otx2 and Gbx2, and the role of this interaction in regulating the expression domains of Otx2 and Gbx2.

It also has been suggested based on a germ-layer-recombination assay in mouse that expression of Otx2 during gastrulation is regulated by positive and negative signals from anterior and posterior mesoderm, respectively (Ang et al., 1994). Retinoic acid (RA), a posteriorizing factor, can repress Otx2 expression in mouse embryos treated in utero at E7.5 (Ang et al., 1994), whereas Gbx2 can be induced by RA in cultured P19 embryonal carcinoma cells and Xenopus embryos (Bouillet et al., 1995; von Bubnoff et al., 1996). However, it remains to be determined whether RA normally plays a crucial role in regulating expression of Otx2 and Gbx2 in vivo.

In order to determine whether an interaction between Otx2 and Gbx2 is required for determining their initial expression domains, we performed a detailed analysis of Gbx2 and Otx2 expression in embryos that lacked Otx2 or Gbx2. We show that the expression domains of Otx2 and Gbx2 are initially established independently of each other, but that by the late headfold stage (LHF) antagonistic interactions between these two genes play a crucial role in maintaining their respective borders of expression at the mes-met junction. To investigate factors that could regulate the early expression pattern of Otx2 and Gbx2, we analyzed expression of these two genes in mouse embryos treated in utero with RA. Expression of Gbx2 is induced anteriorly within 4 hours of RA treatment, and Otx2 is repressed in the midbrain and all but the anterior most forebrain by 24 hours. Significantly, in the absence of Gbx2, Otx2 is not repressed by RA. Furthermore, to study the collective roles of Otx2 and Gbx2 in initiation of Fgf8 and genes that are normally expressed in the mes-met region (mes-met genes), we generated Otx2 and Gbx2 double homozygous mutant embryos. We show that Otx2 and Gbx2 are not required for initiation or maintenance of these genes. Otx2 and Gbx2 are essential, however, for negatively regulating Fgf8 and Wnt1, respectively, and thus subdividing the presumptive mes-met region into two different domains. Finally, given the apparent antagonistic interaction between Otx2 and Gbx2, some of the phenotypes seen in Otx2 or Gbx2 single mutants could result primarily from mis-expression of Gbx2 or Otx2, respectively, rather than a positive requirement for Otx2 or Gbx2. Indeed, we show that Gbx2 plays only a permissive role in r3 development, whereas Otx2 is intrinsically required for forebrain development.
MATERIALS AND METHODS

Generation and genotyping of wild type and mutant mice
Noon of the day on which the vaginal plug was detected was considered as E0.5 in timing of embryos. Staging of embryos before somite formation was based on morphological landmarks (Downs and Davies, 1993). Gbx2 and Otx2 mutant mice were maintained on an outbred background. Genotypes of offspring were determined by PCR analysis as described (Acampora et al., 1998; Wassarman et al., 1997).

Retinoic acid treatment
Retinoic acid was administrated to pregnant females as described previously (Conlon and Rossant, 1995). All-trans retinoic acid (Sigma) was dissolved in DMSO (100 mg/ml) and further diluted to 10 mg/ml with corn oil before use. The mixture was administrated by oral gavage to a final dose of 20 μg/g of body weight of the pregnant females. The administration was performed between 10 am and noon on E7.5, and embryos were dissected and fixed 4, 8 or 24 hours after RA administration.

In situ hybridization
Whole-mount RNA in situ hybridization was performed essentially as previously described (Wilkinson, 1992). Section RNA in situ hybridization was performed as described (Wassarman et al., 1997). The antisense riboprobes for the following genes were used: Bfl1 (Foxg1 – Mouse Genome Informatics) (Tao and Lai, 1992), Otx2 (Ang et al., 1994), human OTX1 (Acampora et al., 1998), Hesx1 (Thomas and Beddington, 1996), Gbx2 (Bouillet et al., 1995), Fgf8 (Crossley and Martin, 1995), Wnt1 (Parr et al., 1993), Enl and En2 (Millen et al., 1995), Six3 (Oliver et al., 1995), Krox20 (Egr2 – Mouse Genome Informatics) (Wilkinson et al., 1989a), Pax2 (Dressler and Douglass, 1992), Hoxa2 and Hoxb1 (Wilkinson et al., 1989b).

RESULTS

An interaction between Gbx2 and Otx2 is required to maintain their anterior and posterior expression limits, respectively, as early as the late headfold stage
To determine the requirement for an interaction between Otx2 and Gbx2 in regulating their expression domains, we performed detailed expression analysis of Otx2 and Gbx2 mutants using morphological landmarks to stage embryos from E7.0 to E8.5 (Downs and Davies, 1993). In order to study the requirement for Otx2 in development of the anterior neuroectoderm, we used a mutant allele, Otx2 trans–/–, in which the human OTX1 protein is expressed in place of mouse Otx2 only in the anterior visceral endoderm, and rescues the gastrulation defects of Otx2 null mutants (Acampora et al., 1998). The OTX1 protein is not produced from this allele in the epiblast and its derivatives, although OTX1 mRNA transcripts are expressed from the Otx2 locus (Acampora et al., 1998).

First, we double labeled for transcripts of Gbx2 and Hesx1, a homeobox gene that is normally expressed in the prospective forebrain at the headfold stage (Thomas and Beddington, 1996). At the early headfold (EHF) stage, Hesx1 was not detected in Otx2 trans–/–;Hesx1–/– embryos (n=3), whereas Gbx2 was readily detected (Fig. 1A). The distance between the anterior limit of Gbx2 expression and the position of the node in the midline of Otx2 trans–/–;Hesx1–/– embryos was comparable with that in wild-type controls. By contrast, by the LHF stage, Gbx2 expression in Otx2 trans–/–;Hesx1–/– embryos was significantly expanded rostrally, based on an increased distance between the

Fig. 1. An interaction between Gbx2 and Otx2 defines the limits of their respective expression domains at the start of somitogenesis.
(A) Expression of Gbx2 and Hexx1 in wild-type and Otx2 trans–/–;Hoxa1–/– embryos at the EHF stage. The anterior limit of Gbx2 expression in the midline (arrow), relative to the position of the node (arrowhead) in Otx2 trans–/–;Hoxa1–/– and wild-type embryos is comparable, although the lateral expression of Gbx2 (asterisk) appears slightly expanded anteriorly in the mutant. (B) Gbx2 expression is significantly expanded anteriorly in Otx2 trans–/–;Hoxa1–/– embryos at the LHF stage, compared with wild-type controls. (C,D) Expression of Otx2 in wild-type and Gbx2+/– embryos at the EHF (C) and LHF (D) stage. Relative to the position of the node (arrowhead), the posterior limit of Otx2 (arrow) is not altered in Gbx2+/– embryos at the EHF stage, but shifted caudally by the LHF stage, particularly in the midline. (E) Expression of Gbx2 is normal in Gbx2+/– embryos at the EHF stage. (F) Hexx1 expression (brackets) is not changed in Gbx2+/– embryos, whereas Gbx2 expression is reduced and its anterior limit (arrow) is shifted caudally compared with wild-type controls at the LHF stage. The apparently stronger staining of Hexx1 in the Gbx2+/– embryo is due to a prolonged color reaction in the mutant compared with the wild type in order to visualize Gbx2 staining. (B,F) Dorsal views of flat-mount embryos.
anterior limit of the Gbx2 domain and the position of the node, as well as a reduced Gbx2-negative region in the anterior ectoderm (Fig. 1B). At the four-somite stage, Gbx2 expression was expanded to the anterior tip of mutant embryos. By the six-somite stage, its expression became restricted to the anterior tip (data not shown) (Acampora et al., 1998). Thus, Otx2 function is not required to determine the initial anterior expression limit of Gbx2 but is required rapidly to maintain it. Furthermore, consistent with a previous study of chimeras composed of Otx2 mutant and wild-type cells (Rhinn et al., 1998), Otx2 is required for Hesx1 expression in the anterior neuroectoderm at a time when it is not required to regulate Gbx2.

Our finding that Gbx2 is expanded rostrally in Otx2hOtx1/hOtx1 embryos by the LHF stage prompted us to examine whether Gbx2 is also required to define the posterior limit of Otx2 expression at the equivalent stage. Otx2 is normally expressed at the EHF stage in the anterior third of embryos with a diffuse posterior limit. A similar pattern of expression was seen in the EHF stage Gbx2–/– embryos (Fig. 1C). However, at the LHF stage, expression of Otx2 was abnormally expanded caudally, primarily in the midline of Gbx2–/– embryos (Fig. 1D).

To determine whether the caudal expansion of Otx2 in Gbx2–/– embryos at the LHF stage is associated with a loss of r1-3 specification, we first examined expression of Krox20, which is initially expressed in r3 from the LHF to three-somite stages (Wilkinson et al., 1989a). In Gbx2–/– embryos, Krox20 expression was not detected between the LHF and three-somite stages (n=3), whereas its expression was readily detected in r3 of wild-type controls (n=3) at the same stages (data not shown). Krox20 expression also was not detected in r3 in Gbx2–/– embryos at the seven-somite stage (see Fig. 7B) (Wassarman et al., 1997). These observations show that Gbx2 is required for initiation of Krox20 expression in r3.

To further examine whether caudal expansion of Otx2 results in a general respecification of the presumptive r1-3 region, we analyzed Gbx2 RNA expression in Gbx2–/– embryos at the LHF stage, using a probe corresponding to 5′ coding sequences not deleted in the mutant allele (Wassarman et al., 1997). In Gbx2–/– embryos, expression of Gbx2 was normal at the EHF stage (Fig. 1E). By contrast, at the late LHF stage in Gbx2–/– embryos, expression of Gbx2 was significantly reduced and its anterior expression limit was shifted posteriorly (Fig. 1F). Interestingly, expression of Gbx2 was greatly reduced in Gbx2–/– embryos at E8.5 (n=2), even in posterior regions of the embryo where development seems unaffected by disruption of Gbx2, suggesting Gbx2 becomes autoregulated.

Taken together, these data demonstrate that establishment of the initial posterior or anterior limit of Otx2 or Gbx2 expression at the EHF stage is not dependent on Gbx2 or Otx2, respectively, but by the LHF stage, an interaction between Otx2 and Gbx2 plays a crucial role in defining their respective expression limits. Disruption of either Otx2 or Gbx2 results in rapid expansion of the Gbx2 or Otx2 expression domains, respectively, which may then lead directly to respecification of the midbrain or r1-3.
Gbx2 is required for repression of Otx2 by exogenous RA

As the expression domains of Otx2 and Gbx2 are initially established independently of each other, it was of interest to explore what molecules might regulate their initial expression patterns. RA has been implicated as a posteriorizing factor during embryonic AP patterning, and has opposite effects on Otx2 and Gbx2 expression (Ang et al., 1994; Bouillet et al., 1995; von Bubnoff et al., 1996). Therefore, we studied the temporal and spatial responses of Otx2 and Gbx2 in mouse embryos 4, 8 and 24 hours after exposure to a teratogenic dose of RA.

Within 4 hours of RA treatment of mouse embryos at E7.5, expression of Gbx2 was rapidly and dramatically induced and expanded anteriorly (Fig. 2A,C). As shown previously (Ang et al., 1994), the expression domain of Otx2 was only slightly reduced in the anterior region of embryos 4 hours after RA treatment (Fig. 2E,G) and its expression domain was further reduced by 8 hours (data not shown). Twenty-four hours after RA treatment, Gbx2 expression was found to be expanded and in a broad anterior region with a small Gbx2-negative domain at the anterior tip of the embryos (Fig. 2B,D). The Gbx2-negative region seemed to correspond to a greatly restricted Otx2 expression domain (Fig. 2F,H).

The rapid induction of Gbx2 by RA and an apparent antagonistic interaction between Otx2 and Gbx2 led us to investigate whether Gbx2 is required for the RA-mediated repression of Otx2. Interestingly, expression of Otx2 in Gbx2−/− embryos was not repressed 4 hours (Fig. 2I) or 24 hours (Fig. 2J) after exogenous RA treatment. Instead, in Gbx2−/− embryos with or without RA treatment, Otx2 was expanded to the presumptive r3/r4 border. These results demonstrate that Gbx2 is required to mediate repression of Otx2 by RA. The studies also indicate that the initial restriction of Otx2 expression to the anterior region of the embryo does not depend on RA signaling, as the expression domain of Otx2 is normal at the EHF stage in Gbx2−/− embryos.

Mes-met genes are induced in a normal temporal order, but in a broad anterior region in Gbx2−/−; Otx2hOtx1/hOtx1 embryos

Based on Meinhardt’s model, the division of the neural ectoderm into anterior Otx2-positive and posterior Gbx2-positive domains could be imperative for the induction of Fgf8 and other mes-met genes. To test this hypothesis, we generated Gbx2−/−; Otx2hOtx1/hOtx1 embryos and investigated whether the mes-met genes are induced in these double mutant embryos at early somite stages.

At the four-somite stage, Fgf8 was detected in the metencephalon (Fig. 3A). In Otx2hOtx1/hOtx1 embryos at the five-somite stage, diffuse Fgf8 expression was detected in a broad domain of the anterior neuroectoderm and slightly stronger expression was seen at the anterior tip of the embryos (Fig. 3B). By the seven-somite stage, Fgf8 expression became restricted to the anterior tip of the mutant embryos (inset in Fig. 3B) (Acampora et al., 1998). In Gbx2−/− mutants the presumptive metencephalon was greatly reduced and shifted caudally (Fig. 3C) and by the seven-somite stage Fgf8 was diffuse and expanded to r4 and fused with expression of Hoxb1, a gene that normally marks r4 (inset in Fig. 3C). Interestingly, at both the four- and six-somite stages in Gbx2−/−; Otx2hOtx1/hOtx1 embryos, Fgf8 was strong and throughout a large anterior domain (Fig. 3D and inset). Therefore, Otx2 and Gbx2 are not required for Fgf8 to be induced.

We next examined another gene expressed at the mes-met junction. Wnt1 is normally expressed across the entire mesencephalon at the four-somite stage and then in a narrow band anterior to the Fgf8 expression domain (Fig. 3E). In Otx2hOtx1/hOtx1 mutants at the five-somite stage, Wnt1 was absent from the mesencephalon (n=2) (Fig. 3F). By the eight-somite stage Wnt1 was found in the lateral edges of the neural fold along the entire AP axis (n=2) (inset in Fig. 3F) (Acampora et al., 1998). In Gbx2−/− embryos, Wnt1 expression was expanded caudally at the five-somite stage (Fig. 3G) (Millet et al., 1999). In contrast to either single mutant, in Gbx2−/−; Otx2hOtx1/hOtx1 embryos at the four-somite stage, Wnt1 expression was patchy and expanded to the anterior tip of the neuroectoderm overlapping with Fgf8 (Fig. 3H).

Pax2 is the earliest known gene expressed in the mes-met region. Pax2 transcripts are first detected in the anterior ectoderm of mouse embryos at the late streak stage (Rowitch and McMahon, 1995). At the LHF stage, Pax2 is normally expressed as a transverse band corresponding to the presumptive mes-met region (Fig. 3I) (Rowitch and McMahon, 1995). At the four-somite stage, in addition to strong Pax2 expression in the mes-met region, Pax2 also is expressed in the anterior neural ridge and presumptive otic vesicles (Fig. 3M) (Hidalgo-Sanchez et al., 2001). In LHF stage Otx2hOtx1/hOtx1 mutants, expression of Pax2 was reduced and expanded anteriorly (Fig. 3J). By the four-somite stage, Pax2 expression was restricted to the anterior tip of the neuroectoderm in Otx2hOtx1/hOtx1 mutants, whereas its expression in the presumptive otic ectoderm appeared normal (Fig. 3N). In Gbx2−/− embryos, expression domain of Pax2 was expanded caudally to the level of the expression in the otic ectoderm (Fig. 3K,O). Interestingly, in LHF stage Gbx2−/−; Otx2hOtx1/hOtx1 embryos, Pax2 expression domain was expanded both anteriorly and posteriorly at the LHF (Fig. 3L), and by the four-somite stage its expression was detected throughout a broad region of the anterior neuroectoderm (Fig. 3P).

En1 is another early marker for the mes-met region and is first detected by the one-somite stage (Joyner et al., 2000). Interestingly, alteration of En1 expression pattern mirrored that of Pax2 expression in the mes-met domain of Gbx2−/−; Otx2hOtx1/hOtx1 and Gbx2−/−; Otx2hOtx1/hOtx1 mutant embryos. At the three-somite stage, En1 expression was reduced and its expression domain was restricted to the anterior tip of Otx2hOtx1/hOtx1 embryos (Fig. 3R). In Gbx2−/− embryos, expression domain of En1 was expanded caudally (Fig. 3S). By contrast, in Gbx2−/−; Otx2hOtx1/hOtx1 embryos, expression of En1 was found in a broad region of the anterior neuroectoderm (Fig. 3T). In summary, in Gbx2−/−; Otx2hOtx1/hOtx1 embryos the expression of mes and met genes is abnormally colocalized in a broad anterior domain, suggesting that the presumptive midbrain and r1 regions are not differentially specified and instead a broad anterior region of neuroectoderm takes on characteristic of both regions. By contrast, in Otx2hOtx1/hOtx1 embryos, the initial anterior expansion of the expression domains of Gbx2 and then Fgf8, and a lack of initiation of Wnt1 expression in the presumptive
Gbx2−/−; Otx2hOtx1/hOtx1 embryos have an anterior deletion and exencephaly

Given the requirement for an interaction between Otx2 and Gbx2 in maintaining their early respective expression domains, some of the phenotypes seen in Otx2 or Gbx2 homozygous mutants could result primarily from the abnormal expansion of the Gbx2 or Otx2 expression domains, respectively. Indeed, the initial gene expression analysis above showed that expression of some midbrain markers, Wnt1 and human OTX1, were restored in a broad anterior region of the neuroectoderm by removing Gbx2 from Otx2hOtx1/hOtx1 embryos at early somite stages. However, this anterior region appeared abnormally specified and composed of molecular attributes of both the mesencephalic and metencephalic regions. Furthermore, there were no consistent morphological differences between Otx2hOtx1/hOtx1 and Gbx2−/−; Otx2hOtx1/hOtx1 embryos at these stages (Fig. 3, compare second and fourth rows), and both

Fig. 3. Expression of mes-met genes in single Otx2 or Gbx2 and double homozygous mutant embryos at early somite stages. (A,B) Expression of Fgf8 at the four-somite stage. Insets in the images show expression of Hoxb1 (arrowheads) and Fgf8 (brackets) in six- to seven-somite stage embryos. Note that in the Gbx2−/− embryo (inset in C), broad and weak Fgf8 expression forms a gradient in the anterior hindbrain with highest expression overlapping with Hoxb1 expression in r4. By contrast, Fgf8 is expressed broadly in the anterior neuroectoderm of Gbx2−/−; Otx2hOtx1/hOtx1 embryos (inset in D) and its posterior limit ends a few cell diameters from Hoxb1 expression in r4. (E-H) Expression of Wnt1 in the posterior hindbrain (arrows) remains unchanged in embryos that lack Otx2 (F), Gbx2 (G) or both (H) at the four- to five-somite stage. The transverse band of Wnt1 expression in the mesencephalon (brackets), however, is affected in these embryos. Inset in F shows an Otx2hOtx1/hOtx1 embryo at the eight-somite stage with Wnt1 expression in the lateral edges of the neural plate extending from the posterior hindbrain to the anterior extreme of the embryo. (I-L) and (M-P) Expression of Pax2 at the LHF stage and four-somite stage, respectively, in embryos lacking Otx2 (J,N), Gbx2 (K,O) or both genes (L,P). The mes-met expression of Pax2 is indicated by a bracket. Expression of Pax2 in the pre-otic ectoderm is marked by arrows. (Q-T) En1 expression (brackets) is shifted anteriorly in embryos that lack Otx2 (R,T), whereas En1 expression is expanded posteriorly in embryos that lack Gbx2 (S,T). The En1 expression level in Otx2hOtx1/hOtx1 embryos is also reduced. (U-W) Human OTX1 is expressed from the Otx2 locus in Otx2 heterozygous (U), Otx2hOtx1/hOtx1 (V) and Gbx2−/−; Otx2hOtx1/hOtx1 (W) embryos. Human OTX1 is expressed only in the anteriormost endoderm and ectoderm (arrowhead), but not in the neuroectoderm of Otx2hOtx1/hOtx1 embryos, whereas in Gbx2−/−; Otx2hOtx1/hOtx1 embryos human OTX1 is expressed in a broad anterior domain of the neuroectoderm. Note that all the Otx2hOtx1/hOtx1 and Gbx2−/−; Otx2hOtx1/hOtx1 embryos have a similar anterior truncation (compare embryos in the second column with those in the forth column).
mutants had a similar anterior truncation. Interestingly, by E9.5, the morphology of the anterior structures of these two types of mutant embryos was significantly different. The anterior neural tube of Gbx2–/–; Otx2hOtx1/hOtx1 embryos failed to close and the anterior neural fold was expanded laterally, whereas in Otx2hOtx1/hOtx1 embryos, the anteriorly truncated neural tube consisted of a thin neural epithelium (data not shown). The majority of embryos deficient for Otx2 died around E10.5. Remarkably, we did manage to recover an Otx2hOtx1/hOtx1 embryo at E12.5 from a single litter (out of a total of 10 litters). At this stage, the most anterior structure of the Otx2hOtx1/hOtx1 embryo was reminiscent of an anterior hindbrain, consisting of the IVth ventricle and the roof of the ventricle (Fig. 4A) (Acampora et al., 1998). Exencephaly persisted in the Gbx2–/–; Otx2hOtx1/hOtx1 embryos at E12.5, and no discernable craniofacial or eye structures developed in these mutants (Fig. 4B). The Gbx2–/–; Otx2hOtx1/hOtx1 embryos, however, appeared to have more anterior tissue compared with the Otx2hOtx1/hOtx1 mutant. Histological analysis showed that in the Gbx2–/–; Otx2hOtx1/hOtx1 embryos there was significantly more head mesenchyme and an additional thin layer of undifferentiated neuroectoderm at the anterior end of the brain, compared with the Otx2hOtx1/hOtx1 embryo (Fig. 4D,E). Taken together, although there was some expansion of anterior tissue by removing Gbx2 in Otx2hOtx1/hOtx1 mutants, development of head structures was greatly impaired, and no discernable midbrain or cerebellar anlage developed in Gbx2–/–; Otx2hOtx1/hOtx1 mutants.

To characterize the regional identity of the anterior neural plate of Gbx2–/–; Otx2hOtx1/hOtx1 embryos, we examined expression of markers that are distinctive for specific brain regions at E9.5. In wild-type embryos, Wnt1 and Fgf8 are expressed in two juxtaposed bands of cells at the mid-hindbrain junction, with Wnt1 in the midbrain and Fgf8 in the hindbrain, whereas En1 is expressed broadly across both regions (Fig. 5A,C,E). Otx2 is normally expressed in the forebrain and midbrain (Fig. 5G). In Gbx2–/–; Otx2hOtx1/hOtx1 embryos, the expression of Wnt1, Fgf8, En1 and human OTX1 was seen to persist in a broad domain of the anterior neuroectoderm of E9.5 double mutant (Fig. 5B,D,F,H). By contrast, expression of Fgf8 and En1 was restricted to the anterior tip of Otx2hOtx1/hOtx1 embryos.

During normal development, when anterior structures develop, the posterior limits of expression of the anterior neural tube markers are successively more posterior (Fig. 6B-D). Expression of En1 and En2 encompassed the Wnt1, Fgf8 and Pax2 expression domains but the posterior En1 and En2 expression limits were extended caudally, with En2 being more posterior (Fig. 6E,F). These results show that a normal spatial relationship of the posterior expression domains of Fgf8, Pax2, En1 and En2 is maintained in Gbx2–/–; Otx2hOtx1/hOtx1 embryos.

Taken together, removal of Gbx2 in Otx2hOtx1/hOtx1 mutant embryos allows some early midbrain genes to be expressed, but does not rescue midbrain development. Persistent broad expression of Fgf8, overlapping with other mes-met genes in the anterior neuroectoderm may disrupt neural tube closure and normal differentiation of the midbrain and hindbrain.

The forebrain fails to develop in Gbx2–/–; Otx2hOtx1/hOtx1 embryos

The abnormal expansion of mes-met genes to the anterior extreme of Gbx2–/–; Otx2hOtx1/hOtx1 embryos at early somite stages indicated that the forebrain failed to develop in these mutants. To verify this, we examined expression of Six3, a homeobox gene that is normally expressed in the forebrain (Fig. 7A) (Olive et al., 1995). Previous studies have shown that Otx2 activity in the anterior neuroectoderm is not essential for

**Fig. 4.** Gbx2–/–; Otx2hOtx1/hOtx1 embryos have an anterior truncation and exencephaly. (A,B) Morphology of wild-type (left in A), Otx2hOtx1/hOtx1 (right in A) and Gbx2–/–; Otx2hOtx1/hOtx1 embryos (B) at E12.5. (C-E) Sagittal sections of wild-type (C), Otx2hOtx1/hOtx1 (D) and Gbx2–/–; Otx2hOtx1/hOtx1 embryos (E) at E12.5. The anterior structures of Otx2hOtx1/hOtx1 and Gbx2–/–; Otx2hOtx1/hOtx1 embryos are greatly truncated. There is more anterior tissue, particularly head mesenchyme (asterisk) in Gbx2–/–; Otx2hOtx1/hOtx1 embryos than in Otx2hOtx1/hOtx1 embryos. There is an additional thin epithelium (bracket) extending from the presumptive hindbrain in Gbx2–/–; Otx2hOtx1/hOtx1 embryos. D.E are at a higher magnification than C. The junction between the spinal cord and hindbrain is marked by arrowheads. Cb, cerebellum; Dt, dorsal thalamus; IV, IVth ventricle; Mb, midbrain; Sc, spinal cord; Tel, telecephalon.
initiation of Six3 expression but is required for maintenance of Six3 expression (Acampora et al., 1998; Kimura et al., 2000). In agreement with these observations, Six3 expression was detected in the anterior headfold of Otx2 hOtx1/hOtx1 and Gbx2–/–; Otx2 hOtx1/hOtx1 embryos at the LHF stage (data not shown). By the three-somite stage, expression of Six3 in the anterior neuroectoderm was not detected in either Otx2 hOtx1/hOtx1 (n=2) or Gbx2–/–; Otx2 hOtx1/hOtx1 embryos (n=2) (Fig. 7C,D). As expected, in Gbx2–/– embryos Six3 expression was normal (Fig. 7B). Furthermore, expression of Bfl (n=2) and Hesx1 (n=1), two other forebrain markers (Thomas and Beddington, 1996; Shimamura and Rubenstein, 1997), was absent in the neuroectoderm of the double homozygous mutants at E8.5 (data not shown). Thus, removal of Gbx2 is not sufficient to allow initiation of forebrain development in Otx2 hOtx1/hOtx1 embryos.

Development of r3, but not r2, is rescued in Gbx2–/– embryos by removing Otx2 function

We have shown that Otx2 expression is expanded posteriorly in Gbx2–/– embryos and Krox20 expression is not initiated in r3, reflecting a loss of specification of r1-3 (see Fig. 1). To investigate whether removal of Otx2 in Gbx2–/– embryos rescues hindbrain development, we examined gene expression in r2 and r3. Expression of Krox20 was examined in embryos at the eight-somite stage. In Gbx2–/– embryos, only a single stripe of cells weakly expressing Krox20 was detected in r5 (Wassarman et al., 1997). Strikingly, in Gbx2–/–; Otx2 hOtx1/hOtx1 embryos, two transverse stripes of Krox20 expression were seen, similar to that in wild-type and Otx2 hOtx1/hOtx1 embryos (Fig. 7A,C,D), suggesting r3 is rescued in Gbx2–/–; Otx2 hOtx1/hOtx1 embryos.

We next examined the expression of Hoxa2, which normally is strongly expressed in r3, r5 and weakly in r2 at E9.5 (Fig. 7E). The neural crest cells migrating out from r4 also express Hoxa2. In Gbx2–/– embryos, Hoxa2 expression in r5 and the migrating neural crest cells from r4 appeared normal, but there was no Hoxa2 expression in r2-3 (Fig. 6F). This result further supports our previous studies showing that the defects in Gbx2 mutants are limited to r1-3. As expected, Hoxa2 expression in Otx2 hOtx1/hOtx1 embryos was essentially normal, although the
expression domains in r2 and r3 appeared slightly expanded (Fig. 7G). Interestingly, in Gbx2−/−; Otx2ΔOtx1/Otx1 embryos, Hoxa2 expression was detected in r3, as well as in r5 and the neural crest cells migrating from r4 (Fig. 7H). In these mutants, expression of Hoxa2 in r3 was slightly expanded as in Otx2ΔOtx1/Otx1 embryos. Significantly, expression of Hoxa2 was not detected in r2 of Gbx2−/−; Otx2ΔOtx1/Otx1 embryos. Taken together, these results show that development of r3, but not r2, was rescued in Gbx2−/−; Otx2ΔOtx1/Otx1 embryos. Therefore, Gbx2 is not directly involved in initiating r3 formation, but instead is required to limit Otx2 expression to the anterior neuroectoderm and thus allow r3 development to proceed.

**DISCUSSION**

In this study, we have focused on the phenotypes of Otx2 and Gbx2 single and double homozygous mutants at early developmental stages. We show that the initial expression domains of Otx2 and Gbx2 are established independently, although their expression rapidly becomes interdependent. We further show that although RA can regulate Otx2 negatively and Gbx2 positively, the repression of Otx2 by RA requires Gbx2. We demonstrate that Otx2 and Gbx2 are not essential for the initiation of mes-met gene expression. Furthermore, although Fgf8 and other mes-met genes are induced in Gbx2−/−; Otx2ΔOtx1/Otx1 embryos, the expression domains of these genes are abnormally co-localized in a broad anterior region of the neuroectoderm (summarized in Fig. 8A), uncovering negative regulatory roles of Gbx2 and Otx2 in midbrain and hindbrain development, respectively. Consistent with this, removal of Otx2 from Gbx2 homozygous mutant embryos rescues segmental development of r3, demonstrating that Gbx2 plays only a permissive role in r3 development by limiting Otx2 expression to the anterior neuroectoderm. By contrast, the forebrain fails to develop in Gbx2−/−; Otx2ΔOtx1/Otx1 embryos, but midbrain gene expression is restored. Therefore, the abnormal anterior expansion of Gbx2 is responsible for the loss of midbrain but not forebrain development in Otx2 mutants.

**Establishment of the expression domains of Otx2 and Gbx2**

During gastrulation, expression of Otx2 and Gbx2 is dynamic and the expression domains of these two genes become complementary. Interestingly, the initial expression patterns of Gbx2 or Otx2 are generally preserved in embryos deficient in Otx2 or Gbx2, respectively, although a reciprocal antagonistic interaction between Otx2 and Gbx2 is crucial in maintaining their expression limits at the presumptive mes-met border as early as the LHF stage. In agreement with these results, the expression domains of Otx2 and Gbx2 do not immediately abut each other at the EHF stage in mouse embryos (J. Y. H. L. and A. L. J., unpublished) and similar observations were recently made in the chick (Garda et al., 2001). Therefore, the initial Otx2-Gbx2 border must be established by external factors. Previous studies have shown that the expression domain of Otx2 is defined by both positive and negative signals (Ang et al., 1994). One possible scenario is that Otx2 and Gbx2 are regulated by the same pathways, but with opposite responses. RA has been considered a candidate for such a signal. Indeed, previous studies and the work presented here have shown that RA can repress Otx2 and induce Gbx2. However, in this study, we demonstrate that repression of Otx2 by RA is dependent on Gbx2. Therefore, RA cannot play an essential role in regulating the initial expression of Otx2 in vivo.

Other posteriorizing factors, like Fgfs, are plausible regulators of early Otx2 and Gbx2 expression. Multiple Fgfs, including Fgf3, Fgf4, Fgf5, Fgf8 and Fgf17 (Sun et al., 1999), are expressed in posterior regions of the mouse embryo during gastrulation. Furthermore, Fgf8-soaked beads can induce Gbx2 and repress Otx2 in the midbrain of chick embryos or in embryonic mouse brain explants (Irving and Mason, 1999; Liu and Joyner, 2001; Liu et al., 1999; Martinez et al., 1999; Garda et al., 2001). Significantly, unlike RA, Fgf8 can repress Otx2 independent of Gbx2 function (Liu and Joyner, 2001). Finally,
in Fgf8-null mutant embryos, which also fail to express Fgf4 in the primitive streak. Otx2 is expressed throughout the epiblast, whereas Gbx2 is not expressed (Sun et al., 1999). These results strongly implicate Fgfs in regulating the initial expression of Otx2 and Gbx2.

**How is the mes-met genetic cascade initiated?**

One surprising result of our study is that expression of Fgf8 and other mes-met genes is induced and maintained in the absence of both Gbx2 and Otx2. Based on analysis of the genetic mechanisms that regulate formation of local organizers in various developmental systems, Meinhardt proposed that an interaction between posteriorizing and anteriorizing signals determine the position of the Otx2/Gbx2 border, as well as a mes-met competence domain by E7.5. The entire presumptive mes-met region is competent to respond to a signal that induces expression of Wnt1, Fgf8 and other mes-met genes. Expression of Otx2 and Gbx2 in the neuroectoderm at E7.75 subdivides the presumptive mes-met region into two distinct domains. Negative regulation of Wnt1 by Gbx2, and of Fgf8 by Otx2 results in the initial restriction of Wnt1 and Fgf8 expression specifically to the Gbx2- and Otx2-negative positive regions, respectively, at E8.5. At later stages, expression of Wnt1 and Fgf8 is maintained only in cells adjacent to each other through mutual positive feedback between Fgf8 and Wnt1 (or an unknown secreted factor in the midbrain) and between all mes-met genes. Otx2 and Gbx2 continue to negatively regulate Fgf8 and Wnt1 expression, respectively. MHB, mid-hindbrain boundary.

The finding that Fgf8 and Wnt1 are induced in a broad domain in Gbx2+/−; Otx2hOtx1/hOtx1 embryos indicates that the presumptive mesencephalon and metencephalon are equally competent to express Fgf8 and Wnt1, as well as other mes-met genes, in response to an inductive signal. One role of Otx2 and Gbx2 is therefore to restrict Fgf8 and Wnt1 expression to their appropriate regions through negative regulation. A key question now is how a mes-met competence domain is initially
specific. Gene expression studies have indicated that specification of the mes-met domain occurs as the neuroectoderm induced. For example, Pax2 is initially expressed in the anterior region of mouse embryos as early as the late streak stage and becomes restricted to a transverse band marking the presumptive mes-met region by the LHF stage (Rowitch and McMahon, 1995). Furthermore, explants of anterior ectoderm from late streak stage mouse embryos express En1 after 2 days in culture, suggesting that induction of En1 is already autonomous to the anterior ectoderm by the late streak stage (Ang and Rossant, 1993). A similar observation was recently made in chick (Muhr et al., 1999).

The initial regionalization of the neural plate is thought to be achieved by two opposing signals: posteriorizing signals released from the node and molecules expressed by the anterior visceral endoderm and mesendoderm that antagonize the posteriorizing signals (Stern, 2001). These two opposing signals may determine the position of the Otx2-Gbx2 border, as well specifying a mes-met competence domain (Fig. 8B). This hypothesis is supported by the fact that mouse mutations in genes that function in the anterior visceral endoderm or/and anterior mesendoderm (Otx2, Lim1, Nodal and Smad2) disrupt development of the mes-met region, as well as formation of more anterior regions (Beddington and Robertson, 1998). Furthermore, it has been shown that both inductive signals derived from the anterior mesendoderm and posterior mesendoderm determine the expression domain of Otx2 (Ang et al., 1994), as well as En1/2 (Muhr et al., 1999). Finally, we have shown in this study that the prospective Otx2-Gbx2 border, as well as a mes-met competence domain is initially defined independent of Otx2 and Gbx2.

The molecular identity of the signal(s) inducing the mes-met cascade is still unknown. Because after initiation of the mes-met cascade, it can probably be maintained by Fgf8 and intricate mutual regulation among the mes-met genes, we predict that the initial inductive signal is transient. Genetic studies have so far failed to identify the initial inductive signal for the mes-met cascade. Fgf4- or Fgf8-soaked beads inserted into the diencephalon or anterior midbrain of chick embryos at the 10-somite stage can initiate a de novo induction of mes-met genes including Fgf8, suggesting Fgf4 and Fgf8 may mimic the normal induction mechanism of the mes-met cascade (Shamin and Mason, 1998; Crossley et al., 1996; Martinez et al., 1999). Paradoxically, Fgf8-soaked beads can not induce Fgf8 or Pax2 in E9.5 mouse brain tissue explants (Liu et al., 1999; Garda et al., 2001). It is not clear, however, whether the neuroectoderm from E9.5 embryos has the same competence as E8.0 embryos, when the mes-met genes are initially induced. Previous studies have shown that Fgf signaling is required for the initiation and maintenance of En1/2 expression in chick epiblast explants (Muhr et al., 1999). Interestingly, Spry2, a likely downstream target of Fgf signaling, is expressed specifically in the presumptive mes-met region at the LHF stage (J. Y. H. L. and A. L. J., unpublished), indicating the presence of Fgf signaling in this region when mes-met gene expression is initiated. Similarly, activated extracellular signal-related kinases, which mediate signaling of various receptor tyrosine kinases including Fgfs, were detected specifically in the presumptive mid-hindbrain boundary and the primitive streak of Xenopus embryos at stage 12.5, a stage before mes-met gene initiation (Christen and Slack, 1999). These observations indicate that the Fgf signaling pathway could be involved in induction of the mes-met cascade.

The location of a mes-met induction signal(s) is also elusive. Fgf4 is expressed in the notochord underlying the presumptive mes-met region in chick embryos and this expression has been implicated to be important for initiation of the mes-met cascade (Shamin et al., 1999). Recently, it has been shown that Fgf18 is transiently expressed in the chick head process (Ohuch et al., 2000). Similar expression of Fgf4 and Fgf18 has not been reported in other species, and the functional significance of these gene expression has not been tested. Several groups have investigated possible source of mes-met inductive signals using in vitro explant culture, in which the naive neuroectoderm is co-cultured with different potential inductive tissues or signaling molecules. It has been shown that the anterior mesendoderm is sufficient to induce expression of both Otx2 and En1/2 in pre-streak stage anterior and posterior ectoderm (Ang and Rossant, 1993; Ang et al., 1994). In agreement with this, surgical removal of anterior midline mesendoderm and ectoderm from E7.5 mouse embryos in culture disrupts initiation of Fgf8 expression in the mes-met region (Camrus et al., 2000). Furthermore, Muhr and colleagues have demonstrated that a rostralizing signal from the anterior mesendoderm, together with Fgfs, and an unknown signal from the paraxial mesoderm are essential for induction of En expression in chick epiblast explants (Muhr et al., 1999). Finally, in terms of later differentiation, neuroectoderm explants have been used, and it has been shown that neuronal differentiation with characteristics of the mid-hindbrain region resulted from an interaction between Fgf8, which is locally expressed in the mid-hindbrain junction, or Fgf4, which is expressed in the primitive streak, with Shh, which is expressed in the floor plate (Ye et al., 1998). These studies indicate that induction of the mes-met cascade may result from convergence of multiple signaling pathways.

**Otx2 and Gbx2 are required for establishment of the normal spatial relationships of mes-met genes**

We have shown that in embryos lacking both Otx2 and Gbx2, expression of mes-met genes, such as En1, Pax2, Fgf8 and Wnt1, is initiated at similar stages to those in wild-type embryos and maintained at strong levels until at least E10.5. However, in such mutants, expression of the mes-met genes is co-localized in a broad domain of the anterior neural plate (Fig. 8A). The entire neuroectoderm anterior to r3 displays molecular markers of both mes and met regions, including human OTX1 (expressed from the Otx2 locus), Fgf8 and Wnt1. Thus, in mouse embryos that lack both Otx2 and Gbx2 the mes-met region develops as a single unit. Otx2 and Gbx2, therefore, act as selector genes to subdivide the prospective mes-met region into two distinct domains.

Previous studies have indicated that Otx2 regulates Wnt1 positively (Rhinn et al., 1999) and Fgf8 negatively (Acampora et al., 1997). Conversely, Gbx2 appears to regulate Wnt1 negatively and possibly Fgf8 positively (Millet et al., 1999; Liu and Joyner, 2001; Katahira et al., 2000). We show that the initial expression domains of Fgf8 and Wnt1 are broad and overlapping in Gbx2- and Otx2-negative domains, respectively (Fig. 8B). Interestingly, expression of
Fgf8 is greatly reduced in Otx2+/Otx1/hOtx1 embryos at early somite stages, whereas the level of Fgf8 expression in Gbx2–/–; Otx2+/Otx1/hOtx1 embryos is initially comparable with that in wild-type embryos and later becomes upregulated. These results indicate that factors other than Otx2 and Gbx2 must be involved in modulating Fgf8 expression.

Normally, the expression domains of Wnt1 and Fgf8 become progressively restricted to two sharp transverse rings immediately next to each other at the mid-hindbrain border by E9.5, after their initial broad and complementary expression at E8.5. Concurrently, the expression domains of Pax2 and Enl become restricted to bands that straddle the mid-hindbrain border. The molecular basis that underlies this compaction of gene expression remains to be elucidated. Recently, it was shown that Fgf8 can induce Wntl expression and maintain Wntl expression only in Gbx2-negative cells (Liu and Joyner, 2001). In a complementary manner, Wntl may also positively regulate Fgf8, as expression of Fgf8 is rapidly lost in the metencephalon of mouse embryos deficient in Wntl (Lee et al., 1997). Consistent with this, previous studies have also implicated a secreted factor from the metencephalon in positive regulation of Fgf8 (Irving and Mason, 1999; Danielian and McMahon, 1996). It is possible that once Wntl and Fgf8 are induced in two adjacent domains, only cells close to their common border maintain Wntl and Fgf8 expression through cell-nonautonomous actions of Fgf8 and Wnt1 (or an unknown factor), respectively, and thus their expression domains become restricted to the boundary region (Fig. 5B). The progressive restriction of Fgf8 and Wntl may also account for the later restriction of Pax2, Enl and En2 to this region, as a synergy between Fgf8 and Wntl appears to be responsible for maintaining their expression (Danielian and McMahon, 1996; Crossley et al., 1996). Based on this, it is likely that in Gbx2–/–; Otx2+/Otx1/hOtx1 embryos, a failure in segregation of the Fgf8- and Wntl-expressing domains prevents the normal compaction of the Fgf8 and Wntl expression domains. Additionally, positive regulatory interactions among the mes-met genes likely contribute to the high levels of mes-met gene expression in Gbx2–/–; Otx2+/Otx1/hOtx1 mutants. It is interesting to note that Wntl is an exception among the mes-met genes in that expression of Wntl is not upregulated in Gbx2–/–; Otx2+/Otx1/hOtx1 embryos at late somite stages. This is in agreement with genetic evidence that Otx2 plays a positive regulatory role in Wntl expression (Rhim et al., 1999).

In conclusion, we demonstrate that a mes-met region is specified independently of Otx2 and Gbx2. However, subdivision of the mes-met region into two distinct units requires Otx2 and Gbx2. Furthermore, Otx2 and Gbx2 primarily play negative regulatory roles in establishing the normal spatial expression domains of mes-met genes, with Otx2 repressing Fgf8 and Gbx2 repressing Wntl. Juxtaposition of Wntl and Fgf8 expression at the mes-met border is probably a prerequisite for establishment of a normal mid-hindbrain organizer.

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