FGF8 can activate Gbx2 and transform regions of the rostral mouse brain into a hindbrain fate

Aimin Liu1,2, Kasia Losos1 and Alexandra L. Joyner1,2,3,*

1Howard Hughes Medical Institute and Developmental Genetics Program, Skirball Institute of Biomolecular Medicine, Departments of 2Cell Biology and 3Physiology & Neuroscience, New York University School of Medicine, 540 First Avenue, New York, NY10016, USA
*Author for correspondence (e-mail: joyner@saturn.med.nyu.edu)

Accepted 10 August; published on WWW 6 October 1999

Summary

The mid/hindbrain junction region, which expresses Fgf8, can act as an organizer to transform caudal forebrain or hindbrain tissue into midbrain or cerebellar structures, respectively. FGF8-soaked beads placed in the chick forebrain can similarly induce ectopic expression of mid/hindbrain genes and development of midbrain structures (Crossley, P. H., Martinez, S. and Martin, G. R. (1996) Nature 380, 66-68). In contrast, ectopic expression of Fg8a in the mouse midbrain and caudal forebrain using a Wnt1 regulatory element produced no apparent patterning defects in the embryos examined (Lee, S. M., Danielian, P. S., Fritzsch, B. and McMahon, A. P. (1997) Development 124, 959-969). We show here that FGF8b-soaked beads can not only induce expression of the mid/hindbrain genes En1, En2 and Pax5 in mouse embryonic day 9.5 (E9.5) caudal forebrain explants, but also can induce the hindbrain gene Gbx2 and alter the expression of Wnt1 in both midbrain and caudal forebrain explants. We also show that FGF8b-soaked beads can repress Otx2 in midbrain explants. Furthermore, Wnt1-Fgf8b transgenic embryos in which the same Wnt1 regulatory element is used to express Fgf8b, have ectopic expression of En1, En2, Pax5 and Gbx2 in the dorsal hindbrain and spinal cord at E10.5, as well as exencephaly and abnormal spinal cord morphology. More strikingly, Fgf8b expression in more rostral brain regions appears to transform the midbrain and caudal forebrain into an anterior hindbrain fate through expansion of the Gbx2 domain and repression of Otx2 as early as the 7-somite stage. These findings suggest that normal Fgf8 expression in the anterior hindbrain not only functions to maintain development of the entire mid/hindbrain by regulating genes like En1, En2 and Pax5, but also might function to maintain a metencephalic identity by regulating Gbx2 and Otx2 expression.

Key words: FGF8, Mid/hindbrain patterning, Gbx2, Otx2, Mouse

INTRODUCTION

Anteroposterior patterning of the vertebrate central nervous system begins to be established early during gastrulation (reviewed by Beddington and Robertson, 1999), and local signaling centers play very important roles in refining the identity of neural tissues at specific anteroposterior positions later during development. Two local signaling centers that have been identified are the anterior neural ridge (ANR, Shimamura and Rubenstein, 1997; Rubenstein et al., 1998) and the junction between the future midbrain and hindbrain (also called the isthmus, see reviews by Joyner, 1996; Wassef and Joyner, 1997). Tissue transplantation experiments between quail and chick embryos have shown that, when isthmic tissue is grafted to ectopic sites in the brain, it not only maintains its own identity but also can induce expression of mid/hindbrain genes such as En2 and Wnt1 in surrounding tissues. Later, ectopic development of midbrain structures occurs in the caudal diencephalon, or cerebellar structures in the caudal hindbrain (Gardner and Barald, 1991; Itasaki et al., 1991; Martinez et al., 1991; Bally-Cuif et al., 1992; Bally-Cuif and Wassef, 1994; Marin and Puelles, 1994). These experiments showed that the isthmic region is determined earlier than adjacent tissue and that it contains signal(s) sufficient to induce ectopic development of midbrain tissue in the caudal forebrain and cerebellar structures in the caudal hindbrain.

Aspects of the molecular mechanisms underlying this organizer activity and patterning of the midbrain and anterior hindbrain (mesencephalon and metencephalon, respectively, referred to as mes/met) have been revealed by genetic studies of a group of transcription factors, EN1, EN2, PAX2, PAX5, OTX2 and GBX2, and of two secreted factors, WNT1 and FGF8, which are expressed in this region (see Fig. 8) and required for midbrain and cerebellar development (reviewed in Wassef and Joyner, 1997). Mice homozygous for null mutations in Wnt1, which is expressed early in the mesencephalon and later in the isthmus, do not develop derivatives of the mesencephalon or metencephalon (McMahon and Bradley, 1990; McMahon et al., 1992; Thomas...
andCapecchi,1990).MutationsinEn1,whichisexpressed
cross the mes/met, cause a similar, but milder phenotype
(Wurst et al., 1994), whereas, mutations in En2 lead to subtle
defects in tectum and cerebellum development (Joyner et al.,
1991; Millen et al., 1994). However, mice with mutations in
both En genes lack the entire mes/met (W. Wurst and A. J.,
unpublished data) and En2 can rescue the En1 mutant brain
defects when it is expressed in place of En1 (Hanks et al.,
1995, 1998), revealing overlapping functions between the two En
genes. A similar deletion of the mes/met also was found when
both mouse Pax2 and Pax5, or the zebrafish Pax2 homolog
pax2.1, are mutated (Schwarz et al., 1997; Urbanek et al., 1997;
Krauss et al., 1992; Brand et al., 1996; Lun and Brand, 1998).
These Pax genes, like the En genes, are expressed in most of
the mes/met.

In contrast to the similar phenotypes produced by mutations
in these mes/met genes, mutations in Otx2 and Gbx2 alter the
midbrain or hindbrain differentially. Otx2 expression in neural
tissue is restricted to the forebrain and midbrain from
presomitic stages (Simeone et al., 1992). The first phenotype
seen in Otx2 mutants, however, arises during gastrulation and
includes deletion of the brain anterior to rhombomere 3
(Acampora et al., 1995; Ang et al., 1996; Matsuo et al., 1995). It
has been shown in chimera and knock-in studies that this
phenotype results from a lack of Otx2 in the visceral endoderm
and that later expression of Otx2 in the anterior neural tube is
essential for maintenance of the forebrain and midbrain (Rhinne
et al., 1998; Acampora et al., 1998). A function of Otx1 and
Otx2 in patterning the forebrain and midbrain was addressed
by analyzing Otx1+/−; Otx2+/− (Suda et al., 1997) or Otx1−/−;
Otx2+/− (Acampora et al., 1997) mutant mouse embryos.
Strikingly, in such mutants, the Gbx2 domain is expanded
anteriorly and the midbrain and caudal forebrain are
transformed into a cerebellar fate. A mutation in Gbx2, which
is expressed posterior to Otx1 and Otx2, causes a
complementary phenotype in which the anterior hindbrain (r1-
r3) is deleted and the Otx2 domain and tectum are expanded
caudally, suggesting that Gbx2 might be involved in
maintaining the fate of the anterior hindbrain (Wassarman et
al., 1997; Millet et al., 1999). The Gbx2 and Otx2 mutant

Fig. 1. FGF8b can induce Gbx2 and
alter Wnt1 in both midbrain and
caudal forebrain explants and
repress Otx2 in midbrain explants.
(A) Schematic diagram showing the
caudal alar plate forebrain tissue,
p1, p2, taken at E9.5 and cultured in
vitro with FGF8b protein-soaked
beads in experiments shown in B-H.
(B-D) After 40 hours in culture, the
expression of En1, En2
and Pax5 (red arrowhead) was seen
induced around the FGF8b-soaked
beads (blue). (E,F) Wnt1 expression
is always several cell diameters away
from the beads (E,F) and
endogenous midline expression
(green arrowheads) was rearranged
when the beads were placed near the
Wnt1 domain (F). (G) Endogenous
Gbx2 is not repressed by BSA
beads. (H) Endogenous Gbx2 is
repressed whereas ectopic Gbx2 is
induced in cells near FGF8b beads.
(I) Schematic diagram showing the
mesencephalic tissue taken at E9.5
and cultured in vitro with FGF8b
protein-soaked beads in experiments
shown in J-M. (J) After 40 hours in
culture, expression of Gbx2 (blue)
was induced in cells around the
FGF8b-soaked beads (pale yellow).
(K) Otx2 expression (blue) was
repressed (arrowheads) in cells
surrounding the FGF8b-soaked
beads (outlined in green). (L) Wnt1
(purple, red arrowhead) is induced
outside the Gbx2 domain (blue,
yellow arrowhead). (M) Gbx2
(purple) is induced in cells where
Otx2 (blue) is repressed. Inset shows
the same explant after Otx2 staining
and before Gbx2 staining.
Fig. 2. Ectopic expression of Fgf8b in transgenic mice produces a more extreme phenotype than Fgf8a. (A-D) E10.5 embryos representing a (A) wild type, (B) Wnt1-Fgf8a transgenic with the most prevalent phenotype, an overgrowth of the di/mes region (structures above the red line). An En2-lacZ transgene is also present in this embryo marking the isthmus with β-galactosidase expression (blue), (C) Wnt1-Fgf8a transgenic embryo showing the exencephaly phenotype and ectopic En2 expression in the lateral edges of the midbrain and dorsal midline of the hindbrain and spinal cord (red arrowheads). Green arrowhead points to the En2 expression across the mes/met. (D) Wnt1-Fgf8b transgenic, showing extreme exencephaly with an open neural tube anterior to rhombomere 4. Yellow arrowhead points to the otic vesicle. (E,F) Lateral view of the whole head of an E15.5 wild-type (E) and a Wnt1-Fgf8b (F) embryo. (G,H) Mid-sagittal section of the same E15.5 wild type (G) and transgenics (H).

Fig. 3. Both En-1 and En-2 are expressed ectopically in E10.5 Wnt1-Fgf8b transgenic embryos. (A,B) Wild-type embryos showing that expression of both En genes is restricted to the mes/met, with En1 occupying a narrower domain (green arrowhead). (C,D) A Wnt1-Fgf8b embryo was bisected in half at the midline and each side is shown in lateral view. (C) En1 and (D) En2 expressions were detected by whole-mount RNA in situ analysis. Expression of both En genes was extended rostrally and caudally and along the dorsal (lateral in exencephalic regions) brain and spinal cord (red arrowheads). (E) En1 expression is shown to be induced in the dorsal spinal cord of an E10.5 Wnt1-Fgf8b embryo (red arrowhead). (F) En2 expression is shown to be induced in an E10.5 Wnt1-Fgf8a embryo that had exencephaly. In both E and F, anterior (a) is up and posterior (p) is down, green arrowheads point to endogenous En expression and yellow arrows indicate the forelimbs (fl).

Fig. 4. Ectopic Fgf8 expression produces abnormal morphology, expanded Wnt1 expression and ectopic induction of En1, En2 and Gbx2 expression in the dorsal spinal cord at E10.5. Cross-sections at the lumbar level are shown. Green arrowheads in A, D and G point to endogenous gene expression. Red arrowheads in B, C, E, F, H and I point to the boundary of gene expression in dorsal spinal cord.
phenotypes together suggest that these genes also are involved in positioning and normal functioning of the organizer.

En2 was first suggested to play a role in patterning the midbrain and cerebellum in vertebrates based on its expression in the isthmic region (Heitkeimino et al., 1994; Crossley and Martin, 1995) and then shown to be capable of mimicking isthmic transplants in the diencephalon (Crossley et al., 1996a). In the chick diencephalon, beads soaked in FGF8b or FGF4 can induce ectopic tectum development, as well as ectopic expression of En2, Fgf8 and Wnt1, whereas in the hindbrain, FGF8b could not induce these genes or cause morphological changes. In contrast to the chick experiments, Wnt1-Fgfl8a transgenic mouse embryos expressing Fgf8a from a Wnt1 enhancer in the mesencephalon and the roof plate were reported to have an enlarged midbrain and caudal diencephalon, without an obvious morphological transformation, although En2 expression was expanded in the dorsal midbrain and hindbrain (Lee et al., 1997). Loss-of-function studies have shown that FGF8 is required for development of both the midbrain and cerebellum. A Fgf8 mutation in zebrafish thought to produce a hypomorphic allele results in loss of the cerebellum (Brand et al., 1996; Reifers et al., 1998). A hypomorphic mutation in mouse Fgf8 leads to a more severe deletion with much of the midbrain and cerebellum lost, while a null mutation causes an earlier gastrulation defect (Meyers et al., 1998).

Many alternatively spliced isoforms of mouse Fgf8 mRNA have been characterized (Tanaka et al., 1992; Crossley and Martin, 1995), and all seem to be found in the various embryonic regions where Fgf8 is expressed. However, cell transformation assays and receptor affinity assays have indicated that isoform b has the strongest activity (MacArthur et al., 1995a,b; Blunt et al., 1997). Thus, one possible explanation for the difference between the in vivo gain-of-function studies in chick and mouse is that different isoforms of FGF8 were used. Indeed, short-term explant studies using 3- to 5-somite mouse anterior brain tissue indicated that FGF8b could induce En2 in posterior regions of the explants within 24 hours (Shimamura and Rubenstein, 1997). We have explored the roles of FGF8a and FGF8b in anteroposterior patterning of the mouse midbrain and cerebellum using both in vitro explant assays and in vivo transgenic analysis. In explant assays, we found FGF8b could not only induce expression of the mid/hindbrain genes En1, En2 and Pax5 in the caudal diencephalon, but it could also induce the hindbrain gene Gbx2 and alter the expression of Wnt1 in both midbrain and caudal forebrain tissues. We also show that FGF8b could repress the anterior gene Otx2 in midbrain explants. To examine whether ectopic expression of Fgf8b is sufficient in vivo to re-specify the central nervous system along the anteroposterior axis, we used the Wnt1 enhancer to generate a new domain of Fgf8a or Fgf8b expression in the midbrain at early somite stages and in the isthmus and roof plate of the hindbrain and spinal cord at later stages. Our results show that early ectopic expression of Fgf8a or Fgf8b in the mesencephalon can cause exencephaly and is capable of inducing expression of mes/met genes in the brain and spinal cord. Furthermore, instead of inducing midbrain structures, FGF8b transformed the midbrain and caudal diencephalon into a metencephalic fate, which correlated with the induction of Gbx2 and repression of Otx2, as early as E8.5. A model is proposed to account for the various chick and mouse gain-of-function studies with FGF8.

**MATERIALS AND METHODS**

**Explant culture**

One package (25 μg, in 1.25 mg BSA) of FGF8b protein (R&D) was reconstituted in PBS (Gibco) to a final concentration of 1 mg/ml FGF8b. Blue gel affigel-gel beads (Pharmacia) or Heparin acrylic beads (Sigma) were rinsed in 50 volume PBS 4-6 times and then about 100 beads were soaked in 12 μl FGF8b solution for 1 hour at 37°C or overnight at 4°C for each type of bead, respectively (Ye et al., 1998; Furuta et al., 1997). The beads were then rinsed in PBS three times and two or three beads were applied to each explant. Control beads were soaked in 50 mg/ml BSA (Sigma) in PBS in the same manner.

Outbred Swiss Webster (SW, Taconic) mouse embryos were dissected in ice-cold L15 medium (Cellgro) at embryonic day 9.5 (E9.5). Explants were made by cutting out the alar plate of either the diencephalic or mesencephalic region along with the overlying ectoderm and mesoderm and placing it on a Microprobe filter membrane (Costar) floating on Dulbecco’s Modified Eagle’s Medium (DMEM, Cellgro) supplemented with 20% Fetal Bovine Serum (Gibco) and 1X Non-essential Amino Acid (100X stock from Gibco). The beads were then added and the explants cultured at 37°C, 10% CO₂ for 16-40 hours.

At the end of culture, the membranes were transferred into fresh 4% paraformaldehyde to let the explant float off the membrane. Then the explants were fixed in 4% paraformaldehyde at 4°C overnight, washed with PBS, 2x 5 minutes, dehydrated through 25%, 50% and 75% methanol and stored in methanol at −20°C.

**Fgf8 misexpression transgenes**

A full-length Fgf8b cDNA (kind gift from Dr MacArthur of Washington University at St Louis; Tanaka et al., 1992) was subcloned into the Wexp3 vector (Wnt1-misexpression vector, Dr McMahon, Harvard University, Echelard et al., 1994). The resulting Wexp3-Fgf8b construct, referred to as Wnt1-Fgfl8b, was released from the plasmid using SalI digestion. The Wexp3-Fgf8a construct (Lee et al., 1997), referred to as Wnt1-Fgf8a, was a kind gift from Dr McMahon and it was also released from the plasmid with SalI.

**Production of transgenic embryos**

Transgenic mice were made as described by Hogan et al. (1994). SW mice (Taconic) were used to generate the fertilized eggs. Injected eggs were cultured overnight in M16 medium and transferred at the 2-cell stage into the oviduct of a pseudopregnant female mouse that had been mated the night before. The day when embryos were transferred was designated E0.5. Staged transgenic and wild-type embryos were dissected at E8.5, E9.5 and E10.5 into PBS and fixed in 4% paraformaldehyde overnight, rinsed with PBS and photographed using a M10 stereo photomicroscope (Leica) using either a Polaroid Digital Microscopic camera or Kodak 64T film. Embryos were then dehydrated through 25%, 50% and 75% methanol in PBS and stored in 100% methanol at −20°C.

The genotypes of embryos were determined by PCR analysis of genomic DNA extracted from embryonic yolk sacs. The primers used for Wnt1-Fgf8b were directed against the lacZ sequences present in the transgene construct; S’ primer, CCG AAC CAT CCG CTG TGG TA and 3’ primer, CAT CCA CGC GCG CGT ACA TC. The following primers were used to identify the Wnt1-Fgf8a transgene; S’ primer, CTG CTC CCT CAC ATG CTG TG and 3’ primer, CCT ATT AGA GCC AGC CTT G 3’. The PCR program included 30 cycles of: 94°C, 1 minute; 61°C, 1 minute; 72°C, 1 minute. PCR products were visualized on ethidium-bromide-stained agarose gels.

To determine whether the transgene was expressed in the CNS as
expected, the tail regions of E9.5-E10.5 embryos were used to do whole-mount RNA in situ hybridization using lacZ- or Fgf8-specific RNA probes (see below). For the Wnt1-Fgf8b transgene, of a total of 225 embryos obtained between E8.5 and E10.5, 75 were transgenic and of these, 38 expressed the transgene, all of which showed the reported phenotype. For the Wnt1-Fgfa transgene, a total of 219 E10.5 embryos were dissected and, of these, 32 carried the transgene and 11 embryos expressed the transgene. 4 of the 11 showed the exencephaly phenotype and the other 7 showed an expanded midbrain and diencephalon.

Whole-mount RNA in situ hybridization

Whole-mount RNA in situ hybridization of embryos was performed as described (Matise and Joyner, 1997). Antisense riboprobes used for in situ hybridization were prepared using previously published mouse sequences. En1, En2 (Millen et al., 1995), Fgf8 (Crossley and Martin, 1995), Gbx2 (Bouillet et al., 1995), Wnt1 (Parr et al., 1993), Pax2, Pax5, Pax8 (Asano and Gruss, 1992), Pax6 (Grindley et al., 1997) and Otx2 (Simeone et al., 1993). Embryos were fixed, washed and dehydrated as described above. The embryos were rehydrated immediately before whole-mount RNA in situ hybridization was performed. After hybridization, embryos were washed in PBT (0.1% Tween-20 in PBS) overnight at 4°C, refixed in 4 % paraformaldehyde overnight at 4°C and stored in 70% glycerol in PBS at 4°C. Modifications were made for double in situ as described by Hecksher-Sorensen et al. (1998).

Section RNA in situ hybridization

Frozen sections were hybridized with 35S-labeled antisense riboprobes using a protocol similar to the one described by Hui and Joyner (1993). The probes used were prepared using previously published mouse sequences; Wnt7b (Parr et al., 1993) and Meis-2 (Toresson et al., 1999), and those used for whole-mount analysis. 35S-labeled sections were counterstained with Toilibine Blue and mounted. Digoxigenin-labeled sections were counterstained with Nuclear Fast Red and mounted. Pictures were taken using a DR MXE microscope (Leica) and Polaroid Digital Camera.

RESULTS

FGF8b protein can induce Gbx2 and alter Wnt1 in both mesencephalic and diencephalic explants and repress Otx2 expression in midbrain explants

As one approach to test whether FGF8b was capable of inducing mes/met genes in the mouse embryonic forebrain, we performed a pilot experiment in which FGF8b-soaked beads were placed in diencephalic explants taken at E8.5, E9.5 and E10.5, and analyzed for En2 expression after 40 hours. At all stages En2 was found to be induced (data not shown). We therefore performed additional explant assays at E9.5 when the morphological landmarks between telencephalon, diencephalon, mesencephalon and metencephalon are clearly visible as constrictions and the nature of the explanted tissue can be confirmed by checking the expression of marker genes specific for each domain. Explants were taken from the dorsal and lateral part of the diencephalon, such that they consisted mostly of prosomere 1 (p1) and p2 (Fig. 1A). To confirm that no midbrain tissue was included in the explants, RNA in situ analysis was done on parallel control explants that were not cultured using the mesencephalonic marker gene Meis-2 (Ceconi et al., 1997; Oulad-Abdelghani et al., 1997, data not shown).

Whole-mount RNA in situ hybridization was done after culturing each explant with two or three FGF8b beads for 16 or 40 hours. After 40 hours of culture, expression of the mes/met genes En1 (in 24 explants), En2 (in 17 out of 18 explants) and Pax5 (in 4 explants) were found to be induced in the cells adjacent to the FGF8b beads (Fig. 1B-D). However, no induction of Pax2 (in 12 explants) or Pax8 (in 5 explants) could be detected (data not shown). Surprisingly, we failed to see Fgf8 expression either after 16 hours (in 6 explants) or 40 hours (in 6 explants) of culture (data not shown). We did however, find that Wnt1 expression was altered by FGF8b beads (in 3 explants). Interestingly, in contrast to the other genes examined, Wnt1 expression was only seen in cells several cell diameters away from the beads (Fig. 1E,F). When beads were placed at a distance from the midline, the induced Wnt1 expression seemed to be separate from the endogenous one, leaving the endogenous expression undisturbed (Fig. 1E). In contrast, when the beads were placed adjacent to the endogenous midline Wnt1-expressing cells (green arrows in Fig. 1E,F), the endogenous expression was altered such that the cells adjacent to the beads were always devoid of Wnt1 expression and Wnt1-positive cells were induced in a ‘halo’ around the beads, several cell diameters from them (Fig. 1F).

We also examined the expression of Gbx2 and Otx2 in diencephalic explant. In control explants Gbx2 was found to be expressed in the lateral region of the explants after 40 hours in culture (in 5 explants, data not shown), consistent with the fact that Gbx2 is normally expressed in the dorsal thalamus at E11.5 (Bouillet et al., 1995). This endogenous expression was not altered by BSA-soaked beads (Fig. 1G, in 2 explants) but was greatly reduced (Fig. 1H, in 2 explants) or abolished (6 explants, not shown) in the presence of FGF8b-soaked beads. In addition, FGF8b induced ectopic Gbx2 expression around the beads (Fig. 1H). Endogenous Otx2 expression in control diencephalic explants was variable with some explants showing Otx2 in patches and there were Otx2-negative cells scattered among Otx2-positive cells (9 explants examined, not shown). Similar results were obtained when the explants were cultured with BSA-soaked beads (in 3 explants, not shown). The expression of Otx2 after 40 hours in culture with FGF8b beads was more variable. In some of the explants (7/21, not shown), Otx2 expression was similar to the control explants, although the expression seemed a little weaker around the beads. In other explants (14/21, not shown), Otx2 expression seemed to be greatly downregulated in most cells in the middle of the explant, but this downregulation did not seem to be restricted to the vicinity of the beads. Because of the variable expression of Otx2 in control and FGF8b-treated explants, it is not clear whether FGF8b can regulate Otx2 in the diencephalon at this stage.

We also analyzed the expression of Otx2, Gbx2 and Wnt1 in mesencephalic explants (Fig. 1I); the expression of En1, En2 and Pax5 was not examined because they are normally expressed in the caudal midbrain. The expression of Gbx2 (in 7 out of 8 explants) and Wnt1 (in 6 explants) in response to FGF8b beads was similar to that in the diencephalon; both genes were induced, Gbx2 adjacent to the beads (Fig. 1J) and Wnt1 at a distance (data not shown). Otx2 was found to be maintained in control cultures (10 explants with BSA beads) and repressed in cells adjacent to FGF8b-soaked beads (Fig. 1K, in 8 explants). Double in situ hybridization showed that the Wnt1 domain was adjacent to the Gbx2 domain, although
The boundary between the two domains was not very sharp (Fig. 1L). Double in situ also showed that Gbx2 was strongly induced only in Otx2-negative cells, but there did not appear to be a complete complementarity in their expression, as there were cells that appeared not to express either gene (Fig. 1M).

Ectopic expression of Fgf8b produces exencephaly and abnormalities in the spinal cord

In contrast to our explant studies with FGF8b, when Fgf8a was ectopically expressed in the midbrain using a Wnt1 enhancer, no disturbance of anteroposterior patterning or ectopic mes/met gene induction was observed in the embryos examined, except for a loss of tectum polarity at late stages. However, 4 embryos at E10.5 were reported to have a more severe non-specific phenotype that was suggested to be due to widespread activation of the transgene (Lee et al., 1997). To determine whether different isoforms of FGF8 account for the different
4833 FGF8 induces anterior hindbrain development

results in vitro and in vivo and to further study the role of Fgf8 in mouse embryonic CNS development, we made transgenics expressing Fgf8b from the same Wnt1 enhancer and also made Wnt1-Fgf8a transgenic embryos for comparison of the phenotypes.

At E10.5, 64% (7/11) of transgenic embryos that expressed the Wnt1-Fgf8a transgene (11/32) showed a slightly enlarged midbrain and caudal diencephalon compared to wild-type littermates (Fig. 2B), similar to the phenotype previously reported (Lee et al., 1997). However, the remaining transgenics (4/11) showed a variable degree of exencephaly (Fig. 2C) and these embryos appeared to show the strongest transgene expression in the dorsal spinal cord based on whole-mount RNA in situ hybridization of tail regions using a transgene-specific lacZ RNA probe (data not shown, see Materials and Methods). However, no expression was observed outside of the CNS, indicating that the severe phenotype was not a result of widespread activation of the transgene.

In contrast to the Wnt1-Fgf8a transgenics, all Wnt1-Fgf8b transgenic embryos that expressed the transgene (10/17) had exencephaly at E10.5 and a more severe phenotype than any of the Wnt1-Fgf8a transgenics (Fig. 2D). Again, the tail region from all the Wnt1-Fgf8b transgenic embryos examined were probed with a lacZ RNA in situ probe and no non-specific transgene expression was found outside the dorsal neural tube where the transgene was expected to be expressed. Gross observation of Wnt1-Fgf8b embryos revealed that, instead of the alar plate of the brain folding inward to form a neural tube, it folded outward such that the neural tube anterior to the otic vesicle was open. An additional striking morphological defect in transgenics was that the head seemed to be truncated. In addition, the branchial arches of Wnt1-Fgf8b embryos seemed underdeveloped, possibly resulting from abnormal development of neural crest cells, since the Wnt1 enhancer expresses in neural crest precursors (Echelard et al., 1994). At E15.5, the exencephaly persisted (Fig. 2F,H) and no eye structure was formed. However, nasal and oral structures were present at this stage (compare Fig. 2G and H).

Ectopic Fgf8b expression in the hindbrain and spinal cord resulted in a morphological abnormality, in contrast to the chick FGF8b bead experiments in which the hindbrain was not altered (Crossley et al., 1996b). A similar spinal cord phenotype also was seen in some (6/11) of the Wnt1-Fgf8a transgenic embryos. The spinal cord had a wavy appearance in dorsal view (Fig. 3E,F). In cross sections, it could be seen that the dorsal neural tube was overgrown such that the central canal that normally assumes a narrow oval shape had either a ‘T’ shape or an inverted ‘L’ shape (Fig. 4). At E15.5, the spinal cord of transgenics appeared normal in ventral regions, but overgrown dorsally (data not shown).
**Mes/met genes are induced by ectopic Fgf8 expression in the brain and more posterior regions**

To determine whether ectopic expression of Fgf8a or Fgf8b in the brain leads to alterations in anteroposterior patterning, transgenic brains were analyzed for mes/met gene expression using whole-mount RNA in situ hybridization. To compare the spatial relationship between the expression of the two En genes, E10.5 Wnt1-Fgf8b embryos (n=2) were bisected along the mid-sagittal plane, followed by whole-mount RNA in situ hybridization using En1- or En2-specific probes on either half of the embryo. Expression of both En genes was clearly induced ectopically in Wnt1-Fgf8b embryos (Fig. 3). Normally, En2 is only expressed in a wide band covering the caudal part of mesencephalon and in the metencephalon (Fig. 3B). En1 is expressed in a similar, but narrower band than En2 at E10.5 (Fig. 3A). In addition, En1 is also expressed in two ventrolateral stripes of postmitotic cells in the hindbrain and spinal cord, as well as in non-neural tissues including the somites and ventral limb ectoderm (Fig. 4A). In transgenic embryos, we found that the anterior border of En1 expression was the same as that of En2, indicating there is at least a rostral expansion of En1 (compare Fig. 3C and D). Another striking ectopic domain of both En1 and En2 expression was along the entire dorsal part of the hindbrain and spinal cord caudally to at least the lumbar level, and rostrally joining the mes/met domain (Fig. 3C-E). The expression of En1 in the hindbrain appeared more restricted to the dorsal lateral part of the opened neural tissue than En2, which appeared to have a wider domain (compare Fig. 3C and D). However, when cross sections were taken at the lumbar level of the same embryo and processed for radioactive RNA in situ hybridization, there seemed to be no apparent difference between the expression domains of En1 and En2 (Fig. 4B,C).

Expression of Wnt1, Fgf8, the transgene (lacZ probe), Pax5 and Gbx2 in the spinal cord was also examined in cross sections using RNA in situ analysis. A Wnt1 probe that detects both Wnt1 and transgene-derived transcripts detected expression in a much wider band of cells than Wnt1 is normally seen in wild types (compare Fig. 4E with D). This could be due to over-proliferation of the dorsal spinal cord cells or induction by FGFB, although Fgf8 and the transgene appeared to be expressed in the same cells as Wnt1 (Fig. 4F,I). Pax5, which normally is expressed only in postmitotic cells in the spinal cord (Asano and Gruss, 1992), was induced in the same dorsal ventricular spinal cord cells that expressed Wnt1, Fgf8, En1 and En2 (data not shown). Interestingly, Gbx2, which is normally clear from the roof plate of the spinal cord (Fig. 4G), was induced in the most dorsal region of the spinal cord of the transgenics (Fig. 4H). The expression of Pax3 in the dorsal spinal cord was not altered in the transgenics (data not shown), indicating that dorsal genes are not repressed by FGFB.

In E10.5 Wnt1-Fgf8a transgenic embryos examined for En2 expression, 2/2 with exencephaly and 2/4 without exencephaly had ectopic expression in the dorsal midbrain and posterior roof plate of the hindbrain and spinal cord (Fig. 2C), similar to Wnt1-Fgf8b transgenics. However, two differences in the expression pattern were observed. First, the ectopic roof plate expression domain of En2 was separate from the normal mes/met expression domain. Second, the morphological abnormality of the spinal cord appeared milder in these Wnt1-Fgf8a transgenic embryos compared to Wnt1-Fgf8b transgenics and En2 expression seemed to fade off posteriorly at the level of the forelimbs (Fig. 3F).

**The midbrain is missing by E9.5 in Wnt1-Fgf8b transgenics**

To explore the neural identity of the cells in the anterior region of En1 and En2 expression, we performed RNA in situ analysis on sagittal sections of E10.5 embryos. Pax6 is normally expressed at this stage in the dorsal forebrain with a posterior boundary at the diencephalon/mesencephalon (di/mes) border, as well as in the ventrolateral hindbrain and spinal cord (Gruss and Walthier, 1992). In adjacent sagittal sections of wild-type embryos, the Pax6 dorsal expression domain is separated from that of En2 by a rostral mesencephalic domain which is negative for both genes (compare Fig. 5A and C). In contrast, the transgenic embryos analyzed had strong En2 expression in a domain that abutted the Pax6 expression domain in the dorsal forebrain (Fig. 5B,D). This indicates that En2 expression is induced in the rostral region of the mesencephalon or that the region is lost in Wnt1-Fgf8b transgenomic embryos.

The extreme deformity of the transgenic embryos at E10.5 made it difficult to characterize the anteroposterior patterning defects in the CNS, based on histology or marker gene analysis. Further analysis of expression of Pax6 and Gbx2, which mark different brain regions, was therefore carried out at E9.5 by examining gene expression in adjacent sections and double staining of whole embryos. Pax6 was used as a marker for the forebrain and di/mes and r2/r3 boundaries (Fig. 5E,I). Gbx2, which is most strongly expressed as a ring in the anterior hindbrain, was used to mark the mes/met border and the metencephalon (Fig. 5G,1). In E9.5 transgenics, Pax6 was expressed in the most rostral part of the CNS with a sharp caudal boundary, as seen at E10.5 (Fig. 5F,J). Gbx2 was detected in two broad dorsolateral bands extending caudal from the posterior border of Pax6 to the otic vesicle (Fig. 5H,J). This observation strongly suggested that the midbrain was absent in Wnt1-Fgf8b transgenics at E9.5.

**Caudal regions of the forebrain are not present at E9.5 in Wnt1-Fgf8b transgenic embryos**

To further analyze the CNS anteroposterior patterning defects in Wnt1-Fgf8b transgenic embryos, RNA in situ hybridization was performed on adjacent sagittal sections at E9.5. Consistent with the results described above with whole-mount and section RNA in situ analysis, Pax6 expression (Fig. 6F) abutted the Fgf8 expression domain (Fig. 6L), providing further evidence that the midbrain was largely missing in transgenics. Most strikingly, Wnt7b, a gene with strongest expression in the dorsal telencephalon and most rostral diencephalon (p3, Fig. 6G), had a posterior boundary in transgenic embryos which was very similar to the posterior boundary of Pax6, abutting the anterior boundary of expression of Fgf8, Wnt1, Pax5 and En2 (compare Fig. 6H to F,J,L,N,P). This finding suggests that the caudal diencephalic region including p1 and p2 is absent in transgenics.

We next compared the expression domains of genes that normally have borders at the mes/met junction (Otx2) or are expressed in the isthmus (Wnt1/Fgf8), in order to determine whether an ‘organizer’ was preserved. Otx2, which normally labels the entire forebrain and midbrain at this stage (see Fig. 6C), was found to be expressed strongly overlapping with Pax6.
only in the transgene and expression, which normally has a rostral border at the along the lateral edges of the neural plate (Fig. 7H). The only expression left in the caudal brain was two weak, thin bands transgenic embryos at 7 somites (Fig. 7H,I). The only 7G), was expressed only in the anterior most forebrain of in the forebrain and mesencephalon in wild-type embryos (Fig. 7E,F). Strikingly, Fgf8 detected in a domain similar to the transgene, as well as in the mid/hindbrain junction (Fig. 7A).

In order to determine when a phenotype could first be detected in Wnt1-Fgf8b transgenics and to determine whether the di/mes is deleted or transformed into metencephalic tissue, we analyzed transgenic embryos at early somite stages, 12 hours after transgene expression is initiated (Echelard et al., 1994). A morphological difference between wild-type embryos and Wnt1-Fgf8b transgenic embryos was already obvious at 5-7 somites. At this stage, the anterior neural plate normally has begun to invaginate in wild-type embryos (Fig. 7A,L). In contrast, in transgenic embryos, the alar plate of the anterior CNS folded out instead of in (Fig. 7B,C,I), and a sharp bend was seen in the anterior neural plate (red arrowhead in Fig. 7B,E,H,K).

To examine anteroposterior patterning in the transgenic embryos, we examined the expression patterns of Gbx2, Otx2, Fgf8 and the Wnt1-Fgf8b transgene using a lacZ probe (see Materials and Methods) in early somite stage embryos. At 7 somites, the transgene was strongly expressed in the dorsal hindbrain and spinal cord, similar to Wnt1 expression in wild-type embryos (compare Fig. 7A and B). Transgene expression in more anterior brain regions was restricted to a transverse band in the caudal forebrain region, as well as two thin lines of weak expression along the lateral edges of the neural folds extending caudally from the transverse band (Fig. 7B). Wild-type embryos, in contrast, express Wnt1 in a transverse band in the caudal region of the midbrain adjacent to the mid/hindbrain junction (Fig. 7A). Fgf8 expression was detected in a domain similar to the transgene, as well as in the domains of endogenous Fgf8 in the ANR and mesoderm (Fig. 7E,F). Strikingly, Otx2, which normally is strongly expressed in the forebrain and mesencephalon in wild-type embryos (Fig. 7G), was expressed only in the anterior most forebrain of transgenic embryos at 7 somites (Fig. 7H,I). The only Otx2 expression left in the caudal brain was two weak, thin bands along the lateral edges of the neural plate (Fig. 7H). Gbx2 expression, which normally has a rostral border at the mid/hindbrain junction (Fig. 7I) was also dramatically altered in the transgenic embryos. The Gbx2 domain was expanded such that its rostral border was shifted to the caudal forebrain region (Fig. 7K), overlapping with the anterior border of the transgene and Fgf8 expression and abutting the caudal Otx2 border (Fig. 7B,E,H,K). Since it is unlikely that FGF8 could alter proliferation to such an extent to account for the changes seen in the Otx2 and Gbx2 expression domains within 12 hours, these studies indicate that, in Wnt1-Fgf8b transgenics, the midbrain and caudal forebrain are transformed into an anterior hindbrain fate during the early somite stages.

**DISCUSSION**

In this study, we explored the role of mouse FGF8 in patterning the CNS along the anteroposterior axis during early embryonic development using both in vitro explant culture experiments and in vivo transgenic techniques. FGF8b was found to induce expression of En1, En2 and Pax5, but not Fgf8 in E9.5 diencephalic explants. It also induced Gbx2 and altered Wnt1 expression in both midbrain and diencephalic explants and repressed Otx2 in midbrain explants. When Fgf8b was ectopically expressed using a Wnt1 regulatory element, a phenotype much more severe than that of Wnt1-Fgf8a was observed. All the transgenic embryos showed exencephaly and a mild morphological change could be recognized as early as the 7-somite stage. A morphological abnormality was also observed in the roof plate of the caudal hindbrain and spinal cord at E10.5. Genes like En1, En2, Pax5 and Gbx2 that normally mark the mes/met were induced in the dorsal hindbrain and spinal cord. More strikingly, the midbrain and caudal forebrain were not present in transgenic embryos at E9.5, and tissue expressing the hindbrain gene Gbx2 was adjacent to the remaining forebrain. This phenotype seems to arise from a transformation of the mesencephalon and diencephalon (at least p1 and p2) into metencephalic tissue at early somite stages, since Gbx2 expression was expanded anteriorly and the Otx2 domain reduced within 12 hours of transgene expression. At the new border region between Otx2 and Gbx2 expression, organizer genes (Fgf8 and Wnt1) were expressed, but not in their normal relative positions. Furthermore, Fgf8 was unable to induce the more rostral brain tissue to form a midbrain and only Gbx2-expressing hindbrain tissue was maintained posterior to the organizer.

FGF8b has been shown in tissue culture systems to have a stronger cell transformation activity and higher affinity to the known FGF receptors than FGF8a (MacArthur et al., 1995a,b; Blunt et al., 1997). Therefore, it has been suggested that the two isoforms might have different functions in vivo and be responsible for the different results obtained in chick and mouse gain-of-function studies in the limb or brain. In our study, the biological functions of the two isoforms were directly compared by expressing them from the same Wnt1 regulatory element. Many factors, such as different protein stability, different affinity for the same receptor or the existence of an unknown receptor, could underlie the functional differences of the two FGF8 isoforms. Our studies, however, strongly indicate that the main difference between the proteins is quantitative, because some Wnt1-Fgf8a transgenics showed exencephaly and a spinal cord phenotype similar to, but milder than, Wnt1-Fgf8b transgenics. Consistent with our studies, a recent comparison of the phenotypic consequences of placing beads soaked in FGF8a or FGF8b in the chick brain reported that both can induce similar changes (Shamim et al., 1999).
Our finding that, in Wnt1-Fgf8b transgenic embryos, the midbrain and caudal forebrain are transformed into a metencephalon, on the surface appears to be different from the results obtained when FGF8b-soaked beads were implanted into the chick diencephalon and ectopic midbrain structures were induced (Crossley et al., 1996). One difference between the two experiments is that ectopic FGF8b was applied at different stages during development. The beads were placed in the chick diencephalon at HH (Hamburger and Hamilton, 1992) stage 9-12, a period when the anterior neural tube is closing, which should correspond to E9 in mouse embryos. In Wnt1-Fgf8b transgenic embryos, transgene expression is initiated in the mesencephalon around E8, an earlier stage during neurogenesis. However, this difference does not likely account for the different phenotypes observed, since we demonstrated that FGF8b-soaked beads can induce Gbx2 expression and repress Otx2 expression in mouse mesencephalic explants taken at E9.5.

A more likely explanation for the phenotypes seen in chick and mouse is that FGF8 was provided in different manners. In the chick experiments, FGF8b beads provided a focal source of FGF8b which likely induced Gbx2 and repressed Otx2 in cells locally, creating an ectopic mes/met border. Similar to our explant results, Wnt1 was likely activated in the ectopic mes/met border region and, En, Pax5 and Fgf8 induced more broadly, creating a new organizer region (Fig. 9A). Consistent with this, since the submission of our paper, it has been reported that FGF8b beads can repress Otx2 expression locally in the caudal diencephalon and mesencephalon and that cells closest to the beads can take on cerebellar characteristics (Martinez et al., 1999). Based on our results, the cells that form the cerebellum likely express Gbx2 and the cells that form ectopic midbrains express Otx2. In contrast, in our studies, FGF8 was initially expressed in most of the midbrain and induced Gbx2 and repressed Otx2 broadly. It is plausible that the transgene then induced its own expression in more anterior forebrain regions through induction of the Wnt1 regulatory elements in the transgene, since we found that FGF8 protein can induce Wnt1 expression in diencephalic explants. Both Wnt1 and Fgf8 became
restricted anteriorly, possibly to the prospective ZLI, in Wnt1-FGF8 transgenics leaving Gbx2-positive metencephalic tissue caudal to the Fgf8 domain (Fig. 9B). The position of the new Otx2/Gbx2 boundary might be set by the position of forebrain tissue (p3 and telencephalon), which cannot adopt a mes/met fate in response to FGF8 induction, based on isthmic transplants and FGF8 bead experiments in chick and mouse (Bloch-Gallego et al., 1996; Crossley et al., 1996; Shimamura and Rubenstein, 1997). Since the rostral forebrain cannot respond to FGF8b to form a midbrain, only metencephalic tissue could be induced in Gbx2-expressing cells posterior to Fgf8/Wnt1 in Wnt1-Fgf8 transgenics.

The patchy expression of Otx2 in regions posterior to the sharp Pax6 caudal boundary at E9.5 in Wnt1-Fgf8 transgenics, and the overlap in expression of midbrain and hindbrain genes in this region, indicate the organizer region is not normal. It is noteworthy that, in Gbx2 mutant embryos, a similar abnormal organizer is formed with overlapping expression of Wnt1, Fgf8 and Otx2 (Wassarman et al., 1997). In Wnt1-Fgf8 transgenics, the organizer region might be compromised by altered gene regulation (i.e. Fgf8 expression under the control of a Wnt1 regulatory element), such that the spatial relationship among different mes/met genes differs from that in wild type.

It is interesting that the phenotype that we observe in early Wnt1-Fgf8 transgenics has similarities to that seen in Otx1+/−, Otx2+/− (Suda et al., 1997) or Otx1+/− Otx2+/− double mutants (Acampora et al., 1997); an early induction of Gbx2 and repression of Otx2 in the midbrain and caudal forebrain. In Otx1−/−; Otx2+/− embryos, an anterior expansion of Fgf8 expression was found to precede an anterior shift of Wnt1 and En1 expression and an anterior retraction of Otx2 expression (Acampora et al., 1997). The Otx mutant studies suggest a certain level of Otx2 expression is necessary to repress expression of Fgf8 in the midbrain and forebrain, and our results suggest that, in addition, expanded Fgf8 expression could contribute to repression of Otx2 expression in the midbrain. A reciprocal negative regulation between Otx2 and Fgf8 might therefore normally contribute to maintaining the Otx2 caudal boundary and positioning the organizer.

Finally, we showed for the first time that Fgf8 can induce Gbx2 in an explant assay and in Wnt1-Fgf8b transgenic embryos. Recent studies show that, in Wnt1-Gbx2 transgenic embryos in which Gbx2 is expressed in the mesencephalon at early somite stages, Otx2 expression in the caudal midbrain is repressed by early somite stages and Fgf8 and Wnt1 expression is shifted anteriorly to the new Otx2/Gbx2 border (Millet et al., 1999). These latter gain-of-function studies demonstrate that Gbx2 can directly, or indirectly, repress Otx2. Consistent with this, in Gbx2 mutant embryos, Otx2 expression is expanded caudally into what would normally be the Gbx2 domain at early somite stages (Millet et al., 1999). Taken together with our present studies, these results suggest that in Wnt1-Fgf8b transgenics Gbx2 could mediate the repression of Otx2 by FGF8b.

We thank Dr A. McMahon for providing Wexp3 and Wexp3-Fgf8a constructs, Dr C. MacArthur for an Fgf8b-SDNA, Drs A. McMahon, M. Frohman, P. Gruss, K. Campbell, and J. Rossant for providing probes for RNA in situ hybridization analysis and the Skirball Institute transgenic facility for support in making transgenics. We also thank Sandrine Millet for many helpful discussions and comments and Gordon Fishell, Yuanhao Li and Michael Matise for comments on the manuscript. This research was supported by a grant from the NINDS (#R01-NS35876). A. L. J. is an investigator of the Howard Hughes Medical Institute.

REFERENCE


Bally-Cuif, L., Alvarado-Mallart, R. M., Darnell, D. K. and Wassef, M. (1999). Axis development and early somite stages (Millet et al., 1999). Taken together with Fgf8 and Otx2+/− double mutants (Acampora et al., 1997); an early induction of Gbx2 and repression of Otx2 in the midbrain and caudal forebrain. In Otx1−/−; Otx2+/− embryos, an anterior expansion of Fgf8 expression was found to precede an anterior shift of Wnt1 and En1 expression and an anterior retraction of Otx2 expression (Acampora et al., 1997). The Otx mutant studies suggest a certain level of Otx2 expression is necessary to repress expression of Fgf8 in the midbrain and forebrain, and our results suggest that, in addition, expanded Fgf8 expression could contribute to repression of Otx2 expression in the midbrain. A reciprocal negative regulation between Otx2 and Fgf8 might therefore normally contribute to maintaining the Otx2 caudal boundary and positioning the organizer.

Finally, we showed for the first time that Fgf8 can induce Gbx2 in an explant assay and in Wnt1-Fgf8b transgenic embryos. Recent studies show that, in Wnt1-Gbx2 transgenic embryos in which Gbx2 is expressed in the mesencephalon at early somite stages, Otx2 expression in the caudal midbrain is repressed by early somite stages and Fgf8 and Wnt1 expression is shifted anteriorly to the new Otx2/Gbx2 border (Millet et al., 1999). These latter gain-of-function studies demonstrate that Gbx2 can directly, or indirectly, repress Otx2. Consistent with this, in Gbx2 mutant embryos, Otx2 expression is expanded caudally into what would normally be the Gbx2 domain at early somite stages (Millet et al., 1999). Taken together with our present studies, these results suggest that in Wnt1-Fgf8b transgenics Gbx2 could mediate the repression of Otx2 by FGF8b.

We thank Dr A. McMahon for providing Wexp3 and Wexp3-Fgf8a constructs, Dr C. MacArthur for an Fgf8b-SDNA, Drs A. McMahon, M. Frohman, P. Gruss, K. Campbell, and J. Rossant for providing probes for RNA in situ hybridization analysis and the Skirball Institute transgenic facility for support in making transgenics. We also thank Sandrine Millet for many helpful discussions and comments and Gordon Fishell, Yuanhao Li and Michael Matise for comments on the manuscript. This research was supported by a grant from the NINDS (#R01-NS35876). A. L. J. is an investigator of the Howard Hughes Medical Institute.

REFERENCE


Bally-Cuif, L., Alvarado-Mallart, R. M., Darnell, D. K. and Wassef, M. (1999). Axis development and early somite stages (Millet et al., 1999). Taken together with Fgf8 and Otx2+/− double mutants (Acampora et al., 1997); an early induction of Gbx2 and repression of Otx2 in the midbrain and caudal forebrain. In Otx1−/−; Otx2+/− embryos, an anterior expansion of Fgf8 expression was found to precede an anterior shift of Wnt1 and En1 expression and an anterior retraction of Otx2 expression (Acampora et al., 1997). The Otx mutant studies suggest a certain level of Otx2 expression is necessary to repress expression of Fgf8 in the midbrain and forebrain, and our results suggest that, in addition, expanded Fgf8 expression could contribute to repression of Otx2 expression in the midbrain. A reciprocal negative regulation between Otx2 and Fgf8 might therefore normally contribute to maintaining the Otx2 caudal boundary and positioning the organizer.

Finally, we showed for the first time that Fgf8 can induce Gbx2 in an explant assay and in Wnt1-Fgf8b transgenic embryos. Recent studies show that, in Wnt1-Gbx2 transgenic embryos in which Gbx2 is expressed in the mesencephalon at early somite stages, Otx2 expression in the caudal midbrain is repressed by early somite stages and Fgf8 and Wnt1 expression is shifted anteriorly to the new Otx2/Gbx2 border (Millet et al., 1999). These latter gain-of-function studies demonstrate that Gbx2 can directly, or indirectly, repress Otx2. Consistent with this, in Gbx2 mutant embryos, Otx2 expression is expanded caudally into what would normally be the Gbx2 domain at early somite stages (Millet et al., 1999). Taken together with our present studies, these results suggest that in Wnt1-Fgf8b transgenics Gbx2 could mediate the repression of Otx2 by FGF8b.

We thank Dr A. McMahon for providing Wexp3 and Wexp3-Fgf8a constructs, Dr C. MacArthur for an Fgf8b-SDNA, Drs A. McMahon, M. Frohman, P. Gruss, K. Campbell, and J. Rossant for providing probes for RNA in situ hybridization analysis and the Skirball Institute transgenic facility for support in making transgenics. We also thank Sandrine Millet for many helpful discussions and comments and Gordon Fishell, Yuanhao Li and Michael Matise for comments on the manuscript. This research was supported by a grant from the NINDS (#R01-NS35876). A. L. J. is an investigator of the Howard Hughes Medical Institute.

REFERENCE
development of the chick embryo. 1951 [original article] [see comments].

Dev Dyn. 199, 251-260.


