Neural patterning of the vertebrate brain starts within the ectoderm during gastrulation and requires the activity of organizer cell populations in the neuroectoderm. One such organizer is located at the prospective midbrain-hindbrain boundary (MHB) and controls development of the midbrain and the anterior hindbrain via the secreted signaling molecule Fgf8. However, little is known about how the ability of neural precursors to respond to Fgf8 is regulated. We have studied the function of the zebrafish spiel-ohne-grenzen (spg) gene in early neural development. Genetic mapping and molecular characterization presented in the accompanying paper revealed that spg mutations disrupt the pou2 gene, which encodes a POU domain transcription factor that is specifically expressed in the MHB primordium, and is orthologous to mammalian Oct3/Oct4. We show that embryos homozygous for spg/pou2 have severe defects in development of the midbrain and hindbrain primordium. Key molecules that function in the formation of the MHB, such as pax2.1, spry4, wnt1, her5, eng2 and eng3, and in hindbrain development, such as krox20, gbx2, fkd3 and pou2, are all abnormal in spg mutant embryos. By contrast, regional definition of the future MHB in the neuroectoderm by complementary expression of otx2 and gbx1, before the establishment of the complex regulatory cascade at the MHB, is normal in spg embryos. Moreover, the Fgf8 and Wnt1 signaling pathways are activated normally at the MHB but become dependent on spg towards the end of gastrulation. Therefore, spg plays a crucial role both in establishing and in maintaining development of the MHB primordium. Transplantation chimeras show that normal spg function is required within the neuroectoderm but not the endomesoderm. Importantly, gain-of-function experiments by mRNA injection of fgf8 and pou2 or Fgf8 bead implantations, as well as analysis of spg-ace double mutants show that spg embryos are insensitive to Fgf8, although Fgf receptor expression and activity of the downstream MAP kinase signaling pathway appear intact. We suggest that spg/pou2 is a transcription factor that mediates regional competence to respond to Fgf8 signaling.

Key words: Competence, Fgf8, pou2, Oct3/4, Pou5f1, MHB, Isthmus, pax2, spiel-ohne-grenzen, Hindbrain, Zebrafish

INTRODUCTION

The organization of the vertebrate brain and its differentiation into functionally and anatomically distinct areas is based on early patterning and regional specification of the neural plate during embryonic development. Both vertical signals that emanate from the mesendoderm and planar signals travelling within the plain of the neuroectoderm itself are thought to be involved in neural plate patterning (Ruiz i Altaba, 1994; Gurdon et al., 1995; Kelly and Melton, 1995; Lumsden and Krumlauf, 1996; Wilson et al., 2002). Embryonic development of the midbrain and the anterior hindbrain in particular depend on an ectodermal population of cells located at the midbrain-hindbrain junction [the mid-hindbrain boundary (MHB) or isthmic organizer]. The organizer potential was initially demonstrated by transplantation experiments in chicken embryos, where isthmic tissue grafts induced midbrain and cerebellum ectopically. In its normal location, the MHB organizer was then proposed to regulate polarized morphological differentiation of the adjacent tectum and elaboration of the cerebellar anlage (Martinez and Alvarado-Mallart, 1990; Marin and Puelles, 1994; Garda et al., 2001; Liu and Joyner, 2001; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001).

Evidence for this concept has come from functional studies of the molecules involved. Several transcription factors of the Pox, Otx, Gbx and Lim class play pivotal roles during development and function of the MHB organizer. In zebrafish, null mutations for the pax2.1 gene (pax2a – Zebrafish Information Network) (no isthmus or noi) or inactivation of the eng2 and eng3 genes causes absence of the midbrain, MHB and cerebellum (Brand et al., 1996; Lun and Brand, 1998; Pfeffer et al., 1998; Scholpp and Brand, 2001), similar to the cognate mouse phenotypes (Mallen et al., 1994; Wurst et al., 1994; Favor et al., 1996; Urbanek et al., 1997; Schwarz et al., 1997). The secreted signaling molecules Wnt1 (McMahon et al., 1992) and Fgf8 are thought to mediate organizer function. Fgf8 in particular is expressed in the MHB organizer and when
added ectopically can mimic the organizing activity (Crossley et al., 1996). Functional studies of the acerbellarfgf8 (ace) mutant in zebrafish and targeted disruption in mice highlight the crucial function of Fgf8 in this process (Brand et al., 1996; Reifers et al., 1998; Meyers et al., 1998; Picker et al., 1999).

Like other Fgfs, Fgf8 is thought to signal through the MAP Reifers et al., 1998; Meyers et al., 1998; Picker et al., 1999). the crucial function of Fgf8 in this process (Brand et al., 1996; Shimamura and Rubenstein, 1997; Meyers and Martin, 1999; Sun et al., 1999; Reifers et al., 2000b; Shanmugalingam et al., 2000; Moon and Capechci, 2000; Streit et al., 2000). Furthermore, Fgf8 is required during formation of the heart field, for limb development, neural induction, telencephalon patterning, left-right asymmetry, gastrulation and ear development, among others (Brand et al., 1996; Shimamura and Rubenstein, 1997; Meyers and Martin, 1999; Sun et al., 1999; Reifers et al., 2000b; Shanmugalingam et al., 2000; Moon and Capechci, 2000; Streit et al., 2000). Thus, Fgf8 elicits very different responses in different embryonic target cells, raising the important question of how the differential competence of the responding cells arises.

We have analyzed the function of the zebrafish spiel-ohne-grenzen (spg) gene during neural development. We find that spg allows the early neuroectodermal cells around the MHB to respond to Fgf8. spg alleles have been isolated in several mutagenesis screens as mutations affecting MHB morphology (Schier et al., 1996a; Burgess et al., 2002). In the accompanying paper (Burgess et al., 2002), we report that spg mutants affect the gene encoding the transcription factor Pou2, an ortholog of the mammalian Oct3/Oct4/Pou5f1 gene. We now show that spg serves a key function during development of the MHB and the hindbrain. Such a function has not been described for the mammalian gene; therefore, we have identified a novel component of the MHB genetic hierarchy. We show that spg/pou2 functions specifically in patterning the neuroectoderm where it is necessary to establish and maintain the MHB organizer and the hindbrain primordium; whereas, it appears dispensable for the earliest stage of subdividing the neuroectoderm. Importantly, RNA injections and Fgf8-bead implantations demonstrate that the early neural primordium of spg mutants is insensitive to the effects of Fgf8. In particular, both Pou2 and Fgf8 are necessary for the initiation of gbx2 expression at the MHB primordium. We, thus, suggest that spg/pou2 is required to make neuroectodermal cells competent to respond to Fgf8, as assayed by their ability to activate the correct target genes.

MATERIALS AND METHODS

Fish maintenance

Zebrafish were maintained under standard conditions (Westerfield, 1994; Brand and Granato, 2001). Embryos were staged as described elsewhere (Kimmel et al., 1995) or by hours post fertilization (hpf) at 28°C.

Fish lines

Alleles of spg^m216, spg^713 and spg^b340 are described elsewhere (Schier et al., 1996b; Burgess et al., 2002), the latter-most allele probably being a null allele (see Burgess et al. (Burgess et al., 2002) for a description of the molecular nature of these alleles). The ace^m282/fgf8 allele has been described previously (Brand et al., 1996; Reifers et al., 1998). Heterozygous double carriers for spg^b2614 and ace^m282 were identified by random intercrosses.

Staining of living embryos

Confocal microscopy of Bodipy-Ceramide (Molecular Probes) was as described previously (Picker et al., 1999). Acridine Orange (2 μg/ml, Molecular Probes) was added into the medium surrounding dechorionated embryos for 4 hours during gastrulation, at the 5 somite stage and at the 12 somite stage.

Analysis of gene expression

Standard methods for whole-mount RNA in situ hybridization (ISH) were used, with laboratory modifications as described elsewhere (Reifers et al., 1998). Probes for the following genes were used: krox20 (egr2 – Zebrafish Information Network) (Oxtoby and Jowett, 1993); pax2.1 (Krauss et al., 1991a); pax6 (Macdonald et al., 1994); eng2 (eng2a – Zebrafish Information Network) (Egger et al., 1992; Fjose et al., 1988); shh (Krauss et al., 1993); efn4 (efn4 – Zebrafish Information Network) (Xu et al., 1994); wnt1 (Kelly et al., 1993); wnt4 (Ungar et al., 1995); ets2 (Mori et al., 1994); gbx2 (Brand et al., 1996); fgf8 (Reifers et al., 1998); spry4 (Fürthauer et al., 2001); sis3 (Seo et al., 1998b); gbx1 and gbx2 (Lun et al., 2002); fkd3 (fxb1.2 – Zebrafish Information Network) (Odenthal and Nusslein-Volhard, 1998); valentino (Moens et al., 1996); wnt8b (Kelly et al., 1995); emx1 (Morita et al., 1995); and oct3/4 (Schoeler et al., 1989).

Immu-no- and histochemistry

Antibody staining against acetylated tubulin was carried out as previously described (Macdonald et al., 1997). Embryos for histological sections were embedded in epoxy resin, sectioned with a microtome (1 μm sections) and stained with Methylene Blue-Toluidine Blue, as described elsewhere (Kuwada et al., 1990). Brain morphology and staining (embryos were mounted in 70% glycerol after in situ hybridization) were documented on a Zeiss axiophot.

RNA injections

cDNA of murine Oct3/4, fgf8, pou2 and nuclear lacZ, subcloned into pCS2+ (Rupp et al., 1994), were linearized and transcribed using the SP6 message mMachine kit (Ambion). The amount of mRNA injected was estimated from the concentration and volume of a sphere of RNA solution (0.25M KCl, 0.2% Phenol Red) injected into oil at the same pressure settings. RNA solution was backloaded into borosilicate capillaries prepared on a Sutter puller and injected into the cytoplasm of one cell of 2-cell stage embryos (about 100 pg fgf8 mRNA or 200 pg of pou2 mRNA per embryo). The injected mRNA has a strong tendency to stay in the progeny of the injected blastomere, as monitored by the unilateral distribution of co-injected lacZ mRNA, detected by staining with anti-β-gal antibody (Promega, 1:500) after in situ hybridization.

Bead implantation

Bead implantation was carried out as previously described (Reifers et al., 2000b). Beads coated with Fgf8b or phosphate-buffered saline (PBS) control beads were implanted at indicated regions of wild-type and spg mutant embryos at the 13 somite stage, embryos were fixed at 26 hpf.

Transplantation

Zygotes of wild-type embryos were labeled by injection of 10% HRP-coupled tetramethylrhodaminextran (M1=10,000, Molecular Probes D-1817) in 0.25M KCl and raised together with unlabeled host embryos from a heterozygous cross of spg carriers. Heterotopic transplantations of wild-type donor cells into host embryos were made between sphere and shield stage using a trimmed borosilicate capillary. Host embryos were fixed at the tailbud stage. After in situ hybridization, transplanted cells were
stained combining the Vectastain ABC system (VectorLabs) and the DAB system (Sigma).

**Inhibitor treatment**

For inhibition of the Fgf pathway, wild-type and spg embryos were treated with the chemical inhibitor SU5402, which blocks activity of all Fgf receptors (Calbiochem) (Mohammadi et al., 1997). The inhibitor was applied at 20 μM into embryo medium at 28°C in the dark and embryos were incubated from end of gastrulation until fixation at the eight-somite stage.

**RESULTS**

**spiel-ohne-grenzen (spg) is required for midbrain-hindbrain boundary and hindbrain development**

At 24 hpf, the MHB of wild-type embryos is marked by a prominent inward fold of the neuroepithelium, which develops into the isthmic constriction of the brain. The formation of this fold is disturbed in living homozygous spg embryos (Fig. 1) (Schier et al., 1996a). Results are based on analysis of the spg<sup>715</sup> allele and the likely null allele spg<sup>hi349</sup>; they give an identical phenotype of slightly variable expressivity, with the exception of the pou2 staining [see Burgess et al. (Burgess et al., 2002), for a discussion of molecular defects of the alleles]. Optical sections of live embryos stained with Bodipy-Ceramide and histological sections show that in spg mutants, both the prominent inward fold at the MHB and the cerebellar primordium which abuts the MHB are missing, and that the tectum opticum is variably reduced in size (Fig. 1C-F,I,J). After 26 hours of development, a small aggregate of cells is visible at the MHB of spg mutants (Fig. 1D,F) that is absent in acerebellar (ace) mutants (Reifers et al., 1998). In addition, spg mutants have smaller otic vesicles with often only one otolith, and a curved, slightly shortened tail with misshaped somites (not shown). From day 4 onwards, 30-50% of mutant larvae show a slightly reduced frequency of heartbeat and develop edema, although both the atrium and ventricle are initially present, unlike in acerebellar embryos (Reifers et al., 2000b). spg mutants feed far less efficiently than wild-type embryos and die after 14 to 19 days for unknown reasons.

Acridine Orange specifically interacts with DNA of non-condensed, fragmented chromatin and can be used in zebrafish to detect cells undergoing cell death (Brand et al., 1996). We detected dying cells in the prospective MHB and tectum of spg mutants from the 14-somite stage onwards until the pharyngula period, most prominently during late somitogenesis (Fig. 1G,H). Cell death is particularly apparent within the hindbrain around the 22 somite stage, in two transverse stripes (Fig. 1H, insert) that probably correspond to r3 and r5 (see Fig. 5). Weaker incorporation of Acridine Orange occurs in the optic stalk, tail-tip and the dorsal midline of the tail and trunk regions (Fig. 1H and not shown). Because dying cells are detectable from mid-somitogenesis stages onwards, cell death probably results from earlier defects. In addition, the cell death probably contributes to the development of the MHB phenotype of spg mutants at 24 hpf. Anti-acetylated-tubulin staining demonstrates that the axonal scaffold of spg embryos is specifically disrupted not only in midbrain, but also hindbrain development: longitudinal and transverse axon bundles, normally located at rhombomeric boundaries, are not tightly fasciculated and show imprecise scaffolding, and the distance between forebrain and hindbrain commissures is reduced in spg embryos, probably owing to tissue elimination by cell death (Burgess et al., 2002) (Fig. 1H). Indeed, spg embryos lack a recognizable trochlear nerve within the MHB (not shown).

**Establishment and maintenance of the MHB is affected in spg mutants**

The above observations, previous data (Schier et al., 1996a) and the expression pattern of pou2 (the gene affected in spg mutants) (Burgess et al., 2002) all suggest that early neural
Fig. 2. The primordia of the MHB and the hindbrain affected in spg embryos. (A'-D', E'-H', I'-N', Q', U'-U') Dorsal views; embryos in the remaining pictures are shown from lateral view. Gene expression, stages and genotypes are noted. Red arrowheads indicate expression of genes at the MHB throughout. (A'-D') In spg embryos, pax2.1 shows reduced expression at the MHB from its onset onwards (A',B'), is lost during midsomitogenesis (C') and re-expressed as a dorsal patch after 24 hpf. Expression of pax2.1 within the otic placode (B-D') is not affected in mutant embryos. (E-H') wnt1 is normally expressed at its onset at 80% of epiboly (not shown), but becomes subsequently downregulated in mutant (E') at the time when pax2.1 is initiated. During somitogenesis (F-G'), the expression of wnt1 at the MHB (arrowhead) and within rhombomeres is downregulated in spg embryos. At pharyngula stages, wnt1 expression is continued within a dorsal patch at the MHB. (H,H') The midsagittal expression in the diencephalon seems unaffected, but MHB expression is reduced to a dorsal patch in mutant embryos. (I-I') fgf8 expression, like that of wnt1, is not affected in spg embryos at its onset of expression at the MHB (not shown), but soon becomes downregulated at around 90% of epiboly. (J) fgf8 expression caudally continues in r1, r2 and r4 in wild-type embryos. (J') In mutant embryos, fgf8 expression is strongly reduced within r1 and abolished within r2 and r4. During somitogenesis, fgf8 expression is completely lost from the MHB but, like pax2.1 and wnt1, recovers at a dorsal patch at the MHB. (M-P') spry4 is not properly initiated in spg embryos. At the four-somite stage, spry4 is strongly reduced at the MHB and in r1, r2 and r4 (N'). MHB expression of spry4 during somitogenesis and pharyngula stages follows the same mode as fgf8 and pax2.1. (Q,Q') en2 is normally initiated at the MHB at the end of gastrulation. In spg mutant embryos, en2 is downregulated from its initiation of expression. (R) en2 is expressed in the prospective tectum in a graded fashion during somitogenesis but is strongly reduced in spg embryos (R'). (S) en3 is encompassed within the en2 domain at the tectum in wild-type embryos. (S') In spg embryos, en3 is downregulated in a similar fashion as en2. (T,T') half sides of transverse sections through the her5 positive domain at the spatial level of the future MHB; arrows point to the neuroectoderm. (T) her5 is normally initiated within the neuroectoderm around 70% of epiboly, overlying mesendodermal expression. (T') her5 is not properly initiated in spg embryos. (U,U') her5 expression at the MHB is reduced in mutant embryos at the end of gastrulation.
development is abnormal in spg mutants. Using in situ hybridization with antisense RNA, we therefore followed in detail the expression of MHB and hindbrain marker genes. Four representative stages are shown to illustrate the results (Fig. 2, summarized in Fig. 3). Expression of pax2.1 is downregulated already at the onset of expression at 80% of epiboly (Fig. 2A'), as reported previously (Schier et al., 1996a; Burgess et al., 2002), as is spry4 (Fig. 2M'). pax2.1 expression remains strongly reduced during early somitogenesis stages (Fig. 2B'), is completely eliminated during midsomitogenesis stages (Fig. 2C') and is re-expressed in a dorsal patch at the prospective MHB after 24 hpf (Fig. 2D'). Similarly, expression of eng2, eng3 and her5 is affected both during initiation and maintenance (Fig. 2Q'-U'). The kinetics of fgf8 expression at the MHB in spg mutants is overall similar to that of pax2.1 and the other markers (Fig. 2I'-L'; red arrowheads). By contrast, however, expression of fgf8 and wnt1 is initiated normally at 70-80% of epiboly, and only become decreased at 80-90% of epiboly (Fig. 2E',I'). During early somitogenesis stages, fgf8 and spry4 are expressed in rhombomeres 1, 2 and 4 (Reifers et al., 1998; Fürthauer et al., 2001). In these rhombomeric domains, fgf8 and spry4 expression is likewise strongly reduced in spg mutants (Fig. 2J',N'). Conversely, initiation of pou2 expression is not affected during gastrulation stages in noi/pax2.1 and ace/fgf8 mutants, or in wild-type embryos in which all Fgf signaling is blocked pharmacologically (data not shown). Beginning at the one- to two-somite stage, pou2 expression is gradually lost in ace mutants or in inhibited embryos, as described previously for many other markers, but pou2 expression remains normal in noi mutants at least until the six-somite stage (data not shown) (Reifers et al., 1998). Thus, spg/pou2 is required to initiate expression of pax2.1, eng2, eng3 and her5, and is required to maintain, but not initiate, expression of wnt1 and fgf8.

Although the tectum expresses otx2 (see Fig. 5Q), tectum development is abnormal in spg mutants. Expression of the engrailed genes eng2 and eng3 is reduced in spg mutants throughout embryonic development (Fig. 2R,S'), consistent

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**Fig. 3.** Marker gene expression at the MHB in spg. development is abnormal in spg mutants. Using in situ hybridization with antisense RNA, we therefore followed in detail the expression of MHB and hindbrain marker genes. Four representative stages are shown to illustrate the results (Fig. 2, summarized in Fig. 3). Expression of pax2.1 is downregulated already at the onset of expression at 80% of epiboly (Fig. 2A'), as reported previously (Schier et al., 1996a; Burgess et al., 2002), as is spry4 (Fig. 2M'). pax2.1 expression remains strongly reduced during early somitogenesis stages (Fig. 2B'), is completely eliminated during midsomitogenesis stages (Fig. 2C') and is re-expressed in a dorsal patch at the prospective MHB after 24 hpf (Fig. 2D'). Similarly, expression of eng2, eng3 and her5 is affected both during initiation and maintenance (Fig. 2Q'-U'). The kinetics of fgf8 expression at the MHB in spg mutants is overall similar to that of pax2.1 and the other markers (Fig. 2I'-L'; red arrowheads). By contrast, however, expression of fgf8 and wnt1 is initiated normally at 70-80% of epiboly, and only become decreased at 80-90% of epiboly (Fig. 2E',I'). During early somitogenesis stages, fgf8 and spry4 are expressed in rhombomeres 1, 2 and 4 (Reifers et al., 1998; Fürthauer et al., 2001). In these rhombomeric domains, fgf8 and spry4 expression is likewise strongly reduced in spg mutants (Fig. 2J',N'). Conversely, initiation of pou2 expression is not affected during gastrulation stages in noi/pax2.1 and ace/fgf8 mutants, or in wild-type embryos in which all Fgf signaling is blocked pharmacologically (data not shown). Beginning at the one- to two-somite stage, pou2 expression is gradually lost in ace mutants or in inhibited embryos, as described previously for many other markers, but pou2 expression remains normal in noi mutants at least until the six-somite stage (data not shown) (Reifers et al., 1998). Thus, spg/pou2 is required to initiate expression of pax2.1, eng2, eng3 and her5, and is required to maintain, but not initiate, expression of wnt1 and fgf8.

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**Fig. 4.** Prosencephalic markers expand posteriorly in spg embryos. (A) emx1 is expressed in telencephalic precursors from end of gastrulation onwards in wild-type embryos. The posterior transverse expression domain marks the di-mesencephalic boundary. pax2.1 expression at the anterior MHB is shown in red. (A') In spg embryos, defined by the impaired expression of pax2.1 at the MHB, emx1 expression is generally elevated but reduced in its spatial lateral extent. The posterior border of emx1 expands caudally. (B) anf1, like emx1, is expressed at the anterior neural border with a patch of expression centering around the midline of the neuroectoderm. gbx2 expression at the posterior MHB is shown in red. (B') anf1 is lost within the midline expression domain in spg embryos, defined by impaired gbx2 expression (see Fig. 3 for gbx2 expression). (C) pax6 is initiated within the forebrain at the end of gastrulation, pou2 expression at the MHB is seen in red. (C') spg embryos, identified by loss of pou2 expression, show a posterior expansion of pax6 expression into the territory of the prospective MHB. (D,D') Double in situ hybridization with fgfr3 (blue) and en3 (red) at the 10-somite stage show the hindbrain domain of fgfr3 (arrow in D,D') is fused with the diencephalic domain of fgfr3, particularly at its ventral aspect. The MHB marker en3 is restricted to a dorsal patch in mutant embryos (D'). (E) During somitogenesis, besides its expression in the forebrain, pax6 is also expressed within the hindbrain and spinal cord in wild-type embryos. (E') The prosencephalic and the rhombencephalic domain nearly fuse in spg mutant embryos mainly owing to strong posterior expansion of the posterior border of the prosencephalic domain of pax6. (F,G) Anterograde filling of whole eyes with DiI (green fluorescence) or DiO (red fluorescence) shows a proper contralateral retinotectal mapping of RGC axons in spg embryos (F). The chiasma opticum is properly formed in spg mutant embryos (G).
with their role as target genes of \textit{pax2.1} (Lun and Brand, 1998; Scholpp and Brand, 2001). Moreover, the tectum-specific ephrins ephrinA5\textalpha and ephrinA5\textbeta, the genes for which are probably the target of engrailed proteins, are strongly restricted to a dorsal patch but never completely abolished in \textit{spg} mutants (not shown). \textit{her5} encodes a bHLH transcription factor expressed at 70\% of epiboly in the MHB primordium and the underlying mesendoderm (Fig. 2T) (Müller et al., 1996; Lun and Brand, 1998). In \textit{spg} mutants, \textit{her5} expression is initially normal in the mesendodermal layer (Fig. 2T\textprime), but is not initiated within the overlying neuroectoderm (arrow in Fig. 2T\textprime,U\textprime). At 90\%, \textit{her5} expression is downregulated in the mesendoderm of wild-type and mutant embryos. The expression of additional MHB markers in \textit{spg} mutants is similar to those above and is summarized in Fig. 3. MHB markers are typically more strongly affected in the ventral MHB of \textit{spg} mutants during early somitogenesis, before the expression is eventually lost completely (see Fig. 8O; and not shown). This is not due to defective midline tissue, as \textit{shh} and \textit{twhh}, which encode two secreted Hedgehog-family members expressed throughout the ventral CNS midline, are expressed normally in \textit{spg} mutants. Tailbud expression of these genes was slightly reduced (not shown), possibly explaining the slightly twisted tail of the mutants.

**Positioning of the MHB is normal in \textit{spg} embryos**

Studies in several vertebrates suggest that \textit{Otx2} and \textit{Gbx2}, which are expressed in mutually exclusive territories of the hindbrain and fore/midbrain, respectively, are involved in positioning the organizer at the MHB (Wasserman et al., 1997; Broccoli et al., 1999; Millet et al., 1999; Rhinn and Brand, 2001). In zebrafish, \textit{gbx1} is the functional equivalent of the murine \textit{Gbx2} gene (Lun et al., 2002). In wild-type embryos, \textit{gbx1} is expressed in the hindbrain primordium, in a domain complementary to the expression of \textit{otx2}, which partially includes the \textit{pax2.1} activation domain (Lun et al., 2002) (see Fig. 5A,B). In \textit{spg} mutants, recognizable by their reduced expression of \textit{pax2.1}, the spatial relationship between \textit{gbx1}, \textit{otx2} and \textit{pax2.1} expression appears normal (see Fig. 5A\textprime,B\textprime), indicating that the initial subdivision of the neuroectoderm into an \textit{otx2}- and a \textit{gbx1}-positive domain occurs normally in \textit{spg} mutants.

**Caudal expansion of prosencephalic gene expression**

Morphological, histological and immunohistochemical inspection at pharyngula stages showed that forebrain architecture was largely normal in \textit{spg} mutants (Fig. 1). \textit{ace/fgf8} mutants show abnormal retinotectal projection and a defective
spg/pou2 mediates competence for Fgf8 optic chiasm (Picker et al., 1999; Shanmugalingam et al., 2000), which prompted us to study forebrain marker expression and to examine specifically the visual system in spg mutants using anterograde fills with DiI. In all of seven examined spg mutants, we observed normal contralateral retinotectal mapping of retinal ganglion cell axons (Fig. 4F) and a properly elaborated decussation of the optic nerve (Fig. 4G). In our marker analysis, we find evidence for abnormal development of the forebrain neural plate, especially of the diencephalic primordium. 

emx1 is expressed in the telencephalic primordium at the end of gastrulation, lining the anteriormost border of the developing brain, and in a bilateral transverse stripe of expression in the posterior diencephalon (Fig. 4A) (Shanmugalingam et al., 2000; Kazanskaya et al., 1997) that is not seen in spg embryos (Fig. 4B'). Furthermore, expression of pax6 at the di-mesencephalic boundary (Macdonald et al., 1994) is upregulated and strongly expanded caudally from its onset of expression (Fig. 4C').

During somitogenesis stages, fgfr3 and pax6 are expressed in wild-type embryos in the diencephalon and in r1, i.e. in territories abutting the midbrain and MHB (Fig. 4D,E) (Krauss et al., 1991b; Sleptsova-Friedrich et al., 2001). In spg embryos, these expression domains almost fuse (Schier et al., 1996a). As strong cell death is not yet detectable at this stage, this fusion may be due to a transformation of the intervening mis-specified midbrain and MHB tissue, rather than a simple elimination. In double in situ hybridization analysis with fgfr3 and eng3 (red staining in Fig. 4D,D'), eng3 is still expressed in a mesencephalic remnant posteriorly adjacent to the diencephalic territory but is reduced to a faint dorsal patch as expected for spg embryos (Fig. 4D'). This suggests that during early somitogenesis, the remnant expression of tectal or MHB markers is still able to specify some rudimentary tissue between the forebrain and the hindbrain, which prevents forebrain gene expression from invading into this distinct dorsal tissue. Later in development, when gene expression is generally absent at the MHB in spg embryos, prosencephalic markers are not only posteriorly expanded on the ventral but also on the dorsal side, exemplified by the expanded dorsal thalamic domain of ephA4 (Fig. 5H). In contrast to telencephalic and diencephalic gene expression domains, midbrain expression domains (i.e. those of engrailed genes or of otx2) are never caudally expanded in spg embryos (Fig. 5Q). Unlike diencephalic marker expression, hindbrain marker expression is not markedly expanded towards the anterior. For example, the anterior limit of the hindbrain expression domains of pax6 or gbx1 (Fig. 4E' and not shown)
appear normal in spg embryos, and even in double in situ hybridization analysis with krox20 and pax6, we found no relative expansion of the anterior border of pax6 expression in the hindbrain of spg mutants (not shown). Similarly, rostral borders of the diencephalic expression domains of dlx2, otx1, wnt8b, emx1, pax6, ephA4, six3 and anf1 were not altered during early somitogenesis stages in spg mutants (not shown).

**Early failure of hindbrain gene expression in spg mutants**

During the analysis of MHB-specific genes such as fgf8 and spry4, we found that these genes are also affected in their hindbrain expression (Fig. 2J’N’). pou2 is itself expressed in the hindbrain primordium at the end of gastrulation (Burgess et al., 2002; Hauptmann and Gerster, 1995) prompting us to examine establishment of gene expression in the hindbrain primordium of spg embryos. We find that gene expression in the hindbrain fails from late gastrula stages onwards, as is most clearly evident from the analysis of gbx2 expression. gbx2 expression is initiated first in the mesendoderm around 80% of epiboly and subsequently around 90% within the overlying hindbrain neuroectoderm (Lun et al., 2002) (Fig. 5C-E). Studies of acerebellar (ace) mutants have shown that both tissue layers absolutely require fgf8 to express gbx2 (Fig. 5E’) (Lun et al., 2002). By contrast, in spg mutants, neuroectodermal expression of gbx2 is not initiated at all (Fig. 5D’), whereas the underlying mesendodermal expression of gbx2 occurs normally (Fig. 5C’D’; arrowhead in D’). In addition to gbx2, ectodermal expression of the forkhead domain transcription factor fkd3 (Odenthal and Nusslein-Volhard, 1998) is absent from the hindbrain primordium of spg embryos (Fig. 5F’). Therefore, although positioning of the MHB in the neuroectoderm appears normal, global gene expression in the hindbrain primordium, a known site of pou2 expression (Burgess et al., 2002), is already severely disrupted before the end of gastrulation in spg mutants.

During the early segmentation period, pou2 expression becomes confined to distinct cell populations in r2 and r4 (Fig. 5L) (Hauptmann and Gerster, 1995). Genes that mark the segmental organization of the hindbrain, such as krox20, ephA4, wnt8b, hoxb1a and hoxb4a are all strongly affected in their expression (Fig. 5G’K’), probably owing to a mixture of a global and a rhombomeric specific requirement for pou2 in the hindbrain. Next to the pou2-expressing rhombomeres r2 and r4, krox20, ephA4 and wnt8b are normally expressed within r3 and r5. In spg mutants, the size of r3 and r5 appears reduced, whereas that of the intermittent r4 appears normal or enlarged (Fig. 4G’I’). Thus, in addition to the early gene expression defects of the hindbrain primordium, rhombomeres also show specific defects during segmentation stages that differ depending on the rhombomere considered. This may reflect a later, rhombomere-restricted function of pou2. six3 expression in the prospective telencephalon and eye field is not altered in spg, whereas ephA4 expression in the otic placode is reduced, and diencephalic expression is posteriorly expanded into the midbrain and MHB (Fig. 5H’), as described above for other diencephalic markers, except for wnt8b, which is not altered (Fig. 5I’). Expression of the Hox genes hoxb1a in rhombomere 4 (r4) and hoxb4a from r7 into the spinal cord (Fig. 5J’) is mildly affected in spg embryos at midsomitogenesis. The hoxb1a domain appears more diffuse compared with the wild type and is even wider towards the end of somitogenesis (Fig. 5K’), but more strikingly, the gap between the hoxb1a and hoxb4a domains in the r4-r7 territory is significantly reduced in spg embryos, as indicated by brackets in Fig. 5J’. At late somitogenesis stages, hoxb1a is more strongly affected in spg mutants. Concomitant with the reduced odd-numbered rhombomeres, r4 apparently enlarges at the expense of r3 and 5, as judged from hoxb1alkrox20 double staining (Fig. 5K,K’). The expression of pou2 itself provides one of the clearest example for a function of spg/pou2 in specific hindbrain rhombomeres. In embryos that carry the spg773 point allele, the discrete patches of pou2 expression in r2 and r4 are strongly reduced or absent in spg mutants (Fig. 5L’). Embryos carrying the apparent null allele spgk340 show complete absence of expression in all pou2 domains (Fig. 5L’’). Expression of the bZIP transcription factor valentino/Kreisler (Manzanares et al., 1999; Cordes and Barsh, 1994) in r5 and r6 is abrogated in r5 but unaffected in r6, including the neural crest streaming from r6 (Fig. 5M’). Consistent with the disorganization of hindbrain commissures (Burgess et al., 2002), expression of fkd3 (Fig. 5N) (Odenthal and Nusslein-Volhard, 1998) at rhombomere boundaries is nearly abolished in spg embryos (Fig. 5N’).

Neurogenesis, as labeled by zathl expression, is reduced in spg mutants in the ventricular zone of the hindbrain ventricle at 24 and 32 hpf (Fig. 5O’P’). zathl expression in the mutants occurs also in a position corresponding to the cerebellar anlage/posterior MHB in the wild-type (arrow). Expression in this tissue might either reflect an expanded rhombic lip, or a partial reformation of the cerebellum at later stages in the mutants. This tissue does not express the fore/midbrain marker ot2 (Fig. 5Q), and we therefore tentatively suggest that this is the result of a partial re-formation of cerebellar tissue after 30 hpf in the spg mutants, explaining some of the observed variation in morphological strength. Earlier stages of neurogenesis, as labeled by the proneural bHLH transcription factor ngnl are also affected. ngnl is expressed in trigeminal precursors and in proneural cell clusters in the brain primordium already at the end of gastrulation, and this expression fails to be initiated in spg mutants (Fig. 5R,R’). Expression in three rostrocaudal rows of cells within the presumptive spinal cord containing the precursors of motoneurons, interneurons and sensory neurons is, however, initiated normally in spg mutants, although the rows are compressed into a narrower space.

**Mesendoderm development in spg embryos**

In addition to the brain phenotype, spg mutants have a curved and malformed tail with misshapen somites (Burgess et al., 2002), suggesting the existence of non-neural defects. We find that myod expression is strongly reduced in somitic precursors (Fig. 5T), but unaffected in adaxial cells. Somitic expression of other markers like snail1 (Hammerschmidt and Nusslein-Volhard, 1993), eng2 (Devoto et al., 1996) and fgf4.1 (Grandel et al., 2000) are also reduced in spg embryos during somitogenesis (not shown). However, unlike in acerebellar mutant embryos (Reifers et al., 1998), somitic alterations are not morphologically distinguishable before the beginning of pharyngula stages. Because induction of muscle pioneers is dependent on signals from the notochord (Halpern et al., 1993), we analyzed markers expressed in the midline mesoderm. The
expression of the pan-mesodermal gene ntl (Schulte-Merker et al., 1994), the early mesendodermal marker wnt8 (Kelly et al., 1995) and the early axial mesoderm marker hhh/znot (Talbot et al., 1995) are not altered at gastrulation stages (not shown). Similar to her5 (Fig. 2T), the expression of the anterior prechordal plate marker gsc (Schulte-Merker et al., 1994) is normal at 70% of epiboly and tailbud stage, but shows reduced midline expression at the four-somite stage in the mutant. The intermediate mesodermal expression of pax2.1 is never affected in spg mutants. By contrast, expression of the endoderm specific marker sox17 (Alexander and Stainier, 1999) is strongly reduced at the tailbud stage (Fig. 5S') but not at its onset around 50% of epiboly, although pou2 expression is restricted to the ectoderm during gastrulation (inset). The reduction in sox17 expression may be due to the general expression of pou2 at pre-gastrula stages. Expression of nkh2.5 in the heart primordium (Chen et al., 1996; Reifers et al., 2000b) is only slightly reduced at the eight-somite stage (not shown).

### Specific requirement for spg/pou2 in the neuroectoderm

The abnormal development of both the endomesodermal and ectodermal layers led us to ask in which germ layer normal spg/pou2 activity is required to allow normal hindbrain development (Fig. 6). We transplanted wild-type cells before onset of gastrulation into the prospective ectoderm or mesoderm of spg mutants. After developing until the tailbud stage, chimeric embryos were examined for expression of gbx2 or pax2.1. Previous data have shown that both mesendodermal and neuroectodermal expression of gbx2 expression requires Fgf8 (Fig. 5E', Fig. 6G) (Lun et al., 2002). By contrast, only the neuroectodermal gbx2 expression requires spg, and expression in the underlying mesendoderm is intact (Fig. 5C',D'). When the transplanted wild-type cells were located in the neuroectoderm, they expressed gbx2 in spg embryos, whereas a location in the mesoderm was not sufficient to restore neuroectodermal expression (Fig. 6A,B,E) (Table 1); Cross-sections confirmed that the expressing cells were confined to the ectoderm (Fig. 6B, bracket). Chimeric spg embryos with neuroectodermal clones fixed during mid-somitogenesis also showed rescue of pax2.1 at the MHB (Fig. 6C,D). We conclude that the neuroectoderm of spg mutants is permissive for proper gbx2 and pax2.1 expression of wild-type cells, whereas wild-type cells located in the mesodermal layer do not support ectodermal expression of these markers (Fig. 6E). Together with the fact that pou2 is expressed only in the neuroectodermal germlayer of the gastrula, these results strongly suggest that spg/pou2 specifically functions in the neuroectoderm during gastrulation, independently of its ubiquitous expression during pre-gastrula stages (Fig. 6G).

### Mouse Oct3/Oct4 can functionally replace zebrafish pou2

While determining the molecular nature of spg alleles, we found that pou2 is the likely to be the zebrafish ortholog of the mouse Oct3/Oct4 gene (Burgess et al., 2002), which is widely known for its involvement in differentiation of the inner cell mass and of germ cells, but for which a role in brain development had not been reported. We therefore examined the expression of Oct3/Oct4 in mouse embryos, and found that Oct3/Oct4 was expressed at E8-8.5 throughout the neural plate, though the expression is apparently not restricted to the midbrain-hindbrain domain (Fig. 6F) (Schoeler et al., 1989), as in zebrafish [see Fig. 4 by Burgess et al. (Burgess et al., 2002) for comparison]. We reasoned that Oct3/Oct4 as an ortholog of pou2 might be able to restore the phenotype of spg mutant embryos if injected, and found that this was indeed the case. Injection of synthetic mRNA for Oct3/Oct4 into one cell at the two-cell stage rescued the expression of pax2.1, which is normally severely reduced in spg/pou2 mutants at this stage (Fig. 6H), in the same manner as does injection of pou2 mRNA (Burgess et al., 2002). These results suggest that Oct3/Oct4 may function in activation of Pax2 also in normal mouse development.

### Combinatorial roles for pou2 and fgf8 in the hindbrain

The phenotypic similarities between aceffg8 and spg/pou2 mutants raised the possibility that these genes might act in the same pathway, or in synergistic pathways. fgf8 transcription is initiated normally in spg mutants at 70% epiboly, but becomes downregulated by the end of gastrulation and is completely lost during somitogenesis (Fig. 2I–K). We therefore injected fgf8 mRNA unilaterally into wild-type and spg two-cell stage embryos to determine if fgf8 was capable of rescuing any aspect of the spg phenotype. We used gbx2 and spry4, known early downstream targets of Fgf8, as markers to assay the effects of fgf8 mis-expression at the end of gastrulation in spg embryos. In wild-type embryos, fgf8 mis-expression caused a strong dorsalization of the whole embryo (Füthauer et al., 1997; Reifers et al., 1998), which is visible as a pronounced dorsoventral expansion of spry4 and gbx2 in the injected half (Fig. 7A,C, arrow). As in the wild type, fgf8-mRNA injection into spg mutants results in strong lateral expansion of the endogenous mesendodermal domain of gbx2 (Fig. 7B), confirming that fgf8 can also exert its dorsalizing activity in spg embryos; the residual, weak expression of spry4 may be similarly expanded (Fig. 7D). Moreover, in the neuroectoderm of wild-type embryos, fgf8 mRNA injection also caused upregulation of the endogenous expression domains of both gbx2 and spry4 (Fig. 7A,C). Unexpectedly, and in contrast to

### Table 1. Summary of fgf8, pou2 and mouse Oct3/Oct4 overexpression studies and transplantation experiments

<table>
<thead>
<tr>
<th>Injected mRNA</th>
<th>Amount injected</th>
<th>Genotype of treated embryo</th>
<th>Embryonic response*</th>
<th>Number injected</th>
<th>Rescue of expression</th>
</tr>
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<tr>
<td>fgf8</td>
<td>25 pg</td>
<td>spg+/–</td>
<td>100%</td>
<td>56 (total)</td>
<td>0</td>
</tr>
<tr>
<td>pou2</td>
<td>250 pg</td>
<td>ace+/–</td>
<td>100%</td>
<td>40 (total)</td>
<td>0</td>
</tr>
<tr>
<td>luc2</td>
<td>500 pg</td>
<td>0</td>
<td>0</td>
<td>32 (total)</td>
<td>0</td>
</tr>
<tr>
<td>Mouse Oct3/Oct4</td>
<td>300 pg</td>
<td>spg+/–</td>
<td>25 (total)</td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>

Total numbers indicate mutant embryos only.

*Embryos misexpressing fgf8 mRNA are typically dorsalized from mid-gastrulation onwards. Embryos misexpressing pou2 mRNA show altered cell movements during gastrulation and lateral expansion of MHB expression domains.

Of 72 mutant embryos with transplanted cells in the MHB region, 64 show ‘rescue’ of gbx2 expression at tb; of 23 mutant embryos with transplanted cells in the mesendoderm, none shows ‘rescue’ of gbx2 expression at tb; and of 19 mutant embryos with transplanted cells in the MHB region, all show ‘rescue’ of pax2.1 expression at 20 s.
the mesendodermal expression domain, the neuroectodermal expression of gbx2 and spry4 could not be initiated (gbx2) or restored to the wild-type level (spry4) in spg embryos injected with fgf8 mRNA (Fig. 7B,D). The gbx2 expression seen in Fig. 7B is the mesendodermal domain that is unaffected in spg mutants (see also Fig. 5C,D). Equivalent results were obtained with fgf8 injection when wild-type and spg mutant embryos were fixed at early and mid-somitogenesis stages (not shown). Thus, the hindbrain and MHB primordium of spg/pou2 mutants appear to be insensitive to Fgf8 signaling. In mice, Fgf8 and Gbx2 are thought to act in a feedback loop (Garda et al., 2001); the loop could simply be interrupted between fgf8 and gbx2 by the absence of pou2, if pou2 acts within this loop upstream of gbx2. We therefore tested whether injection of pou2 mRNA into ace embryos could restore gbx2 expression, and found that this was not the case (Fig. 7E,F). These findings show that both Fgf8 and Pou2 are required for gbx2 and spry4 expression in the ectoderm. In addition to gbx2 embryos were fixed at early and mid-somitogenesis stages (not shown).

Fig. 6. spg/pou2 requirement in the neuroectoderm. (A) Transplanted wild-type cells (brown) in spg embryos express gbx2 cell autonomously. All blue cells carry the brown transplantation marker. The right half of the embryo serves as a control: it is devoid of wild-type cells. As is normally seen in spg mutants (compare with Fig. 5D), gbx2 expression is found only in the mesendoderm, but not the overlying neuroectoderm. Dorsal view of a spg chimera, anterior is upwards. The white line indicates the plane of the transversal section in B along the gbx2 domain. (B) Cross-section of the embryo in A showing that the transplanted wild-type cells expressing gbx2 (bracket; arrow indicates the unaffected non-neural ectoderm domain, see also Fig. 5D) are located in the neuroectoderm. Other cells that are only brown lie outside the normal domain of gbx2 expression. (C,D) Transplanted wild-type cells (brown) in spg embryos also express pax2.1 normally at the MHB. Arrows point to the residual pax2.1 expression at the MHB which is retained in spg embryos until late stages of somitogenesis. (E) Clones of wild-type cells within the mesoderm cannot restore gbx2 expression in spg mutant embryos at the tailbud stage. The plane of section is similar to B. Arrows point to the unaffected non-neural ectoderm domain. (F) Mouse Oct3/Oct4/Pou5f1 is globally expressed within the neural plate at day 8.0 p.c. (dorsal view, anterior to the left). (G) spg/pou2 might be required to activate Fgf8-dependent gbx2 expression either for a planar or vertical signal. The transplantation experiments presented here show a requirement in the neuroectoderm. (H) Mouse Oct3/Oct4/Pou5f1 mRNA and lacZ mRNA were co-injected into one cell of a two-cell stage zebrafish embryo. pax2.1 expression can be restored in spg mutant embryos by mouse Oct3/Oct4 mRNA (arrow, lacZ expression is indicated by the brown color) (Burgess et al., 2002). nec, neuroectoderm; mes, mesendoderm; tb, tailbud stage.

Fig. 7. Relationship between pou2 and fgf8. All embryos are depicted dorsally with the exception of the embryo in F, which is depicted laterally. (A-D) Gain of fgf8 function by unilateral misexpression of fgf8 mRNA into one cell of two-cell stage embryos. To determine the effect caused by fgf8 overexpression, gbx2 (A,B) and spry4 (C,D), both markers for the prospective hindbrain, were used. The activity of misexpressed fgf8 can be judged from dorsoanteriorization of the embryos indicated by lateral expansion of endogenous gbx2 and spry4 expression, indicated by arrows (A-E) at the injected side of the embryo. Deposition of co-injected lacZ mRNA is visualized by staining for anti-β-gal antibody (brown), reflecting the location of injected fgf8 mRNA (not visualized). Distribution of injected mRNA is restricted to one half of the embryo, allowing for comparison with the contralateral side as a control. In spg embryos, neither expression of gbx2 (B) nor spry4 (D) could be rescued or upregulated, respectively, by fgf8 overexpression. (E,F) In a reversed experiment, pou2 mis-expression into ace embryos (carried out in the same unilateral fashion described for fgf8 injection above), pou2 overexpression and fgf8 itself can provoke dorsoanteriorization of the injected half of the embryo (obviously seen in the wild-type embryo in E, but not in the ace embryo in F, owing to complete loss of the readout marker gbx2) but cannot rescue expression of gbx2 in ace mutant embryos. (H) A bead soaked with Fgf8 protein can not rescue the morphology of the isthmic constriction at the MHB but can evoke ectopic spry4 expression in wild-type and spg embryos (G,H; white circles indicate the implanted bead).
and spry4, we also found transcriptional activation of fkd3 within the hindbrain primordium to be dependent on both pou2 and fgf8 (not shown).

To further test the idea that spg embryos might be regionally insensitive to Fgf8, we implanted beads soaked with Fgf8 protein into the prospective MHB territory of spg mutants. For technical reasons, these implantations were made at the 13-somite stage. In ace mutants, this treatment rescues the formation of the MHB constriction, and leads to re-expression of the target gene spry4 (F. Reifers and M. B., unpublished) (Fürthauer et al., 2001). In wild-type embryos examined at 26 hpf, the MHB constriction is clearly visible. The localized source of Fgf8 protein provided by the bead was not able to restore the MHB constriction in spg embryos. However, after in situ staining, ectopic expression of spry4 was readily observed both in wild-type and in mutant embryos (Fig. 7G,H; compare with Fig. 2P,P’). This finding corroborates the results of the fgf8 mRNA injections and furthermore indicates that the MAP kinase pathway through which Fgf8 exerts its effect on spry4 induction is functional at least at later stages of MHB development in spg embryos. We found normal expression of the known Fgf receptors 1, 3 and 4 at tailbud stage in spg mutants (not shown), suggesting that the pathway is also intact around the normal time of gbx2 and spry4 onset. In summary, analysis of the loss- and gain-of-function experiments in spg and ace embryos suggests that Fgf8 and Pou2 do not act in a simple linear pathway, but genetically act in parallel in a stage- and tissue-specific manner, in order to initiate and maintain the developing MHB (Fig. 8Q).

To examine how specific pou2 function might be for Fgf8, we studied the phenotype of spg/ace double homozygous mutants. At 90% epiboly, spg/ace double mutants embryos show no gbx2 expression, in the same way as ace single mutants (not shown; see also Fig. 5E’). At later stages, however, the double mutants are easily distinguishable, because their MHB and their ear and tail phenotypes are stronger than that of either single mutant. The prospective tectal region is strongly reduced in size, and the otic placodes are extremely small and never develop into otic vesicles (Fig. 8J). In situ analysis shows that in the double mutants, pax2.1 expression is already almost completely abolished at the MHB during early somitogenesis stages, whereas it is still recognizablely expressed in either single mutant embryo (Fig. 8E,H,K). This finding suggests that at later stages, pou2 might also function independently of Fgf8, possibly in conjunction with other Fgfs. Given the often redundant nature of Fgf signaling, a stronger phenotype might arise from a pou2 requirement for mediating the effects of Fgfs other than Fgf8 that are also expressed at the MHB (Reifers et al., 2000a). We therefore compared the double mutant phenotype to the phenotype of embryos where all Fgf signaling is blocked, owing to pharmacological inhibition with SU5402 in a spg/pou2 mutant background (Fig. 8M-P). At the eight-somite stage pax2.1 expression is reduced at the MHB in inhibitor-treated wild-type embryos, resembling pax2.1 expression in ace mutants at the same age (Reifers et al., 1998), although the expression domain is more reduced than in ace mutants (Fig. 8N). Inhibitor treatment of spg mutant embryos, which normally show a dorsally restricted pax2.1 MHB domain at the eight-somite stage, leads to complete abrogation of pax2.1 expression at the MHB (Fig. 8O,P), almost mimicking the

Fig. 8. The double mutant spg/ace shows a more severe brain phenotype (J, living embryo) than each mutant alone (D, G, living embryos). (B,C,E,F,H,I,K,L,M-P) pax2.1 expression. At midsomitogenesis, the MHB expression of pax2.1 is severely reduced and completely missing at pharyngula stages in the double mutant embryos (K,L). (M-P) Phenocopy of the double mutant phenotype produced by blocking Fgf receptors using the inhibitor SU5402. (N,P) spg embryos treated with the inhibitor (+ SU5402, P) reveal strong similarity to spg/ace double mutant embryos, which is reflected by pax2.1 staining (compare P with K; expression of pax2.1 within the otic placode is also strongly reduced by inhibition (S. Léger and M. B., unpublished)). (Q) During the first steps of regionalization of the MHB and the hindbrain, positioning of the MHB is independent of Pou2 (a). During the establishment phase of the MHB organizer, Pou2 is upstream of several cognate MHB markers (b). In the hindbrain primordium, spg/pou2 and ace/fgf8 serve a combinatorial role in initiation of gbx2 and spry4.

spg/ace double mutant phenotype (Fig. 8K). These findings suggest that both additional Fgfs and non-Fgf-dependent pathways contribute to the enhanced phenotype of spg/ace double mutant embryos.

**DISCUSSION**

We have analyzed the function of spg/pou2 during zebrafish brain development, and have found that spg/pou2 is essential for proper development of the mid-hindbrain boundary and hindbrain territories. Our present analysis of early marker genes shows that spg/pou2 functions during the initial establishment of these brain regions, and may also function
during their maintenance, in particular in hindbrain rhombomeres 2 and 4. In addition, Spg/pou2 functions also during development of the forebrain, in particular the diencephalon, and in differentiation of the paraxial mesoderm and endoderm. Most importantly, the results of our cell transplantsations, mRNA injections and bead implantation experiments show that Spg embryos are regionally insensitive to Fgf8 in the early hindbrain neuroectoderm. We therefore suggest that Spg/pou2 encodes the first example of a tissue-specific competence factor for Fgf8 signaling. In the accompanying paper, we show that Spg mutations affect the pou2 gene, an ortholog of mammalian Oct3/Oct4 (Burgess et al., 2002).

**Spg functions during establishment of the MHB- and hindbrain neuroectoderm**

Key molecules that control MHB development, such as Fgf8, Pax2.1 and Wnt1, are already expressed during the earliest, establishment, phase of MHB organizer development (Reifers et al., 1998; Lun and Brand, 1998). Investigation of *no isthmus* (*noi*)/Pax2.1 and *acerebellar* (*ace*)/Fgf8 mutant embryos has revealed that Pax2.1, Fgf8 and Wnt1 define three separate and independent signaling pathways during this initial phase of MHB development at around 80% of epiboly (reviewed by Rhinn and Brand, 2001). During early somitogenesis, these genes become mutually dependent, demarcating the transition from the establishment to the maintenance phase of MHB development. Fgf8 serves a key function both in the hindbrain primordium and during maintenance of the MHB organizer (Reifers et al., 1998; Fürthauer et al., 2001; Raible and Brand, 2001), and the phenotype of Spg/pou2 mutants suggests that the function of Spg/pou2 is closely related, but not identical to that of ace/fgf8.

Given that Pou2 is also expressed maternally and in the preeagula zygote (Takeda et al., 1994; Hauptmann and Gerster, 1995), it was important to determine whether this preeagula expression phase influences the later neuroectodermal function of Spg/pou2. The loss of endodermal Sox17 expression that we have observed in Spg mutants may reflect a Spg/pou2 function at the preeagula-stage, because after the onset of gastrulation, Pou2 is no longer expressed in the endomesoderm. This phenotype requires further examination. Mild Oct4 overexpression in mouse ES cells triggers endoderm and mesoderm differentiation, also suggesting a possible role of this gene in endoderm development (Niwa et al., 2000). Interestingly, Pou-type transcription factors often heterodimerize with HMG-domain proteins (Kamachi et al., 2000). Pou2 might therefore be a binding partner for the zebrafish HMG domain protein Casanova, a crucial regulator of endoderm development and Sox17 expression (Dickmeis et al., 2001; Kikuchi et al., 2001). With respect to neuroectodermal development, the results of the transplantation experiments, together with the expression pattern of Pou2, suggest that the effect on the neuroectoderm is a specific function of Spg/pou2, and not a secondary consequence of altered endodermal development. For MHB development, a crucial event is the positioning of the organizer in the gastrula neuroectoderm. In mice and chick, positioning is reflected in formation of a molecular interface between the Otx2 and Gbx2 genes (Hidalgo-Sanchez et al., 1999; Millet et al., 1999; Broccoli et al., 1999). In zebrafish, this situation is very similar, but not identical, as the function of gbx2 appears to have switched to gbx1 (Lun et al., 2002; Rhinn and Brand, 2001). In this respect, our observation that the Otx2/gbx1 interface is formed normally in Spg mutants is important, as is the finding that expression of fgf8 and Wnt1 is initiated in the correct spatial domain in Spg mutants. Together, this shows that the neuroectoderm is not generally defective in Spg mutants.

Shortly after the initial formation of the Otx2/gbx1 interface (around 70% of epiboly), the gene expression program in Spg mutants becomes specifically abnormal in the MHB and the hindbrain primordium, coincident with the time and place of restricted Pou2 expression in the neuroectoderm. By contrast, anterior neural plate markers such as six3 or otx2 are not only mildly affected in the mutants, consistent with the notion that Spg/pou2 acts specifically within the MHB and hindbrain primordium. The strong reduction in Pax2.1 staining and Wnt1 staining illustrates the function in midbrain development (Fig. 8Q). In fact, given its expression profile and requirement in Pax2.1 activation, Spg/pou2 encodes the first candidate regulator of Pax2.1 expression; this regulation may well be direct, as a functional Pax2.1 promoter fragment contains putative Pou protein binding sites (Picker et al., 2002) (A. Picker and M. B., unpublished). The requirement for early hindbrain development is most clearly seen by the effects on the markers gbx2, fkd3 and spry4, all of which become activated at this stage in the hindbrain primordium. Expression of these marker genes has been clearly linked to Fgf signaling (Chambers and Mason, 2000; Liu et al., 1999; Fürthauer et al., 2001; Darlington, 1999) (K. Lun and M. B., unpublished), further strengthening the case for a relation between Spg/pou2 function and Fgf8 signaling. Given that expression of these genes and Fgf8, Wnt1 and Her5 becomes abnormal from 80% of epiboly onwards, this marks the time when Pou2 first exerts a crucial function in the MHB and hindbrain neuroectoderm. These genes could require Spg/pou2 directly or indirectly for their expression. Many of the gene expression defects we observed at later stages in Spg mutants are also likely to be due ultimately to this early failure to express gbx2, spry4, Pax2.1 and fkd3 (e.g. the reduced Eng2 and eng3 expression is probably due to loss of Pax2.1 expression, since Pax2.1 is absolutely required for eng gene expression) (Lun and Brand, 1998). In summary, our results show that Spg is required for proper development of the MHB organizer and the hindbrain primordium, positively regulating expression of Pax2.1, Krox20, gbx2, fgf8, spry4 and fkd3 at the end of gastrulation.

**Competence to respond to Fgf8 in the early hindbrain requires Spg/pou2**

Fgf8 is expressed in several domains in or around the early neuroectoderm, and the same molecule functions differently in different tissues. The different potential to respond must therefore be encoded by the developmental state or history of the target tissue, referred to as competence to respond in a specific way to an inductive signal, in this case Fgf8. The factors that mediate competence to respond to Fgf8 are so far unknown; our analysis suggests that Pou2 is one such factor. Our transplantation assays revealed that Spg/pou2 is cell autonomously required in the neuroectoderm, in accordance with its expression pattern and the function of Pou2 as a transcriptional regulator. While addressing the mechanism by which Pou2 exerts its effects in the earlier neuroectoderm
through fgf8 mRNA injection and bead implantation assays, we found that spg mutants were regionally insensitive to the effects of fgf8 expression. Providing fgf8 mRNA or Fgf8 protein to spg embryos was not sufficient to restore expression of the target genes gbx2 and spry4 to spg mutants, although other effects that characteristically result from Fgf8 treatment, e.g. doralization, were still evident. Conversely, providing pou2 mRNA back to acerfgf8 mutants, which normally lack gbx2 expression, failed to restore gbx2 and fkd3 expression, although pou2 injections clearly rescued the spg mutant phenotype (Burgess et al., 2002). Together with the results of the spg/ace double mutant phenotype, these data suggest that pou2 and fgf8 do not act in a simple linear pathway that leads to gbx2, fkd3 and spry4 activation, but rather are both required to synergistically activate these genes (Fig. 8Q).

Mechanistically, Pou2 might, for example, require an activating signal that is under the control of Fgf8, such as phosphorylation, for its activity (this signal would be absent in ace mutants) or both a target of the MAP kinase pathway and Pou2 could act together in a transcriptional complex controlling gbx2 expression. Further evidence to support the theory that pou2 is not simply downstream of fgf8 comes from the analysis of embryos where Fgf signaling has been pharmacologically inhibited, and from studying pou2 expression in ace mutants. In both conditions, pou2 expression is initially normal, and becomes only reduced from somitogenesis stages onwards, when maintenance mechanisms start to operate (G. R., unpublished). In summary, initial somitogenesis stages onwards, when maintenance mechanisms is initially normal, and becomes only reduced from mutants and Fgf-inhibitor treated embryos, suggests that signaling pathway that is thought to mediate the effects of Fgf8 expression or dorsalization, suggesting that the MAP kinase pathway that is thought to mediate the effects of Fgf8 is not generally disrupted in the mutants. An obvious possibility is that Spg/Pou2, as a transcription factor, might control expression of some component of the MAP kinase cascade in a tissue-specific manner. However, at least with respect to the Fgf receptors 1, 3 and 4 we have not detected any abnormal expression in the early hindbrain primordium of spg mutants (G. R., unpublished). POU type homeodomain transcription factors assemble into transcription factor complexes that include, for example, ETS type transcription factors that serve to integrate the activity of several signaling pathways, including Fgf signaling (Fitzsimmons et al., 1996; Raible and Brand, 2001). Oct4 specifically forms a complex with Ets2, and thus silences transcription of the tau interferon promoter in trophoderm (Ezashi et al., 2001). An appealing mechanism of action is therefore that Spg/Pou2 might be necessary to form a stable transcription factor complex that serves as a target for Fgf signaling in downstream gene activation or repression.

**spg/pou2 functions during maintenance of the MHB and hindbrain primordium**

The lack of pou2 also has consequences for later stages of MHB development. The requirement of spg during the maintenance phase of MHB development can be subdivided into two aspects. During early somitogenesis stages, spg seems required for MHB development along the entire dorsoventral axis at the prospective MHB, as MHB marker expression is lost from this region in mutant embryos, increasing from ventral to dorsal. This difference may reflect a graded requirement for Fgf signaling along the dorsoventral axis (Reifers et al., 1998; Köster et al., 1997; Carl and Wittbrodt, 1999). In contrast to ace mutants, however, which exhibit a gradual narrowing of MHB markers from dorsal to ventral, MHB gene expression in spg mutants is completely lost from the ventral part, but always remains detectable in a dorsal patch in the dorsal neural tube. Alternatively, this phenotype may be a later consequence of the early failure of MHB- and hindbrain gene expression domains to fuse at the midline that is already evident by the end of gastrulation. Midline marker gene expression, e.g. of shh, is not altered, raising the possibility that perception of midline signals might be affected in spg/pou2 embryos. During pharyngula stages, we observed that MHB markers recover in their expression in the dorsal-most neural tube. Morphologically, this coincides with a partial dorsal re-formation of the isthmic fold, as reflected by expression of otx2 and zath1. This recovery is observed in both weak and strong spg alleles, suggesting that alternative and pou2-independent regulatory mechanism(s) might exist that allow for later induction of dorsal parts of the MHB.

The second aspect of spg/pou2 function during the maintenance period is related to the specific subdomains in the hindbrain that express pou2. From the beginning of somitogenesis until the seven-somite stage, pou2 is expressed specifically in rhombomeres 2 and 4 of the hindbrain (Takeda et al., 1994; Hauptmann and Gerster, 1995; Burgess et al., 2002). In particular, gene expression of krox20, ephA4, wnt8 or val in odd numbered rhombomeres 1, 3 and 5 strongly requires pou2, suggesting that pou2 may act on these rhombomeres in a non-autonomous fashion via a diffusible signal. Notably, fgf8 expression is strongly reduced in r2 and r4, making Fgf8 an excellent candidate for the signal controlled by pou2. The
situation is likely to be more complex, as development of the even-numbered rhombomeres itself becomes abnormal, and signaling molecules like Wnt8 in odd-numbered rhombomeres are also affected. In contrast to odd-numbered rhombomeres, which are reduced in size, r2 and 4 are not, yet they show strong downregulation of pou2 expression. Moreover, at late somitogenesis stages, r4 spatially expands at the expense of r3 and 5, as indicated by hoxb1a staining. Furthermore, we found indications that pou2 is necessary to maintain rhombomeric integrity. This is reflected by the loss of fkd3, a marker for inter-rhombomeric boundaries, and by the strongly reduced distance between r4 and 7, which further illustrates the reduced size of r5. A key question that remains to be addressed is of course to what extent the defects in rhombencephalon development during early somitogenesis stages are due to the specific requirement of spg/pou2 within rhombomere 2 and 4, as might be suspected on the basis of its expression, or due to the failure to express early markers of the hindbrain primordium at the end of gastrulation, as we describe here. Similarly, it will be interesting to determine whether pou2 functions directly in proneural gene activation of ngn1 clusters in the rhombencephalon.

One consequence of the failure to specify the MHB and hindbrain primordia properly in spg/pou2 mutants is that these tissues are not or only partially formed in a pharyngula stage embryo. The actual loss of tissue is probably the result of two very different basic mechanisms. The localized cell death we observe in the forming midbrain and hindbrain during late somitogenesis stages may well be a direct consequence of the earlier mis-specification of these tissues. In addition, however, we also observe that neighboring territories to the most strongly affected areas appear expanded in size. This is particularly noticeable for the posterior forebrain (Fig. 4D,Y,E’) and more weakly also for the anterior hindbrain, and within the hindbrain for the rhombomeres bordering on the most strongly deleted rhombomeres r2 and r4. The processes maintaining the major brain subdivisions are poorly characterized, but seem to require integrity of neighboring brain regions. Posterior forebrain expansion is, for example, also seen in the murine Pax2/Pax5 double mutants (Schwarz et al., 1997) and in the zebrafish noi/pax2.1 mutants (S. Scholpp and M. B., unpublished), which exhibit loss of the midbrain, the MHB and the cerebellum, coupled to a posterior expansion of the rostral pax6 domain and partial fusion with the pax6 hindbrain domain. This may result from the lack of eng2 and eng3 gene expression in the mutants, as misexpression of engrailed-type genes can suppress forebrain development during chick and Medaka development (Araki and Nakamura, 1999; Ristoratore et al., 1999). The lack of MHB expression during mid-late somitogenesis in spg therefore probably contributes to the observed fusion of gene expression domains of forebrain and hindbrain markers as a secondary consequence. Interestingly, however, expansion of forebrain markers is already evident during the establishment phase of the MHB primordium, raising the possibility that pou2 on its own has an active role in suppression of forebrain markers. Better fate maps and proliferation assays will be needed to address this issue further.

Control of totipotency versus differentiation switch

In the accompanying paper (Burgess et al., 2002), we have presented evidence that spg/pou2 is likely to be the zebrafish ortholog of the mammalian Oct3/Oct4/Pou5f1 gene, which is suggested to control totipotency of stem cells, i.e. the inner cell mass or ES cells derived from it, and germline determination (Pesce and Scholer, 2000). We have not yet addressed a possible function in germline development in spg mutants. A brain-specific function of Oct3/Oct4 is not known for the mouse gene; the conventional deletion of this gene causes developmental arrest of mouse embryogenesis around implantation, which has so far precluded studying a possible later role in neural development. Although it is conceivable that the brain specific function was lost in the mammalian lineage, or was secondarily acquired in the teleost lineage, our results argue that this is less likely to be the case. We find that injection of mouse Oct3/Oct4 mRNA into spg mutant zebrafish embryos rescues pax2.1 expression (Fig. 6H, arrow; Table 1), and that in mice, Oct3/Oct4 is strongly expressed throughout the neural plate until day 8.0-8.5 p.c. (Fig. 6F). However, gene expression is not confined to the midbrain-hindbrain area in mice, as it is seen for the zebrafish ortholog pou2. Either Oct3/Oct4 functions in a different way in the mouse neuroectoderm, or not at all, or the mechanism is slightly different. For example, a pairing partner of Oct3/Oct4, e.g. a Sox or Ets protein, could provide the spatial specificity in the mouse neural plate, which would alleviate the need to restrict expression to the midbrain-hindbrain domain in the mammalian lineage. Regardless of the exact evolutionary origin, the phenotype of spg mutants appears more specific than would be expected for a gene that controls totipotency in all embryonic cells (Pesce and Scholer, 2000). In zebrafish, spg/pou2 is clearly shut down in much of the neuroectodermal primordium during early somitogenesis stages, and appears to function as a transcriptional regulator for specific target genes in the cells in which it is expressed. Many, but not all cells either begin or have already undergone a significant differentiation at the time when they still express pou2. Therefore, if spg/pou2 were to perform a similar function in controlling totipotency in zebrafish as in mice, this function would very likely be restricted to a specific, early step of differentiation. Instead of controlling totipotency, spg/pou2/Oct3/Oct4 might serve more generally as a switch that controls the ability to respond to signals like Fgf8, and probably other signals of the Fgf subfamily, that act repeatedly during several developmental decisions. It is interesting to note that Fgfs are also important signals in the initial cell divisions of the mouse embryo (Chai et al., 1998). Accordingly, the decision to follow the embryonic fate, and eventually the germline fate, would be specific to the type of binary decisions controlled by this gene.

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REFERENCES


di-mesencephalic boundary by repressing diencephalic fate. Development 126, 5127-5135.


Lun, K., Röhle, M., Thirse, B., Thirse, C., Amores, A., Yan, Y. L., Postlethwait, J. and Brand, M. (2002). Isolation and characterization of...
zebrafish gbx genes and their function in development of the midbrain-hindbrain boundary organizer, in preparation.


