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spiel ohne grenzen/pou2 is required during establishment of the zebrafish midbrain-hindbrain boundary organizer

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SUMMARY

The vertebrate midbrain-hindbrain boundary (MHB) organizes patterning and neuronal differentiation in the midbrain and anterior hindbrain. Formation of this organizing center involves multiple steps, including positioning of the MHB within the neural plate, establishment of the organizer and maintenance of its regional identity and signaling activities. Juxtaposition of the *Otx2* and *Gbx2* expression domains positions the MHB. How the positional information is translated into activation of *Pax2*, *Wnt1* and *Fgf8* expression during MHB establishment remains unclear. In zebrafish *spiel ohne grenzen* (*spg*) mutants, the MHB is not established, neither isthmus nor cerebellum form, the midbrain is reduced in size and patterning abnormalities develop within the hindbrain. In *spg* mutants, despite apparently normal expression of *otx2*, *gbx1* and *fgf8* during late gastrula stages, the initial expression of *pax2.1*, *wnt1* and *eng2*, as well as later expression of *fgf8* in the MHB primordium are reduced. We show that *spg* mutants have lesions in *pou2*, which encodes a POU-domain transcription factor. Maternal *pou2* transcripts are distributed evenly in the blastula, and zygotic expression domains include the midbrain and hindbrain primordia during late gastrulation. Microinjection of *pou2* mRNA can rescue *pax2.1* and *wnt1* expression in the MHB of *spg/pou2* mutants without inducing ectopic expression. This indicates an essential but permissive role for *pou2* during MHB establishment. *pou2* is expressed normally in *noi/pax2.1* and *ace/fgf8* zebrafish mutants, which also form no MHB. Thus, expression of *pou2* does not depend on *fgf8* and *pax2.1*. Our data suggest that *pou2* is required for the establishment of the normal expression domains of *wnt1* and *pax2.1* in the MHB primordium.

Key words: Hindbrain, MHB, Midbrain, Isthmus, *engrailed*, *fgf8*, *gbx2*, *otx2*, *pax2.1*, *spg*, *wnt1*, POU domain, *Danio rerio*

INTRODUCTION

Regionalization of the vertebrate nervous system involves the establishment of local organizing centers that serve as signaling sources for patterning and neuronal differentiation. The MHB, or isthmic organizer, is located at the junction of midbrain and hindbrain. During development, the MHB organizer secretes factors that regulate anteroposterior patterning and specification of the neighboring tectum and cerebellum. The organizing activity of the MHB was initially identified by its ability to induce mes- and metencephalic development when transplanted into the caudal diencephalon (Martinez et al., 1991; Marin and Puelles, 1994). FGF8 was later shown to be capable of mimicking the activities of isthmic transplants in the diencephalon, revealing that FGF8 can induce isthmic organizer formation and mediate some of its patterning activities (Crossley et al., 1996).

Formation of the MHB organizer involves discrete steps, including (1) anteroposterior patterning providing positional information to place the MHB within the neural plate, (2) establishment of the MHB during late gastrulation, and (3) maintenance of its regional identity and signaling activities during further development (Joyner at el., 2000; Rhinn and Brand, 2001). Recent studies have begun to elucidate how these steps are accomplished. Transplantation experiments in chick have shown that juxtaposition of midbrain and hindbrain tissues is sufficient to generate a MHB (Irving and Mason, 1999). At a molecular level, this interface is reflected by the expression boundaries of *Otx1/2* and *Gbx2*, which are the first genes known to be expressed in a restricted manner in the

mid-/hindbrain. Analyses of mutant mouse embryos showed that the *Otx2/Gbx2* border specifies the position of the MHB organizer (Wassarman et al., 1997; Millet et al., 1999; Broccoli et al., 1999; Joyner et al., 2000; Rhinn and Brand; 2001). How positional information provided by the *Otx2/Gbx2* expression border leads to MHB establishment remains unclear. As the anterior notochord of both *Xenopus* and chick (Hemmati-Brivanlou et al., 1990; Darnell and Schoenwolf, 1997) and the anterior mesendoderm in mouse (Ang and Rossant, 1993) can induce expression of *En2*, vertical signals from axial mesoderm underlying the mes-metencephalic primordium have been suggested to contribute to MHB-specific gene expression. In zebrafish, however, mutant embryos that completely lack axial mesoderm still form a MHB, arguing that planar signals within the neurectoderm may be sufficient for its induction (Rhinn and Brand, 2001).

From late gastrulation onwards, *Fgf8*, *Wnt1*, *Pax2/5* and the *Engrailed* genes are expressed in the MHB region (Wassef and Joyner, 1997; Joyner et al., 2000). *Wnt1* is expressed in the midbrain just anterior to the *Fgf8* domain in the hindbrain. *Pax2/5* and the Engrailed genes are expressed in the mid-/hindbrain region surrounding the isthmus. Gain-of-function studies have shown that misexpression of *En1/2* or *Pax2/5* in the posterior forebrain of chick or fish results in ectopic expression of MHB genes, including *Fgf8*, and induction of ectopic MHB development (Araki and Nakamura, 1999; Funahashi et al., 1999; Okafuji et al., 1999; Ristoratore et al., 1999). Loss-of-function studies in mouse and zebrafish have demonstrated that *Fgf8*, *Wnt1*, *Pax2* and *En1* are required for normal mid-/hindbrain development (McMahon and Bradley, 1990; Urbanek et al., 1994; Wurst et al., 1994; Brand et al., 1996; Schwarz et al., 1997; Lun and Brand, 1998; Meyers et al., 1998; Reifers et al., 1998).

Work in mice and zebrafish indicates that at least three parallel pathways are activated during MHB establishment, involving *wnt1*, *pax2.1* and *fgf8* (McMahon et al., 1992; Lun and Brand, 1998). *wnt1* and *pax2.1* appear to be activated independently in partially overlapping domains, and *wnt1* expression in *noi* mutants is unaltered until early somitogenesis (Lun and Brand, 1998). In addition, *Pax2* is activated normally in *Wnt1* mouse mutants (McMahon et al., 1992; Rowitch and McMahon, 1995). In zebrafish, the initial expression of *fgf8* in the anterior hindbrain does not overlap with that of *pax2.1* or *wnt1* (Reifers et al., 1998), while in mice the initial *Fgf8* expression at the MHB, but not that of *Wnt1* and *En2*, depends on *Otx1* and *Otx2* gene dosage (Acampora et al., 1997). During somitogenesis, *Fgf8*, *Pax2*, *Wnt1*, and *En1/2* engage in a regulatory loop that maintains the MHB and its signaling activities during further development (Lun and Brand, 1998; Reifers et al., 1998; Broccoli et al., 1999; Joyner et al., 2000; Rhinn and Brand, 2001).

In this report, we investigate the role of *spiel ohne grenzen* (*spg*) (Schier et al., 1996) during zebrafish MHB organizer development. *spg* mutant embryos develop patterning defects, including a deletion of the isthmic region and cerebellum, and a reduction of the tectum. We have identified *pou2*, which encodes a POU-domain transcription factor (Takeda et al., 1994; Hauptmann and Gerster, 1995), as the gene affected by *spg* mutations. *pou2* is transiently expressed during late gastrulation and early somitogenesis in the midbrain and anterior hindbrain primordia. We have investigated the effects of a new amorphic *spg* mutation, spg^{m793}, on MHB formation. We find that expression of *otx2*, *gbx1* (functional homolog of mouse *gbx2* in zebrafish), and *fgf8* is initiated normally in *spg* mutants, while MHB expression of *wnt1* and *pax2.1* is reduced. We propose that *pou2* is required to achieve the *pax2.1* and *wnt1* expression necessary for MHB organizer establishment during late gastrulation.

MATERIALS AND METHODS

Genetics and strains

The *spg* alleles spg^{m216} and spg^{m308} have previously been briefly described (Schier et al., 1996). The new spg^{m793} allele was isolated following mutagenesis of AB strain fish with ethyl-nitrosourea (Artinger et al., 1999). Other zebrafish mutations used were *acerebellar* (ace^{ti282a}) and *no isthmus* (noi^{tu29a}), a null allele of *noi* (Brand et al., 1996).

Molecular identification of *spg* mutations

To determine genetic linkage, an spg^{m793}/+ male (G0) was crossed with an India (IN) strain female. Gynogenetic diploid F2 progeny were obtained from spg^{m793}/+ F1 fish by the 'early pressure' technique (Westerfield, 1994). A panel of 40 spg^{m793} mutant F_2 embryos was typed with centromere linked SSLP markers (Knapik et al., 1998). *spg* was localized to linkage group 21. A map panel of 2418 spg^{m793}/spg^{m793} F_2 embryos was generated for high resolution genetic mapping, and the *spg* locus was placed between the SSLP marker Z12068 and Z21718 in the proximity of an AFLP marker (Vos et al., 1995) named 'CCA/C'. The 'CCA/C' marker was used to isolate PACs from an arrayed PAC library. Three PAC clones were identified that span the entire *spg* genomic region: PAC A, BUSMP706B14149; PAC B, BUSMP706M22111; and PAC C, BUSMP706E23115. *pou2* sequences were identified in both PAC A and PAC B. DNA encoding *pou2* was isolated from genomic DNA (AB strain, India strain, spg^{m793}, spg^{m308} and spg^{m216}) and from cDNA generated from 30% epiboly stage embryos. Sequencing of the PCR products revealed separate point mutations that generate restriction polymorphisms in the *pou2* transcribed region of each allele: spg^{m793}, *Mse*I site absent; spg^{m308}, *Bfa*I site absent; and spg^{m216}, *Xba*I site absent. These polymorphisms were scored on 4% metaphor agarose gels (BioWhittaker, Rockland, MD) to verify the presence of the mutations predicted by sequence analysis (Fig. 2D) and for genotyping experimental embryos.

The following *pou2* antisense morpholino (GeneTools, Corvallis USA) was used: 5′-CGCTCTCTCCGT CAT CTTTCCGCTA-3′ (start ATG underlined). A four mismatch control oligo 5′-CGGT-CTGTCCGTCATCTATCCCCTA-3′ was used to assess specificity of the knock-down phenotype.

Gene expression analysis

Whole-mount in situ hybridization was performed to visualize gene expression (Hauptmann, 1999). Control embryos denoted wild type in the figures are phenotypically wild-type siblings of mutant embryos shown in the same experiment. The genotype of *noi* mutant embryos was determined by assaying for reduced expression of *eng2* (Brand et al., 1996). cDNAs used have been described (see references in text) except for zebrafish *gbx1*, which is the functional homolog of mouse *Gbx2* (Rhinn and Brand, 2001). While zebrafish *gbx1* has greater sequence similarity to mouse *Gbx1* than it does to mouse *Gbx2* (Bulfone et al., 1993; Rhinn and Brand, 2001) (B. Thisse and C. Thisse, unpublished), the expression of zebrafish *gbx1* more closely resembles that of mouse *gbx2* during late gastrulation.

Overexpression of *pou2* by mRNA microinjection into embryos

The *pou2* cDNA (Hauptmann and Gerster, 1995) was subcloned into

the pCS2+ vector and transcribed using the Sp6 MessageMachine (Ambion). In vitro synthesized mRNA was dissolved at 2 to 40 ng/μl in water and microinjected into one-cell stage embryos. The actual amounts (2-40 pg) injected into embryos were estimated from the injection volume visualized.

RESULTS

Mutations in *spg* disrupt MHB formation

spg mutant embryos are easily identified by the absence of the isthmic constriction which normally demarcates the position of the MHB (Fig. 1) (Schier et al., 1996; Driever et al., 1997). During a recent mutagenesis screen, we identified an allele of *spiel ohne grenzen* (*spg*^{m793}; Fig. 1K) with more severe defects than the previously reported alleles *spg*^{m216} (Fig. 1F) and *spg*^{m308} (not shown; phenotype similar to *spg*^{m216}). *spg*^{m793} mutants develop morphological defects in three major parts of the body.

(1) Mid-/hindbrain: The tectum is reduced in anteroposterior extent and develops an atypical globular morphology. The anteroposterior extent of the ventral midbrain is similarly reduced. The isthmic constriction is absent, and a visually discernable cerebellum does not form. Correspondingly, the distance between eye and ear is reduced by about one-third.

(2) Rhombencephalon and ear: Morphological aspects of segmentation are disturbed and the hindbrain is shorter. The otic vesicle is round instead of oval-shaped and often has only a single otolith (hindbrain segmentation abnormalities seen in *spg* mutants are analyzed in a separate manuscript (Hauptmann et al., unpublished)).

(3) Trunk and tail: Defects are observed during tail development, somite morphology is abnormal, and the number of primary neurons in the spinal cord is reduced (data not shown). We consider these multiple phenotypes to be pleiotropic activities of *spg*, mediated by independent expression domains of *spg/pou2* (see below).

Analysis of gene expression in the MHB region of *spg*^{m793} mutants supports the findings from morphological observation of living embryos. At 24 hpf, *otx2* is expressed in parts of the diencephalon and the midbrain including the tectal primordium (Fig. 1B) (Li et al., 1994). In *spg*^{m216} mutants (Fig. 1G), we observed a slight reduction of the midbrain; while in *spg*^{m793} mutants (Fig. 1L), *otx2* expression indicates that the caudal tectum lies above the rostral hindbrain. *fgf8* is expressed in a thin stripe in the MHB at 24 hpf (Fig. 1C) (Fürthauer et al., 1997; Reifers et al., 1998). In *spg*^{m216} mutants (Fig. 1H), *fgf8* expression is reduced to a small patch within the dorsal MHB. Isthmic *fgf8* expression is completely absent in *spg*^{m793} mutants (Fig. 1M). The POU-domain transcription factor *zp50* is expressed at high levels in the cerebellum (Fig. 1D) (Hauptmann and Gerster, 1996). In *spg*^{m216} mutants, the *zp50* expression domain in the cerebellum is still present, albeit reduced in size (Fig. 1I). No cerebellar expression of *zp50* is found in *spg*^{m793} mutant embryos (Fig. 1N). Thus, in the absence of a cerebellum, the tectum reaches the hindbrain ventricle in *spg*^{m793} mutants. In agreement with our morphological observations, the expression of *otx2*, *fgf8*, and *zp50* indicate that *spg*^{m793} is a stronger allele than *spg*^{m216}.

In the absence of pronounced morphological landmarks in the ventral MHB region, the gap between the discrete expression domains of the transcription factor *zash1a* in the posterior tegmentum (ventral midbrain) and the basal rhombomere 1 (r1) is a useful marker of ventral isthmic territories (Fig. 1E) (Allende and Weinberg, 1994). In both *spg*^{m216} and *spg*^{m793} mutants, these *zash1a* expression domains close in on each other, indicating the absence of the ventral portion of the MHB region (Fig. 1J,O).

pou2 is mutated in *spiel ohne grenzen*

To identify the gene affected in *spg*, we first mapped *spg* to LG21 and identified molecular markers tightly linked to the locus (Fig. 2B). Fine mapping of the *spg* genomic region indicated that the *pou2* gene (Fig. 2A), previously mapped to LG 21 (Postlethwait et al., 1998), is contained within the *spg* interval (Fig. 2B). We sequenced genomic DNA from three *spg* alleles, *spg*^{m793}, *spg*^{m308} and *spg*^{m216}, and found a single point mutation within the transcribed region of *pou2* in each allele (Fig. 2C,D). In *spg*^{m793}, the splice acceptor site of exon 2 contains a transition from A to G. Aberrant splicing at this site leads to a shift in the reading frame resulting in a stop codon at the sixth codon downstream of the splice acceptor site. This generates an open reading frame that lacks both the POU-specific domain and the POU-homeodomain. The aberrant splice product was verified by sequencing cDNA from *spg*^{m793} mutant embryos. The mutation in *spg*^{m308} is an A-to-T transversion in the splice acceptor site of exon 5. In these mutants, splicing of *pou2* occurs at an alternative splice site located further downstream. An identical naturally occurring splice variant of *pou2* has been previously described (Takeda et al., 1994); it disrupts the homeodomain and renders the *pou2* protein unable to bind the canonical octamer POU-binding motif (Takeda et al., 1994). The homeobox is also affected in *spg*^{m216}, where a transition from T to A replaces a leucine with a proline at position 16 in helix 1 of the homeodomain. This leucine is highly conserved among homeodomain proteins (Scott et al., 1989) and is thought to interact with hydrophobic amino acid residues of helix 3 that contact the DNA target sequence (Qian et al., 1989). As proline residues break the secondary structure of α-helices, this mutation is likely to disrupt the three-dimensional structure of the *pou2* homeodomain. Morphologically, *spg*^{m216} and *spg*^{m308} mutants closely resemble each other and display a milder phenotype compared with *spg*^{m793} mutants (Fig. 1; also data not shown). The residual activity of these alleles is likely mediated through the POU-specific domain still present in these proteins.

Expression of *pou2* in wild-type and mutant embryos

pou2 is a maternally expressed gene and through mid-gastrulation *pou2* mRNA is widely distributed in the blastoderm (Fig. 3A) (Takeda et al., 1994; Hauptmann and Gerster, 1995). The earliest regionalized zygotic expression of *pou2* is seen at 80% epiboly adjacent to the midline and in an area corresponding to the midbrain and hindbrain primordia (Fig. 3B). By tailbud stage (Fig. 3C), *pou2* expression has condensed into a butterfly shape, with a lower level of expression in the prospective midbrain and a higher level in the anterior hindbrain. As *pax2.1* expression in the midbrain (Krauss et al., 1991) co-localizes with the lower level of *pou2* expression (Fig. 3D), the border between low and high levels of *pou2* expression is located at or near the MHB.

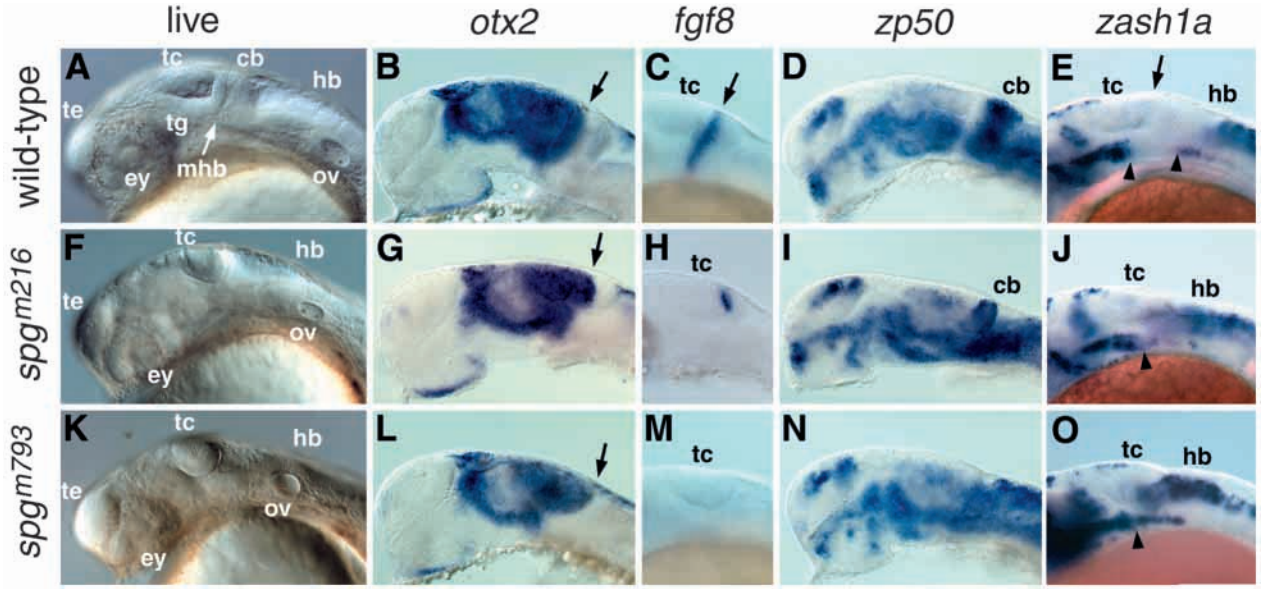

Fig. 1. Mutations in *spiel ohne grenzen* (*spg*) affect MHB formation. (A-E) Wild-type, (F-J) *spg*^{m216} and (K-O) *spg*^{m793} mutant live (A,F,K) or whole-mount embryos at 24 hours post fertilization (hpf). (B,G,L) *otx2* expression in the pretectum and tectum; (C,H,M) *fgf8* expression at the midbrain-hindbrain boundary; and (D,I,N) strong *zp50* expression in the cerebellum. *zp50* is expressed in a complex pattern in all major subdivisions of the brain. (E,J,O) *zash1a* expression in the tegmentum and ventral hindbrain. Arrows indicate the position of MHB. Arrowheads indicate the limits of *zash1a* expression in the tegmentum and ventral rhombomere 1. In all embryos, anterior is towards the left and dorsal is upwards. cb, cerebellum; ey, eye; hb, hindbrain; mhb, midbrain-hindbrain boundary; ov, otic vesicle; tc, tectum; te, telencephalon; tg, tegmentum.

pou2 expression in *spg*^{m216} mutant embryos resembles that of wild-type embryos during gastrulation (data not shown). By the one-somite stage, however, the pattern of *pou2* expression is slightly altered in that the chevron shaped expression domains in prospective rhombomere 2 and 4 seen in the wild type (Fig. 3D) appear more diffuse (Fig. 3I). By contrast, *pou2* mRNA levels are strongly reduced in *spg*^{m793} mutants from as early as 50% epiboly onwards (compare Fig. 3A with 3F). *pou2* expression is further diminished by the end of gastrulation in *spg*^{m793} mutants (Fig. 3C,H) and could not be detected during somitogenesis stages (data not shown). The absence of *pou2* RNA in *spg*^{m793} mutant embryos may be due to instability of the aberrant splice product, and the *pou2* message detected early may predominantly correspond to maternal transcripts. Both the truncated Pou2 protein predicted in *spg*^{m793} mutants, and the lack of *pou2* expression at the end of gastrulation, suggest that *spg*^{m793} acts as a null allele with complete absence of zygotic *pou2* activity.

To determine whether the maternal contribution may modify the *spg* MHB phenotype, we injected a *pou2*-morpholino antisense oligonucleotide (Nasevicius and Ekker, 2000) to repress translation of maternal message. Injection of about 100 pg per embryo resulted in reduction or absence of *pax2.1* expression at three- to six-somite stages, while *emx1* (Patarnello et al., 1997) expression in the forebrain appeared normal (data not shown). This is similar to the phenotype observed in zygotic *spg*^{m793} mutants. At high concentrations (approx. 1 ng/embryo), we observed arrest of gastrulation (data not shown). Our data point at separate functions of *pou2* during early gastrulation and MHB development. In zygotic *spg* mutants, maternal contribution of wild-type *pou2* message appears to rescue gastrulation defects, while MHB development depends on zygotic gene function.

Expression of MHB patterning genes in *spg* mutants

In wild-type embryos, *wnt1* is expressed from 90-100% epiboly in the midbrain and MHB primordium (Fig. 4A,B) (Molven et al., 1991). In *spg*^{m793} mutants, *wnt1* expression is activated at 90-100% epiboly, but only at a reduced level (Fig. 4E,F). The anteroposterior extent of the bilateral *wnt1* expression domains is reduced, with the greatest reductions in medial regions. *wnt1* expression in the MHB primordium gradually ceases during somitogenesis, while expression along the dorsal midbrain and neural tube is maintained (Fig. 4G,H).

In wild-type embryos, *pax2.1* expression is activated in the MHB primordium at about 80-90% epiboly in two lateral domains (shown at 90-100% epiboly, Fig. 4I) (Krauss et al., 1991). By the end of gastrulation, the bilateral *pax2.1* domains have merged at the midline (Fig. 4J). In *spg*^{m793} mutants, *pax2.1* expression appears nearly absent from the medial neural plate (Fig. 4M,N). The anteroposterior extent of the remaining lateral patches of *pax2.1* expression is shorter than normal, and the expression level is strongly reduced. Similar to that of *wnt1*, *pax2.1* expression in the MHB primordium of *spg*^{m793} mutants ceases during mid-somitogenesis (Fig. 4O,P).

Changes in the expression of the *engrailed* genes in *spg*^{m793} mutants are similar to those seen in *pax2.1* expression. In wild-type embryos, *eng2* expression is initiated in bilateral domains at 90-100% epiboly (Fig. 4Q) that merge during early somitogenesis (Fig. 4R,S). At the five-somite stage, *eng2* is expressed in the MHB primordium and reaches into the posterior portion of the midbrain (Fig. 4S). In *spg*^{m793} mutants, initial *eng2* expression is detectable only at reduced levels and in smaller domains corresponding to the reduced *pax2.1* domains (Fig. 4U,V). At the five-somite stage, only a small dorsal expression domain of *eng2* remains in the midbrain (Fig.

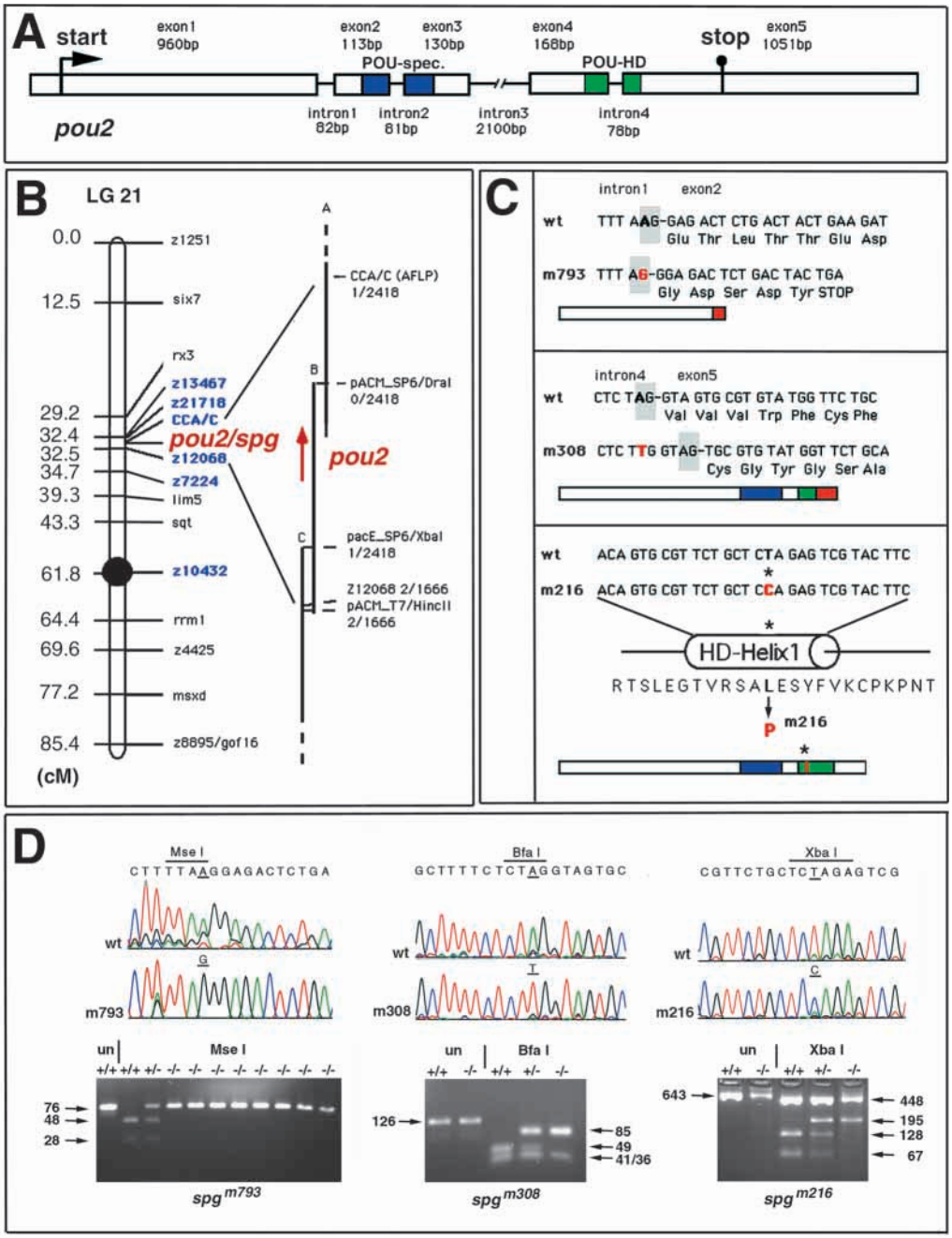

Fig. 2. The *spiel ohne grenzen* mutations are linked to point mutations in *pou2*. (A) Genomic structure of the transcribed region of the *pou2* locus. The coding region is interrupted by four introns. Translational start site and stop codon are indicated. The POU-specific domain is shown in blue, and the POU-homeodomain is shown in green. (B) Mapping of the *spg* locus, and demonstration of tight linkage to the *pou2* gene. Molecular markers used for meiotic mapping are indicated in blue. PACs isolated in the genomic walk are designated A, B and C (see Materials and Methods). Recombination frequencies of the polymorphic markers used are indicated. The map positions of the markers are from the Stanford HS panel (Kelly et al., 2000), except for Z13467 (six recombinants/168 meioses), Z21718 (2/556), Z12068 (2/1666) and Z7224 (21/556), which are from the MGH panel (Shimoda et al., 1999). Z21718 maps on the HS panel at 33.1 cM. (C) Putative Pou2 protein products generated by *spg* mutant alleles. Wild-type and mutant proteins are shown at top and bottom, respectively. Foreign sequences, generated by frameshift, are indicated in red. The asterisk shows the position of the missense mutation in helix 1 of the homeodomain in *spg*^{m216}. The splice acceptor sites in *spg*^{m793} and *spg*^{m308} are highlighted in gray. (D) Top: sequence comparison of *pou2* genomic DNA from wild-type embryos and mutants. Mutated positions are underlined. Bottom: genotype analysis of single embryos. Each *spg* mutation eliminates a restriction site. As expected, these restriction site polymorphisms segregate with the mutant phenotypes. Genotypes are indicated by '+' (wild type) and '-' (mutant). wt, wild type; un, undigested PCR product.

4W). At 1 dpf, *eng2* expression can no longer be detected in the midbrain or the MHB region of *spg*^{m793} mutants (Fig. 4X). Expression of *eng1* and *eng3* is similarly affected in *spg*^{m793} mutants (data not shown). The reduced size of the *engrailed* expression domains in *spg*^{m793} mutants may account for the smaller size of the tectum observed at 1 dpf (Fig. 1K). Our analysis indicates that *spg/pou2* function is required during MHB establishment in late gastrulation. As the initial expression of *pax2.1* and *wnt1* is reduced in the MHB primordium in *spg*^{m793} mutants, *spg/pou2* may be involved in the activation of *pax2.1* and *wnt1* expression. To further analyze the epistatic relationship between *pou2* and *pax2*.1, we examined the expression of *pou2* in *noi/pax2.1* mutant embryos (Fig. 3E,J), and found that it was normal. These findings suggest that *pou2* is required upstream of *pax2.1* for MHB establishment.

Overexpression of *pou2* can rescue the *spg* MHB phenotype

To test whether wild-type *pou2* mRNA can rescue the gene expression defects at the MHB in *spg* mutants, we injected *pou2* mRNA into wild-type and *spg*^{m793} mutant embryos at the one-cell stage and assayed the expression of *pax2.1* during early somitogenesis (Fig. 5; Table 1). While injection of 2 to 5 pg of

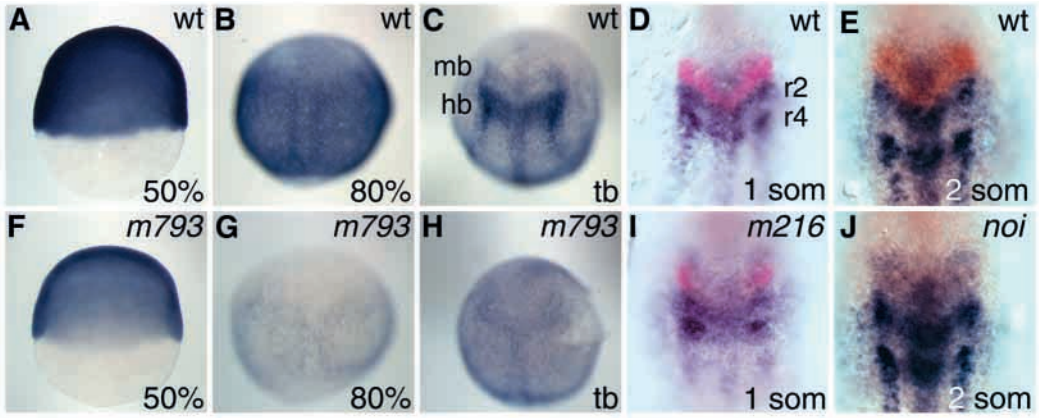

Fig. 3. *pou2* expression in wild-type, *spg* and *noi* mutant embryos. Expression of *pou2* in wild-type (A-E), *spg*^{m793} (F-H), *spg*^{m216} (I), and *noi*^{tu29a} (J) mutant embryos. MHB expression of *pax2.1* (red; D,I) or *eng2* (orange; E,J) relative to that of *pou2* (blue). Dorsal (B-E,G-J) or lateral (A,F) views of whole-mount embryos with animal pole/anterior at the top; (D,E,I,J) dorsal views of the neural plate at higher magnification. Embryonic genotypes are indicated in the top right-hand corner of each image; developmental stages are indicated in the bottom right-hand corner. hb, hindbrain; mb, midbrain; %, % epiboly; tb, tailbud stage; som, somites (stage).

pou2 mRNA did not result in significant rescue of gene expression, some *pax2.1* expression was seen in the MHB region of *spg*^{m793} mutants after injection of 10 pg of *pou2* mRNA. Injection of 20 or 40 pg of *pou2* mRNA was able to rescue *pax2.1* MHB expression, and thus MHB establishment, in most mutant embryos (Fig. 5C; Table 1). Occasionally, we observed malformed embryos (eight out of 117 embryos), that showed various degrees of disorganization, broadening of the neural plate and blisters in the trunk region. These phenotypes are consistent with the interference of high doses of *pou2* with gastrulation processes in the early embryo (Takeda et al., 1994). We also assayed injected embryos for the expression of *pax2.1* and *wnt1* at 24 to 26 hpf (Fig. 5F,I). In five out of seven injected *spg*^{m793} mutants, expression of *pax2.1* and *wnt1* was very similar to or indistinguishable from that of wild-type embryos. Thus, overexpression of *pou2* mRNA injected at the one-cell stage can lead to stable expression of MHB-specific genes.

pax2.2/5/8 expression is absent from the MHB in *spg*^{m793} mutants

In wild-type zebrafish embryos, *pax2.2*, *pax5*, and *pax8* are expressed in the MHB onwards from their activation between

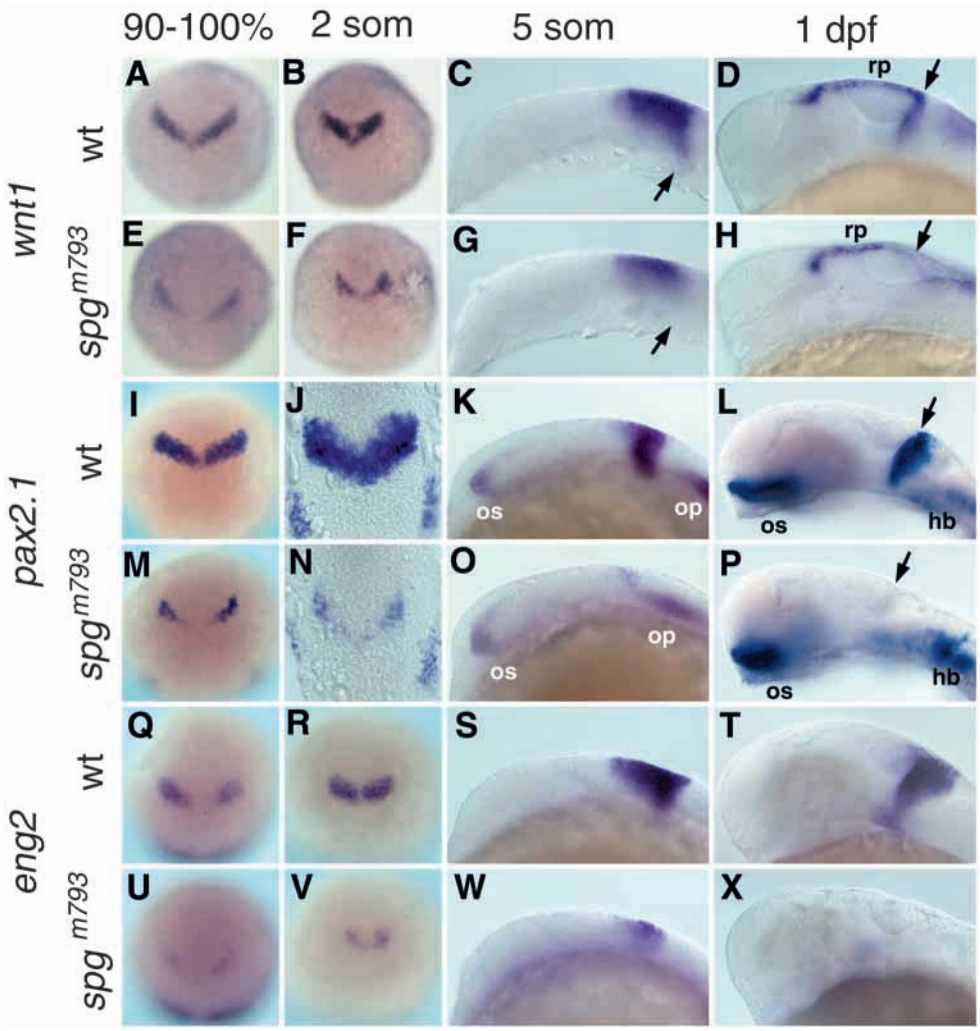

Fig. 4. Expression of MHB genes is reduced in *spg*^{m793} mutants. Expression of *wnt1* (A-H), *pax2.1* (I-P) and *eng2* (Q-X) in wild-type and *spg*^{m793} mutant embryos. Embryonic genotypes are indicated at left, developmental stages, above. 90-100% epiboly and the two-somite stage embryos are shown in dorsal views, anterior/animal pole is at top. 90-100% epiboly stage embryos were genotyped by allele-specific PCR (see Materials and Methods). Five-somite stage and 1 dpf embryos are depicted in lateral views of the head region, anterior towards the left and dorsal upwards; images are focused at a mid-sagittal plane. 90%-100%, 90%-100% epiboly; som, somite (stage); hb, hindbrain; os, optic stalk; op, otic placode; rp, roof plate.

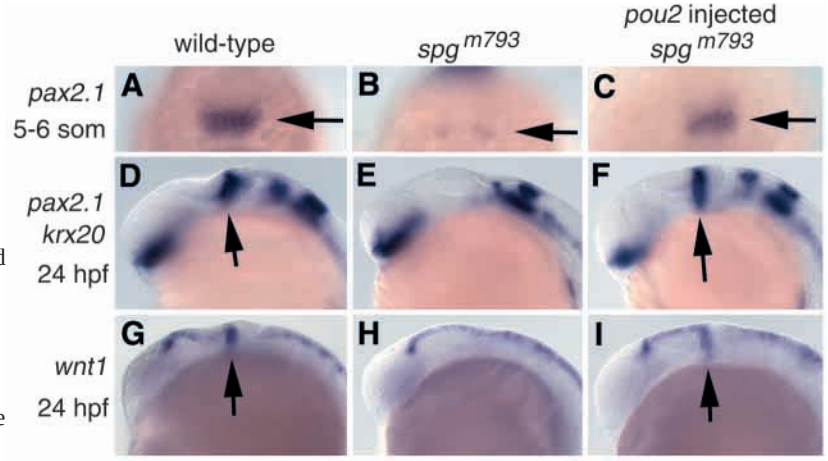

Fig. 5. Rescue of the *spg* mutant phenotype by *pou2* mRNA injection. Wild-type and *spg*^{m793} mutant embryos were injected at the one-cell stage with *pou2* mRNA, fixed at the five- to six-somite stage (A-C) or at 24 hpf (D-I), and assayed for the expression of *pax2.1* (A-C), *pax2.1* and *krx20* (D-F), or *wnt1* (G-I). All embryos shown were genotyped by allele-specific PCR. Arrows indicate the position of the MHB. Orientation: (A-C) dorsal views, anterior at the top; (D-I) lateral views, anterior towards the left.

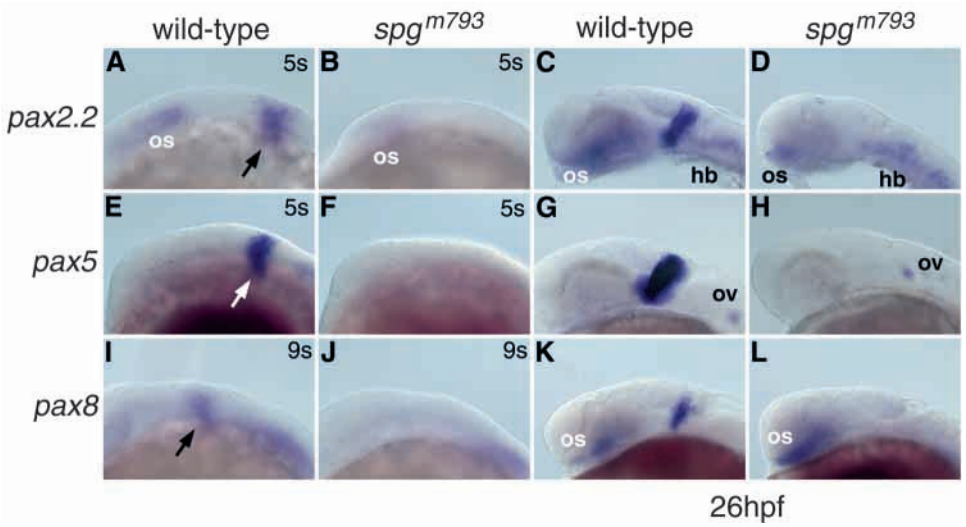

Fig. 6. Expression of *pax2.2*, *pax5* and *pax8* is absent from the MHB region of *spg*^{m793} mutant embryos. Expression of *pax2.2* (A-D), *pax5* (E-H) and *pax8* (I-L) at the five-somite stage (A,B,E,F), the nine-somite stage (I,J) and at 26 hpf (C,D,G,H,K,L) is shown. *pax8* expression is first detected in wild-type embryos at the nine-somite stage (Pfeffer et al., 1998). Embryonic genotypes are indicated at top. Embryos are shown in lateral view, anterior towards the left and dorsal upwards. hb, hindbrain; os, optic stalk; ov, otic vesicle; s, somite (stage). Arrows indicate position of MHB.

Table 1. Rescue of *spg*^{m793} mutants by injection of *pou2* mRNA

	Amount of *pou2* mRNA injected (approx.)	Total number of embryos	MHB gene expression		% rescued (calculated)
			pax2.1 expressed	Very weak or no *pax2.1*	
Assayed at five to six somites	40 pg	117*	116 (99%)	1 (1%)	96
	20 pg	99	96 (97%)	3 (3%)	88
	10 pg	65	56 (86%)	9 (14%)	44
	5 pg	62	56 (90%)	6 (10%)	60
	2 pg	60	50 (83%)	10 (17%)	32
	None	59	43 (73%)	16 (27%)	0
Assayed at 24 hpf	40 pg	124‡	121 (98%)	3 (2%)	90
	20 pg	116	110 (95%)	6 (5%)	80
	10 pg	64	60 (94%)	4 (6%)	76
	5 pg	52	45 (87%)	7 (13%)	48
	2 pg	64	48 (75%)	16 (25%)	0
	None	56	42 (75%)	14 (25%)	0

Heterozygous *spg*^{m793} parents were crossed, and progeny embryos at the one-cell stage were injected with *pou2* mRNA. Non-injected embryos served as experimental controls. The actual number and calculated percentage of embryos that expressed *pax2.1* in the MHB, and the percentage of embryos with the *spg* mutant phenotype of little or no *pax2.1* expression, are shown for each experimental condition. A fraction of less than 25% of the embryos showing weak or no *pax2.1* expression in the MHB indicates rescue of mutant embryos.

The right-most column shows the percentage of rescued embryos calculated from the difference between the expected Mendelian ratio of *spg/pou2* mutants and the observed fraction of injected embryos in which defective *pax2.1* expression was observed. Rescue of *spg*^{m793} embryos was also determined by allele-specific PCR (Figs 2, 5).

*Eight of these embryos showed gastrulation defects or broadening of neural plate associated with expansion of the *pax2.1* expression domain.

‡Two of these embryos showed broadening of the brain, and five embryos showed reduction in the optic stalk, as assessed by expression of *pax2.1*.

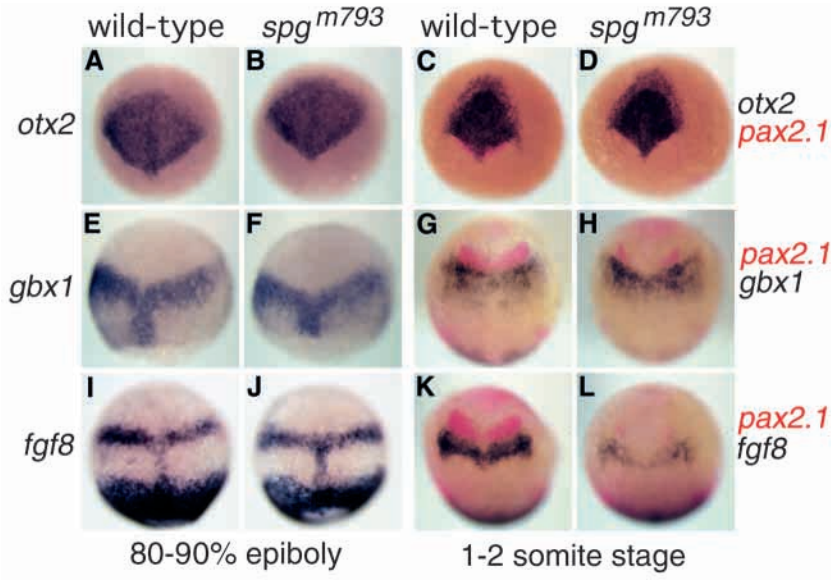

Fig. 7. Expression of *otx2* and *gbx1* and initial expression of *fgf8*, appear normal in *spg*^{m793} mutant embryos. Expression of *otx2*, *gbx2*, *fgf8* (dark blue), and *pax2.1* (red) in wild-type and *spg*^{m793} mutant embryos. Embryonic genotypes are indicated at the top; the genes for which expression is shown are on the left and right; and embryonic stages are indicated below. The genotype of embryos shown in A,B,E,F,I,J was determined by allele-specific PCR. Embryos are viewed from the dorsal side, anterior towards the top.

the 5- and 9-somite stages (Pfeffer et al., 1998). In *noi/pax2.1* mutants, *pax5* and *pax8* are not activated in the MHB region, while *pax2.2* is activated, albeit in a smaller domain. In *spg*^{m793} mutants, *pax5* and *pax8* expression is absent from the MHB primordium, although these genes are expressed normally in other regions (e.g. optic stalks, ear; Fig. 6F,H,J,L). This suggests a requirement of *spg/pou2* for MHB expression of these genes, which is probably mediated through *pax2.1*. In contrast to *noi/pax2.1* mutants, *pax2.2* expression is absent from the MHB region of *spg*^{m793} mutants (Fig. 6B,D), suggesting that *spg/pou2* is required for the normal activation of both *pax2.1* and *pax2.2* genes in zebrafish.

Initiation of *otx2, gbx1 and fgf8* expression is independent of *spg/pou2*

As mouse *Otx2* and *Gbx2* position the MHB, we also examined expression of their homologs in *spg* mutants. In wild-type mid-gastrula embryos, *otx2* is expressed in the forebrain and midbrain primordia (Fig. 7A,C) (Li et al., 1994). Zebrafish *gbx1* (the functional equivalent of mouse *Gbx2*) (Rhinn and Brand, 2001) is expressed directly posterior to the *otx2* domain in the anterior hindbrain primordium with the shared *otx2*/*gbx1* expression boundary marking the position of the future MHB (Fig. 7E,G). In mice and chick, FGF8 has been suggested to maintain a metencephalic identity by activating *gbx2* and repressing *otx2* (Liu et al., 1999; Martinez et al., 1999). Similar to *gbx1*, *fgf8* is also activated in the prospective anterior hindbrain at 70% epiboly (Fig. 7I,K) (Lun and Brand, 1998). During gastrulation in *spg*^{m793} mutants, expression of *otx2* (Fig. 7B), *gbx1* (Fig. 7F) and *fgf8* (Fig. 7J) appears normal. Thus, *pou2* is not required for the initial expression of *otx2*, *gbx1* and *fgf8*. Subsequently, however, at the beginning of somitogenesis, *fgf8* expression at the MHB fades (Fig. 7L), while expression of *otx2* and *gbx1* still appears normal (Fig. 7D,H). As early *fgf8* expression is independent of *spg/pou2*, we assayed *pou2* expression in *ace/fgf8* mutants to see if *fgf8* acts upstream of *pou2*. Comparison of *pou2* expression in *ace/fgf8* mutants and their wild-type siblings at the tailbud stage (50 embryos examined) and the two-somite stage (26 embryos examined) did not yield visually detectable differences (data not shown). This indicates that *pou2* expression does not depend on *fgf8*.

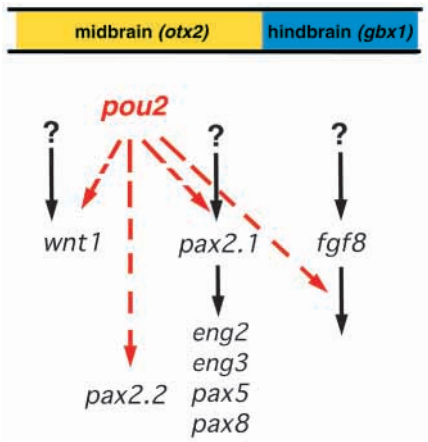

Fig. 8. Function of *spg/pou2* during MHB establishment. Model showing *spg/pou2* activity positioned within the cascade of known MHB patterning genes in zebrafish. Three parallel pathways are involved in the activation of *wnt1*, *pax2.1* and *fgf8* in the mid-/hindbrain (Lun and Brand, 1998) during MHB establishment. Our data suggests that *spg*/*pou2* functions upstream of both *pax2.1* and *wnt1*. *fgf8* is initiated normally in the anterior hindbrain during late gastrulation, but requires *pou2* for its continued expression during MHB establishment. Arrows do not necessarily indicate direct interactions.

DISCUSSION

Experiments in various organisms (Hemmati-Brivanlou et al., 1990; Martinez et al., 1991) as well as the analyses of mutant phenotypes in mice and zebrafish (Rhinn and Brand, 2001) have led to the identification of genes that contribute to the positioning of the MHB in the neural plate during gastrulation. Furthermore, they established the importance of *Pax2*,

Engrailed genes, *Fgf8* and *Wnt1* in maintenance of the MHB organizer from somitogenesis onwards. By contrast, the mechanisms that activate *Pax2, FGF8*, and *Wnt1* expression during the establishment of the MHB organizer remain unclear. So far, four mutant loci have been identified in zebrafish to affect MHB formation: the phenotypes observed in *ace/fgf8* and *noi/pax2.1* are consistent with the results obtained from experimental studies in chick and mutations in mice (Brand et al., 1996; Reifers et al., 1998). The molecular basis of the *aussicht* mutation is so far not understood, but it appears to be involved in the regulation of *fgf8* expression (Heisenberg et al., 1999). We demonstrate that *spiel ohne grenzen* (*spg*) mutations affect the *pou2* gene, and show that *pou2* is required for MHB establishment.

Role of *spg/pou2* in MHB development

MHB development proceeds through three steps: positioning, establishment and maintenance. We discuss roles that *spg/pou2* might play during each of these steps.

Positioning of the MHB organizer

Anteroposterior positioning of the MHB organizer in the neural plate does not depend on *pou2*. The expression of *otx2* and *gbx1* during MHB positioning and establishment appears to be normal in spg^{m793} mutants, demonstrating that the activation of *otx2* and *gbx1* does not depend on zygotic *pou2* function. In addition, the reduced and transient expression domains of *pax2.1* and *wnt1* in the MHB primordia of spg^{m793} mutants appear to be located at the correct anteroposterior position. In mice and chick, FGF8 has been suggested to activate *Gbx2* and repress *Otx2* (Liu et al., 1999; Martinez et al., 1999). During late gastrulation, we observe that expression of *fgf8* in the hindbrain primordium is not affected in *spg* mutants. Thus, the formation of the initial *fgf8* expression domain in the hindbrain primordium is independent of *pou2*. We found normal *pou2* expression in *ace/fgf8* mutants, indicating that *pou2* is independent of *fgf8*, and that both genes may function in parallel pathways during late gastrula stages.

Establishment of the MHB organizer

In late gastrulation, MHB establishment is characterized by the onset of expression of *pax2.1*, *wnt1*, *fgf8* and the Engrailed genes in the mid-/hindbrain. Three parallel pathways have been proposed to control MHB establishment, activating *pax2.1*, *wnt1* and *fgf8*. Mutations in *pou2* affect all three pathways (Fig. 8). In spg^{m793} mutant embryos, the initial expression of *pax2.1* and *wnt1* in the MHB during late gastrulation is reduced, indicating that *pou2* is required for the normal activation of these genes and their continued expression during the establishment phase. Several additional findings suggest that *pou2* acts earlier and upstream of *pax2.1*. First, regionalized expression of *pou2* in the MHB primordium begins at 80% epiboly, while we observe that of *pax2.1* from 90% epiboly onwards. Second, in strong *noi*/*pax2.1* mutants, expression of genes that act in MHB establishment, with the exception of the Engrailed genes, appears normal until early somitogenesis. By contrast, in spg^{m793} mutants, the initial expression of MHB genes is reduced in late gastrulation. Third, expression of *pou2* appears to be unaltered in *noi/pax2.1* mutants until at least the five-somite stage (data not shown).

Although the initial expression of *fgf8* in the hindbrain primordium appears normal in *spg* mutants, it is reduced in the MHB by the two-somite stage and progressively ceases during somitogenesis. Thus, *pou2* or *pou2*-dependent activities appear to be required for maintenance of *fgf8* expression, rather than its establishment. Alternatively, the defect in *fgf8* maintenance in spg^{m793} mutants may be secondary to the abnormal expression of other MHB genes, and an early consequence of the failure to establish the regulatory maintenance loop. In *noi/pax2.1* mutants, normal levels of *fgf8* expression have been reported until the five-somite stage (Lun and Brand, 1998). Thus, *pou2* appears to be required before *pax2.1* to maintain normal *fgf8* expression during MHB establishment.

Pax2 directly regulates the Engrailed genes in the mouse MHB primordium (Song et al., 1996). In *noi*/*pax2.1* zebrafish mutants, MHB expression of *eng1/2/3* is strongly reduced, suggesting that *pax2.1* is also a direct regulator of the Engrailed genes in zebrafish (Brand et al., 1996; Lun and Brand, 1998). In spg^{m793} mutants, expression of all *engrailed* genes (Fig. 4; data not shown) is reduced from its onset. Generally, expression of *eng2* and *eng3* remains weaker in *noi* mutants (Lun and Brand, 1998) than in spg^{m793} mutants. Furthermore, the residual expression domains of *eng2* and *eng3* in spg^{m793} mutants correspond to those of *pax2.1*. These observations suggest that the remaining *pax2.1* activity present in spg^{m793} mutants is responsible for the residual dorsal expression of *eng2* and *eng3*, and that *pou2* is not a direct regulator of the *engrailed* genes.

Maintenance of the MHB organizer

In zebrafish, MHB maintenance is thought to begin near the five-somite stage, when *wnt1* and *fgf8* expression become dependent on the function of *pax2.1* (Lun and Brand, 1998). By early somitogenesis, *pou2* expression in the MHB region disappears. Thus, *pou2* is unlikely to be directly involved in maintenance of the MHB organizer. In spg^{m793} mutants, gene expression during MHB establishment appears to be disrupted in such a way that the regulatory circuit involving *wnt1, fgf8, pax2.1*, and *eng2* is not properly established. Overexpression of *pou2* by injection of *pou2* mRNA into one-cell stage spg^{m793} mutants can induce continued expression of *pax2.1* and *wnt1* in the MHB until at least 28 hpf. These findings suggest a transient requirement for *pou2* to contribute to the activation of early-acting genes, including *wnt1* and *pax2.1*, which, together with *fgf8* and *eng2*, later maintain MHB development.

MHB expression of *pax2.2/5/8* is activated during early somitogenesis, and, in spg^{m793} mutants, is affected from its onset. Analysis of *noi/pax2.1* mutants showed that *pax2.1* is required for the initiation of *pax5* and *pax8* expression in the MHB, but that *pax2.2* MHB expression is independent of *pax2.1* (Pfeffer et al., 1998). Although the role of *pax2.2* in MHB development is unclear, it does not appear to compensate for *pax2.1* function (Pfeffer et al., 1998). As expression of *pax2.2/5/8* begins at a time when *pou2* is no longer expressed in the MHB primordium, *pou2* is not likely to directly regulate these genes. In addition, the defects in *pax5* and *pax8* expression at the MHB in spg^{m793} mutants are very similar to those observed in *noi/pax2.1* mutants, raising the possibility that the requirement of *spg/pou2* for Pax gene expression is mediated entirely through the activity of *pax2.1*. As *pax2.2* expression is dependent on *pou2* but not on *pax2.1* function, activation of *pax2.1* and *pax2.2* present distinct requirements for *pou2*.

Permissive requirement for spg/pou2 during MHB establishment

Injection of *pou2* mRNA into *spg*^{m793} mutant embryos can restore expression of *pax2.1* and *wnt1* at the MHB. As we did not observe ectopic expression of *pax2.1* or *wnt1* after injecting even large amounts of *pou2* mRNA, the ability of *pou2* to induce expression of these genes appears to be limited to the MHB region. Expression of *pax2.1* and *wnt1* in other regions, such as the optic stalk, otic vesicle, midbrain and spinal cord, appeared unaltered by *pou2* overexpression. Thus, the requirement for *pou2* in the MHB appears permissive: while *pou2* is essential for expression of *pax2.1* and *wnt1* at the MHB, it is insufficient to induce ectopic expression of these genes. This apparent permissive requirement for *pou2* suggests either that its function requires the presence of additional factors in the MHB, or suppression of Pou2 activity outside of the MHB region. Other gene products required for MHB establishment may directly interact with Pou2, or indirectly provide competence within the MHB to respond to Pou2. Interaction with co-factors has been shown to determine activity as well as specificity of POU transcription factors (Ryan and Rosenfeld, 1997).

A requirement for interactions between Pou2 and one or more co-factors may serve to integrate different developmental patterning pathways that work during MHB establishment. Interestingly, the *spg* mutant phenotype shows a dorsoventral gradient in severity, and is most pronounced in the ventral MHB. Thus, *pou2* may interact with midline regulatory pathways, although expression of genes required in midline development, such as *shh* and *axial*, appears normal in *spg* mutants (data not shown). *Fgf4* is transiently expressed in the chick axial mesoderm underlying the midbrain and hindbrain and may thus contribute a vertical signal to MHB establishment. Accordingly, exogenous FGF4 can induce expression of engrailed in the chick neural plate (Shamim et al., 1999). If so, *pou2* activity and FGF signaling may cooperate to establish the ventral MHB primordium. Vertical signals from axial mesoderm do not appear to play a significant role in zebrafish MHB establishment, however, as mutants devoid of axial mesoderm still form a MHB organizer (Rhinn and Brand, 2001). Alternatively, the midline defects observed in *spg* mutants may be caused by impaired convergence movements. Consistent with this idea, the neural plate and neural keel in the area of the MHB appear slightly wider in *spg*^{m793} mutants than in wild-type embryos (data not shown). Other zebrafish mutants that exhibit much more severe convergence defects, such as *knypek* and *trilobite* double mutant embryos (Marlow et al., 1998), form a MHB, however, suggesting that the minor impairment in convergence seen in *spg* mutants cannot explain the extensive MHB defects observed.

Evolutionary aspects

In this study, we have presented direct evidence for a novel role of the *POU*-domain transcription factor Pou2 in the establishment of the isthmic organizer in zebrafish. This raises the question of whether other vertebrate classes possess a *pou2* ortholog and whether the function of this ortholog has been conserved during vertebrate evolution. Zebrafish *pou2* is most similar to the mammalian transcription factor *Pou5f1* (formerly *Oct3/4*) (Schöler et al., 1989), a Class V POU gene that shares 79% and 74% of amino acid identity within the *POU*-specific and *POU*-homeodomain, respectively (Takeda et al., 1994). Two lines of evidence suggest that *Pou5f1* may be an ortholog of *pou2*. First, zebrafish *pou2* maps on LG 21 in proximity to the *complement factor B* gene (*BF*) (Postlethwait et al., 1998). This synteny has been conserved in both human and mouse: in mice, *Pou5f1* and *H2-Bf* map to chromosome 17, only 0.35 cM apart (Woods et al., 2000; Blake et al., 2000). Second, zebrafish *pou2* and mammalian *Pou5f1* share important features of their early expression patterns. Both genes are expressed maternally, and early zygotic expression is first found in pluripotent embryonic cells. In contrast to *pou2*, however, *Pou5f1* becomes progressively restricted to the germ line (Pesce et al., 1998) and expression in the neural plate has not been reported. Thus, if *pou2* and *Pou5f1* are true orthologs, their later functions seem to have diverged between the teleost and mammalian lineages, and the role of *pou2* in establishing the isthmic organizer may be unique.

Other *POU* domain transcription factors appear to regulate development of the mammalian MHB. In mouse, a canonical *POU* domain binding site has been shown to be essential for the MHB expression of *Pax5* (Pfeffer et al., 2000). However, *pax5* appears to be controlled by distinct mechanisms in zebrafish and mice (Pfeffer et al., 2000) and *pax5* is unlikely to be a direct target of *pou2*. The phenotypes of *Pax2/5* double mutants in mice suggest that the regulatory relationships governing the establishment of the isthmic organizer have undergone various changes during vertebrate evolution (Schwarz et al., 1997). Thus, distinct POU-domain proteins may act in the establishment of the isthmic organizer in fish and mouse.

In conclusion, *pou2* appears to be required for MHB establishment in zebrafish as, in the absence of *pou2* function, none of the genes implicated in MHB establishment that we have examined is properly activated. By contrast, lack of *pou2* does not interfere with the positioning of the MHB primordium. We, thus, surmise that *pou2* is involved in the translation of positional information provided by the *otx2*/*gbx1* boundary into successful establishment of the MHB.

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